Investigations of Antimicrobial Peptides Piscidin 1 and Ixosin using Circular Dichroism and Nuclear Magnetic Resonance Spectroscopy

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by

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# Contents

Acknowledgments iii

List of Figures v

List of Tables vi

Abstract v

1 Introduction 1
   1.1 Antimicrobial Peptides and Secondary Structure ................. 1
   1.2 Current Research on Antimicrobial Peptides ....................... 5
   1.3 Goals and Objectives ........................................ 6

2 Theory 11
   2.1 Circular Dichroism .......................................... 11
   2.2 Nuclear Magnetic Resonance Spectroscopy ......................... 16

3 Experimental Technique 22
   3.1 Peptide Purification and Dialysis ............................. 22
   3.2 Liquid Circular Dichroism ................................... 23
   3.3 Oriented Circular Dichroism .................................. 23
   3.4 Ultraviolet-Visible Spectroscopy .............................. 24
3.5 Oriented NMR Sample Preparation ......................................... 25

4 Results and Conclusions ............................................................ 26
  4.1 Oriented Circular Dichroism of P1 Wild Type and P1 mutant ........ 26
  4.2 Ultraviolet-Visible Spectroscopy of Ixosin .............................. 29
  4.3 Liquid Circular Dichroism of Ixosin ....................................... 32
  4.4 NMR Spectroscopy of Ixosin .............................................. 37
  4.5 Conclusions and Future Work ............................................ 40

Bibliography .............................................................................. 41
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List of Figures

1.1 Hydrogen Bonds in an α-helix ................................................. 2
1.2 Alpha Helix Secondary Structure ........................................... 2
1.3 Structure of a Parallel β-sheet ............................................. 3
1.4 Structure of an Anti-parallel β-sheet ...................................... 3
1.5 Proposed Membrane Mechanisms of AMPs ............................... 4
1.6 Structure of Histidine ....................................................... 7
1.7 Structure of Different Lipids ............................................... 8
1.8 Visualized α-helix of Ixosin ............................................... 9

2.1 Visualized Ellipse of Circular Dichroism ................................. 12
2.2 Transition Dipoles of Circular Dichroism ................................. 13
2.3 Visualized CD graphs by Secondary Structure ............................ 14
2.4 Angular Dependence of Oriented CD ...................................... 16
2.5 Energy Diagram for a Nucleus with a spin 1/2 ............................ 17
2.6 Visualization of Tensor Elements in Oriented NMR Samples ............ 19
2.7 Visualization of the Dipolar Coupling .................................... 20
2.8 2D NMR Reference Spectra of P1G13 1:20 with 3:1 POPC:POPG .......... 20

4.1 Piscidin 1 Wild Type ............................................................ 27
4.2 Piscidin 1 Mutant H17G ...................................................... 28
4.3 Ixosin with Ni^{2+} Pre-Centrifugation and Post-Centrifugation ........... 30
4.4 Ixosin with Ni²⁺ Pre-Centrifugation and Post-Centrifugation - Zoom-In 31
4.5 Ixosin to 3:1 POPC:POPG 33
4.6 Ixosin to 0.8:2.2:1 PLPE:POPC:POPG 34
4.7 Ixosin with Cu²⁺ to 3:1 POPC:POPG 35
4.8 2D HETCOR NMR spectra of Ixosin with Ni²⁺ 1:30 with 3:1 POPC:POPG 38
4.9 ³¹P NMR spectra of Ixosin with Ni²⁺ to 3:1 POPC:POPG with comparison to Oriented Lipid Blank of 2.6:0.4:1 POPC:AldoPC:POPG . . 39
List of Tables

4.1 Percent Helical Content - Ixosin with 3:1 POPC:POPG . . . . . . . . 33
4.2 Percent Helical Content - Ixosin with 0.8:2.2:1 PLPE:POPC:POPG . 34
4.3 Percent Helical Content - Ixosin with $Cu^{2+}$ and 3:1 POPC:POPG . . 36
Abstract

Throughout this project, we investigated two different antimicrobial peptides (AMPs). AMPs are biomolecules that kill bacterial cells and have potential for medical applications. They have distinct structure-function relationships, which we strove to study through experimentation to determine secondary structures under varied environmental conditions. The first barrier an AMP must face when trying to kill a bacterial cell is the membrane. To understand the mechanism of action, determination of how the peptide interacts with lipid bilayers is key. In the first stage of our experiments, we looked at the AMP Piscidin 1, found in nature from hybrid striped bass. We scrutinized the importance of position 17 in its amino acid sequence in its ability to interact and orient in lipid bilayers through oriented circular dichroism. The results supported our hypothesis that position 17, and the histidine there, is vital to peptide orientation in the lipid bilayer. We also investigated Ixosin, an AMP found in the thick saliva of ticks, and looked at its membrane activity with varying amounts of lipid present and when bound to copper. In these experiments we tested secondary structure with two different lipid system compositions and metallation of the peptide, as well as an attempt to determine what portion of the peptide in solution binds the membrane. We ran several experiments consisting of liquid circular dichroism, ultraviolet-visible spectroscopy, and nuclear magnetic resonance spectroscopy. The data showed potential aggregation of the peptide when there is high concentration, and when there is low peptide concentration with a high peptide to lipid ratio, α-helical content was observed.
Chapter 1

Introduction

1.1 Antimicrobial Peptides and Secondary Structure

When we challenge ourselves to find ways to aid in the functions of a biological system like the human body, the best inspiration can come from models that already exist in nature. One such model that scientists have found is the antimicrobial peptide (AMP) [1], which comes in many different forms but serves the same function throughout - to fight and kill bacteria as the name would suggest. It is not a novel concept to observe that organisms have survived and evolved for many years, perfecting the science of fighting off disease and bacteria that would threaten life. However, understanding exactly how these complex mechanisms work in vivo and how we might be able to transfer their approach in a medical sense is a challenging task. This is the ultimate goal of our research; to understand the mechanism of AMPs and its implications on the potential medical applications that exist.

To understand our investigation of AMPs, some background regarding peptides and secondary structure is necessary. Peptides are biomolecules that are made of a carbon-carbon-nitrogen backbone and have different R-groups bonded to every other carbon, while the other carbon is a carbonyl with a double bond to oxygen [2]. We
can visualize this structure in Fig. 1.1.

![Image of Hydrogen Bonding in an α-helix secondary structure with visualization of the peptide backbone](image1)

**Figure 1.1:** Hydrogen Bonding in an α-helix secondary structure with visualization of the peptide backbone [2].

The sequence of a peptide is the order along the backbone in which the R-groups, which can be any of the different amino acids, are bound. Amino acids each have different properties resulting from their chemical composition and structure making them hydrophobic, charged, non-polar or polar in nature. Hydrogen bonds can form between amide and carbonyl groups of the same peptide or between different peptides forming a secondary structure that allows the amino acids bonded to orient in space in an energetically favorable way depending on the environment to which the peptide is exposed. Two types of secondary structures that are important to note are α-helices and β-sheets. An α-helix forms when hydrogen bonds form within a singular peptide, forming a helical structure shown in Fig. 1.2.

![Image of Ribbon model of the structure of an α-helix](image2)

**Figure 1.2:** Ribbon model of the structure of an α-helix [2].
The other important secondary structure is a $\beta$-sheet, which is created when hydrogen bonds form between different strands of the same peptide and can be parallel or anti-parallel. Both types are shown in Figures 1.3 and 1.4.

Understanding these secondary structures is important to understanding the peptides being studied because there is a structure-function relationship. Some AMPs have been found to form an amphipathic $\alpha$-helix, where amino acids are oriented so hydrophobic residues are on one side of the helix and hydrophilic residues are on the other [3]. This structure forms and allows the peptide to interact with lipid bilayers.
which are also amphipathic. This interaction at the membrane surface is the area of interest because the membrane is the first surface the peptide interacts with when trying to kill a bacterial cell. The orientation and structure play a vital role in our understanding of AMPs’ mechanism of action, because if it is inserted and parallel versus being perpendicular and on the surface, it gives evidence to support the different theories of the interaction between peptide and membrane which are shown in Fig. 1.5.

Figure 1.5: Different proposed membrane mechanisms of antimicrobial peptides [4].

Throughout our investigation of these AMPs, understanding its secondary structure will be the initial question. From there, we can question the required environment to form its secondary structure, mainly questioning the required peptide-to-lipid ratio for the peptide to predominantly be in the secondary structure which means an interaction with the bilayers present. The type of bilayer and other environmental conditions are variables that can also be questioned and are important to consider when trying to mimic the environment in vivo as much as possible.
1.2 Current Research on Antimicrobial Peptides

Antimicrobial peptides date back to about the 1940s, when the AMP now known as gramicidin was found to hinder the infection of mice with *Streptococcus pneumoniae* bacteria [5]. Since then, many AMPs have been discovered and used in drug research for topical aids in for wounds, drug delivery systems and possible antibiotics [1, 6]. With the practical use of these applications, the questions have become how can we improve the effectiveness and do more with these peptides? Recent research has linked *in vivo* conditions like the presence of copper with the mechanism of function for some AMPs, showing higher affinities for membrane penetration and even the formation of oxidized lipids through reactive oxygen species (ROS) formation [7, 8].

Further research has shown that AMPs may also have unique properties that can target oxidized lipids [9]. Oxidized lipids have been linked to many neurological medical conditions, like Alzheimers and Parkinsons [10], and has shown a significant relation to how membrane bound proteins are able to interact and function [11]. Oxidized lipids stem from oxidative stress which has long since been known to cause major cell damage that is most likely the reason that we age, and the reason DNA can be damaged - causing a whole host of other issues [12]. Oxidized Lipids in particular, are just reaching the forefront of how we can understand diseases of the brain, which are difficult to treat and often fatal [10]. Understanding the impact of oxidized lipids on pathways, and the ability of AMPs to target oxidized lipids - there is the potential to target malfunctioning cells that cause neurological disease to kill them or deliver specialized drugs [10]. The potential interactions of AMPs with metal ions and oxidative stress could forge the path to new understanding and applications as AMPs are just beginning to delve into the realm of medicine beyond killing bacteria.
The AMPs that are most interesting to this development, are amphipathic helical peptides with an Amino Terminal Copper and Nickel Binding (ATCUN) motif. An ATCUN motif means that there is a Histidine at position 3 in the AMPs amino acid sequence allowing it to coordinate a metal ion [13]. Peptides like Piscidin 1 and 3 found in fish, and Ixosin found in the thick saliva of ticks, fall into this category and hold much promise [14, 15, 16]. The ability of these peptides to bind Cu$^{2+}$ is important because metal ions are able to form ROS, and the presence of these highly reactive molecules can cause phospholipid peroxidation by attacking the double bonds in the hydrocarbon tails changing the ability of the lipids to organize in the bilayer [8]. Piscidin 1 and Piscidin 3 were even found to form oxidized lipids unbound to copper in thiobarbituric acid (TBA) assays on planktonic E. coli [17].

These characteristics are important because they are the basis that binding copper, forming ROS leading to oxidized lipids [13] that disrupt the membrane is key to the method of how these peptides work, and consequently why these peptides could have potential medical applications [14, 12]. We can scrutinize their amino acid sequence and hypothesize about which side chains may affect their mechanisms. This is how Ixosin was found to have the ATCUN motif and led to research that shows how this affects the function of the peptide [13]. Based on this exciting new research, we can also investigate how oxidized lipids, and other different lipid systems might impact the peptide’s mechanism of action.

1.3 Goals and Objectives

Based on prior studies of Piscidin 1 (P1) and Ixosin, the main goals of my research were to determine the importance of the Histidine at position 17 in the sequence of P1 and determine the predominant secondary structure relating to the function of Ixosin under varying conditions. These two AMPs are similar in that they both have an
ATCUN motif, but differ in that they are found in different species and have different sequences. The amino acid sequence of P1 is FFHHIFRGIVHVGKTIHRLVTG [16]. We can see how at position 3, there is an H, which is histidine, and there is also one at position 17. Looking at the structure of the amino acid Histidine as shown in Fig. 1.6, we can see how it has a unique ring structure and the ability to hydrogen bond.

![Histidine Structure](image)

Figure 1.6: The structure of histidine and potential ionization at physiological pH [2].

The amino acid sequence of Piscidin 3 (P3) is FIHHIFRGIVHAGRSIGRFLTG, and we can see there is a glycine at position 17, which is just a hydrogen. Research has shown that P1 is better at disrupting the membrane, where P3 is better at attacking DNA [18]. One hypothesis is that the crucial difference in their sequences lies at position 17. Our goal is to compare P1 in its natural form to a mutated version where instead of the histidine at position 17, there is a glycine. If we observe a difference in how the peptide interacts with lipid bilayers, then there would be evidence to suggest the histidine plays a vital role.

Furthering beyond Piscidin, the goals of my research were also to determine how the structure of Ixosin plays a role in its mechanism, determine how it is interacting with the lipid bilayer, and how additional factors may impact these findings.
One interesting element of the environment in which Ixosin is found is that the tick species, *Ixodes sinensis* is not a vector for *Borrelia burgdorferi*, which is more commonly known as Lyme disease [19]. This bacteria that causes Lyme disease has a unique membrane composition that is about 12% linoleic acid, which contains 2 double bonds in its hydrocarbon tail - making it a target for oxidation [20]. Generally when we investigate a peptide’s interaction with a phospholipid bilayer, we use 3:1 POPC:POPG, which is a bilayer consisting of 25% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 75% 1-palmitoyl-2-oleoyl-sn-glycero-phosphoglycerol (POPG). This composition is a membrane mimic to *Escherichia Coli*, a general well-known bacteria. We can visualize the difference between these membranes by comparing the different components, where *Borrelia burgdorferi* contains POPC and POPG like *Escherichia Coli*, but it also contains some linoleic acid, which we can mimic with 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine (PLPE). These lipid structures are shown in Fig. 1.7, where we can see the additional double bond in the PLPE that differs from the POPC and POPG.

Figure 1.7: The structure of the different lipids that will be used in our investigation: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-phosphoglycerol (POPG), and 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine (PLPE) [21].
What is interesting about this structure of PLPE, is that it ties in well with the concept of the ATCUN motif and lipid peroxidation because there are more double bonds to be oxidized by ROS. It would be interesting to see if there is a difference in how Ixosin interacts with a membrane mimic to *Borrelia burgdorferi* bound to copper and not bound to copper. However, before we begin to manipulate environmental conditions, the initial goal is to determine if Ixosin is indeed an amphipathic α-helix. If we scrutinize its amino acid sequence, which is GLHKVMREVLGYERNSYKKFFLR [19], we can see the Histidine at position 3 representing its ATCUN motif and if we imagine the first three residues coordinating Cu$^{2+}$, we can visualize a hypothetical α-helix. When this is done, we can observe what the potential organization of the residues would and this is shown in Fig. 1.8.

![Figure 1.8](image_url)

*Figure 1.8: Visualization of the hypothetical α-helix Ixosin would form if the first three residues are removed from a wheel diagram, as we imagine it is coordinating a copper ion [22].*

Looking the what the hypothetical secondary structure would be, we can see a
general organization of hydrophobic and hydrophilic residues of opposite sides of the α-helix, meaning it would form an amphipathic α-helix. From this, the goal will be to determine if this secondary structure forms, and if so what is the needed peptide to lipid ratio? We will also investigate the impacts of using different lipids systems and the binding of Cu$^{2+}$ to observe any effects the formation of the secondary structure. We can investigate both peptides and these specific aspects of their mechanisms through a global and molecular approach involving circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy.
Chapter 2

Theory

2.1 Circular Dichroism

One of the methods in which biomolecules are studied to gain global information about secondary structures is circular dichroism. This method is based on the absorbance of light in specific directions by the molecule being tested. In this method, the light being passed through the sample is circularly polarized. Unfiltered light moves in all directions and since light is a wave, we can imagine that unfiltered light has oscillations in every plane. Thus polarized light refers to light that has been filtered so it is moving in a specific direction or within a specific plane of space. Circularly polarized refers to light that moves in a circular fashion, so instead of imagining a sine wave moving up and down in a plane that is straight up and down, the line of light moves in a helical fashion through space.

At any point along the axis of rotation, we can envision that light is pointing outward at varying degrees, and in this polarization, there are left and right forms of the circularly polarized light that are mirror images of each other. This method is very effective when trying to discern in a sample differences in chirality because enantiomers of chirally active molecules absorb the left and right circularly polarized light differently, where leftly-circularly polarized light and rightly-circularly polarized
light are absorbed by left and right versions of chiral molecules. The signal measured is the difference in the left and right circularly polarized light that is absorbed [23]. We can describe this relationship by the equation,

$$\delta A = A_L - A_R$$  \hspace{1cm} (2.1)

where $A_L$ and $A_R$ are the left and right polarized absorbance components of the sample. This value is often given by the ellipticity, described through the relationship,

$$\theta = \tan^{-1}\left(\frac{b}{a}\right)$$  \hspace{1cm} (2.2)

where $\theta$ is the ellipticity in degrees, and $b$ and $a$ are the major and minor axes of the ellipse [23]. We can visualize this ellipse created by the absorbance of the left and rightly circularly polarized light in Fig. 2.1 below.

![Figure 2.1: The left (L) and right (R) circularly polarized light components: (I) the two components have the same magnitude (II) the two components have different magnitudes and the resulting combination is elliptically polarized as shown by the dashed line [23].](image)

We will use CD, it will be to gain information about the secondary structure of peptides. When looking at a single molecule versus a structure, the principle is similar
because the secondary structure of a β-sheet or α-helix interacts with the circularly polarized light in such a unique way that, based on the amount of signal at specific wavelengths, a secondary structure can be generalized for the entire sample [24].

The reason that α-helices and β-sheets produce these differences in CD signal is due to the n → π* and π → π* transitions that occur between 190 to 250 nm [25]. The strength of the π → π* transition is dependent upon the torsion angles, φ and ψ, of the bond [24]. For an α-helix, there is a regular pattern due to its structure that allows these dipole transitions to orient in the same directions, adding to one another, which is what yields the strong negative band at 208 nm [25]. For a β-sheet, there is more variability in these torsion angles, which is why it is zero or positive at 208 nm, thus showing the π → π* transition. The n → π* transition shows in the negative band of an α-helix at 222 nm and the negative band of a β-sheet at 218 nm [24]. We can visualize these dipole transitions in the peptide backbone and the unique graphs for each secondary structure in Figure 2.2 and Figure 2.3.

Figure 2.2: The dipole transitions for circular dichroism: n → π* and π → π* in the peptide backbone [25].

At 208 nm and 222 nm, α-helices have negative bands, and at 218 nm, β-sheets have negative bands [26], as we can see in Fig. 2.3. So when analyzing our data, we can observe the graphs of different samples and compare the global structure of one to
Figure 2.3: Unique CD signal graphs for different secondary structures shown in a, given by biomolecules with known secondary structures shown in b [26].

another. When comparing these graphs, there is a distinction at 208 nm that strongly differentiates between an α-helix and a β-sheet. For an α-helix, the signal is negative - showing the characteristic band for its structure. However, for a β-sheet the signal at 208 nm is zero or almost positive, differing greatly from the α-helix. This ability to compare samples and structure is key in our data analysis because it allows us to test for the optimum peptide to lipid ratio, so when we prepare NMR samples, we know exactly how much lipid is necessary for the peptide in question to saturate the membrane because it forms the its secondary structure only in the membrane-bound state.

This method can be done either in solution or oriented samples. The difference between these two methods is that in solution, large unilamellar vesicles (LUVs) are tumbling in 3-dimensional space, so the data collected reports strictly on the average secondary structure of the peptide and is not dependent upon orientation. To analyze
data from liquid circular dichroism, two values will be calculated. The data will be analyzed by calculating the molar ellipticity for each data point collected across the span of wavelengths from 190 nm to 260 nm as described by the equation,

\[
[\theta] = \frac{100 \times \theta}{n \times c \times l}
\]  

(2.3)

where \(\theta\) is the CD signal in millidegrees, \(n\) is the number of amide bonds in the peptide, \(c\) is the concentration of the peptide in the cuvette, and \(l\) is the path length of the cuvette [23].

The molar ellipticity at 222 nm will be used to calculate the percent \(\alpha\)-helical content of the sample, using established reference points of 100% helical and 0% helical content values of molar ellipticity at 222 nm shown in the equation,

\[
\% \alpha - Helical \ Content = \frac{[\theta]_{222} - [\theta]_{0\%}}{[\theta]_{100\%} - [\theta]_{0\%}} \times 100
\]

(2.4)

where \([\theta]_{222}\) is the molar ellipticity of the respective peptide to lipid ratio at 222 nm, \([\theta]_{0\%}\) is the standard molar ellipticity of a 0% helical sample, which is -2,000 deg cm\(^2\) dmol\(^{-1}\), and \([\theta]_{100\%}\) is the standard molar ellipticity of a 100% helical sample, which is -32,000 deg cm\(^2\) dmol\(^{-1}\) [27].

In an oriented sample, we study the orientation of the peptide by orienting our bilayers with their long axis parallel to the light beam by thinly spreading the sample in a quartz slide that is put perpendicular to the light beam. When the peptide forms an \(\alpha\)-helix, the data collected reflects the tilt of the axis of symmetry and shows an angular dependence [28]. We can visualize this through Fig. 2.4 which shows the different orientations and the graphs they produce.

This method can show membrane activity and whether a peptide is fully inserted, tilted or parallel to the lipid membrane surface. These orientations relate to the mechanism of action, which is unknown but has several proposed forms. We can visualize how the orientations correspond to the different proposed membrane mechanisms.
Figure 2.4: Different orientations of the peptide and the resulting CD graphs [29].

in Fig. 1.5. This information then helps determine conditions and expectations for samples that are used for NMR.

2.2 Nuclear Magnetic Resonance Spectroscopy

NMR Spectroscopy is a powerful method that can be used in many ways to gain atomic-level knowledge about molecules and larger structures. NMR spectroscopy can be used to determine what a sample consists of, the structure of the atoms in question and can even give knowledge of how structures are oriented in a sample. We are able to discover all of these things because NMR detects nuclei in atoms. The principle is that when a nuclei that has a spin and a magnetic moment is placed in a strong magnetic field, it will precess. This means that when the axis of the magnetic moment is tilted with respect to the static external magnetic field, the axis moves in a circle about the applied field - similar to how a top spins just before it falls.

The precession of the nuclei occurs at a specific frequency, and when radio waves are applied at the same frequency, the precession of that nuclei can be detected
within a sample. This frequency is called the Larmor Frequency and is described by the equation,

\[ v = -\frac{\gamma B_0}{2\pi} \tag{2.5} \]

where \( B_0 \) is the static external magnetic field and \( \gamma \) is the gyromagnetic ratio [30]. When a nucleus has a spin 1/2, that means it has two possible energy states when exposed to a magnetic field, which is shown in Fig. 2.5.

**Energy levels for a nucleus with spin quantum number 1/2**

![Energy levels diagram](image)

Figure 2.5: The energy diagram for a nucleus with a spin 1/2 [31].

The separation between these lower and higher, or \( \alpha \) and \( \beta \), states is an energy gap, \( \Delta E \), which is given by the equation,

\[ \Delta E = \hbar v = \frac{\gamma B_0 \hbar}{2\pi} \tag{2.6} \]

where \( \hbar \) is Planck’s constant, and we can relate this energy to the Larmor frequency given by equation 2.5, shown earlier [31]. When a smaller magnetic field (radio frequency), \( B_i \) is applied, it allows spins to move from the \( \alpha \) state to the \( \beta \) state and return to equilibrium when \( B_i \) is removed. The magnetization goes from the \( z \)-axis before applying \( B_i \) to \( (x, y) \) plane after applying \( B_i \) [32]. The magnetization in the \( (x, y) \) plane is the source of the current and NMR signal collected.

The chemical shift is basically describing slight changes that occur in the bulk magnetic field because when the nuclei precess, the electrons in the nuclei, have
angular velocity which creates a magnetic field of its own that opposes the bulk applied field, \( B_0 \). Depending on the nuclei and its orientation, the electrons in specific orbitals will generate larger or smaller magnetic fields of their own which can shield that nucleus from the applied magnetic field. This nuclear shielding and the slight change in the magnetic field near that nuclei based on the created opposing field can be measured, and this is what affects the chemical shift in parts per million in a NMR spectra which show the slight changes in the Larmor frequency. We can describe the chemical shift through the shielding parameter by the equation,

\[
v_s = v_0(1 - \sigma) \tag{2.7}
\]

where \( \sigma \) is the shielding constant and \( v_0 \) is the frequency in the absence of shielding [32]. We can describe the chemical shift in parts per million by the equation,

\[
\delta = \frac{v_{sample} - v_{ref}}{v_{ref}} \times 10^6 \text{ ppm} \tag{2.8}
\]

where \( v_{ref} \) is the reference frequency [32].

Solution NMR is a common method used to determine structure and dynamics, however in order to determine how the peptide interacts with the lipid membrane, we use oriented samples, so the peptide is in a fixed orientation [33]. The chemical shift (CS) is measured at a “tagged” \(^{15}\)N position of the peptide backbone at specific residues. A nitrogen in the backbone must be tagged with an \(^{15}\)N, because these have a spin 1/2 and will yield signal. In an oriented sample, the CS is given by the equation,

\[
CS = \sigma_{11}\cos^2 \phi \sin^2 \theta + \sigma_{22}\sin^2 \phi \cos^2 \theta + \sigma_{33}\cos^2 \theta = \sigma_{iso} + (\Delta \sigma_{CS}/2)[(3\cos^2 \theta - 1) + \eta(\sin^2 \theta \cos^2 \phi)] \tag{2.9}
\]

where \( \sigma_{11}, \sigma_{22}, \text{ and } \sigma_{33} \) are the tensor elements of the signal in each direction along the axis, \( \theta \) is the angle of the angle between \( B_0 \) and the long axis of symmetry of the
peptide, \( \phi \) is the angle between \( \sigma_{11} \) tensor element and the project of \( B_0 \) into the plane of perpendicular to the long axis of symmetry, and \( \eta \) is the asymmetry value [34]. We can visualize these tensor elements in the Fig. 2.6.

\[ \Delta v_{obs} = \frac{\gamma_I \gamma_S h}{4\pi^2 r_{IS}^5} \mu_o (3 \cos^2 \theta' - 1) \]  

(2.10)

where \( \gamma_I \) is the gyromagnetic ratio of nitrogen, \( \gamma_S \) is the gyromagnetic ratio of hydrogen, \( r_{IS} \) is the bond length between nitrogen and hydrogen, and \( \theta' \) is the angle between the N-H bond and the bulk magnetic field, \( B_0 \) [35]. We can visualize this relationship in the Fig. 2.7.

Then by measuring the \( ^{15}\text{N}-^1\text{H} \) dipolar coupling (DC), we have the plane in space of that bond. The equation for the dipolar coupling is,

When both the chemical shift and the \( ^{15}\text{N}-^1\text{H} \) dipolar coupling are measured, the angle \( \theta' \) can be calculated from equation 2.10, and then \( \phi \) and \( \theta \) can be calculated from equation 2.9, because the CS tensor has a known orientation with the N-H bond. So \( \theta \) can be found from \( \theta' \) as they differ by about 12 degrees. When this is done at each N position in the backbone, the data can be pieced together to give a full structure of the peptide in space, by looking at the repeating pattern of the dipolar values.
This data analysis is called the dipolar wave, and is used to calculate the angle of the oriented peptide with respect to the lipid membrane parallel to the bulk magnetic field and the angle of rotation of the peptide along its axis.

When we analyze 2-dimensional HETCOR spectra generated from this process, when one $^{15}$N position is tagged we expect to see one peak in the $^{15}$N chemical shift and two peaks in the $^1$H chemical shift, where the gap between them is the dipolar coupling representing two transitions as different energy spacings [36]. A reference spectra is shown in Fig. 2.8. When looking at this figure, we can see two distinct
dots in the spectra, represented a clear and uniform orientation and structure of the peptide in this sample, which is Piscidin 1 tagged at position 13. In the event that the peptide in the sample is not oriented or uniform, we would observe what is called a powder pattern, which is a broad peak representing many orientations or structures.
3.1 Peptide Purification and Dialysis

The peptide was purchased in a crude form from the University of Texas South Western, meaning that before samples could be prepared using the ordered peptide, it had to be purified to ensure manipulations had exact concentrations of peptide without impurities like truncated forms of the peptide from synthesis, counterions that could affect results or interact with other elements of the sample negatively, etc. In order to purify the crude peptide, it was run through a Waters high performance liquid chromatography, or HPLC, with trifluoroacetic acid in nanopure water at 0.1% and acetonitrile with 0.1% trifluoroacetic acid through a C-18 column. First, the amount of crude peptide was weighed and dissolved in an 85% nanopure water and 15% acetonitrile solution for a concentration of about 2-3 mg per mL for purification of Piscidin. For purification of Ixosin, the peptide was dissolved in 90% nanopure water and 10% acetonitrile solution for a concentration of about 2 mg per mL. Next, the sample was filtered with low-protein binding filters with a pore size of 13 mm, and run through HPLC in 5 mL increments and peaks were collected in a round bottom flask. To remove the organic solvents, the collected sample was then rotovapped. It was then collected in tubes, frozen with liquid nitrogen and lyophilized to remove
remaining water for about 24 hours. After lyophilization, a dry and pure sample was recovered with about a 10% yield.

### 3.2 Liquid Circular Dichroism

Liquid CD was performed to obtain global secondary structure for Ixosin. Samples were run at varying ratios of peptide to lipid to obtain membrane activity in the presence of two different lipid systems. The first system was 3:1 POPC:POPG, and samples were made at 1:0, 1:4, 1:20 and 1:50 peptide:lipid ratios. The second lipid system was 2.2:0.8:1 POPC:PLPE:PG, and samples were made at 1:0, 1:4, 1:20, 1:30, 1:40 and 1:50 peptide:lipid ratios. For each sample, a blank was run where no peptide was present, but the corresponding volumes of lipid were to account for signal attributed to lipid presence. The lipids were prepared as a 5 mM stock of large unilamellar vesicles (LUVs) through extrusion with 0.2 µm membranes. Samples were hydrated with 5 mM phosphate buffer to achieve a peptide concentration of about 18 µM in the cuvette that was inserted for CD. For metallated peptide samples, 5 mM BisTris Buffer was used and samples were hydrated to achieve a peptide concentration of about 32 µM. Circular Dichroism data was collected from 260 nm to 190 nm with increments of 0.4 nm and averaged over 4 collections. The resulting data was then analyzed for α-helical content.

### 3.3 Oriented Circular Dichroism

Oriented CD was performed to determine the global secondary structure of Piscidin 1. Samples for oriented CD were prepared at varying ratios of peptide to lipid for Piscidin 1 and mutated Piscidin 1. First, the amount of peptide and lipid required to achieve ratios of 1:25, 1:16, and 1:8 were calculated from the concentrations of stock solutions of peptide and lipid. Lipid films were prepared for 3:1 POPC:POPG
for each ratio and pipetted into glass vials. Lipid vials were dried using a stream of Argon gas to remove chloroform, then frozen with liquid nitrogen and lyophilized to dry further. Peptide volumes were also pipetted into individual glass vials for each ratio as well, frozen with liquid nitrogen and lyophilized to dry. To achieve desired intensities, about 0.1 mg of peptide was used, and about 0.5-0.1 mg of lipid was used per slide. Once collected from the lyophilizer, peptide volumes were hydrated with 10 mM Bis Tris buffer, vortexed and pipetted to corresponding ratio lipid vials. They were then vortexed and incubated for about 10 minutes to ensure peptide-lipid interaction. From there, the samples were ready to be placed on thoroughly cleaned quartz slides. The slides were cleaned with phosphate free soap and excess nanopure water, then dried with pure Argon gas to avoid dust. Each sample was spread on a clean slide and left partially uncovered to dry. Once dry, each slide was rehydrated before being run by placement in a humidity chamber of 99% relative humidity using $K_2SO_4$. Each samples was then placed in the OCD wheel chamber that is also kept at 99% humidity and inserted into the CD. Signal was collected in each run from 260 nm to 190 nm at increments of 0.4 nm and averaged over 4 collections. This was done at 8 different angle positions of the wheel, where each rotation was 45 degrees. For each sample, data at each angle position is averaged for an overall signal spread to account to slight variations of distribution of peptide detected at each angle rotation.

3.4 Ultraviolet-Visible Spectroscopy

Ultraviolet-visible (uv-vis) spectroscopy was run to determine peptide concentrations in solution. Samples were from sequential steps in the NMR sample preparation, and are specified in Chapter 4. Samples were run in cuvettes, cleaned with nanopure water and shaken to dry. Before each sample a blank was run, of the mixture of buffer and water present without peptide or lipid. The sample was dissolved in 2
mL of water and 8 mL of 3 mM BisTris buffer, so the blank was a 1:4 water:buffer solution. Before each sample, the spectrometer collected the blank, then the cuvette was cleaned with water and the sample placed in with a pipette. Each spectra was collected from 190 nm to 700 nm.

### 3.5 Oriented NMR Sample Preparation

Oriented NMR samples were prepared to obtain specific peptide data for Ixosin. For proper signal, about 6 mg of pure peptide was weighed and used for each sample. Next, the peptide was dissolved in water and the pH was adjusted to be about 7.6. From peptide weight, lipid volumes were calculated dependent on the lipid system used in that sample. For Ixosin one lipid systems was used, 3:1 POPC:POPG. The lipid film was then prepared by pipetting volumes together for the sample into a round bottom flask, dried with argon gas, frozen with liquid nitrogen and placed on the lyophilizer to dry. Once the lipid film was dry, the peptide was hydrated with about 4 mL of 3 mM Bis Tris Buffer and added to the lipid film to incubate overnight, stirring every so often. Next, the sample was centrifuged at 100,000 g and collected sample was spread onto cleaned slides. Slides were left to dry partially covered and then hydrated in the 99% relative humidity chamber for 2-4 days. Next slides were stacked carefully, rehydrated to 50% by weight with the collected supernatant, sealed with beeswax and placed in the oven overnight.
Chapter 4

Results and Conclusions

4.1 Oriented Circular Dichroism of P1 Wild Type and P1 mutant

To scrutinize the importance of the histidine at position 17 in the amino acid sequence of piscidin 1, two different oriented circular dichroism experiments were conducted. First, with wild type piscidin 1 - meaning the peptide as it is found in nature with no changes made to its amino acid sequence - was run at three different peptide to lipid ratios: 1:8, 1:16, and 1:25 using the lipid system of 3:1 POPC:POPG. This was also done for piscidin 1 with a mutated position 17 where the histidine was replaced with a glycine. Following the experimental procedure for oriented circular dichroism as described in Section 3.3, the data was collected from 190 nm wavelength to 260 nm for each peptide at the respective peptide to lipid ratios. Data was analyzed by averaging the 8 different angle positions for each ratio to generate a graph of CD signal across the span of wavelengths. Signal data was normalized to have equal intensity for comparison to be accurate across both experiments with the different peptides. To normalize the data, the minimum value in millidegrees of signal across every ratio was chosen to be $\theta_{\text{min}}$, and then each of signal was normalized to through
the equation,

$$\theta_{\text{norm}} = \theta_i \times \left[\frac{\theta_{\text{min}}}{\theta_{\text{min},i}}\right]$$ (4.1)

where $\theta_i$ is the CD signal at each increment of the wavelength measured for each peptide to lipid ratio and $\theta_{\text{min},i}$ is the minimum value obtained for that peptide to lipid spectra. The normalized signals are shown in Fig. 4.1 and Fig. 4.2 below for the piscidin wild type and mutant piscidin.

![Piscidin 1 Wild Type at Varying Ratios of Peptide to Lipid with 3:1 POPC:POPG](image)

**Figure 4.1**: Oriented circular dichroism at varying peptide to lipid ratios of Piscidin 1, wild type (no mutations in the amino acid sequence) with the lipid system 3:1 POPC:POPG

Looking at Fig. 4.1 and Fig. 4.2, we can see distinct differences in the bands of signal at 208 nm between the mutated form of piscidin compared to the wild type for each peptide to lipid ratio. For the mutated piscidin, at 208 nm the signal is about the same for each peptide to lipid ratio. For the wild type piscidin, the bands at 208 nm decrease in value as the amount of lipid increases, with the lowest value being found at the 1:25 peptide to lipid ratio. This suggests that the peptide in the mutated
Figure 4.2: Oriented circular dichroism at varying peptide to lipid ratios of Piscidin 1, mutated at position 17, with the lipid system 3:1 POPC:POPG

form is less affected by the changing peptide to lipid ratios, and does not orient in the bilayer to tilt in a state that disrupts the bilayer. This suggests that it is less membrane active. Piscidin 1 is membrane active past a peptide to lipid ratio of 1:20, so the mutated peptide shows some difference in orientation compared to the wild type that allows it tilt more easily. The data suggests that with the histidine at position 17, piscidin 1 tilts more readily. This supports our hypothesis, since the structure of histidine suggests it may play an important role in the dynamic interaction of Piscidin 1 with the bilayer. As P1 tilts more readily than the mutated P1, it suggests that the histidine at position 17 is playing a critical role in the peptide’s interaction with the bilayer to reach the active state. There may be more factors to consider to further investigate this in the future.
4.2 Ultraviolet-Visible Spectroscopy of Ixosin

Moving forward from Piscidin to Ixosin, the initial question to answer was what is Ixosin’s affinity to bind the lipid membrane? To understand the secondary structure or lack thereof in the presence of lipids from the circular dichroism data, we want to know what portion the peptide present binds the lipids. This is important because if 100% of the peptide present bind but we observe only 30% to be in a secondary structure, that suggest that the peptide does not form a secondary structure when it it binds the lipids. The same can be said conversely, where if we observe 30% to bind the lipids and 30% is forming a secondary structure, it suggests that when membrane active the peptide forms that secondary structure. So to investigate this, ultraviolet-visible spectroscopy was performed twice on a sample of Ixosin with Ni$^{2+}$ and at a peptide to lipid ratio of 1:30 with 3:1 POPC:POPG.

An absorbance spectrum was obtained from 190 nm to 700 nm before the addition of the lipids and centrifugation, and then another was obtained on the supernatant after lipids were added and the sample was centrifuged. This process is described in Section 3.4 and 3.5. The absorbance spectra of the two solutions from 190 nm to 400 nm is shown in Figure 4.3.

This figure clearly shows the major decrease in signal at about 280 nm, showing the backbone of the peptide going from a high presence to a much lower presence. What it hard to see in this figure it the nickel that is bound to Ixosin, which shows a much lower absorbance peak at about 400 nm. The comparison of these peaks are shown in Fig. 4.4 below.

Looking at this figure, the increasing signal for the post-centrifuge sample is greater than the peak absorbance observed for the nickel before centrifugation. This coupled with rise in signal moving from about 230 nm to 190 nm suggests there is
Figure 4.3: Ultraviolet-Visible Spectroscopy spectrum of the solution of Ixosin bound to Ni$^{2+}$ before the addition of lipids and centrifugation, and the spectrum of the supernatant, post-centrifugation and addition of to 3:1 POPC:POPG lipid system. Some background lipid signal that could be impacting the absorbance of the post-centrifugation sample spectrum. While a blank was used, it was not able to account for potential lipid presence, it only accounted for background signal from the buffer and water that the peptide was initially dissolved in. This is something to keep in mind as we do calculations with this data.

To determine the peptide concentration from the ultraviolet-visible spectroscopy data, the absorbance at 280 nm was used to calculate the concentration based on the two tyrosine amino acids in the sequence of Ixosin using the equation,

$$c = \frac{A_{280}}{\varepsilon_{Tyr} \times l} \tag{4.2}$$

where $c$ is the concentration of the peptide in M, $A_{280}$ is the absorbance at 280 nm, $\varepsilon_{Tyr}$ is the molar extinction coefficient for Tyrosine at 280 nm at pH 7.4, and $l$ is the path length of the cuvette used. Accounting for the two Tyrosines in the sequence of Ixosin, the $\varepsilon_{Tyr}$ value used was 2560 M$^{-1}$ cm$^{-1}$. After converting to $\mu$M, before
addition of lipids the absorbance at 280 nm was 0.488 giving a peptide concentration was 190.6 µM, which was on very close to the expected concentration calculated from the mass of peptide used to make the solution which was 5.54 mg. From this mass and a solution volume of 10 mL, the expected concentration was 193 µM. The absorbance of ixosin in the supernatant at 280 nm was 0.242, yielding a calculated peptide concentration of 94.5 µM. This yielded a percentage of ixosin remaining in solution to be about 50%, meaning hypothetically about 50% of the peptide bound the lipids and was in the pellet after centrifugation. However, realizing that blank for the post-centrifugation data was not accounting for background lipid signal, this suggests that there is less peptide present in the supernatant than the calculations show, meaning based on the significant decrease in signal shown in the comparison...
data in Fig. 4.3 there is likely much less than 50 % of peptide left from the initial concentration. In order to be more accurate, future work would have to use a blank that can take in to account background lipid signal or peptide concentration should be determined through amino acid analysis to be certain.

4.3 Liquid Circular Dichroism of Ixosin

The first experiment with Ixosin was to determine its predominant secondary structure and the conditions that accompany its highest expression. Liquid circular dichroism (CD) was performed after peptide purification that is outlined in Section 3.1, with varied conditions to obtain information. First, liquid CD was run with two lipid systems - 3:1 POPC:POPG and 0.8:2.2:1 PLPE:POPC:POPG. The second lipid system was used to mimic the Lyme disease bacteria, Borrelia burgdorferi’s membrane composition to observe any potential impacts in the affinity to bind between the two lipid systems. Differences observed in that data might suggest Ixosin’s higher adapted function to fight off Lyme disease given it’s discovery in the thick saliva of ticks that do not spread the Lyme disease bacteria.

The data was analyzed as described by Eq. 2.3 and Eq. 2.4 to calculate the molar ellipticity and percent α-helical content. The resulting spectra from the two lipid systems is shown in Fig. 4.5 and Fig. 4.6 below. The percent α-helical content for each experiment is shown in Tables 4.1 and 4.2 below.

Looking at the two different lipid systems, 3:1 POPC:POPG at 1:50 had a α-helical content of about 32%, and 0.8:2.2:1 PLPE:POPC:POPG at 1:50 had a α-helical content of about 29%. This difference of about 3% does not seem significant considering potential error, so moving forward for consistency the 3:1 POPC:POPG lipid system was used. One important aspect of the experimentation with two lipid systems is that the peptide concentration in the cuvette was about 18 µM, which
Figure 4.5: Liquid circular dichroism done at varying peptide to lipid ratios of Ixosin with the lipid system 3:1 POPC:POPG

Table 4.1: The percent helical contents of Ixosin with 3:1 POPC:POPG by the varied peptide to lipid ratios

<table>
<thead>
<tr>
<th>Peptide to Lipid Ratio (1 to x)</th>
<th>Molar Ellipticity at 222 nm (deg cm$^2$/dmol)</th>
<th>α-Helical content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-1328</td>
<td>-2.2</td>
</tr>
<tr>
<td>4</td>
<td>-1218</td>
<td>-2.6</td>
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<td>-6732</td>
<td>15.8</td>
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<tr>
<td>50</td>
<td>-11594</td>
<td>32.0</td>
</tr>
</tbody>
</table>

Table 4.1: The percent helical contents of Ixosin with 3:1 POPC:POPG by the varied peptide to lipid ratios

is fairly low. The ideal amount for CD is 20 μM or above, so moving forward the peptide concentration was increased.

When considering the amino acid sequence of ixosin, if the first three residues are
Figure 4.6: Liquid circular dichroism done at varying peptide to lipid ratios of Ixosin with the lipid system 0.8:2.2:1 PLPE:POPC:POPG

Table 4.2: The percent helical contents of Ixosin with 0.8:2.2:1 PLPE:POPC:POPG by the varied peptide to lipid ratios

<table>
<thead>
<tr>
<th>Peptide to Lipid Ratio (1 to x)</th>
<th>Molar Ellipticity at 222 nm (deg cm$^2$/dmol)</th>
<th>α-Helical Content (%)</th>
</tr>
</thead>
<tbody>
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<tr>
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</tr>
<tr>
<td>20</td>
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<tr>
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</tr>
<tr>
<td>50</td>
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Table 4.2: The percent helical contents of Ixosin with 0.8:2.2:1 PLPE:POPC:POPC by the varied peptide to lipid ratios

ignored and an α-helix is visualized as shown by Fig. 1.8, and the organization of amino acids suggests an amphipathic helix. So the hypothesis drawn was, if ixosin
is bound to metal with the ATCUN motif present as its first three residues, then the secondary structure of an α-helix will be more predominant due to stabilization. Therefore, the second experiment conducted with Ixosin was liquid CD with the first lipid system, 3:1 POPC:POPG, and Ixosin metallated with Cu$^{2+}$. The data was analyzed using Equation 2.3 and Equation 2.4, as was done before except that the first three residues that are bound in the ATCUN motif are accounted for reducing the number of amide bonds in the calculation. The results are shown in Figure 4.7 and Table 4.3 below.

![Molar Ellipticity for Varying Ratios of Ixosin bound to Cu$^{2+}$ to 3:1 POPC:POPG](image)

Figure 4.7: Liquid circular dichroism done at varying peptide to lipid ratios of Ixosin metallated with Cu$^{2+}$ with the lipid system 3:1 POPC:POPG

Looking at Figure 4.7 and Table 4.3, it is interesting that the highest percent α-helical content is present at 1:20 peptide to lipid ratio which presented almost 0% before the peptide was metal bound. However, looking closely at the Fig 4.7, the most important wavelengths to look at for distinction between α-helix structure versus a β-sheet structure is 208 nm and 220 nm. While the calculated α-helical percentage is
Table 4.3: The percent helical contents of Ixosin with $Cu^{2+}$ and 3:1 POPC:POPC by the varied peptide to lipid ratios

<table>
<thead>
<tr>
<th>Peptide to Lipid Ratio (1 to x)</th>
<th>Molar Ellipticity at 222 nm (deg cm$^2$/dmol)</th>
<th>α-Helical content (%)</th>
</tr>
</thead>
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<td>50</td>
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<td>24.5</td>
</tr>
</tbody>
</table>

Table 4.3: The percent helical contents of Ixosin with $Cu^{2+}$ and 3:1 POPC:POPC by the varied peptide to lipid ratios

high at 1:20, the molar ellipticity at 208 nm is almost in the positive range, suggesting no α-helix structure at all, and rather fully β-sheet content with the band at 220 nm being present for 1:20. So despite the drop in α-helical content past a ratio of 1:20, as the lipid concentration is increased, looking at Fig 4.7, the signal at 208 nm shown decreases. This means there is a distinct "dip" or band at 208 nm for 1:30, 1:40 and 1:50 peptide to lipid ratios - meaning there is α-helical content present. This seems to suggest that the peptide forms an aggregate or β-sheet structure initially, and past a threshold of lipid presence it forms an α helix secondary structure to interact with the bilayer.

Now let’s take a moment to consider this in the context of all of the liquid CD data obtained. Looking at Figs. 4.5 and 4.6, for a ratio of 1:20 there is little to no band at 220 nm, meaning that despite the same peptide to lipid ratio, this data shows no β-sheet content like the content seen in Fig. 4.7. Is it the metal that is the
defining characteristic? Well, one significant aspect between the samples of the two lipid systems and the metallated peptide is that they were run at different peptide concentrations. In the metallated liquid CD experiment, the peptide concentration in the cuvette was almost 36 µM, where for the other liquid CD it was 18 µM. This suggests that when the peptide concentration is high enough, it actually aggregates and forms β-sheet structure and past a lipid threshold it forms α-helix secondary structure, as the 208 nm band grew more pronounced at higher peptide to lipid ratios. Aggregation of the peptide would explain this trend in the CD data, but it would also explain the appearance of the sample post-centrifugation to test the peptide concentration as described in Section 4.2. After centrifugation, the sample had a distinct layer of peptide in the pellet that was separate from the lipid. This made homogenization of the peptide and lipid difficult. Knowing that in Section 4.2, the peptide concentration was very high, at about 190 µM, it would follow the trend of high peptide concentration supporting aggregation and β-sheet formation. To further investigate this, NMR was performed.

4.4 NMR Spectroscopy of Ixosin

Furthering our investigation of Ixosin, an NMR sample was prepared as described in section 3.5, for Ixosin bound to Ni²⁺ at a peptide to lipid ratio of 1:30 with 3:1 POPC:POPG. The first spectra taken was a 2-dimensional $^{15}$N - $^1$H HETCOR (Heteronuclear Correlation) spectra, shown in Figure 4.8.

Looking at fig. 4.8, we see peaks that are not well defined and thinking back to the reference spectra shown in Fig.[add ref here], we would expect to see six different points in this two dimensional spectra if the peptide is membrane bound and oriented. Three peaks in the $^{15}$N chemical shift for the three labels at position 5, 9, and 11, and then the dipolar coupling for each being seen in the $^1$H chemical shift. However,
Figure 4.8: A 2-dimensional NMR $^{15}$N - $^1$H HETCOR spectra collected on Ixosin with Ni$^{2+}$ at a peptide to lipid ratio of 1:30 with 3:1 POPC:POPG

instead we see potentially three peaks and their dipolar coupling, but there is a lot of noise in the data showing potential peaks over a wide range. This could be due to the common issue of hydration, but also could stem or be compounded with a different issue. To determine if hydration was correct for the sample, meaning enough of the supernatant was added to orient the lipids, a $^{31}$P spectra was collected and is shown with a lipid blank comparison spectra of 2.6:0.4:1 POPC:AldoPC:POPG in Figure 4.9 below.

Based on Fig. 4.9, we can see the lipid orientation in the sample, which should show a sharp peak at about 35 ppm for the oriented lipid head groups, instead shows broad span with a few sharp peaks. This is likely due to hydration, and additional hydration should be attempted for this sample, but what if the noise seen in Fig. 4.8 is caused by several factors? As stated earlier, there were concerns for peptide aggregation and
Figure 4.9: A $^{31}$P NMR spectra collected on Ixosin with Ni$^{2+}$ at a peptide to lipid ratio of 1:30 with 3:1 POPC:POPG to show lipid orientation (or lack thereof) shown in red, with overlay comparison of an oriented lipid blank of 2.6:0.4:1 POPC:AldoPC:POPG in blue during sample preparation, after centrifugation homogeneity of the peptide with the lipids was difficult and the sample remained cloudy after hydration and incubation.

Generally, NMR samples prepared for Piscidin 1 are clear and homogeneous after hydration and incubation, and don't aggregate to the point it cannot be rehomogenized after centrifugation. This, however, does seem to be a factor for Ixosin when preparing an NMR sample. Looking at the difficulty of the lipids to orient and the noise in the 2-D spectra, the issue of aggregation seems to be confirmed. In the CD data, there is a fair amount of noise underlying the signal, which is not present in Piscidin 1 or 3 CD spectra. If the peptide is aggregated, then there would be a correlation between the peptide concentration and orientation, or lack thereof. It
could have affected the NMR sample more drastically because the concentration of the peptide was about 190 µM for NMR but only about 18-30 µM for liquid CD. This is an issue to investigate in future work.

### 4.5 Conclusions and Future Work

After analysis of oriented CD data of Piscidin 1 wild type and mutant data, we found that the mutated form the P1 was less membrane active than the wild type. As signal showed no change dependent upon the changing peptide to lipid ratios, we saw no orientation of the mutated version in the bilayers like we observed for the wild type. This supports our hypothesis that the Histidine at position 17 is crucial to Piscidin 1’s ability to tilt and orient in the membrane. Following our investigation of Ixosin, liquid circular dichroism and NMR data supported α-helical content at low peptide concentrations and a high peptide to lipid ratio, and aggregation with β-sheet content at high peptide concentrations. Distinct bands observed in the liquid CD data show no α-helical content until a peptide to lipid ratio of 1:30 or higher, but at 1:20 at high enough peptide concentration, a negative band at 222 nm supports β-sheet content. NMR data suggests aggregation is preventing distinct signals for the three tagged 15N positions, and hydration and aggregation could be affecting the ability of the lipids to orient.

Aggregation of Ixosin could be studied through CD at varied concentrations of peptide with a constant lipid. Another NMR sample could be attempted at a much lower peptide concentration and higher peptide to lipid ratio to counter the peptide’s affinity to aggregate, so just α-helical content could be studied. Repeated 31P spectra could be taken at varied peptide to lipid ratios and hydration until lipids align and the sample becomes homogeneous, which we could observed directly by the sample becoming clear. Continued research would be exciting especially for the metal-bound
peptide in the presence of oxidized lipids and even investigating these factors with potential synergy of Ixosin with Ixosin-B. Previous research shows that there is a potential synergy between Ixosin and Ixosin-B, another strain of AMP derived from the thick saliva of the tick species *I. sinensis*, where when Ixosin is copper bound it promotes peroxidation of phospholipids [13]. Ixosin-B was found to have a strong affinity to bind and attack cells where oxidized lipids were present in the membrane. The synergy proposed is that, Ixosin forms oxidized lipids increasing the function of Ixosin-B.
Bibliography


[22] Image generated from RZ labs from the University of California, Riverside.  
http://rzlab.ucr.edu/scripts/wheel/wheel.cgi?sequence=ABCDEFGHIJLKMNOP&submit=Submit


