Deep UV Resonance Raman Spectral Properties of Alpha Helical Membrane Proteins

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Doctor of Philosophy

by

Anahita Zare

Dr. Renee D. JiJi, Dissertation Supervisor

JULY 2017
The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

**Deep UV Resonance Raman Spectral Properties of Alpha Helical Membrane Proteins**

presented by Anahita Zare,

a candidate for the degree of doctor of philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

________________________________________
Professor Renee D. Ji Ji

________________________________________
Professor Kent Gates

________________________________________
Professor C. Michael Greenlief

________________________________________
Professor Gavin King
To Mom and Dad
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<td>Am</td>
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<td>CHIP</td>
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<td>DDM</td>
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<td>DUVRR</td>
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<tr>
<td>STEM</td>
<td>Science, technology, engineering, and mathematics</td>
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<td>TM</td>
<td>Transmembrane</td>
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Deep UV Resonance Raman Spectral Properties of Alpha Helical Membrane Proteins

Anahita Zare

Dr. Renee D. JiJi, Dissertation Supervisor

ABSTRACT

Membrane protein function and structure determination is vital for pharmaceutical development and disease prevention. Despite the various methods of protein structure elucidation in use, a need still exists for techniques that are of adequate resolution, rapid, inexpensive, and compatible with the membrane environment of these proteins. Deep UV resonance Raman (dUVRR) spectroscopy is an emerging structurally sensitive spectroscopic technique for analyzing membrane protein structure. The backbone amide modes are resonance enhanced in dUVRR spectra while the membrane’s lipid features are not, resulting in strong membrane protein spectral features in near native environments. In order to better define dUVRR spectral features of membrane proteins, a series of model helical peptides, poly(LA)$_7$, were designed. By substituting select residues of the transmembrane region of poly(LA)$_7$, the propensity of helical structure within a membrane has also been studied. Using these model membrane peptides in increasingly dehydrated environments (aqueous, surfactant, and bilayer), hydration depended changes in the spectra are characterized.
Chapter I: An Introduction to Membrane Proteins

1.1 Background of Transmembrane (TM) Proteins

Communication of information between intracellular and extracellular domains is reliant on the ability of transmembrane (TM) proteins to bridge the cellular phospholipid membrane. Stimuli will cause structural changes of TM proteins enabling them to perform functions such as ion transport, chemical signaling, and disease development.\(^1,2,3\) To that end, pharmaceuticals commonly interact with the cell membrane, TM protein, or both causing a structural change of the TM protein to induce specific functions. Considering that it is a fundamental axiom of biology that protein structure dictates function, it is surprising that only about 2% of solved protein structures belong to membrane proteins.\(^4,5,6\)

It is known that membrane-embedded \(\alpha\)-helices are more structurally uniform than their aqueous counterparts and thus can be concluded that structure is impacted by the environment.\(^7\) However, it is not clear as to what extent environment and amino acid sequence are responsible for the observed differences in structural heterogeneity between transmembrane and globular proteins.

1.2 Protein Structure

As previously stated, protein function is dictated by structure.\(^4,5\) Protein structure is confined to four distinct levels: primary, secondary, tertiary, and quaternary. There are 20 naturally occurring amino acids used by cells to construct proteins. Primary structure refers to the sequence of these residues covalently bonded together to form the polypeptide chain. A protein’s secondary structure is the local structural conformations. Tertiary structure is an entire protein’s overall three-dimensional shape. Quaternary
structure refers to the structure of multiple polypeptide units and how they are arranged and interact with one another. Techniques for monitoring transmembrane proteins after insertion into their natural lipid environment are limited. Structure determination using X-ray crystallography has proven difficult for transmembrane proteins, yielding the aforementioned 2% resolved membrane protein structures. Difficulties arise from: the rarity of high yields of functioning transmembrane proteins, insolubility in aqueous solution, and the complex, dynamic and heterogeneous nature of the membrane environment. Optical spectroscopies, such as circular dichroism (CD), infrared spectroscopy (IR), and Raman spectroscopy, are being utilized for rapidly evaluating a protein’s conformation, both in and out of membranes.

1.2.1 Secondary Structure

Secondary structure is determined by the set of dihedral angles of the backbone of a polypeptide or protein: $\phi$, $\psi$ and $\omega$ (Figure 1.1). Due to the planar nature of the amide bond, the $\omega$ angle adopts either the cis configuration of 0° or the more stable trans configuration of 180°. Thus, secondary structure is determined by repeating values of the more flexible $\phi$ and $\psi$ angles. All naturally occurring amino acids, with the exception of glycine, have chiral carbon groups thus can occur as left-handed (L or levo) or right-handed (D or dextro). However, proteins are almost entirely composed of L-amino acids. This stereochemistry bias thus influences secondary structure. The two most common secondary structures are $\alpha$-helices and $\beta$-sheet, but additional secondary structures have been identified.
1.2.1.1 Helices

Most helical structures have a hydrogen bond stretching from the carbonyl oxygen to the amide hydrogen three to five residues away causing the backbone to trace a spiral path along a helix. Helices tend to be right-handed, as they are almost entirely comprised of L-amino acids, which energetically favor the right-handed orientation. The most common, stable helix is the $\alpha$-helix, discovered by Linus Pauling. The $\alpha$-helix has 3.6 residues per turn that span 1.50 Å along the axis and flexible $\phi$ and $\psi$ angles centered around $-60^\circ$ and $-45^\circ$, respectively. A tighter helix, also discovered by Pauling, is the $\pi$-helix. The $\pi$-helix, which has 4.1 residues per turn that span 1.15 Å along the axis, and has flexible $\phi$ and $\psi$ angles centered around $-57^\circ$ and $-69^\circ$, respectively. Lastly, the 3,10-helix has 3 residues per turn that span 2.00 Å along the axis and was discovered by Max Perutz. This is a looser helix with flexible $\phi$ and $\psi$ angles centered around $-49^\circ$ and $-26^\circ$, respectively, and is the second most common helical structure.
1.2.1.2 β-Sheet

β-sheet structures have alternating orientation of the backbone carbonyl and amides, yielding two possible hydrogen-bonding schemes: parallel and anti-parallel. Parallel strands propagate the same direction, moving from N to C terminus, while anti-parallel strands alternate their direction. Parallel β-sheet strands are less stable, but have a closer distribution around \(-119^\circ\) and \(113^\circ\) for the \(\phi\) and \(\psi\) angles, respectively.\(^{18}\) Anti-parallel β-sheet strands have flexible dihedral angles about \(-139^\circ\) and \(135^\circ\) for the \(\phi\) and \(\psi\) angles, respectively.

1.2.1.3 Disordered or unfolded structure and turns

Lastly, the absence of any of these structures is disordered or unfolded, in which the primary structure exhibits random, sterically allowed \(\phi\) and \(\psi\) angles. Often, disordered structure cannot be resolved through high-resolution structural determination methods due to the large spatial sampling of disordered residues. Turns are abundant and non-repetitive areas with great conformational flexibility where the protein reverses its general direction. Studies have shown that there are seemingly disordered proteins with local regions of order. Polyproline-II (PPII) is a secondary structure that occurs with repeating proline residues and is seemingly disordered because of its lack of internal hydrogen bonding.\(^{19}\)

1.3 Cellular membranes and membrane mimicking environments

As previously stated, transmembrane protein conformations are dependent on the environment of the protein, therefore, it is important to properly mimic the membrane environment. Cell membranes are a fluid and dynamic environment comprised of lipids, proteins and sterols.\(^{20}\) The phospholipids that make up the majority of a membrane have
hydrophilic head groups and hydrophobic tail groups, which orient away from solvent to form a hydrophobic region within the lipid bilayer. To monitor protein folding within a membrane, the transmembrane proteins are inserted into a lipid environment comprised of phospholipids. DLPG (1,2-didodecanoyl-sn-glycero-3-phospho-(1’rac-glycerol)) is a two tailed, anionic lipid that forms liposomes after an extrusion process. Membrane insertion can be monitored through fluorescence, denoted by a blue-shift from 350 nm, of an anchoring tryptophan residue.\textsuperscript{21}

Hydrophobic matching is a major factor to take into account when developing an artificial membrane system for the structural analysis of membrane proteins. Hydrophobic mismatch refers a difference between the hydrophobic thickness of the lipid membrane and the transmembrane portion of the protein.\textsuperscript{22} When hydrophobic mismatch occurs, either the membrane, protein, or both will adjust structure to correct for the mismatch.\textsuperscript{22}

Detergent was introduced into the proteoliposome solution to monitor the effect of a hydrating environment on the transmembrane peptides. Detergents are amphipatic molecules with a polar head group and a hydrophobic chain which spontaneously form spherical micelles.\textsuperscript{9} One commonly used detergent is n-dodecyl β-D-maltoside (DDM), which is a mild, non-denaturing, nonionic detergent often used in the isolation and purification of proteins and is important for membrane protein recrystallization.\textsuperscript{23} DDM has minimal spectroscopic features and is commonly used because it disrupts lipid-lipid or protein-lipid interactions rather than protein-protein interactions.
As detergent is introduced into a proteoliposome solution, a three-phase solubilization of the liposomes occurs based on the concentration of the detergent added (Figure 1.2).\textsuperscript{9,23,24} The first phase occurs below solubilization detergent concentrations, the detergent partitions between the liposomes and solution. In phase two, at higher concentrations of detergent, micellization occurs. Once the detergent concentration is higher than that necessary for complete solubilization, phase three yields a reduction in the size of the formed mixed micelles. The hydrophobic chain of the detergent binds to the hydrophobic region of the peptide which exposes the peptide to solution due to the polar head group of the detergent molecule, thus introducing the peptide to a hydrated environment.\textsuperscript{25}

1.4 Model Helical Peptides

To investigate membrane proteins, a model membrane peptide system has been designed. The synthesized peptide, poly (LA)$_7$, is made of repeating leucine and alanine residues between two pairs of lysine residues at the N- and C- termini. Lysine residues were placed at each termini to ensure transmembrane orientation through ionic interactions between the anchoring residues and the lipid headgroups at both sides of the membrane bilayer. Additionally, the lysine residues promote solubility at low concentrations in an aqueous solution. The leucine-alanine repeating core was chosen because poly-leucine peptides have been shown to form spontaneous hydrophobic $\alpha$-
helices within a membrane. Replacing half of the residues with alanine, a smaller and less hydrophobic α-helix forming amino acid, encourages helix formation but the resulting structure is expected to be less stable and more distortable.

1.5 Spectroscopic methodologies for characterization of structure and environment

1.5.1 Circular dichroism (CD)

Currently, the standard approach for secondary structure analysis of proteins is CD spectroscopy, but its sensitivity to specific non-helical structural configurations is low. CD spectroscopy measures the unequal absorption of right- and left-handed polarized light. Since α-helical, β-sheet, and disordered content have distinct spectral features, CD spectroscopy is a good technique for identifying the protein’s dominating secondary structure. A protein with pure α–helix will have large CD bands with a positive ellipicity at 193 nm and negative ellipticity at 222 and 208 nm. Pure β-sheet will have broad CD bands with a positive ellipicity at 195 nm and negative ellipticity at 218 nm. Pure disordered secondary structure will have low, positive ellipicity above 210 nm and a negative band at 195 nm.

1.5.2 Oriented Circular dichroism (OCD)

Oriented CD (OCD) is used to determine the orientation of a helix embedded in a membrane by measuring the CD of a multilayer system at a series of oblique angles using a specialized OCD sample chamber (Figure 1.3). OCD samples are dried proteoliposomes on a quartz slide. As the hydration of the sample decreases, the liposomes reorganize into a bilayer, which is parallel to the slide’s surface, a process that does not occur with detergent micelles.
An OCD spectrum, depicted as an average of each rotated scan, with a decreased intensity at this wavelength is indicative of a protein’s backbone axis’s preferred orientation is parallel to the incident light. The fingerprint region for OCD is at 207 nm. An increased intensity at 207 nm indicates that the protein’s backbone axis’s preferential orientation is perpendicular to the incident light and thus laying along the surface of the membrane bilayer (Figure 1.4).

Figure 1.3: OCD sample chamber.
1.5.3 Deep UV resonance Raman (DUVRR) spectroscopy

Although using circular dichroism will provide the predominate secondary structure, deep-ultraviolet resonance Raman (dUVRR) spectroscopy is emerging as a structurally sensitive technique for analyzing membrane protein structures.  

DUVRR spectroscopy reveals four vibrational, or amide, modes when excited with wavelengths below 230 nm that provide insight into the folded protein’s secondary structure. These modes arise from the protein’s backbone vibrations: amide I (C=O stretch), amide II (N-H bending and C-N stretch), amide III (a mixture of N-H, C-H bend/stretch, and C-N stretch), and amide S (a coupled in plane bending of N-H and C-
H). The amide I band manifests at 1625-1750 cm$^{-1}$, amide II manifests at 1475-1575 cm$^{-1}$, amide III manifests at 1200-1300 cm$^{-1}$ and amide S manifests at 1300-1425 cm$^{-1}$. DUVRR is not limited to amide modes, but also selectively enhances the protein aromatic amino acids: tyrosine and phenylalanine. The Raman bands associated with tyrosine manifest at: 832 cm$^{-1}$ (Fermi resonant doublet), 852 cm$^{-1}$ (symmetric ring stretch), 1180 cm$^{-1}$ (in-plane CH bend), 1210 cm$^{-1}$ (symmetric stretch), 1236 cm$^{-1}$ (ring CCH in-plane bend), 1263 cm$^{-1}$ (C-O stretching and symmetric ring deformation), 1601 cm$^{-1}$ (in-plane ring stretching), and at 1617 cm$^{-1}$ (in-plane ring stretching). The Raman band associated with phenylalanine manifest at: 790 cm$^{-1}$ (symmetric ring breathing), 1000 cm$^{-1}$ (symmetric ring stretch), 1030 cm$^{-1}$ (in-plane CH bend), 1182 cm$^{-1}$ (in-plane CH bend), 1207 cm$^{-1}$ (phenyl-C stretch), 1586 cm$^{-1}$ (in-plane ring stretching), and 1606 cm$^{-1}$ (in-plane ring stretching).
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Chapter II: Helical Stability of Membrane Proteins

2.1 Abstract

It is known that membrane-embedded α-helices are more uniform structurally than their aqueous counterparts. Despite this uniformity, distortions and localized unfolding are thought to be common in these proteins in order for them to conduct their cellular tasks. However, how amino acid sequence facilitates these conformational shifts remains unknown, as methods for investigating structural heterogeneity in transmembrane proteins are limited. Circular dichroism (CD) is often used to characterize the secondary structure of proteins, but the broadly overlapped spectral features limit its sensitivity. Deep-ultraviolet resonance Raman spectroscopy (dUVRR) is an emerging structurally sensitive spectroscopic technique for analyzing membrane protein structure. The backbone amide modes are resonance enhanced in dUVRR spectra while lipid features are not, resulting in strong membrane protein spectral features in near native environments.

Using model leucine-alanine peptides in increasingly dehydrated (surfactant and bilayer) environments, hydration dependent changes in the amide modes are characterized. The amide S (AmS) mode in the dUVRR spectra of these peptides increased with increasing dehydration, while the amide III$_3$ (Am III$_3$) red shifted. These results indicate that the dehydration of the peptide backbone is accompanied by an increase in helical structure. Additionally, the incorporation of helix breaking residues (HBRs), proline or glycine, in these model peptides promoted helical instability in lipophilic environments, as indicated by an increase in the AmS and AmIII$_3$ modes.
2.2 Introduction

Helices are one of the most common secondary structure of proteins, making up 40% of solved proteins.\(^1\) It is known that membrane-embedded $\alpha$-helices are more structurally uniform than their aqueous counterparts and thus can be concluded that structure is impacted by the environment.\(^2\) However, it is not clear to what extent environment and amino acid sequence is responsible for the observed differences in structural heterogeneity between transmembrane and globular proteins.

Transmembrane (TM) protein conformations are dependent on the environment of the protein; therefore, it is important to properly mimic the membrane environment. To monitor folding behavior within a membrane, investigated TM peptides were inserted into cell membrane mimicking environments. Liposomes, bicelles, micelles, and nanodiscs are some commonly used membrane mimics.\(^3,4\) This study has been limited to observing structural heterogeneity in liposomes and detergent solubilized micelles to negate any scattering effects from non-spherical environments.

The lipid, 1,2-didodecanoyl-sn-glycero-3-phospho-(1’rac-glycerol) (DLPG), forms liposomes after an extrusion process. DLPG is a two-tailed, anionic lipid chosen for its good hydrophobic matching to the studied peptide. Hydrophobic mismatch is a difference between the hydrophobic thickness of the lipid membrane and the transmembrane portion of the protein.\(^5\) Membrane insertion is monitored through oriented circular dichroism (OCD).

Detergent was introduced into the proteoliposome solution to monitor the effect of a hydrating environment on the transmembrane peptides. Detergents are amphipatic molecules with a polar head group and a hydrophobic chain which spontaneously form
spherical micelles.\textsuperscript{3} N- dodecyl β –D-maltoside (DDM) is a mild, non-denaturing, nonionic detergent often used in the isolation and purification of proteins and is important for membrane protein recrystallization.\textsuperscript{6} DDM has minimal spectroscopic features and is commonly used because it disrupts lipid-lipid or protein-lipid interactions rather than protein-protein interactions.

As detergent is introduced into a proteoliposome solution, a three-phase solubilization of the liposomes occurs based on the concentration of the detergent added.\textsuperscript{3,6,7} The first phase occurs below solubilization detergent concentrations, the detergent partitions between the liposomes and solution. In phase two, at higher concentrations of detergent, micellization occurs. Once the detergent concentration is higher than that necessary for complete solubilization, phase three yields a reduction in the size of the formed mixed micelles. The hydrophobic chain of the detergent binds to the hydrophobic region of a membrane peptide, exposing it to solution due to the size discrepancy between the polar head groups of the detergent and lipid molecules, thus introducing the peptide to a hydrated environment (Figure 2.1).\textsuperscript{8}

\textbf{Lipid Bilayer} \hspace{1cm} \textbf{Detergent Micelle}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.1.png}
\caption{Detergent solubilization of liposomes into micelles with radius of liposomes and micelles as determined by dynamic light scattering.}
\end{figure}

\textit{Liposome Radius} = 38 ± 2 nm  \hspace{1cm} \textit{Micelle Radius} = 33 ± 3 nm
Proline is unique among amino acids in that the amide nitrogen does not form the normal backbone hydrogen bond but rather its side chain cyclizes back to its backbone amide. This forces the dihedral angle, \( \phi \), to be fixed at -65° resulting in a ‘kink’ in the helix.\(^{9,10}\) On the other end of the spectrum, glycine is unique in that it consists of a single hydrogen as a side chain, yielding a large area allowed to glycine on the Ramachandran plot.\(^{11}\) Proline and glycine are also of interest because they are believed to be functionally important in transmembrane helices.\(^{12}\) Transport proteins within a membrane include proline residues, which have been tied to the protein’s functionality.\(^{13}\) Glycine’s influence within a membrane is also of interest because it introduces a dynamic hinge into helices, which has been shown to operate as a gating mechanism.\(^{12,14}\) However, the extent at which these amino acids affect helical structure in a membrane is unknown.

To investigate the propensity of proline and glycine as helix breakers within a lipophilic environment, a set of de novo designed transmembrane peptides with single and double substitutions of these amino acids have been constructed. The peptide sequences are found in Table 2.1. The synthesized peptide, poly(LA)\(_7\), is made of repeating leucine and alanine residues between two pairs of lysine residues at the N- and C- termini. Lysine residues were placed at each termini to ensure transmembrane orientation through ionic interaction between the anchoring residues and the lipid headgroups at both sides of the membrane bilayer. The leucine-alanine repeating core was chosen because poly-leucine peptides have been shown to form spontaneous hydrophobic \( \alpha \)-helices within a membrane.\(^{15}\) Replacing half of the residues with alanine, a smaller and less hydrophobic \( \alpha \)-helix forming amino acid, encourages helix formation but the resulting structure is expected to be less stable and more distortable.\(^{16}\)
Techniques for monitoring transmembrane proteins after insertion into their natural lipid environment are limited. Difficulties with high resolution techniques arise from: the rarity of high yields of functioning transmembrane proteins, insolubility in aqueous solution, and the complex, dynamic, and heterogeneous nature of the membrane environment. Spectroscopies, such as circular dichroism (CD) and Raman spectroscopy, are being utilized for rapidly evaluating a protein’s conformation, both in and out of membranes.

Deep-ultraviolet resonance Raman (dUVRR) spectroscopy is emerging as a structurally sensitive technique for analyzing membrane protein structures. DUVRR spectroscopy reveals four vibrational, or amide, modes that provide insight into the folded protein’s secondary structure. These modes arise from the protein’s backbone vibrations: amide I (C=O stretch), amide II (N-H bending and C-N stretch), amide III (a

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide Sequence</th>
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</thead>
<tbody>
<tr>
<td>Poly(LA)$_7$</td>
<td>KKLALALALALALALAKK$W$</td>
</tr>
<tr>
<td>P End Poly(LA)$_7$</td>
<td>KKLAPALALALALALAKK$W$</td>
</tr>
<tr>
<td>PP End Poly(LA)$_7$</td>
<td>KKLAPPLALALALALAKK$W$</td>
</tr>
<tr>
<td>PP Mid Poly(LA)$_7$</td>
<td>KKLAPALALPLALAKK$W$</td>
</tr>
<tr>
<td>G End Poly(LA)$_7$</td>
<td>KKLAGALALALALALAKK$W$</td>
</tr>
<tr>
<td>GG End Poly(LA)$_7$</td>
<td>KKLAGGLALALALALAKK$W$</td>
</tr>
<tr>
<td>GG Mid Poly(LA)$_7$</td>
<td>KKLALAGALALGLALAKK$W$</td>
</tr>
</tbody>
</table>

**Table 2.1: Sequences of studied peptides.**
mixture of N-H, C-H bend/stretch, and C-N stretch), and amide S (a coupled in plane bending of N-H and C-H) \(^{19}\). The amide I band manifests at 1625-1750 cm\(^{-1}\), amide II manifests at 1475-1575 cm\(^{-1}\), amide III manifests at 1200-1300 cm\(^{-1}\) and amide S manifests at 1300-1425 cm\(^{-1}\) \(^{9}\).

The aims of this study were to establish a response of the amide modes of dUVR spectroscopy to helix uniformity and to identify spectral features associated with helix deformation due to HBRs as well as environment. This study also monitored modified peptide conformations within a membrane by identifying the changes in amide mode intensities.

2.3 Materials and Methods

2.3.1 Materials

1,2-Dilauroyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DLPG) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). \(n\)-Dodecyl \(\beta\)-D-maltoside (DDM) was obtained from EMD Chemicals (Philadelphia, PA).

Synthetic peptides were purchased from Atlantic Peptides (Lewisburg, PA) at a purity of \(>95\%\) and used without further purification. All peptides were comprised of repeating leucine and alanine residues, with two lysine residues at the \(N\)- and \(C\)- termini, which ensured the peptides would spontaneously associate with the anionic surface of the liposomes and micelles. Poly(LA)\(_7\) sequences that included proline and glycine insertions were designed to characterize the dUVR amide modes in lipophilic environments (Table 2.1). The leucine-alanine repeating core generates a sparingly soluble peptide in aqueous environments, while promoting spontaneous formation of \(\alpha\)-helices within membrane mimicking environments.\(^{15,16}\) Tryptophan has been shown to have a
stabilizing role in poly-leucine peptides; therefore, a tryptophan residue for environment monitoring was placed at the C-terminus to limit its influence on each peptide’s folded conformation.

**2.3.2 Liposome and Micelle Preparation**

DLPG was dissolved in a 65:35:8 by volume chloroform:methanol:water solution and distributed among test tubes to yield 12.5 mg of lipid in each tube. Chloroform solution was dried off with a stream of argon then placed in desiccator overnight. To suspend lipids for liposome preparation, 0.55 mL of 20 mM phosphate, 5 mM NaCl solution is pipetted into each test tube, yielding a 22.7 mg/mL solution. Tubes were sonicated for 1 hour at 50°C. The resulting solution was extruded through a 200 nm filter using a Lipofast (Avestin Inc., Ottawa, Ontario, Canada). Liposomes had uniform hydrodynamic radius of roughly 50 nm, as confirmed by dynamic light scattering (DLS) (Dyna Pro, Wyatt Technology Corp., Santa Barara, CA) measurements. Liposomes were prepared in 10 mM phosphate buffer at pH 7.4 and lipid concentration was verified by a Rouser assay.

Detergent micelles were prepared by adding n-dodecyl β-D-maltoside (DDM) (EMD Chemicals, Philadelphia, PA) to proteoliposome solutions at a concentration of 0.01 µM. The resulting solution was placed on a shaker at 4°C for one hour.

**2.3.3 Proteoliposome Preparation**

Peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Sigma Aldrich, St. Louis, MO), and distributed among test tubes so that each tube holds 0.05 mg of peptide. The HFP was then dried off with a stream of argon while rotating the test tube to evenly dry the peptide.
A 9.9 mM liposome solution was combined with dried peptide for a final concentration of 15.6 µM peptide to produce proteoliposomes. The resulting solution was stored at room temperature overnight to ensure equilibration. The proteoliposome solutions were centrifuged at 45,000 rpm for 120 minutes at 4°C with a Sorval T-1250 rotor. The proteoliposome pellets were resuspended in 20 mM phosphate buffer (pH 7.0) and the internal intensity standard, 1 M sodium perchlorate (Sigma Aldrich, St. Louis, MO), to reach a final peptide concentration of 0.5 mM and an internal standard concentration of 0.05 µM. UV-Vis absorption intensity tryptophan was monitored using a Cary Bio 50 UV/Vis (Varian, Palo Alto, CA) at 278 nm to determine peptide concentration. Liposome insertion was verified by tryptophan fluorescence using a Cary Eclipse fluorometer (Varian, Palo Alto, CA). DUVRR spectra of poly(LA)$_7$ peptides and background liposome were collected at an excitation wavelength of 197 nm.

### 2.3.4 Circular Dichroism (CD)

Circular dichroism (CD) is the difference in absorption of right- and left- handed polarized light. Proteins have a strong CD spectra arising from the chiral nature of the protein backbone. Furthermore, α-helical, β-sheet, and disordered structures have distinct spectral features, making CD spectroscopy a good technique for identifying a protein’s dominant secondary structure though the sensitivity to specific non-helical structural configurations is low. A pure α-helical protein will have a strong positive feature at 193 nm and two negative features at 222 and 208 nm.

CD spectra were collected on a Jasco J-710 CD spectropolarimeter (Easton, MD) using a 1 mm path length quartz cuvette (Hellma, Plainview, NY). Each spectrum was the average of five replicate scans with a scan speed of 50 nm/min and a response time of
4 seconds for each sample. A background CD spectrum of DLPG was subtracted from the CD spectra of peptide in liposomes. All CD spectral processing was carried out in the MATLAB environment (7.1, MathWorks, Natick, MA).

2.3.5 Oriented Circular Dichroism (OCD)

For membrane proteins, oriented CD (OCD) is the CD spectrum of a multilayer system at a series of oblique angles. OCD is used to determine a helical protein’s preferential orientation (parallel, perpendicular or tilted) with respect to a membrane. OCD samples were prepared by drying proteoliposomes on a quartz slide. As the hydration of the sample decreases, the proteoliposomes reorganize into bilayers parallel to the slide’s surface, a process which does not occur for detergent micelles. OCD spectra were generated by averaging the CD spectra of multilayer proteoliposomes at six different angles.

An increased negative ellipticity at 222 nm with respect to 208 nm indicates the helix is parallel to the incident light. Alternatively, if there is a decrease in intensity at 222 nm with respect to 208 nm indicates that the helix is perpendicular to the incident light and thus laying along the surface of the membrane bilayer.

OCD spectra were collected on a Jasco J-710 CD spectropolarimeter (Easton, MD) using a quartz plate with 1 mm thickness and a diameter of 22 mm (Hellma, Plainview, NY). Plates were prepared by drying 400 µL of sample, using a micropipette tip to improve homogenous spreading, onto quartz plate in a dessicator with 80% ZnCl₂ overnight. The dried sample was slowly hydrated for three hours and the quartz plate was then assembled in a Jasco OCD sample chamber. The Jasco OCD sample chamber is a cylindrical chamber with an aluminum frame (Figure 1.3). The cell contains a ring
shaped cavity surrounding a central bore for two quartz slides, one of which holds the oriented sample. OCD spectra were recorded every 60 deg rotation of the cell, as an average of five replicate scans with a scan speed of 50 nm/min and a response time of 4 seconds. All OCD spectral processing was carried out in the MATLAB environment (7.1, MathWorks, Natick, MA).

2.3.6 Deep UV resonance Raman (dUVRR) spectroscopy

The DUVRR instrument has been described previously by Wang et al.\textsuperscript{29} UVRR spectra were obtained using the fourth harmonic of a 4kHz frequency quadrupled Ti:Sapphire laser (Coherent Inc., Santa Clara, CA). The Ti:Sapphire laser was pumped using a diode-pumped frequency-doubled Nd:YLF laser (Coherent Inc., Santa Clara, CA). Samples were excited at 197 nm; the average power at the sample was approximately 0.5 mW to reduce the potential for degradation.

The sample solution was circulated using a model 75211-10 gear pump (Cole Palmer, Vernon Hills, IL). A wire-guided (Small Parts Inc., Miramar, FL) stream of sample was purged with a steady stream of nitrogen gas to remove ambient oxygen. Raman scattering from the sample was collected at 135° backscattering geometry and dispersed using a 1.25 m spectrograph (Horiba Jobin Yvon, Edison, NJ) fitted with 3600 groove/mm grating. The spectrometer was equipped with a back illuminated, phosphor coated, liquid nitrogen cooled Symphony CCD camera (Horiba Jobin Yvon, Edison, NJ) with a chip size of 2048 x 512 pixels. The maximum resolution of the instrument is, approximately, 0.6 cm\textsuperscript{-1}. Pixels were binned in the horizontal direction in increments of four, for a final resolution of approximately 2.4 cm\textsuperscript{-1}. Spectra were collected and
exported using SynerJY software (Horiba Jobin Yvon, Edison, NJ). Spectra were calibrated using a standard cyclohexane spectrum.

2.3.7 Analysis

The data was processed using MATLAB 7.1 (MathWorks, Natick, MA). Pre-processing of raw spectra included removal of cosmic rays using a program written in-house followed by averaging of 96 replicates of five 30 s scans (four hours total) for each sample. The baseline of each averaged spectrum was then fit using MATLAB’s curve fitting toolbox and subtracted from each spectrum. The blank lipid spectrum was fit to a series of Gaussian/Lorentzian distributions using a non-linear least squares (NLLS) function as described previously. The modeled blank lipid spectrum from subtracted each sample spectrum using the characteristic DLPG feature at 1740 cm\(^{-1}\) to scale the lipid spectrum. A modeled lipid spectrum was used to avoid introduction of non-random noise features into the sample data.

2.4 Results

2.4.1 Spectral Response to the Impact of Environment on Helices

Amphiphilic peptides such as the β-amyloid and pH low insertion peptide (pHLIP) are disordered in aqueous environments and spontaneously adopt helical structure in membrane mimicking environments. Both leucine and alanine promote helical structure; the leucine-alanine motif should promote helical structure and prompt spontaneous insertion into a membrane. Micelles, bicelles and liposomes are often used to mimic natural lipophilic environments. Extrusion of lipids produces uniform liposomes that mimic a natural bilayer environment. DUVRR studies of the antimicrobial peptide valinomycin in non-extruded and extruded lipids indicated that valinomycin was
more hydrated in the non-extruded lipid environment.\textsuperscript{35} The effect of hydration on the secondary structure of poly(LA)$_7$, as determined by CD, is represented in Figure 2.2. In Figure 2.2, the CD spectra of poly(LA)$_7$ in a detergent micelle versus poly(LA)$_7$ in a lipid bilayer environment are shown. Both spectra show predominately $\alpha$-helical structure, as indicated by a maximum at 195 nm, and two minima at 208 nm, and 222 nm. The decreased intensities at these three wavelengths of interest for the poly(LA)$_7$ in detergent micelles spectrum indicate that solubilizing the lipid bilayers with poly(LA)$_7$ into detergent micelles will decrease the helicity of the peptide.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.2.png}
\caption{Circular dichroism spectra of poly(LA)$_7$ in a liposome (black) and in a detergent solubilized micelle (blue).}
\end{figure}
The dUVRR spectra of poly(LA)$_7$ in a lipid bilayer (DLPG) and detergent micelles (DDM) are shown in Figure 2.3. The spectrum for poly(LA)$_7$ in detergent micelles shows a red-shift in the amide III$_3$ and an increase in the amide S modes, indicating a decrease in ideality of the helix.$^{36}$ Also, poly(LA)$_7$ in a detergent micelle has decreased amide I and amide II intensities.
Figure 2.3: Deep UV resonance Raman spectra of poly(LA)$_7$ in a liposome (black) and in a detergent solubilized micelle (blue).
2.4.2 Structural Impact of Proline on Transmembrane Proteins in a Lipid Bilayer

The OCD data for all studied peptides show that all poly(LA)\textsubscript{7} peptides are transmembrane, however the introduction of the HBR proline induces an observed tilt. Since polyLA\textsubscript{7} will adopt predominately helical secondary structure in a lipophilic environment, circular dichroism was employed to verify this secondary structure for each sequence.\textsuperscript{34} The comparative CD spectra of poly(LA)\textsubscript{7}, P end poly(LA)\textsubscript{7}, PP end poly(LA)\textsubscript{7}, and P Mid poly(LA)\textsubscript{7} in a lipid bilayer environment are shown in Figure 2.3. All spectra in Figure 2.3 suggest that the studied peptides have a predominately α-helical structure, indicated by a maximum at 195 nm and two minima at 208 nm and 222 nm.\textsuperscript{17} The CD spectra of peptides with proline residues have a decrease in intensity at these three points of interest, pointing to a decrease in the helicity from polyLA\textsubscript{7}. 
A comparison of the dUVRR spectra of poly(LA)\textsubscript{7}, P end poly(LA)\textsubscript{7} and PP end poly(LA)\textsubscript{7} in DLPG is shown in Figure 13. For the Amide III band (1200-1300 cm\textsuperscript{-1}), the P end poly(LA)\textsubscript{7} behaves similarly to poly(LA)\textsubscript{7}. However, PP end poly(LA)\textsubscript{7} has a decreased intensity for Amide III from poly(LA)\textsubscript{7}. This is also the case for the Amide II region (1475-1575 cm\textsuperscript{-1}), P end poly(LA)\textsubscript{7} and poly(LA)\textsubscript{7} have similar peak intensities, but PP end poly(LA)\textsubscript{7} has a decreased intensity for the Amide II. The Amide I band

Figure 2.4: Circular dichroism spectra of poly(LA)\textsubscript{7} (black), P end poly(LA)\textsubscript{7} (light blue), PP end poly(LA)\textsubscript{7} (purple), and P mid poly(LA)\textsubscript{7} (green).
(1625-1750 cm⁻¹), however, has a decreased intensity from poly(LA₇) to P end poly(LA₇) and from P end poly(LA₇) to PP end poly(LA₇).

Figure 2.5: DUVRR spectra of poly(LA₇) (black), P end poly(LA₇) (yellow), PP end poly(LA₇) (blue), and P mid poly(LA₇) (green).
2.4.3 Structural Impact of Glycine on Transmembrane Proteins in a Lipid Bilayer

The OCD data for all studied peptides show that all poly(LA)$_7$ peptides are transmembrane, however the introduction of the HBR, glycine, induces a tilt. Since polyLA$_7$ will predominately adopt helical secondary structure in a lipophilic environment, circular dichroism was employed to verify this secondary structure for each sequence. The comparative CD spectra of polyLA$_7$, G end polyLA$_7$, pLA$_7$GG, and pLA$_7$Gmid in a lipid bilayer environment are shown in Figure 2.6. All spectra in Figure 2.6 have a predominately $\alpha$-helical shape, indicated by a maximum at 195 nm and two minima at 208 nm and 222 nm. The CD spectra of peptides with glycine residues have a decrease in intensity at these three points of interest, pointing to a decrease in the helicity from polyLA$_7$.

![Figure 2.6: Circular dichroism spectra of poly(LA)$_7$ (black), G end poly(LA)$_7$ (red), GG end poly(LA)$_7$ (yellow), and G mid poly(LA)$_7$ (purple).](image-url)
A comparison of the dUVRR spectra of Poly(LA)$_7$, G end poly(LA)$_7$, GG end poly(LA)$_7$, and G mid in DLPG is shown in Figure 2.7. The Amide III band (1275 cm$^{-1}$) has an increase in intensity for G end poly(LA)$_7$ from poly(LA)$_7$, which is indicative of a decrease in helicity. The Amide S band, manifesting 1300-1425 cm$^{-1}$, is also a marker for helicity. The slight increase seen for the interrupted peptides from poly(LA)$_7$ indicates that there is a decrease in helicity. The Amide I band has a decreased intensity from poly(LA)$_7$ for both glycine interrupted poly(LA)$_7$. 
Figure 2.7: DUVRR spectra of poly(LA)$_7$ (black), G end poly(LA)$_7$ (yellow), GG end poly(LA)$_7$ (blue), and G mid poly(LA)$_7$ (green).
2.5 Discussion

To investigate the spectral response of environment on membrane peptide, poly(LA)$_7$, was monitored in a lipid bilayer and detergent micelle. Membrane proteins embedded in detergent solubilized micelles are considered to be more hydrated since there is great mismatch between the head groups of detergent and lipids. The OCD spectra confirmed that poly(LA)$_7$ in a lipid bilayer is embedded and transmembrane. The CD data depicted that both environments depict helical peptide structure, however a decrease in intensity for poly(LA)$_7$ in a detergent micelle is due to a loss of helical structure from poly(LA)$_7$ in a lipid bilayer. This loss in helicity is due to greater mobility afforded poly(LA)$_7$ in a detergent micelle.

The dUVRR spectra for poly(LA)$_7$ in a lipid bilayer versus a detergent micelle yields no increase in the helical marker of dUVRR, the AmS mode. This indicates that when in a detergent micelle, poly(LA)$_7$ remains helically constrained. The red-shift of the AmIII$_3$ mode indicates that the alpha helix is moving to a 3,10, or extended, helix in a hydrated environment. A 3,10 helix has three residues per turn that span 2.00 Å along the axis.$^{37}$

For poly(LA)$_7$ and poly(LA)$_7$ with HBR mutations, all peptides were embedded in a lipid bilayer, as confirmed by OCD. Additionally, all peptides are helical, as shown with the CD spectra. Peptides with HBR mutations have a decrease in intensity, again a loss of helical structure from poly(LA)$_7$.

The dUVRR spectra for poly(LA)$_7$ versus poly(LA)$_7$ with proline mutations does not have an increase in the helical marker of dUVRR, the AmS mode. However, the increase in intensity of the AmIII$_3$ indicates that glycine promotes a lack of ideality of a helix.
This indicates that within a lipid bilayer, membrane peptides with proline may remain helical overall, but locally a kink is induced with the proline substitutions. The dUVRR spectra for poly(LA)$_7$ versus poly(LA)$_7$ with glycine mutations does have an increase in the helical marker of dUVRR, the AmS mode and an increase in intensity of the AmIII$_3$ indicates that glycine promotes a lack of ideality of a helix and is not helically constrained.
2.6 References


Chapter III: Helix Breaking Residues and the Amide I band of Deep UV Resonance Raman Spectroscopy

3.1 Abstract

It is known that membrane-embedded a-helices are more uniform structurally than their aqueous counterparts. Despite this uniformity, protein dynamics are thought to be common in these proteins in order for them to conduct their cellular tasks. However, how amino acid sequence facilitates these dynamics remains unknown, as methods for investigating structural heterogeneity in transmembrane proteins are limited. Circular dichroism (CD) is often used to characterize the secondary structure of proteins, but its sensitivity to specific non-helical structural configurations is low. Deep-ultraviolet resonance Raman spectroscopy (dUVRR) is a structurally sensitive spectroscopic technique emerging for analyzing membrane protein structure.

Using a model leucine-alanine peptide in a lipophilic environment, changes in the amide I mode in dUVRR spectra were observed to determine the impact of sequence. A set of model leucine-alanine peptides with proline or glycine residues, which are known helix breaking residues in globular proteins, were designed to test how helical instability impacts the AmI mode. The secondary structure of each peptide was monitored via dUVRR and CD spectroscopies. Introduction of helix breaking residues (HBRs) into a membrane peptide causes a decrease in the amide I, indicating that HBRs disrupt the hydrogen bonding of the protein backbone carbonyl.
3.2 Introduction

Elucidating the structure of membrane proteins within their lipophilic environments has proven very difficult. Deep ultraviolet resonance Raman spectroscopy is an emerging technology for investigating secondary structure as the backbone amide modes are resonance enhanced in dUVRR spectra while lipid features are not resulting in strong membrane protein spectral features in near native environments.

There are four amide modes which arise from the protein’s backbone vibrations: amide I (C=O stretch), amide II (N-H bending and C-N stretch), amide III (a mixture of N-H, C-H bend/stretch and C-N stretch), and amide S (a coupled in-plane bending of N-H and C-H). This study focuses on the amide (Am) I mode. The AmI arises between 1600 and 1700 cm\(^{-1}\) from the vibrations of the protein backbone’s carbonyl stretch in combination with some minor C-N stretching and Ca-C-N deformation. Due to the unique hydrogen geometry of each secondary structure and the carbonyl’s hydrogen bonding capability, the AmI band is very structurally sensitive. For an \(\alpha\)-helix, the AmI band will arise between 1645 and 1655 cm\(^{-1}\) and will be enhanced over other secondary structures. The AmI band for beta-sheet or disordered proteins arises between 1660 and 1682 cm\(^{-1}\). Furthermore, membrane proteins have an enhanced AmI over globular protein spectra. With infrared (IR) spectroscopy, the AmI mode is more intense so it is exclusively used for structural determination.

Often the Am I is a broad band that is difficult to fully characterize. To address this issue, a set of synthesized peptides with repeating leucine and alanine residues between two lysine residues at the N- and C- termini was designed. This poly(LA)\(_7\) peptide set has a very sharp Am I band allowing for easier characterization. Single and
double substitutions of proline and glycine were used to investigate their impact on the Am I band.

This study aims to extend understanding of the factors that affect the AmI mode in deep UV resonance Raman spectroscopy. Transmembrane peptides with helix breaking residue substitutions were studied to see how hydrogen bonding along a helix impacts the AmI mode.

3.3 Materials and methods

3.3.1 Materials

1,2-Dilauroyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DLPG) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). n-Dodecyl β-D-maltoside (DDM) was obtained from EMD Chemicals (Philadelphia, PA).

Synthetic peptides were purchased from Atlantic Peptides (Lewisburg, PA) at a purity of >95% and used without further purification. All peptides were comprised of repeating leucine and alanine residues, with two lysine residues at the N- and C- termini, which ensured the peptides would spontaneously associate with the anionic surface of the liposomes and micelles. Poly(LA)$_7$ sequences that included proline and glycine insertions were designed to characterize the dUVRR amide modes in lipophilic environments and listed in Table 3.1. The leucine-alanine repeating core generates a sparingly soluble peptide in aqueous environments, while promoting spontaneous formation of α-helices within membrane mimicking environments.$^{4,5}$ Tryptophan has been shown to have a stabilizing role in poly-leucine peptides,$^6$ therefore, a tryptophan residue for environment monitoring was placed at the C-terminus to limit its influence on each peptide’s folded conformation.
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<thead>
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<tbody>
<tr>
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For membrane proteins, oriented CD (OCD) is the CD spectrum of a multilayer system at a series of oblique angles. OCD is used to determine a helical protein’s preferential orientation (parallel, perpendicular or tilted) with respect to a membrane\textsuperscript{10}. OCD samples were prepared by drying proteoliposomes on a quartz slide. As the hydration of the sample decreases, the proteoliposomes reorganize into bilayers parallel to the slide’s surface,\textsuperscript{10,11,12} a process which does not occur for detergent micelles. OCD spectra were generated by averaging the CD spectra of multilayer proteoliposomes at six different angles.

An increased negative ellipticity at 222 nm with respect to 208 nm indicates the helix is parallel to the incident light.\textsuperscript{13} Alternatively, if there is a decrease in intensity at 222 nm with respect to 208 nm indicates that the helix is perpendicular to the incident light and thus laying along the surface of the membrane bilayer.
OCD spectra were collected on a Jasco J-710 CD spectropolarimeter (Easton, MD) using a quartz plate with 1 mm thickness and a diameter of 22 mm (Hellma, Plainview, NY). Plates were prepared by drying 400 µL of sample, using a micropipette tip to improve homogenous spreading, onto quartz plate in a dessicator with 80% ZnCl₂ overnight. The dried sample was slowly hydrated for three hours and the quartz plate was then assembled in a Jasco OCD sample chamber. The Jasco OCD sample chamber is a cylindrical chamber with an aluminum frame. The cell contains a ring shaped cavity surrounding a central bore for two quartz slides, one of which holds the oriented sample. OCD spectra were recorded every 60º rotation of the cell, as an average of five replicate scans with a scan speed of 50 nm/min and a response time of 4 seconds. All OCD spectral processing was carried out in the MATLAB environment (7.1, MathWorks, Natick, MA).

3.3.6 Deep UV Resonance Raman (dUVRR) Spectroscopy

The DUVRR instrument has been described previously by Wang et al. UVRR spectra were obtained using the fourth harmonic of a 4kHz frequency quadrupled Ti:Sapphire laser (Coherent Inc., Santa Clara, CA). The Ti:Sapphire laser was pumped using a diode-pumped frequency-doubled Nd:YLF laser (Coherent Inc., Santa Clara, CA). Samples were excited at 197 nm; the average power at the sample was approximately 0.5 mW to reduce the potential for degradation.

The sample solution was circulated using a model 75211-10 gear pump (Cole Palmer, Vernon Hills, IL). A wire-guided (Small Parts Inc., Miramar, FL) stream of sample was purged with a steady stream of nitrogen gas to remove ambient oxygen. Raman scattering from the sample was collected at 135º backscattering geometry and
dispersed using a 1.25 m spectrograph (Horiba Jobin Yvon, Edison, NJ) fitted with 3600 groove/mm grating. The spectrometer was equipped with a back illuminated, phosphor coated, liquid nitrogen cooled Symphony CCD camera (Horiba Jobin Yvon, Edison, NJ) with a chip size of 2048 x 512 pixels. The maximum resolution of the instrument is, approximately, 0.6 cm\(^{-1}\). Pixels were binned in the horizontal direction in increments of four, for a final resolution of approximately 2.4 cm\(^{-1}\). Spectra were collected and exported using SynerJY software (Horiba Jobin Yvon, Edison, NJ). Spectra were calibrated using a standard cyclohexane spectrum.

3.3.7 Data Analysis

The data was processed using MATLAB 7.1 (MathWorks, Natick, MA). Pre-processing of raw spectra included removal of cosmic rays using a program written in-house followed by averaging of 96 replicates of five 30 s scans (four hours total) for each sample. The baseline of each averaged spectrum was then fit using MATLAB’s curve fitting toolbox and subtracted from the each spectrum. The blank lipid spectrum was fit to a series of Gaussian/Lorentzian distributions using a non-linear least squares (NLLS) function as described previously.\(^{16}\) The modeled blank lipid spectrum from subtracted each sample spectrum using the characteristic DLPG feature at 1740 cm\(^{-1}\) to scale the lipid spectrum. A modeled lipid spectrum was used to avoid introduction of non-random noise features into the sample data.
3.4 Results

3.4.1 Spectral Effect on the Amide I Band of DUVRR Spectroscopy of Membrane Proteins with Proline

For peptides with proline substitutions, the AmI mode has a decrease in intensity. The AmI mode for P end poly(LA)$_7$ had a greater intensity than the AmI mode than either PP end poly(LA)$_7$ or P mid poly(LA)$_7$. Lastly, the AmI mode for PP end poly(LA)$_7$ has a decrease in intensity from P mid poly(LA)$_7$.

3.4.2 Spectral Effect on the Amide I Band of DUVRR Spectroscopy of Membrane Proteins with Glycine

Focusing on the AmI, which is predominately a C=O stretch, all poly(LA)$_7$ with glycine substitutions were positioned in the range typical of a-helices (1650-1660 cm$^{-1}$). For peptides with glycine mutations, the AmI mode has a decrease in intensity from poly(LA)$_7$ without any substitutions. The AmI mode for G mid poly(LA)$_7$ had a greater intensity than the AmI mode than either G end poly(LA)$_7$ or GG end poly(LA)$_7$. Lastly, the AmI mode for GG end poly(LA)$_7$ has a decrease in intensity from G end poly(LA)$_7$. 
Figure 3.1: Deep UV resonance Raman spectra of proline and glycine substituted peptides. Spectra has been normalized to the Am II mode.
### Figure 3.2: Amide I mode positions and intensity.

#### 3.5 Discussion

The OCD data for all peptides studied have confirmed that these are embedded membrane peptides. Additionally, the CD data shows that all peptides depict helical structure, however with the introduction of HBRs has an intensity loss which is due to loss of ideality of helical structure from poly(LA)$_7$.

In comparison to poly(LA)$_7$, peptides with a HBRs had a decrease in intensity of the AmI mode. Considering that the AmI mode arises predominately from the protein backbone carbonyl stretch, the loss of ideality of the studied helices would disrupt the hydrogen bonding along the helix thus causing the observed decrease in intensity.
3.6 References


Chapter IV: Aromatic Modes in DUVRR of Membrane Proteins

4.1 Abstract

A protein’s environment can affect its structure and consequently its function. The aromatic residue, tryptophan, has been used extensively in protein dynamics to correlate local changes in environment with structural changes. However, tryptophan modes are not enhanced in deep-ultraviolet (below 200 nm) resonance Raman (dUVRR) spectra of proteins, where the structurally sensitive backbone amide modes are strongest. Environmentally sensitive markers would be useful in dUVRR studies of proteins, especially membrane proteins. Previous studies have shown that the intensities of the ν7a', ν7a, and ν9a tyrosine and phenylalanine modes change with solvent environment but their response to more subtle changes including lipid bilayer versus surfactant environment has yet to be characterized. Phenylalanine and tyrosine were incorporated into a set of model leucine-alanine peptides that adopt α-helical structure in membrane mimicking environments. As expected, the ring modes of phenylalanine and tyrosine (ν7a, and ν9a) increased in intensity upon going from aqueous to membrane mimicking environment. More interestingly, the intensity of the 1237 cm⁻¹ band of tyrosine’s ν7a' mode was greatly decreased in the surfactant environment and absent in the bilayer environment. Aromatic modes were found to be highly sensitive to the presence of solvent water, making aromatics an ideal indicator of localized or partial protein hydration in membrane mimicking environments in the deep ultraviolet.
4.2 Introduction

Of the three naturally occurring aromatic amino acids (tryptophan, phenylalanine, and tyrosine), tryptophan is least prevalent at 1.1% within proteins followed by tyrosine (at 3.3%) and phenylalanine (at 4.4%)\(^1\). However, tryptophan is preferred over phenylalanine and tyrosine as playing a prominent role in studying protein folding and environmental monitoring of membrane proteins since tryptophan can be substituted into proteins by site-directed mutagenesis, with minimal effect on structure and activity.\(^2\) Tryptophan fluorescence is a frequently utilized tool for studying protein structure and function.\(^3\) Tryptophan’s fluorescence emission maximum is blue-shifted to \(~360\) nm from \(~325\) nm and increases in intensity as tryptophan moves from aqueous to membrane environments.\(^3\) Tryptophan fluorescence can be utilized as a monitor of protein unfolding under a wide variety of conditions\(^4\) but structural information must be obtained using a secondary structurally sensitive technique such as circular dichroism, infrared absorption, or Raman scattering.

Deep UV resonance Raman (dUVRR) spectroscopy has advantages over other methods in that it can provide environmental and structural information simultaneously\(^5,6\). As in fluorescence, tryptophan modes are environmentally sensitive and can be used to monitor protein folding/unfolding\(^6,7\). Unfortunately, resonance enhancement of tryptophan modes occurs well above the excitation wavelengths needed to enhance the structurally sensitive backbone amide modes, around 220-230 nm. As the excitation wavelength is lowered to below 200 nm where the amide modes dominate, the Raman scattering of tryptophan is diminished\(^8,9\). However, both tyrosine and
phenylalanine modes are resonance enhanced in this region and have shown some sensitivity to environment.\textsuperscript{10,11,12,13}

Phenylalanine has seven aromatic modes: 838 cm\(^{-1}\) (\(\nu_1\), symmetric ring breathing), 1002 cm\(^{-1}\) (\(\nu_{12}\), symmetric ring stretch), 1030 cm\(^{-1}\) (\(\nu_{18a}\), in-plane CH bend), 1188 cm\(^{-1}\) (\(\nu_{9a}\), in-plane CH bend), 1205 cm\(^{-1}\) (\(\nu_7a\), phenyl-C stretch), 1581 cm\(^{-1}\) (\(\nu_{8b}\), in-plane ring stretching), and 1600 cm\(^{-1}\) (\(\nu_{8a}\), in-plane ring stretching). Tyrosine also has seven aromatic modes: 2\(\nu_{16a}\) at 829 cm\(^{-1}\) arising from Fermi resonance doublet, \(\nu_1\) at 855 cm\(^{-1}\) arising from Fermi resonance doublet with symmetric ring stretch, \(\nu_{9a}\) at 1182 cm\(^{-1}\) arising from in-plane CH bend with \(\text{C}_6\text{H}_5\)-C stretch, \(\nu_{7a}\) at 1213 cm\(^{-1}\) arising from totally symmetric stretch, \(\nu_{7a'}\) at 1263 cm\(^{-1}\) arising from C-O stretching and symmetric ring deformation, \(\nu_{8b}\) at 1600 cm\(^{-1}\) arising from in-plane ring stretching, and \(\nu_{8a}\) at 1616 cm\(^{-1}\) arising from in-plane ring stretching. In addition to these aromatic modes, which are enhanced below 200 nm, proteins have four amide modes in dUVRR spectroscopy. These modes arise from the protein’s backbone vibrations: amide I (C=O stretch), amide II (N-H bending and C-N stretch), amide III (a mixture of N-H, C-H bend/stretch, and C-N stretch), and amide S (a coupled in plane bending of N-H and C-H).\textsuperscript{14} The amide I band manifests at 1625-1750 cm\(^{-1}\), amide II manifests at 1475-1575 cm\(^{-1}\), amide III manifests at 1200-1300 cm\(^{-1}\) and amide S manifests at 1300-1425 cm\(^{-1}\).\textsuperscript{15}

This study aims to investigate environmental sensitivity of phenylalanine and tyrosine aromatic modes using dUVRR spectroscopy. Using transmembrane peptides with phenylalanine or tyrosine residues in increasingly dehydrated environments, let to the environmental sensitivity in the aromatic modes. Additionally, the tyrosine mode \(\nu_{7a'}\) is evident in aqueous and detergent micelles, but is not evident in a lipid bilayer.
4.3 Materials and Methods

4.3.1 Materials

1,2-Dilauroyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DLPG) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). n-Dodecyl β-D-maltoside (DDM) was obtained from EMD Chemicals (Philadelphia, PA).

Synthetic peptides were purchased from Atlantic Peptides (Lewisburg, PA) at a purity of >95% and used without further purification. All peptides were comprised of repeating leucine and alanine residues, with two lysine residues at the N- and C- termini, which ensured the peptides would spontaneously associate with the anionic surface of the liposomes and micelles. Poly(LA)$_7$ sequences that included phenylalanine and tyrosine insertions were designed to characterize the dUVRR aromatic modes in lipophilic environments (Table 4.1). The leucine-alanine repeating core generates a sparingly soluble peptide in aqueous environments, while promoting spontaneous formation of α-helices within membrane mimicking environments. Tryptophan has been shown to have a stabilizing role in poly-leucine peptides; therefore, a tryptophan residue for environment monitoring was placed at the C-terminus to limit its influence on each peptide’s folded conformation.
4.3.2 Liposome and Micelle Preparation

DLPG was dissolved in a 65:35:8 by volume chloroform:methanol:water solution and distributed among test tubes to yield 12.5 mg of lipid in each tube. Chloroform solution was dried off with a stream of argon then placed in desiccator overnight. To suspend lipids for liposome preparation, 0.55 mL of 20 mM phosphate, 5 mM NaCl solution is pipetted into each test tube, yielding a 22.7 mg/mL solution. Tubes were sonicated for 1 hour at 50°C. The resulting solution was extruded through a 200 nm filter using a Lipofast (Avestin Inc., Ottawa, Ontario, Canada). Liposomes had uniform hydrodynamic radius of roughly 50 nm, as confirmed by dynamic light scattering (DLS) (Dyna Pro, Wyatt Technology Corp., Santa Barara, CA) measurements. Liposomes were prepared in 10 mM phosphate buffer at pH 7.4 and lipid concentration was verified by a Rouser assay.

Detergent micelles were prepared by adding \( n \)-dodecyl \( \beta \)-D-maltoside (DDM) (EMD Chemicals, Philadelphia, PA) to proteoliposome solutions at a concentration of 0.01 µM. The resulting solution was placed on a shaker at 4°C for one hour.

4.3.3 Proteoliposome Preparation

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(LA)(_7)</td>
<td>KKLALALALALALAKKW</td>
</tr>
<tr>
<td>Poly(LA)(_7)F</td>
<td>KKLALFLALALFLAKKW</td>
</tr>
<tr>
<td>Poly(LA)(_7)Y</td>
<td>KKLALYALALALYLAHKKW</td>
</tr>
</tbody>
</table>

Table 4.1: Sequences of peptides studied.
Peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (Sigma Aldrich, St. Louis, MO), and distributed among test tubes so that each tube holds 0.05 mg of peptide. The HFP was then dried off with a stream of argon while rotating the test tube to evenly dry the peptide.

A 9.9 mM liposome solution was combined with dried peptide for a final concentration of 15.6 µM peptide to produce proteoliposomes. The resulting solution was stored at room temperature overnight to ensure equilibration. The proteoliposome solutions were centrifuged at 45,000 rpm for 120 minutes at 4°C with a Sorval T-1250 rotor. The proteoliposome pellets were resuspended in 20 mM phosphate buffer (pH 7.0) and the internal intensity standard, 1 M sodium perchlorate (Sigma Aldrich, St. Louis, MO), to reach a final peptide concentration of 0.5 mM and an internal standard concentration of 0.05 µM. UV-Vis absorption intensity tryptophan was monitored using a Cary Bio 50 UV/Vis (Varian, Palo Alto, CA) at 278 nm to determine peptide concentration. Liposome insertion was verified by tryptophan fluorescence using a Cary Eclipse fluorometer (Varian, Palo Alto, CA). DUVRR spectra of poly(LA)₇ peptides and background liposome were collected at an excitation wavelength of 197 nm.

4.3.4 Circular dichroism (CD)

Circular dichroism (CD) is the difference in absorption of right- and left-handed polarized light.²⁹ Proteins have a strong CD spectra arising from the chiral nature of the protein backbone. Furthermore, α-helical, β-sheet, and disordered structures have distinct spectral features, making CD spectroscopy a good technique for identifying a protein’s dominant secondary structure though the sensitivity to specific non-helical structural
configurations is low.\textsuperscript{20,21} A pure $\alpha$–helical protein will have a strong positive feature at 193 nm and two negative features at 222 and 208 nm.

CD spectra were collected on a Jasco J-710 CD spectropolarimeter (Easton, MD) using a 1 mm path length quartz cuvette (Hellma, Plainview, NY). Each spectrum was the average of five replicate scans with a scan speed of 50 nm/min and a response time of 4 seconds for each sample. A background CD spectrum of DLPG was subtracted from the CD spectra of peptide in liposomes. All CD spectral processing was carried out in the MATLAB environment (7.1, MathWorks, Natick, MA).

4.3.5 Oriented Circular Dichroism (OCD)

For membrane proteins, oriented CD (OCD) is the CD spectrum of a multilayer system at a series of oblique angles. OCD is used to determine a helical protein’s preferential orientation (parallel, perpendicular or tilted) with respect to a membrane.\textsuperscript{22} OCD samples were prepared by drying proteoliposomes on a quartz slide. As the hydration of the sample decreases, the proteoliposomes reorganize into bilayers parallel to the slide’s surface,\textsuperscript{22, 23, 24} a process which does not occur for detergent micelles. OCD spectra were generated by averaging the CD spectra of multilayer proteoliposomes at six different angles.

An increased negative ellipticity at 222 nm with respect to 208 nm indicates the helix is parallel to the incident light.\textsuperscript{25} Alternatively, if there is a decrease in intensity at 222 nm with respect to 208 nm indicates that the helix is perpendicular to the incident light and thus laying along the surface of the membrane bilayer. The angle of each peptide was calculated using Equation 4.1:

$$\sin^2 \theta = \frac{2 I_{208}}{3 I_{222}}$$

Equation 4.1
where $\theta$ is the angle between the helix and the incident light and $I$ is the amplitude at that wavelength with results shows in Table 4.2.\textsuperscript{24,25} A peptide with a backbone orientation that is perpendicular to the lipid bilayer would have an angle of $0^\circ$ and a peptide with backbone orientation parallel to the bilayer would have an angle of $180^\circ$.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Tilt angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(LA)$_7$</td>
<td>0$^\circ$</td>
</tr>
<tr>
<td>Poly(LA)$_7$:F</td>
<td>46.89$^\circ$</td>
</tr>
<tr>
<td>Poly(LA)$_7$:Y</td>
<td>47.06$^\circ$</td>
</tr>
</tbody>
</table>

**Table 4.2:** Tilt angle of peptides studied in lipid bilayer.

OCD spectra were collected on a Jasco J-710 CD spectropolarimeter (Easton, MD) using a quartz plate with 1 mm thickness and a diameter of 22 mm (Hellma, Plainview, NY). Plates were prepared by drying 400 $\mu$L of sample, using a micropipette tip to improve homogenous spreading, onto quartz plate in a dessicator with 80% ZnCl$_2$ overnight. The dried sample was slowly hydrated for three hours and the quartz plate was then assembled in a Jasco OCD sample chamber. The Jasco OCD sample chamber is a cylindrical chamber with an aluminum frame.\textsuperscript{26} The cell contains a ring shaped cavity surrounding a central bore for two quartz slides, one of which holds the oriented sample. OCD spectra were recorded every $60^\circ$ rotation of the cell, as an average of five replicate scans with a scan speed of 50 nm/min and a response time of 4 seconds. All OCD spectral processing was carried out in the MATLAB environment (7.1, MathWorks, Natick, MA).
4.3.6 Deep UV Resonance Raman (dUVRR) Spectroscopy

The DUVRR instrument has been described previously by Wang et al.\textsuperscript{27} UVRR spectra were obtained using the fourth harmonic of a 4kHz frequency quadrupled Ti:Sapphire laser (Coherent Inc., Santa Clara, CA). The Ti:Sapphire laser was pumped using a diode-pumped frequency-doubled Nd:YLF laser (Coherent Inc., Santa Clara, CA). Samples were excited at 197 nm; the average power at the sample was approximately 0.5 mW to reduce the potential for degradation.

The sample solution was circulated using a model 75211-10 gear pump (Cole Palmer, Vernon Hills, IL). A wire-guided (Small Parts Inc., Miramar, FL) stream of sample was purged with a steady stream of nitrogen gas to remove ambient oxygen. Raman scattering from the sample was collected at 135° backscattering geometry and dispersed using a 1.25 m spectrograph (Horiba Jobin Yvon, Edison, NJ) fitted with 3600 groove/mm grating. The spectrometer was equipped with a back illuminated, phosphor coated, liquid nitrogen cooled Symphony CCD camera (Horiba Jobin Yvon, Edison, NJ) with a chip size of 2048 x 512 pixels. The maximum resolution of the instrument is, approximately, 0.6 cm\textsuperscript{-1}. Pixels were binned in the horizontal direction in increments of four, for a final resolution of approximately 2.4 cm\textsuperscript{-1}. Spectra were collected and exported using SynerJY software (Horiba Jobin Yvon, Edison, NJ). Spectra were calibrated using a standard cyclohexane spectrum.

4.3.7 Data Analysis

The data was processed using MATLAB 7.1 (MathWorks, Natick, MA). Pre-processing of raw spectra included removal of cosmic rays using a program written in-house followed by averaging of 96 replicates of five 30 s scans (four hours total) for each
sample. The baseline of each averaged spectrum was then fit using MATLAB’s curve fitting toolbox and subtracted from the each spectrum. The blank lipid spectrum was fit to a series of Gaussian/Lorentzian distributions using a non-linear least squares (NLLS) function as described previously. The modeled blank lipid spectrum from subtracted each sample spectrum using the characteristic DLPG feature at 1740 cm\(^{-1}\) to scale the lipid spectrum. A modeled lipid spectrum was used to avoid introduction of non-random noise features into the sample data.

The per-residue cross-sections for the aromatic modes were calculated using Equation 4.2:

\[
\sigma_{\lambda,\text{mode}} = \frac{\sigma_{\text{ClO}_4^-}}{n} \frac{l_{\text{mode}}}{l_{\text{ClO}_4^-}} \left( \frac{\nu_{\text{exc}} - \nu_{\text{ClO}_4^-}}{\nu_{\text{exc}} - \nu_{\text{mode}}} \right)^4 \frac{C_{\text{ClO}_4^-}}{C_{\text{mode}}} \left( \frac{A_0 + A_{\text{mode}}}{A_0 + A_{\text{ClO}_4^-}} \right)
\]

Equation 4.2

where the subscript ClO\(_4^-\) refers to the internal standard parameters and mode refers to aromatic mode of interest, \(\sigma\) is the Raman cross-section, \(n\) is the number of amino acid residues, \(l\) is the Raman intensity (peak height) of the internal standard ClO\(_4^-\) and aromatic mode of interest, \(\nu_{\text{exc}}\) is the excitation frequency and \(\nu\) is the observed Raman shift of the internal standard and aromatic mode of interest in cm\(^{-1}\), \(C\) is the concentration in M, \(A_0\) is the absorption of the sample at the excitation wavelength (197 nm) and \(A\) is the sample absorption at the Raman shifted wavelengths for the internal standard (201 nm) and aromatic mode of interest.\(^{29,30}\)

4.4 Results

4.4.1 Oriented Circular Dichroism Spectral Data
It is known that deep UV resonance Raman spectra of aromatic modes is dependent on environment\(^5\). Thus, the orientation of the peptides with respect to the membrane could have an effect on the exposure of the aromatic residues to solvent water. Previous studies investigating the secondary structure of membrane proteins have assumed the environment of the protein in question. However, with the use of oriented circular dichroism, it is known that the peptide studied was transmembrane. For example, if the backbone orientation (helix) were parallel to the membrane surface, solvent water would have greater access to the peptide versus a perpendicular orientation. Oriented circular dichroism (OCD) spectra of poly(LA)\(_7\), poly(LA)\(_7\)F, and poly(LA)\(_7\)Y were collected to determine if the peptides were perpendicular, parallel or tilted with respect to the membrane surface. The angle of the helix within the membrane can be determined from the ratio of intensities at 208 and 222 nm (Equation 4.1).

The intensity at 222 nm in the OCD spectrum of pLA\(_7\) was much greater than the intensity at 208 nm, which corresponds to a tilt angle of 0° according to Equation 1. The ratio of the intensities at 208 and 222 nm in the OCD spectrum for poly(LA)\(_7\)F, and poly(LA)\(_7\)Y were quite close and correspond to a tilt angle of 47° for both peptides (Table 4.2).

**4.4.2 Circular Dichroism Spectral Data**

Circular dichroism was employed to verify the helical structure of the peptide poly(LA)\(_7\) in DLPG lipid bilayers (Figure 4.1). The CD spectrum of poly(LA)\(_7\) is characteristic of a predominately \(\alpha\)-helical protein, which is indicated by a maximum at 195 nm and two minima at 208 and 222 nm.\(^20\) The CD spectra of poly(LA)\(_7\)F and poly(LA)\(_7\)Y indicate that incorporation of aromatic residues reduces the overall helicity
of the peptide, as indicated by a decrease in the intensity the negative features at 208 and 222 nm (Figure 4.1). Incorporation of tyrosine (poly(LA)$_7$Y) also resulted in a decrease in the intensity of the positive feature at 195 nm.

![Circular dichroism spectra](image)

**Figure 4.1: Circular dichroism spectra of poly(LA)$_7$ (black), poly(LA)$_7$F (purple), and poly(LA)$_7$Y (orange) in a lipid bilayer.**

Detergent micelles are often used as a model for membrane environments. In a detergent solubilized micelle, the minima at 208 and 222 nm in the CD spectrum of poly(LA)$_7$ are less intense than in the bilayer environment indicating that poly(LA)$_7$ is less helical in micelles (Figure 4.2). Although incorporation of aromatic residues decreases the intensity of the CD spectra of poly(LA)$_7$F and poly(LA)$_7$Y in detergent
solubilized micelles, the effect is much less pronounced than in lipid bilayers (Figure 4.2).

The CD spectra of poly(LA)$_7$ suggested that the peptide was less helical in the micellar versus bilayer environment. However, previous studies have shown that an overall loss of intensity in the CD spectra of helical proteins may be associated with distortion rather than unfolding of the helix.$^{12}$

![Graph showing CD spectra](image)

Figure 4.2: Circular dichroism spectra of poly(LA)$_7$ (black), poly(LA)$_7$F (purple), and poly(LA)$_7$Y (orange) in a detergent solubilized micelle.

4.4.3 DUVRR Spectral Data
Using the amide modes of dUVRR spectra, the secondary structure of the studied peptides can be further characterized. There are four amide modes which arise from the protein’s backbone vibrations: amide I (C=O stretch), amide II (N-H bending and C-N stretch), amide III (a mixture of N-H, C-H bend/stretch and C-N stretch), and amide S (a coupled in-plane bending of N-H and C-H)\(^{14}\). The amide I (Am I) mode manifests at 1625-1750 cm\(^{-1}\), amide II (Am II) manifests at 1475-1575 cm\(^{-1}\), the amide III (Am III) manifests at 1200-1300 cm\(^{-1}\), and the amide S (Am S) manifests at 1300-1425 cm\(^{-1}\)\(^{14}\). The Am I mode for poly(LA)\(_7\)F in a lipid bilayer is located at 1646 cm\(^{-1}\), the Am II mode is located at 1532 cm\(^{-1}\), and the Am S mode is located at 1389 cm\(^{-1}\). The Am III mode has three features (the Am III\(_1\), III\(_2\), and III\(_3\)), which are located at 1266, 1297, and 1333 cm\(^{-1}\), respectively. The Am I mode for poly(LA)\(_7\)F in a detergent micelle is located at 1657 cm\(^{-1}\), the Am II mode is located at 1543 cm\(^{-1}\), and the Am S mode is located at 1389 cm\(^{-1}\). The Am III\(_1\), III\(_2\), and III\(_3\) are located at 1247 cm\(^{-1}\) at 1291 cm\(^{-1}\) at 1330 cm\(^{-1}\), respectively. The Am I, III\(_2\), and III\(_3\) modes are dwarfed by aromatic modes in poly(LA)\(_7\)Y in a lipid bilayer and a detergent micelle. The Am II mode for poly(LA)\(_7\)Y in a lipid bilayer is located at 1554 cm\(^{-1}\), the Am III\(_1\) mode is located at 1343 cm\(^{-1}\), and the Am S mode is located at 1437 cm\(^{-1}\). The Am II mode for pLA\(_7\)Y in a detergent micelle is located at 1554 cm\(^{-1}\), the Am III\(_1\) mode is located at 1338 cm\(^{-1}\), and the Am S mode is located at 1437 cm\(^{-1}\).

The Am S mode of dUVRR spectra is a useful marker for helicity.\(^{31}\) Increased intensity of the Am S corresponds to a loss of helicity. The Am S for poly(LA)\(_7\)F in liposomes and micelles is strong, indicating a loss of helical structure. This loss likely arises from the large aromatic side group disrupting the helix. The intensity of the Am S
modes for poly(LA)$_7$Y in liposomes and micelles mirrors the intensity seen for poly(LA)$_7$F suggesting that the loss of helicity can be attributed to the aromatic ring.

The seven aromatic modes of phenylalanine are seen in the spectrum of poly(LA)$_7$F in detergent micelles. Within a detergent micelle, the $v_1$ manifests at 824 cm$^{-1}$, $v_{12}$ at 996 cm$^{-1}$, $v_{18a}$ at 1019 cm$^{-1}$, $v_{9a}$ at 1182 cm$^{-1}$, $v_{7a}$ at 1196 cm$^{-1}$, $v_{8b}$ at 1575 cm$^{-1}$, and $v_{8a}$ at 1594 cm$^{-1}$. In comparison to the phenylalanine in an aqueous environment, all aromatic modes are red shifted.

The seven aromatic modes of phenylalanine are seen in the spectrum of poly(LA)$_7$F in lipid bilayers. Within a lipid bilayer, the $v_1$ manifests at 824 cm$^{-1}$, $v_{12}$ at 996 cm$^{-1}$, $v_{18a}$ at 1022 cm$^{-1}$, $v_{9a}$ at 1179 cm$^{-1}$, $v_{7a}$ at 1199 cm$^{-1}$, $v_{8b}$ at 1575 cm$^{-1}$, and $v_{8a}$ at 1597 cm$^{-1}$. In comparison to the phenylalanine in an aqueous environment, all aromatic modes are red shifted. In comparison to poly(LA)$_7$F in detergent micelles, all aromatic modes of poly(LA)$_7$F in lipid bilayers are red shifted.

The seven aromatic modes of tyrosine are seen in the spectrum of poly(LA)$_7$Y in detergent micelles. Within a detergent micelle, the $2v_{16a}$ manifests at 829 cm$^{-1}$, $v_1$ at 856 cm$^{-1}$, $v_{9a}$ at 1176 cm$^{-1}$, $v_{7a}$ at 1209 cm$^{-1}$, $v_{7a'}$ at 1270 cm$^{-1}$, $v_{8b}$ at 1610 cm$^{-1}$, and $v_{8a}$ at 1631 cm$^{-1}$. In comparison to the tyrosine in an aqueous environment, the $v_{9a}$ and $v_{7a}$ modes are red shifted while the $2v_{16a}$, $v_1$, $v_{7a'}$, $v_{8b}$, and $v_{8a}$ modes are blue shifted.

The seven aromatic modes of tyrosine are seen in the spectrum of poly(LA)$_7$Y in lipid bilayers. Within a lipid bilayer, the $2v_{16a}$ manifests at 829 cm$^{-1}$, $v_1$ at 856 cm$^{-1}$, $v_{9a}$ at 1176 cm$^{-1}$, $v_{7a}$ at 1209 cm$^{-1}$, $v_{7a'}$ at 127 cm$^{-1}$, $v_{8b}$ at 1610 cm$^{-1}$, and $v_{8a}$ at 1631 cm$^{-1}$. In comparison to the detergent environment, there is no change in these band positions. In comparison to the tyrosine in an aqueous environment, the $v_{9a}$ and $v_{7a}$ modes of
poly(LA)$_7$Y in lipid bilayers are red shifted while the $2\nu_{16a}$, $\nu_{1}$, $\nu_{7a'}$, $\nu_{8b}$, and $\nu_{8a}$ modes are blue shifted. There is no Raman shift change when comparing aromatic modes of poly(LA)$_7$Y in detergent micelles to those of poly(LA)$_7$Y in lipid bilayers.

<table>
<thead>
<tr>
<th>Phenylalanine Amide Modes</th>
<th>Raman Shift (cm$^{-1}$) for DLPG</th>
<th>Raman Shift (cm$^{-1}$) for DDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amide I</td>
<td>1646</td>
<td>1657</td>
</tr>
<tr>
<td>Amide II</td>
<td>1532</td>
<td>1543</td>
</tr>
<tr>
<td>Amide III$_1$</td>
<td>1266</td>
<td>1247</td>
</tr>
<tr>
<td>Amide III$_2$</td>
<td>1297</td>
<td>1291</td>
</tr>
<tr>
<td>Amide III$_3$</td>
<td>1333</td>
<td>1330</td>
</tr>
<tr>
<td>Amide S</td>
<td>1389</td>
<td>1389</td>
</tr>
</tbody>
</table>

Table 4.3: DUVRR amide mode shifts of poly(LA)$_7$F in a lipid bilayer, and poly(LA)$_7$F in a detergent micelle.
<table>
<thead>
<tr>
<th>Phenylalanine Aromatic Mode</th>
<th>Aq Raman Shift (cm(^{-1}))</th>
<th>DLPG Raman Shift (cm(^{-1}))</th>
<th>DDM Raman Shift (cm(^{-1}))</th>
<th>Cross Section (aq)</th>
<th>Cross Section (DLPG)</th>
<th>Cross Section (DDM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\nu_{12})</td>
<td>1002</td>
<td>996.3</td>
<td>996.3</td>
<td>178.64</td>
<td>285.20</td>
<td>228.67</td>
</tr>
<tr>
<td>(\nu_{18a})</td>
<td>1030</td>
<td>1019</td>
<td>1022</td>
<td>74.12</td>
<td>140.19</td>
<td>139.74</td>
</tr>
<tr>
<td>(\nu_{9a})</td>
<td>1188</td>
<td>1182</td>
<td>1179</td>
<td>76.88</td>
<td>96.45</td>
<td>96.73</td>
</tr>
<tr>
<td>(\nu_{7a})</td>
<td>1205</td>
<td>1196</td>
<td>1199</td>
<td>137.20</td>
<td>189.63</td>
<td>183.46</td>
</tr>
<tr>
<td>(\nu_{8b})</td>
<td>1581</td>
<td>1575</td>
<td>1575</td>
<td>129.51</td>
<td>127.90</td>
<td>128.78</td>
</tr>
<tr>
<td>(\nu_{8a})</td>
<td>1600</td>
<td>1594</td>
<td>1597</td>
<td>138.89</td>
<td>138.64</td>
<td>137.64</td>
</tr>
</tbody>
</table>

**Table 4.4:** DUVRR aromatic mode shifts and cross sections for phenylalanine, poly(LA)-F in a lipid bilayer, and poly(LA)-F in a detergent micelle.
Tyrosine Amide Mode | DLPG Raman Shift (cm\(^{-1}\)) | DDM Raman Shift (cm\(^{-1}\))
--- | --- | ---
Amide I | N/A | N/A
Amide II | 1554 | 1554
Amide III\(_1\) | 1343 | 1338
Amide III\(_2\) | N/A | N/A
Amide III\(_3\) | N/A | N/A
Amide S | 1437 | 1437

Table 4.5: DUVRR amide mode shifts of poly(LA)\(_7\)Y in a lipid bilayer, and poly(LA)\(_7\)Y in a detergent micelle.
<table>
<thead>
<tr>
<th>Tyrosine Aromatic Mode</th>
<th>Raman Shift (cm(^{-1})) for aq</th>
<th>Raman Shift (cm(^{-1})) for DLPG</th>
<th>Raman Shift (cm(^{-1})) for DDM</th>
<th>Cross Section (aq)</th>
<th>Cross Section (DLPG)</th>
<th>Cross Section (DDM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2\nu_{16a})</td>
<td>829.3</td>
<td>829.8</td>
<td>829.8</td>
<td>49.40</td>
<td>46.12</td>
<td>45.98</td>
</tr>
<tr>
<td>(\nu_1)</td>
<td>854.9</td>
<td>855.7</td>
<td>855.7</td>
<td>96.57</td>
<td>112.35</td>
<td>98.41</td>
</tr>
<tr>
<td>(\nu_{9a})</td>
<td>1182</td>
<td>1176</td>
<td>1176</td>
<td>66.01</td>
<td>78.21</td>
<td>65.42</td>
</tr>
<tr>
<td>(\nu_{7a})</td>
<td>1213</td>
<td>1209</td>
<td>1209</td>
<td>254.94</td>
<td>256.33</td>
<td>260.72</td>
</tr>
<tr>
<td>(\nu_{7a}')</td>
<td>1263</td>
<td>1270</td>
<td>1270</td>
<td>112.97</td>
<td>128.30</td>
<td>115.94</td>
</tr>
<tr>
<td>(\nu_{8b})</td>
<td>1600</td>
<td>1610</td>
<td>1610</td>
<td>115.75</td>
<td>130.30</td>
<td>116.90</td>
</tr>
</tbody>
</table>

Table 4.6: DUVRR aromatic mode shifts and cross sections for tyrosine, poly(LA)\(_7\)Y in a lipid bilayer, and poly(LA)\(_7\)Y in a detergent micelle.
Figure 4.3: DUVRR spectra of phenylalanine (grey), poly(LA)$_7$F in a lipid bilayer (black), and poly(LA)$_7$F in a detergent micelle (purple).
Figure 4.4: DUVRR spectra of tyrosine (grey), poly(LA)$_7$Y in a lipid bilayer (black), and poly(LA)$_7$Y in a detergent micelle (orange).
4.5 Discussion

While the OCD data for poly(LA)$_7$F and poly(LA)$_7$Y show that the peptides are more tilted than poly(LA)$_7$Y, all poly(LA)$_7$ peptides are transmembrane. The CD data shows that all peptides depict helical structure, however poly(LA)$_7$F and poly(LA)$_7$Y have a loss of which is due to a loss of helical structure from poly(LA)$_7$. This loss in helicity is due to the large aromatic rings of phenylalanine and tyrosine, and can be seen through the increase of the AmS and AmIII$_3$ modes of the dUVRR spectra for poly(LA)$_7$F. A similar increase cannot be seen for the dUVRR spectra for poly(LA)$_7$Y as the tyrosine aromatic modes eclipse the Am S and Am III$_3$ modes.

The dUVRR spectra for tyrosine in aqueous solution, poly(LA)$_7$Y in a detergent micelle, and poly(LA)$_7$Y in a lipid bilayer shows an increase in intensity of tyrosine modes as the residue moves from a hydrated to dehydrated environment. DUVRR spectra for phenylalanine in aqueous solution, poly(LA)$_7$F in a detergent micelle, and poly(LA)$_7$F in a lipid bilayer also shows increases in intensities of phenylalanine modes as the residue moves from a hydrated to dehydrated environment.
4.6 References


Chapter V: Shining Light on Proteins

When someone talks about protein, it often is in reference to steak, chicken, or eggs. People think about protein as a nutritional requirement but do not consider what protein actually is. In reality, proteins are molecules found throughout the body that perform specialized functions. These molecules are the work horses of every living organism and their complexity is still not fully understood.

In my lab, we look at a specific class of proteins called membrane proteins. Membrane proteins are found in cell walls, which protect the cell against invasion and contains the components inside of the cell. These proteins can work as shuttles moving molecules across the membrane. They can also be messengers that relay information on what is happening outside of the cell to the inside. Membrane proteins are also really important for our health because most pharmaceuticals target these membrane proteins. If you, or a family member, take asthma, cholesterol, or acid reflux medicine, you are most likely putting your membrane proteins to work.

Knowing exactly how these proteins work and interact with pharmaceuticals can be very important. The key to understanding a protein’s function is to be able to see what structure or shape it takes. There are three structures that all proteins are made up of: helix, sheet, or unfolded. A helix is a spiral, like a Slinky®, a sheet is a strand that is folded back and forth on itself, and unfolded proteins have no real defined shape. If we can see the structure a protein takes, then we can understand how it functions.
There are two barriers to seeing the shape of proteins: the size of the protein and the membrane that conceals them. Proteins are molecules that are too small to be seen with the naked eye. The average protein is about 3 nm in diameter. To put that into perspective, you would have to break a grain of sand into one million pieces to make it the same size as the average protein.

Next, we have to deal with that membrane. A cell membrane is mostly comprised of molecules called phospholipids. Each phospholipid has a head that is hydrophilic, or “water-loving,” and tails that are hydrophobic, or “water-fearing.” Proteins often embed themselves into the hydrophobic region of the cell membrane so they are completely hidden from view. This can be compared to trying to look at the people inside of a house from an aerial photo; you would only see the roof. Researchers want to see proteins in their native environments, otherwise we are not getting the full picture of how they function. Without the native environment, it would be similar like placing a person in the middle of the ocean and assuming they spend their whole life swimming. To solve these problems, my lab uses an interesting solution: light!
Light can be used to see objects that are too small or too far away to see. It works similarly to how you can get information about an object from its shadow. Light shines on an object and a shadow is produced. You may not be able to get as clear of an image as if you looked directly at the object, but you can see if the object is a person or animal, stationary or in motion. Using the interaction between matter and light is called spectroscopy.

Let’s say that you are looking at a shadow of a person and you wanted to know exactly how tall this person was, you would need some kind of legend. You can use a set of shadows, knowing the height of the objects that cast the shadows to create this legend. This is what my research entails. I use a set of known proteins to create the legend on my group’s laser to make a legend of how different shapes look in the output.

The specific kind of light my group uses is Raman spectroscopy. Raman spectroscopy is a vibrational spectroscopy, meaning that the proteins we study use the light provided as energy and they start to vibrate from absorbing this energy. Then the vibrations are recorded by a camera, which has an output that looks like a graph. Over
time, we can start to see patterns in the graphs of different proteins, which relate to the location and height of the peaks of the graph, which help us identify signature features that give information about any protein studied. For example, each of the three structures that make up all proteins have a signature graph.

To wrap up, my research is to make a legend to a technique called Raman spectroscopy in order for researchers to look at membrane proteins within their native membrane environment. This is important for us to fully understand how, and why, proteins function the way that they do. Furthermore, this can help researchers make better pharmaceuticals.
Chapter VI: The Chemistry Immersion Program (CHIP)

6.1 Abstract

The Chemistry Immersion Program (CHIP) at the University of Missouri is a two-week summer opportunity designed for both high school students and teachers. During the first week of the CHIP program, teachers undergo training and laboratory development with CHIP staff. Laboratories are specifically designed to include an array of measurement and spectroscopic technologies. Teachers experience key laboratories in general collegiate chemistry and biochemistry and have time to adapt those laboratories for use in their own classrooms. The developed labs are then implemented in the second week of the summer program with CHIP high school students, who are incoming college freshman or rising high school seniors. The goals for the CHIP summer program are to: improve teachers’ and students’ skills and comfort with laboratory technology, introduce spectroscopy and spectroscopic measurement concepts, to refresh knowledge of chemistry and to engage students with investigations into chemistry and biochemistry through a total of eight two-hour laboratory investigations in both fields. This study used pre- and post- content exam data to gauge content knowledge previous to attending CHIP and to demonstrate any growth in content knowledge. At the end of CHIP, students and teachers participants were given surveys consisting of Likert and short answer questions to analyze program factors besides content knowledge. Within the four years of CHIP completed, results indicate that student’s content knowledge improved after participating in the program, and students and teachers felt they had a positive experience in the program overall.
6.2 Introduction

The Chemistry Immersion Program (CHIP) at the University of Missouri is a multifaceted program designed to provide quality science outreach to students, teachers, and the community. The initial phase of CHIP focused solely on students who are rising college seniors or incoming college freshman. Subsequent phases expanded to include high school science teachers and broad community outreach. Currently, CHIP is in its fifth year of providing science outreach with an overarching goal of increasing student interest in STEM careers and boost retention in STEM disciplines.

This goal is accomplished by enhancing students’ comfort levels in collegiate chemistry and biochemistry by engaging the students in a week of laboratory investigations highlighting measurement and spectroscopic technology or lab equipment. High school teachers participate in a two-week summer program. For the first week, teachers briefly refresh on key collegiate chemistry and biochemist concepts, increase personal comfort with laboratory equipment, and work with CHIP colleagues to adapt the CHIP laboratories for use in their own classrooms. The following week, CHIP teachers implement the Week One laboratories with the CHIP high school participants, allowing for more seamless transitions into their own high school courses. Since Dr. Emily Harbert, who began the program, previously discussed the inaugural year of CHIP, the data compiled here is for the years two through four of CHIP (2014-2016).\(^1\) This study used pre- and post- content exam data to gauge content knowledge previous to attending CHIP and to demonstrate any growth in content knowledge. At the end of CHIP, students and teachers participated in focus group interviews and were given surveys
consisting of Likert and short answer questions to analyze program factors besides content knowledge.\textsuperscript{2}

High school teachers were not included in the inaugural year of CHIP (2013), however in subsequent years they have been included to extend the impact of the program. For outreach to the general community, CHIP has created programming for a dozen events, which have served nearly 7,000 participants. The programming created is available in Appendices A-E.

6.3 Student Participants

In years two through four of CHIP, 93 students and 13 teachers have participated, of which 66 students and 11 teachers are from Missouri. The remaining students and teachers are from eight states. Of the student participants, 61\% were female and 39\% were male and 65\% were incoming college freshman and 35\% were rising high school seniors. The majority of students (58\%) chose not to disclose their ethnicity, but 27\% self identified as Caucasian, 7\% identified as Asian, 5\% identified as African-American and 3\% identified as Hispanic.

6.4 Program Design and Implementation

Four chemistry and four biochemistry topics were covered during the course of CHIP. For chemistry, the topics were stoichiometry, ideal gas law, thermochemistry, and chemical bonding. Students were expected to have already had some understanding or familiarity with these topics, as most general high school chemistry classes cover them. These labs, which have been previously published,\textsuperscript{1} were designed to focus on
introduction of technology and scientific content that is used in collegiate chemistry. For biochemistry, the topics covered were pipetting and dilution, protein quantification, polymerase chain reaction (PCR), and gel electrophoresis. Unlike chemistry, biochemistry is not a common high school course and thus no previous knowledge is expected of the students.

A second facet of CHIP was to expose students to various STEM-related careers and undergraduate opportunities. To this end, evening research seminars introduced students to University of Missouri researchers or undergraduate students who were conducting research along with guidance materials for college students interested in pursing research. Additionally, every CHIP participant is given a STEM career worksheet, which is available in Appendix F. Lastly, participants are gives tours of University of Missouri research labs where they met graduate students and professors conducting research.

6.5 Results and Discussion

6.5.1 Content Evaluations

Pre- and post-program evaluations, which have been previously published,¹ were identical and designed to gauge knowledge of STEM careers and conceptual understanding of the aforementioned eight CHIP topics. These evaluations were given to CHIP students and teachers. Responses were scored individually then the score difference between the pre- and post-program evaluation were calculated. The average percent difference was determined and is shown in Figure 6.2 and Figure 6.3. According to the students’ and teachers’ overall scores, conceptual understanding of chemistry and
biochemistry topics increased through the course of the program for CHIP 2014 through 2016. Additionally, it is seen that CHIP students experienced a greater increase in conceptual understanding than CHIP teachers, which could be expected, as CHIP teachers have a deeper conceptual understanding of chemistry and biochemistry. When comparing growth through the duration of CHIP, biochemistry had a greater increase in conceptual understanding as is expected since biochemistry is not as widely available as a high school course.

![Years 2014-2016 Percent Difference in Evaluations](image)

**Figure 6.1:** Percent increase in overall points between pre- and post- CHIP evaluation for student and teachers.
Figure 6.2: Percent increase in points between pre- and post- chemistry evaluations for student and teachers for years 2-4 of CHIP.

Figure 6.3: Percent increase in points between pre- and post- biochemistry evaluations for student and teachers for years 2-4 of CHIP.
6.5.2 Likert Survey

Teachers were given two Likert surveys, one at the end of each week of CHIP, to gauge satisfaction with factors beyond content and comfort of implementation. CHIP students were given a program survey at the end of the week as well. The Likert response scores were as follows: Disagree – 1, Somewhat Disagree – 2, Neither Agree nor Disagree – 3, Somewhat Agree – 4, and Agree – 5. Table {students} displays the average Likert responses for each question for CHIP student surveys for years 2014 through 2016.
<table>
<thead>
<tr>
<th>Statement</th>
<th>Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>The content of the CHIP program chemistry investigations was relevant to my interests in science.</td>
<td>4.4</td>
</tr>
<tr>
<td>I felt the pace of the laboratory investigations was just right. I did not feel rushed and I was not bored.</td>
<td>3.9</td>
</tr>
<tr>
<td>The chemistry laboratory investigations planned and implemented by the chemistry teacher team for the morning CHIP session were interesting and well organized.</td>
<td>4.4</td>
</tr>
<tr>
<td>Chemistry investigations were interesting and well organized the morning laboratory sessions. The CHIP Coordinators were always available and able to help everyone understand the protocol and also how to use the equipment.</td>
<td>4.6</td>
</tr>
<tr>
<td>Key concepts in chemistry were explained in a way which was easy to understand.</td>
<td>4.3</td>
</tr>
<tr>
<td>The biochemistry laboratory investigations planned and implemented by the biochemistry team for the afternoon CHIP session were interesting and well organized.</td>
<td>4.1</td>
</tr>
<tr>
<td>The pace of the afternoon session was appropriate and presented in a way which was easy to understand</td>
<td>4.0</td>
</tr>
<tr>
<td>I enjoyed working with other students during the activities and investigations.</td>
<td>4.3</td>
</tr>
<tr>
<td>I felt I learned a good deal from the CHIP program.</td>
<td>4.6</td>
</tr>
<tr>
<td>I would recommend the CHIP program to my colleagues as an interesting and enjoyable way to refresh chemistry and biochemistry knowledge.</td>
<td>4.6</td>
</tr>
<tr>
<td>Housing was comfortable and supplied the materials needed to make my stay comfortable.</td>
<td>4.5</td>
</tr>
<tr>
<td>The Dining Hall offered a wide culinary diversity and the food was good.</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Table 6.1. Averaged Student Likert Survey Responses. (n=83)
6.6 Summary

Years two through four of the Chemistry Immersion Program (2014-2016) have been very successful in that for the over 100 participants, there has been an increase in conceptual understanding for chemistry and biochemistry. Feedback on the program is overwhelmingly positive. Providing the teachers with all CHIP materials and two weeks to develop and troubleshoot laboratories to implement in their classrooms has allowed for an increased exposure of collegiate chemistry and biochemistry concepts and technology. The inclusion of high school teachers in the CHIP has also given great insight into the issues encountered when educating high school students.
7.6 References


Appendix A: DNA Extraction

Strawberry DNA Extraction

1. Take everything out of your Ziplock bag

2. Peel and place the strawberry in a Ziplock bag and smush using your hands.

3. Add the liquid from the plastic cup to the Ziplock bag. This is soapy salt water. It is water, Dawn dish soap, and salt mixed together in it.

4. Swish the solution in the smashed strawberry and let sit for a few minutes.

5. While you are waiting, set up your filter.

6. Take the coffee filter and fold the filter around the edges of the little cup that has soapy salt water in it. Put a rubber band around the cup to keep the filter in place, as shown:

7. After a few minutes have passed, slowly pour the smashed strawberry with soapy salt water into the filter paper.

8. After the liquid is filtered out of the mush, tilt your cup gently to one side and pipette or pour the rubbing alcohol (glass bottle)
down the side of the cup. You must be very careful to not mix the two liquids.

9. You should see small strands of DNA come out of the red liquid into the rubbing alcohol. Use a pipette to collect the DNA strands by pulling up only the clear rubbing alcohol sitting on top of the strawberry liquid.
Appendix B: Chromatography

Chromatography

Although on paper different inks may look the same (like a Sharpie versus Crayola), they are actually a mixture of different dyes. We can identify which dyes make up ink by using a technique called chromatography. Chromatography is a way to analyze mixtures by separating the chemicals that make up the mixture. To separate the chemicals from ink, we put a dot of ink near the bottom of special paper called chromatography paper. When we put just an edge of the paper in a liquid, like water, the liquid will climb up the paper while dragging the dot of ink. As the ink drags, the different dyes will climb up the paper at different speeds.

We have 4 suspects for the crime of stealing artwork. They each had a black marker on them when caught:

<table>
<thead>
<tr>
<th>Suspect</th>
<th>Ink</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sharpie</td>
</tr>
<tr>
<td>B</td>
<td>Crayola</td>
</tr>
<tr>
<td>C</td>
<td>Wet Erase Marker</td>
</tr>
<tr>
<td>D</td>
<td>Dry Erase Maker</td>
</tr>
</tbody>
</table>

Using ink samples from ransom note, you will use chromatography to be our forensic scientist! First, you will separate the known samples using chromatography. Then you will separate the samples taken from the ransom note. From there, you can determine which suspect stole the art by matching the pattern on the chromatography papers after separation.
Ink Identification
1. Take out your 2 pieces of paper and use a pencil to draw a line $\frac{1}{2}$ inch from the bottom.
2. Mark each paper once with each pen to mark on the line and label the marks with pencil.

<table>
<thead>
<tr>
<th>Dot</th>
<th>Ink</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sharpie</td>
</tr>
<tr>
<td>2</td>
<td>Crayola</td>
</tr>
<tr>
<td>3</td>
<td>Wet Erase Marker</td>
</tr>
<tr>
<td>4</td>
<td>Dry Erase Maker</td>
</tr>
</tbody>
</table>

3. Each paper should have 4 marks. Both papers should now look like this:

4. Using a pencil, label one sheet of paper in the top left corner with “W” for water and label the other sheet with “A” for acetone.

5. Clip a binder clip to the top of each paper and then slip a pencil into the binder clip. This will keep your paper from falling into the water and acetone that develops the ink.

6. You should have 2 glass beakers, one will be used for water and the other will be for acetone (nail polish remover). Water and acetone have different properties that will cause the ink to develop differently.
7. Place both sheets of chromatography paper into their beakers. The pencil will balance on the lip of beaker.

8. Use a pipette to squirt water into the beaker with the W paper in it. When you add the water, squirt into the side of the beaker, you do not want to get your paper wet. Only add enough water so that it is below the line of dots. Someone will come by to add acetone into your other cup. Now your cups should look like this:

![Image of beakers with chromatography paper]

9. Watch the liquids move up the paper, dragging the different inks with them.

10. When the wet line is just below the binder clip, remove the paper to let it dry.

11. Repeat steps 3-6 with the ransom note samples that has been made for you. You should have 2, one is for water and one is for acetone.

12. Do any of the markers have the same pattern as the ransom note? If so, then you have identified what kind of ink was used!

13. Are the acetone and water strips that matched up with the ransom note made by the same marker?
Appendix C: pH of Paper

pH of Paper

We can usually tell that a book is old because the paper is yellow. That yellow comes from light and air mixing with the paper and slowly turning it *acidic* over time. *Acids* are bad for paper and cause it to degrade, or fall apart, over time. To stop paper from becoming *acidic* too fast, it is usually treated with chemicals so that it is *basic*, which is the opposite of *acidic*. Scientists can use this to test if artwork is old (*acidic*) or new (*basic*). We can use universal indicator to test because it turns different colors depending on how *acidic* or *basic* something is like this:

![pH Color Chart]

We need you to be a forensic scientist! Analyze 3 samples, one that we know is old, one that we know is new, and one that claims its old artwork but we suspect is counterfeit! Use universal indicator to find out if the paper is counterfeit or authentic!
Testing the pH of Paper

1. You should have 3 pieces of paper (one is new paper, one is old paper and one is paper from the artwork) and a cup with a lid for each piece of paper.

2. Rip up each piece of paper and put it in its cup. The paper needs to be ripped up enough to fit, but does not need to be really small pieces.

3. Use the transfer pipette to squirt in 2 squirts of water into each cup.

4. Put the lid on each cup. Make sure the lid is completely closed. You do not want any water to escape.

5. Swirl all three cups for 30 seconds. This will make any acids or bases in the paper move to the water so we can test it.

6. Now carefully open the cup. Again, you don’t want to lose any water. You do not need to worry about any paper stuck to the lid of the cup.

7. Now add 2 drops of indicator to the new paper cup. Swirl gently to get the water and indicator to mix. What color did the water turn for the new paper? What is the pH for the paper?
8. Now add 2 drops of indicator to the old paper cup. Swirl gently to get the water and indicator to mix. What color did the water turn for the old paper? What is the pH for the paper?

9. Now add 2 drops of indicator to the crime scene paper cup. Swirl gently to get the water and indicator to mix. What color did the water turn? What does this mean about the pH of the paper? Is it old (authentic) or new (counterfeit)?
Light Science

**Frequency** is how often something happens within a given or set period of time.

For example, if a person eats at 6:00 AM, 2:00 PM and then again at 10:00 PM, every day, they are eating with a frequency of 3 times per day or once per 8 hours.

Scientists write this out as an equation with the number of occasions the event happens on the top and the amount of time on the bottom.

\[
\text{Frequency} = \frac{\# \text{ of occasions}}{\text{unit of time}}
\]

If we put a 1 on the top and an 8 on the bottom, we can see that this person eats at frequency of \(\frac{1}{8}\) per hour.

\[
\text{Frequency} = \frac{1}{8} \text{ hours}
\]

Now let's try an experiment. Have someone time you and bounce a ball for 10 seconds. Record the number of bounces in the box below. Your value should be somewhere between 10 and 50.

\[
\text{Frequency} = \frac{\# \text{ of bounces}}{10 \text{ seconds}} = \_ \_ \cdot \_ \_ \text{ bounces per second}
\]

When we divide by 10, we put a period (decimal) in front of the last number. Re-write the number of bounces in the two spaces on the right, putting the period between the two numbers. **This is your frequency!**

Electromagnetic radiation (light) travels through space as a wave. The **frequency** of light is equal to the number of waves that passes a fixed point in space each second. The distance between waves is called the **wavelength**.

The frequency of light is proportional to its **energy** and the wavelength is inversely proportional.

When light of a certain wavelength interacts with a molecule, a small amount of energy is sometimes transferred to the molecule from the light. We can observe this energy transfer by measuring the how much the wavelength has been **shifted** from its original wavelength. We call this the **Raman shift** after the Indian physicist, Sir Chandrasekhar Venkata Raman, who first observed this effect.

**Light Speed**
All light travels at the same speed. In space light travels at 300,000 km per second.
Light Science

The amounts of energy transferred from the light to the molecule will correspond to the energies of the various **chemical bonds** in the molecule. A stronger (higher energy) bond will absorb more energy, so the wavelength the light will become longer (lower energy). Each type of molecule will have its own **Raman spectrum (or fingerprint)** like the ones below for the chemicals aspirin and maltol.

Forensic chemists can use these characteristic spectra to detect things like explosives, counterfeit medicine or environmental contamination. **Can you identify the unknown compound from its spectrum?**

**Aspirin**

Acetylsalicylic acid is the active ingredient in aspirin. A natural version of aspirin comes from the bark of willow trees, which are high in salicylates. **Looking for alternatives with few side effects, chemists at Bayer synthesized acetylsalicylic acid in the late 1800’s.**

**Maltol**

Maltol is a naturally occurring flavor and odor. The smell of maltol is caramel like. Maltol can be isolated from the bark of larch trees.

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**Chemical bond**: A uniting force between two atoms

**Frequency**: The number of waves that passes a fixed point each second

**Energy**: The capacity for doing work

**Molecule**: The smallest particle of a substance, made up of bonded atoms, having all the characteristics of the substance

**Spectrum**: Separation of light into its colors or wavelengths

**Wavelength**: The distance (as from crest to crest) in the line of advance of a wave from any one point to the next corresponding point

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All definitions are from [www.wordcentral.com](http://www.wordcentral.com)
**WHICH STEM CAREER IS RIGHT FOR YOU?**

Careers in science, technology, engineering, and mathematics, or STEM careers, are crucial to the Nation’s health and economy. This worksheet is designed to help you investigate STEM careers which may be suited to your talents and interests.

First we will discover what your individual STEM Career personality is by filling out a short quiz. This quiz is designed to match you with either science, technology, engineering, or mathematics based on your answers to a few short questions. The quiz can be found at the following website:

http://forgirlsinscience.org/career-personality-quiz/

Now that you’ve identified a general field, let’s investigate different careers within that field. If you were matched with Science, investigate potential careers at:

http://www.sciencebuddies.org/science-engineering-careers

If you were matched with Technology, use Google to find the "Top Ten" tech jobs for 2013. If you were matched with Engineering, follow this link to read about various career paths in engineering:

http://educatingengineers.com/career-specialties

If you were matched with Math, investigate potential careers at:

http://www.maa.org/careers

A second quiz which will match you with a potential STEM career can be found by going to:

http://www.qub.ac.uk/sites/STEM/futurize-me/

Again, now that we’ve been given a general career, let’s investigate how to pursue a career in your match at the University of Missouri. If you were matched as an engineer, go to:

http://engineering.missouri.edu/academic-departments/

For all other matches go to:

http://coas.missouri.edu/departments.shtml

and find the discipline you were matched with. After navigating to your match’s
website, find the answers to the following questions:

1. Are there any admission requirements to this major?

2. Which courses are required for an undergraduate degree in this major?

3. What scholarship opportunities are available? Some major specific scholarships can be found at the discipline's website but more general scholarship opportunities can be found at:

   http://coas.missouri.edu/scholarships/

4. Are there any student organizations affiliated with this major?

5. Is there a freshman interest group (FIG) for this major at Mizzou? Information about FIGs can be found at:

   http://reslife.missouri.edu/fig-list

Now that you have investigated how to receive a degree, let us look into careers involving your match. Listed below are various job search engines. Go through a few of them and search for jobs available in your chosen match.

   http://www.nature.com/naturejobs/science/
Hopefully you were able to find some careers corresponding to your match. If you are interested in investigating these matches future you can look at the MU career center.

http://career.missouri.edu
VITA

Anahita Zare was born on February 7th, 1988 in Clearwater, Florida and grew up in Orlando, Florida. She attended Florida State University where she obtained her B.S. in Chemistry in 2011 with a minor in Mathematics. She received a Ph. D. in Chemistry under the supervision of Dr. Renee JiJi, at the University of Missouri-Columbia, with a graduation date of Summer 2017.