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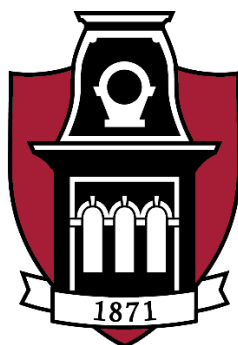
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Effect of charged lipids on the ionization behavior of glutamic acid containing transmembrane helices

An Honors Thesis submitted in partial fulfillment of the requirements for Honors Studies in Chemistry.

**By
Brooke Nunn**



UNIVERSITY OF
ARKANSAS[®]

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Department of Chemistry & Biochemistry

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The University of Arkansas

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Abstract

Transmembrane proteins make up critical components of living cells. Protein function can be greatly impacted by the charged state of its respective components, the side chains of amino acid residues. Thus far, in the lipid membrane, little is known about the properties of residues such as glutamic acid. To explore these properties, I have included glutamic acid in a suitable model peptide-lipid system for fundamental biophysical experiments. Within the system, I have placed a glutamic acid residue instead of leucine in the L14 position of the helical hydrophobic peptide GWALP23 (acetyl-GGALWLALALALAL¹⁴ALALWLAGA-amide). Substitutions of glutamine and aspartic acid serve as controls for the properties of the peptide helix in lipid bilayer membranes. The GWALP23 peptide derivatives are placed in various lipid bilayer environments.

Specifically, I investigated the impact of glutamic acid (position E14) when differently charged lipids are present in the bilayer. The underlying importance is to understand the charged or neutral state behavior of glutamic acid under conditions where it is important for the functioning of several types of membrane proteins, such as ion channels, drug transporters and others. For the experimental plan, core alanine residues of GWALP23 were labeled with deuterium to enable detection of helix characteristics by solid-state ²H NMR spectroscopy. The peptide-lipid samples included primarily the neutral lipid DMPC, 1,2-dimyristoylphosphatidylcholine, (with 14-carbon acyl chains), along with 10% of a charged lipid. For each membrane system, I confirmed lipid bilayer formation for the particular peptide-lipid mixture by solid-state ³¹P NMR. The charged lipids consisted of the negatively charged lipid DMPG, 1,2-dimyristoylphosphatidylglycerol, and the positively charged lipid DMTAP, 1,2-dimyristoyl-3-trimethylammonium-propane. These charged lipids were found to influence the

properties of the GWALP23 helix when E14 was present. DMTAP, in particular, improves the ^2H NMR spectra and the prospects for characterizing helix dynamics when a glutamic acid residue is present. While some experiments were cut short due to a global emergency, the results show promise for characterizing glutamic acid in model helices and actual membrane proteins.

Abbreviations

A, Ala, alanine

CD, circular dichroism

DMTAP, 1,2-dimyristoyl-3-trimethylammonium-propane

DMPC, 1,2-dimyristoylphosphatidylcholine

DMPG, 1,2-dimyristoylphosphatidylglycerol

DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine

Fmoc, fluorenyl methoxycarbonyl

GALA, geometric analysis of labeled alanines

G, Gly, glycine

GWALP23, acetyl-GGALW(LA)6LWLAGA-[ethanol]amide

HPLC, high-performance liquid chromatography

kHz, kilohertz

L, Leu, leucine

MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight

MtBE, methyl-t-butyl ether

NMR, nuclear magnetic resonance

E, Glu, glutamic acid

TFA, trifluoroacetic acid

TFE, 2,2,2-trifluoroethanol

TIPS, triisopropylsilane

W, Trp, tryptophan

Q, Gln, glutamine

Introduction

The study of transmembrane proteins is a crucial topic when investigating any aspect of the cell membrane. These proteins are key for maintaining proper cell function to prevent disease. Transmembrane proteins in the cellular lipid bilayer play major roles in the functioning of the cell in processes such as cell signaling, ion transport and adhesion activities¹. These proteins make up around 30% of proteins in the cell membrane². The structure of transmembrane proteins is generally made up of a stretch of hydrophobic residues, and it is speculated that the proteins are anchored in the membrane with aromatic residues that flank the protein by interacting with the charged lipid head groups³. These confer an α -helical formation in the hydrophobic region of the lipid bilayer membrane. Transmembrane proteins may oligomerize to form larger complexes of proteins, connected with a flexible loop region^{1,4}. The polarity of amino acid residues present in the interior of the bilayer determine the structure and function of these proteins in the cell.

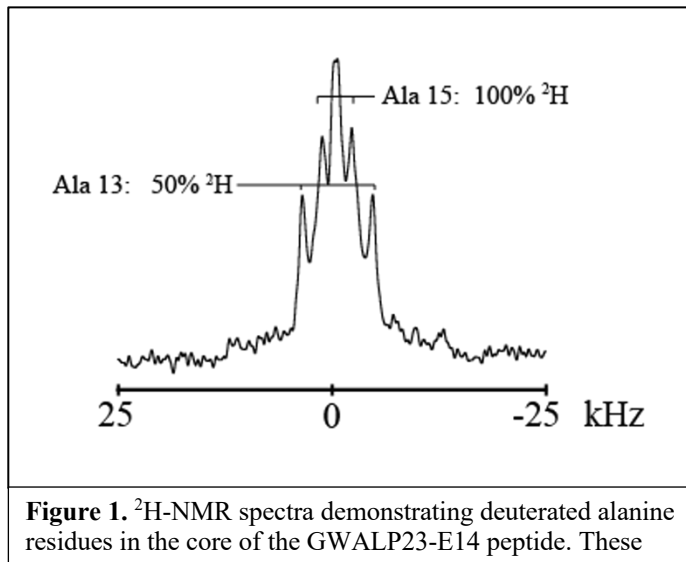
Transmembrane proteins are a significant area of biochemical research due to their roles in cellular function and disease. Around fifty percent of currently marketed pharmaceuticals target membrane proteins, due to the nature of their transport and signaling processes¹. These targeted proteins are involved in many different diseases, including Alzheimer's⁴. Some of these proteins require molecular chaperone, but some are able to fold into their active conformations based solely on their amino acid composition¹. Aiming to understand the fundamental principles of this folding in proteins based on amino acid sequence allows the possibility of computational models to be built to predict the structure and function of these transmembrane proteins from the sequence alone, as the lipid environment greatly complicates these processes⁵. Along with this, the effect of different membrane environments must also be considered, as the cellular lipid

bilayer environment is made up of a variety of lipids and other materials, such as cholesterol and cardiolipin⁴.

In order to better understand how transmembrane proteins with certain modifications may interact in a cellular environment, their behavior is studied using model systems. Using a system of model peptides has shown to be an effective way to simplify and study isolated aspects of the complex protein-lipid system⁴. To examine the basic principles for these interactions, a synthetic model can be used⁶. Specifically, this project uses a synthetic GWALP peptide model system. This system was initially modified from the WALP model, containing tryptophan residues which function as anchors to secure the peptide in the membrane³. Characteristics of this model, such as its high dynamic averaging, make it a poor model to study single residue substitutions¹. Further studies adapted the original WALP model into a GWALP23 peptide model system. This system has a higher stability and a defined orientation that serves to make it better suited for studying single residue substitutions¹. Instead of containing four tryptophan residues to secure the transmembrane helix, this model contains two located at positions 5 and 19. The core of this model peptide contains repeated leucine-alanine units which create a highly hydrophobic core with an α -helical conformation⁷. This core allows the peptide to span the hydrophobic interior portion of the membrane, and the tryptophan residues secure the peptide in place at the polar exterior portions of the membrane⁷. This characteristic positioning allows the peptide to be observed using solid-state NMR spectroscopy. Different substitutions to the model can then be made and analyzed in comparison to examine changes in tilt and rotation within the lipid bilayer.

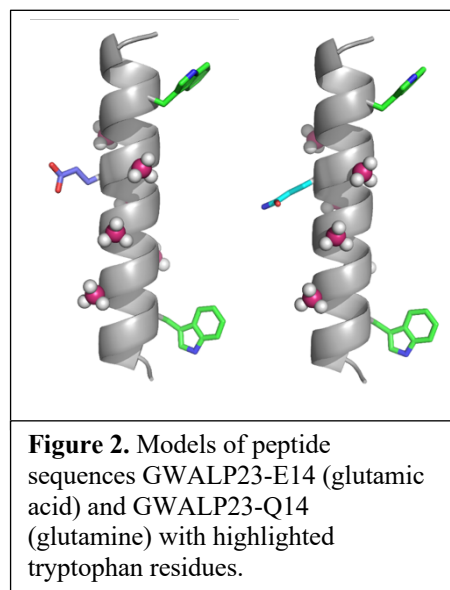
In order to study this system using solid-state NMR spectroscopy, deuterium labelled alanine residues are utilized. A pair of these deuterated alanines are introduced at specific locations in the core of the GWALP23 model peptide, one in 50% and the other in 100% isotope

abundance. By selecting the specific percent abundancies of 50% and 100%, the residues can clearly be identified on ^2H -NMR spectra due to their concentrations which present different peak heights upon analysis, as shown in Figure 1. Utilizing this method of analysis, previous

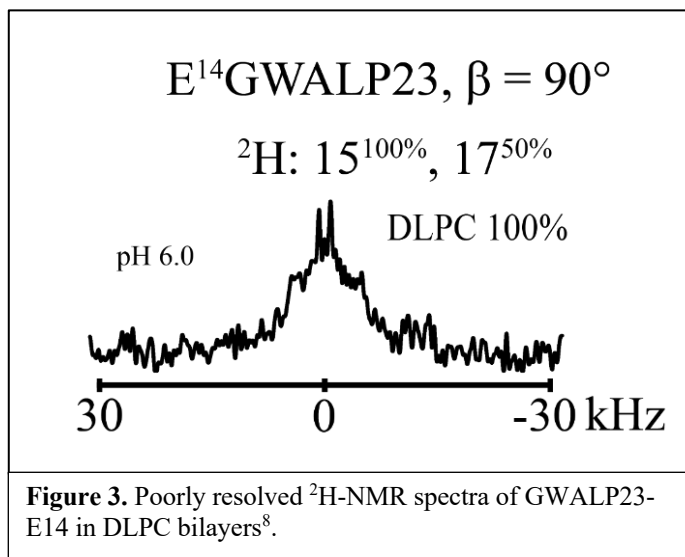


experimentation has focused on analyzing the impact of substituting charged residues, such as arginine, histidine and glutamic acid, in different positions on the GWALP23 model peptide in order to study the impacts these substitutions may have on behavior in the lipid bilayer system.

In this project, the GWALP23 model serves as a control for the system. Glutamic acid, an amino acid found within membrane proteins, is negatively charged in water solution at a neutral pH, but its ionization properties may change if glutamic acid is submerged in a lipid membrane. The interaction of this amino acid with the lipids in the cell membrane can therefore impact the behavior of certain transmembrane proteins and thereby influence cell function. Glutamic acid residues can be placed at various points in the GWALP23 model. This protein can then be introduced into different lipid bilayer environments to observe the behavior in the presence of the transmembrane protein. This behavior will be observed using core alanine residues labeled with



deuterium to detect helix characteristics by solid-state NMR spectroscopy. ^{31}P -NMR spectroscopy will also be used in order to analyze the lipid bilayer orientation with the peptide inserted into the membrane. This is done to ensure that proper bilayers are formed. Previous studies



with glutamic acid substitutions have focused on the study of neutral lipids. The orientation of the helix, with a polar residue at position 14 in GWALP23, has also been determined to be affected in DLPC [2]. DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine, is a neutral lipid like DMPC. These experiments suggest that the pK_a of the E14 peptide is high or that the host helix, containing the E14 residue, does not respond to the titration of the side chain⁸. The results of this study with DLPC resulted in noisy spectra with poorly resolved peaks, as demonstrated in Figure 3.

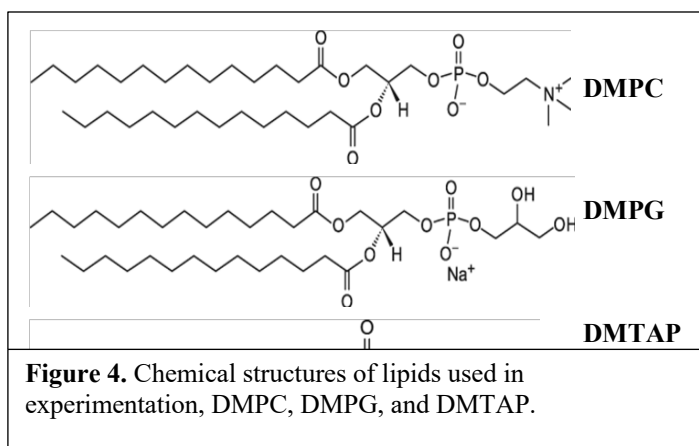
The objective of this project is to analyze the influence of inserting the glutamic acid residues and the impact of pH on the ability of the lipids to form properly oriented bilayers and potentially find a model similar to the cell membrane that will suit the glutamic acid side chain. For this project, a glutamic acid residue will be substituted at the L14 position in the helical model. This residue is polar and negatively charged. GWALP23-E14 will then be introduced into a membrane model. A list of relative peptides and their sequences is shown in Table 1.

Peptide	Sequence
GWALP23	Ac-GGALWL <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> WLAGA-amide
GWALP23-Q14	Ac-GGALWL <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>Q</u> L <u>A</u> L <u>A</u> L <u>A</u> WLAGA-amide
GWALP23-E14	Ac-GGALWL <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>A</u> L <u>E</u> L <u>A</u> L <u>A</u> L <u>A</u> WLAGA-amide

Table 1. Names and sequences of relevant peptides. GWALP23 was not actually synthesized but is shown in order to compare sequences with experimentally prepared peptides.

While cellular lipid bilayers are mostly made up of neutral lipids, charged lipids are also present. In order to better mimic this type of environment the impact of using charged lipids will be studied. These will consist of the positively charged lipid, DMTAP (1,2-dimyristoyl-3-trimethylammonium-propane), the negatively charged lipid DMPG (1,2-dimyristoylphosphatidylglycerol), and the neutral lipid, DMPC (1,2-dimyristoylphosphatidylcholine). The structures of these lipids are shown in Figure 4. The model will consist of the neutral DMPC with a percentage of the charged lipid. The results will be examined using solid-state ^2H -NMR spectroscopy. Deuterated alanine labels will be incorporated into the model peptide at various positions within the core helix in order to study the effects of pH variation on the system. These labels will be used to determine the tilt of the helix assessed with solid-state ^2H -NMR

spectroscopy. This project will monitor changes in the quadrupolar splittings of the neighboring deuterated alanines in order to determine if there is a change. The



outcome of this experimental analysis will be helpful in understanding various properties of glutamic acid containing peptides in cell membranes as well as understanding the behavior of glutamic acid in different lipid environments.

Materials and Methods

Solid Phase Peptide Synthesis

The peptides of interest were synthesized using an Applied Biosystems 433A Peptide Synthesizer from Life Technologies⁹. All side chain protected amino acids were purchased from Novabiochem (San Diego, CA) for use in the synthesizer, with the exception of the deuterated alanine residues. This residue was formed and recrystallized for use in the laboratory¹⁰. For the peptide synthesis the Fmoc, fluorenyl methoxycarbonyl, method was used. This is a stepwise method where each amino acid is added individually while attached to a stabilizing resin and then the byproducts of the attachment reaction are removed through washing¹¹. The amino (N) terminus will be capped with an acetyl group and the carboxy (C) terminus will be capped with an amide group. This will neutralize the charges on either end of the peptide, thus removing the N and C-terminus from consideration in the results of the experiment. Peptide synthesis began by weighing out 0.57 mmol of each amino acid required for the sequence in separate, labelled cartridges using an analytical balance. These cartridges were then capped. Two deuterium labelled alanine residues were selected to replace alanine residues in the sequence, one in 50% abundance and the other in 100% abundance. To measure out the 50% abundance, Fmoc-ala-d₄, the laboratory synthesized deuterated alanine, only half of the measured 0.57 mmol of alanine was replaced in the cartridge with Fmoc-ala-d₄. To measure out the 100% abundance, the 0.57 mmol of alanine was entirely replaced by measured Fmoc-ala-d₄. These residues were placed in pairs at positions 3 and 21, 13 and 15, and 9 and 11 in GWALP23-E14 and in a pair at position 13 and 15 in GWALP23-Q14. Once properly measured out and sealed, these cartridges were placed in the delivery chamber of the peptide synthesizer from C-terminus to N-terminus in

sequence for the proper peptide. The resin was also placed into the synthesizer where the peptide began to form from the C-terminus. Ensuring proper synthesis is vital to spectral determination¹².

Peptide Cleavage

Once synthesized, the peptide must be cleaved from the stabilizing resin. This was done using TFA, trifluoroacetic acid, cocktail. This cocktail is composed of an 85:5:5:5 ratio of TFA:TIPS:H₂O:phenol¹¹. To begin, 100 mg of peptide-resin mixture was weighed out. The TFA cocktail was then added to the mixture, sealed, and shaken for 3 hours. The mixture was then removed from the shaker and filtered through a Pasteur pipette filled with glass wool. This filtration effectively removed the peptide from the resin. The filtered peptide solution was then dried under N₂ flow until a quarter of the original volume was present. Next, the peptide was transferred to a 50 mL centrifuge tube and precipitated from solution by adding 30 mL MtBE(methyl-t-butyl ether):hexane solution. The mixture was submerged in ice for 30 minutes, reaching a temperature of 4°C. This was then centrifuged at this temperature for 10 minutes at a speed of 1800 rotations per minute (RPM). After centrifugation, the supernatant was removed, another 30 mL of MtBE:hexane solution was added, and the previous precipitation steps repeated twice more. Finally, the precipitated product was dried under N₂ flow, ensuring a low flow to not lose peptide by being blown out of the tube, 2 mL of acetonitrile added, and lyophilized, or freeze dried, overnight to obtain a white powder product.

Crude Peptide Purification and Quantitation

The cleaved peptide product was purified using reverse-phase HPLC in order to remove any contaminants. The white powder product was dissolved in 0.8 mL of TFE and 0.1 mL of

acetic acid. This solution was filtered through a 0.22 μm yellow filter which was rinsed with 100 μL of TFE. This ensured no particulate matter was present before purification in the HPLC, a Hitachi L1700 system with a Zorbax RX-C8 column (9.4 mm x 250 mm) from Agilent Technology. The column was rinsed overnight. A test run was performed with 10 μL of solution in order to determine the proper gradient for the best separation, usually a gradient of 86-90% methanol. Once determined, injections of 250-350 μL were performed, and the resulting product collected in a 50 mL centrifuge tube. This solution was dried under N_2 flow. Then, 1 mL of acetonitrile was added, and the solution lyophilized. The process was repeated twice more and the final lyophilization left overnight.

The peptide was quantified using UV-Vis Spectroscopy and confirmed for mass and deuteration using MALDI-TOF mass spectrometry. The tryptophan residues in the purified peptide fluoresce at 280 nm, allowing the use of a diode array UV-Vis spectrophotometer to characterize the peptide. The lyophilized peptide was dissolved in 2 mL of TFE. The UV-Vis instrument was zeroed using a blank of MeOH (methanol). Then, 10 μL of peptide solution was mixed with 990 μL of MeOH. The cuvette was then filled with this solution and run at 280 nm. The optical density measured from the sample was used to calculate the amount of peptide present. 1.33 μL aliquots and 0.0625 μL aliquots of peptide solution were placed in separate, labelled glass tubes and dried under N_2 flow. These were then sealed and stored in the refrigerator until further experimentation.

Circular Dichroism and Fluorescence Spectroscopy

0.0625 μL aliquoted peptide samples were combined with each lipid in a 1:60 ratio, dried under N_2 flow, and placed under vacuum for 48 hours. Then, 1 mL of deuterated water was added, and the solution was sonicated for around 1 hour in order to form vesicles, which contain the peptides. The sample was centrifuged, and the supernatant collected for analysis. The fluorescence data of the sample was analyzed, and CD spectra recorded in order to determine if the peptide was α -helical in conformation.

Oriented Sample Preparation

In order to perform solid-state NMR spectroscopic analysis, oriented samples were prepared. Peptide samples were combined with lipids in a 1:60 peptide to lipid ratio. This was completed by adding 80 μmol of lipid to the 1.33 μL aliquot. The lipids used for this were DMPC, DMPG and DMTAP. DMPC was used as a control due to its neutral nature. Charged samples were created by adding 5-20% of charged lipid with 80-95% DMPC. Once the lipids were added to the peptide and vortexed, the peptide-lipid mixture was dried under N_2 flow. Two clean glass plates were obtained and labelled accordingly. 36 glass slides were arranged between the two plates. Then, 850 μL of a 1 mL solution of 47:47:6 methanol to chloroform to water was added to the peptide-lipid film. The mixture was then added to the slides by transferring 25 μL with a 50 μL Hamilton syringe to each slide, leaving two slides blank to be used as cover slides during hydration. The slides were allowed to dry and then placed under vacuum in a desiccator and shielded from light for 48 hours.

After two days, the plates were removed from the vacuum line and hydrated with a buffer at the desired pH for experimentation. The amount of buffer required was determined by the molar mass of the peptide, lipids and number of slides for hydration at 45%. A 0.2-2 μL pipette was used to distribute the proper amount of buffer across the slides in 3 drops, while simultaneously stacking the plates on top of each other. Three slides maximum were hydrated at a time before stacking to ensure proper hydration. Using forceps, pressure was applied to the stack in order to mechanically create bilayers in the slides. Finally, once all plates were hydrated, they were placed into a cuvette for analysis and the cuvette was sealed with a glass cover and epoxy glue. The glue was allowed to dry, and the sample placed in a heating block at 40°C for at least 48 hours to incubate. Incubation ensures that the sample can acquire a liquid-crystalline state that allows for proper bilayer formation. Figure 5 illustrates this process.

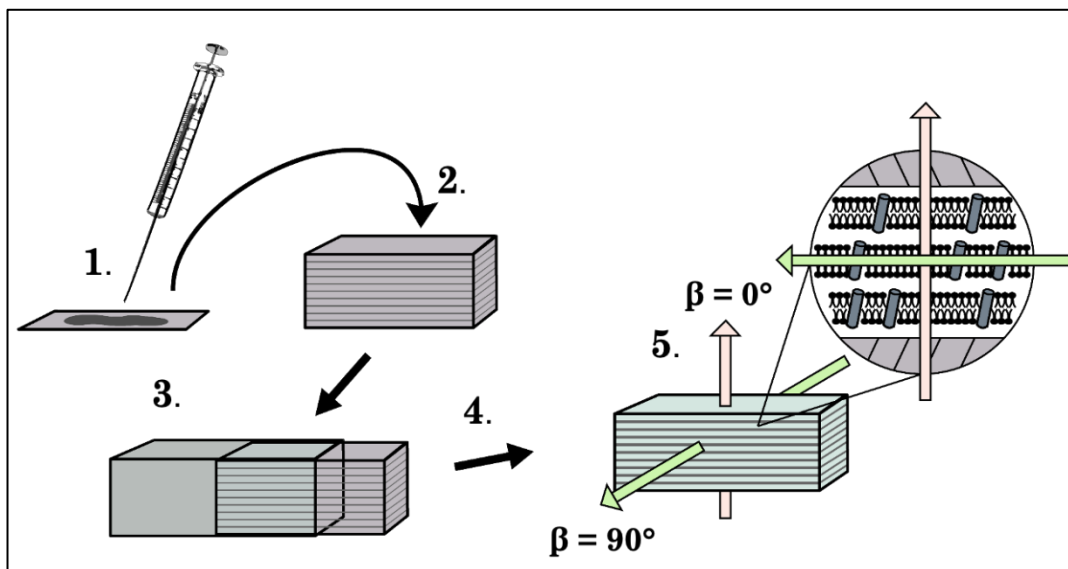


Figure 5. Visual depiction of oriented sample preparation.

1. Peptide and lipid mixture was distributed evenly on 34 glass slides and dried under vacuum for 48 hours.
2. Plates were then hydrated and stacked.
3. The hydrated stack was placed in the cuvette & sealed.
4. Sample was incubated at 40°C for 48 hours.
5. NMR experiments were performed at $\beta = 0^\circ$ and 90° .

Solid-State ^2H -NMR Spectroscopy

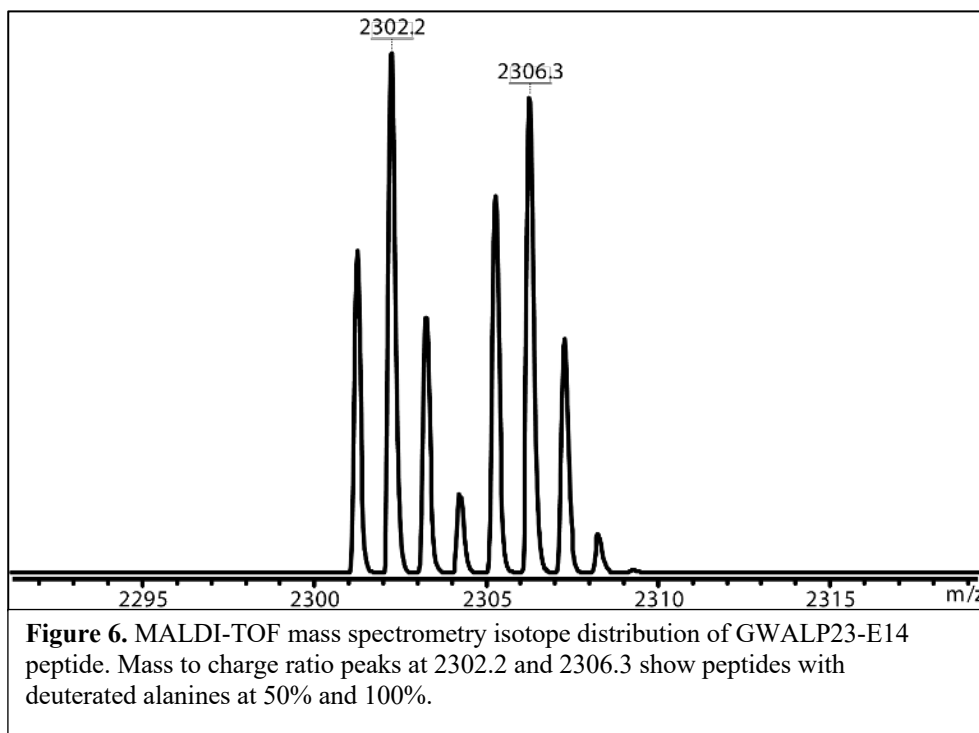
Oriented samples were placed in a Bruker Avance 300 spectrometer and run twice, at $\beta=0^\circ$ and $\beta=90^\circ$ in respect to magnetic field. $\beta=90^\circ$, indicates the bilayer is perpendicular to the magnetic field and $\beta=0^\circ$ indicates the bilayer is parallel to the magnetic field. The displayed spectra produce characteristic quadrupolar splittings, distance between the deuterated alanine peaks, that can be quantified to determine peptide orientation. These spectra were recorded and processed for data analysis.

Solid-State ^{31}P -NMR Spectroscopy

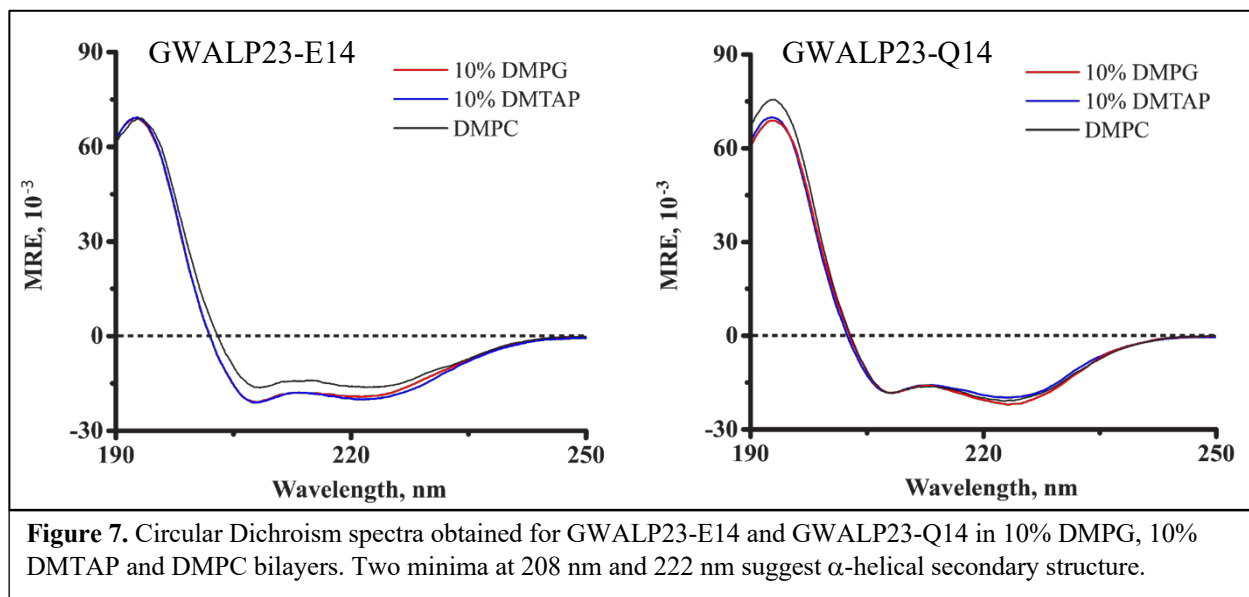
This method is very similar to the solid-state ^2H -NMR spectroscopy. Oriented samples were placed in a Bruker Avance 300 spectrometer and run twice at $\beta=0^\circ$ and $\beta=90^\circ$ in respect to magnetic field. The displayed spectra serve to interpret if the bilayer is properly aligned by analyzing the phosphorous-containing head groups of the lipids. Lipid-only oriented samples were also analyzed with this method. These samples were prepared in the same method, omitting the addition of peptide.

Results

In order to carry out further experimentation, the first step of analysis was to confirm the GWALP23-E14 peptide had been synthesized with the intended deuterium-labelled alanines through MALDI-TOF mass spectrometry. The expected mass of GWALP23-E14 was 2275 Da. The two peaks labelled in Figure 6 occur at 2302.2 and 2306.2 Da corresponding to the 50% and 100% deuterated alanine labels, as expected for the peptide molecular mass plus a sodium ion plus four deuterons (50% of sample) or eight deuterons (100% of sample). In light of this, the method confirms the expected results.



The secondary structure of GWALP23-E14 was examined using Circular Dichroism (CD) Spectroscopy. The hydrophobic core is expected to be α -helical in structure when inserted into the interior of the membrane. There is a characteristic spectral shape for this α -helical structure, that is in fact aligned with the spectra for GWALP23-E14 and GWALP23-Q14, which was used as a control, as pictured in Figure 7, with double minima at 208 nm and 222 nm¹³. This is further quantified in Table 2.



$\epsilon_{222}/\epsilon_{208}$			
Peptide	DMPC	DMTAP	DMPG
E14	0.9958	0.9519	0.9157
Q14	1.1236	1.0746	1.1741

Table 2. Numerical data for Circular Dichroism spectra of GWALP23-E14 and GWALP23-Q14 in 10% DMTAP, 10% DMPG and DMPC lipid bilayers. The numerical data suggests α -helical secondary structure.

The positioning of tryptophan residues was investigated using Fluorescence Spectroscopy in order to analyze the fluorescence maxima in each of the bilayers. The characteristic wavelength expected to be observed was at or near 333 nm. Figure 8 shows these spectra obtained for GWALP23-E14 and GWALP23-Q14 in 10% DMTAP, 10% DMPG and DMPC bilayers. Table 3 details the maxima of the spectra to confirm that it is at or near 333 nm indicating that the tryptophan residues are situated at the lipid bilayer interface in order to anchor the transmembrane peptide in the lipid bilayer¹⁴.

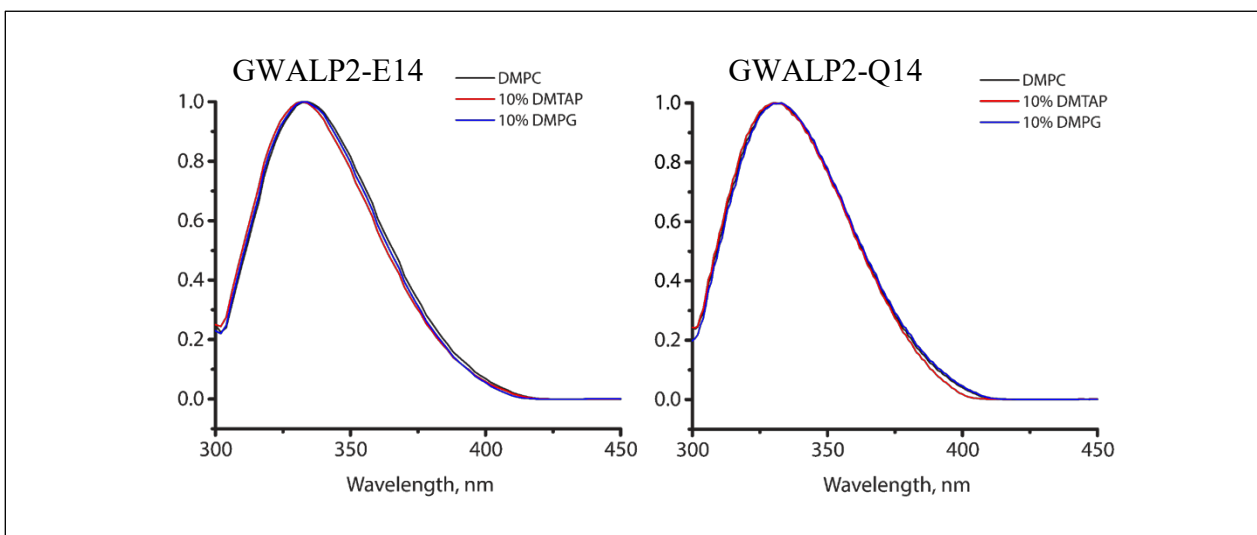


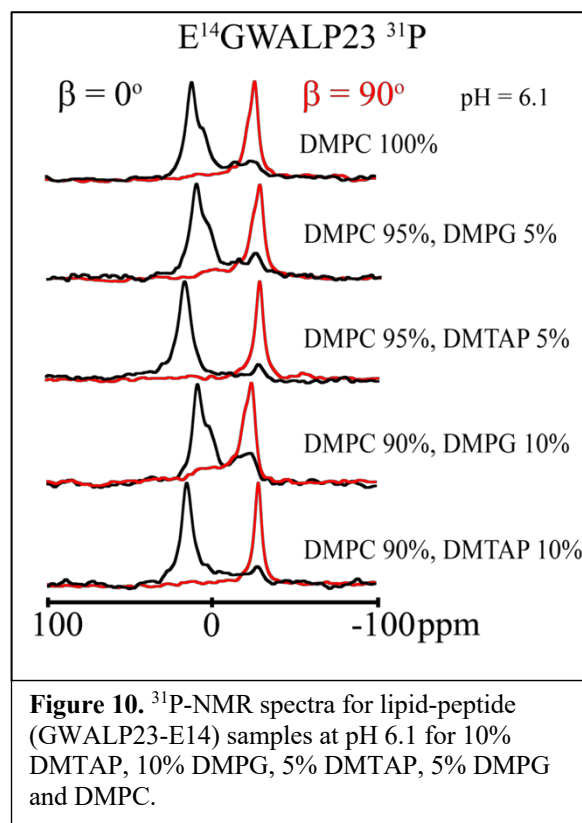
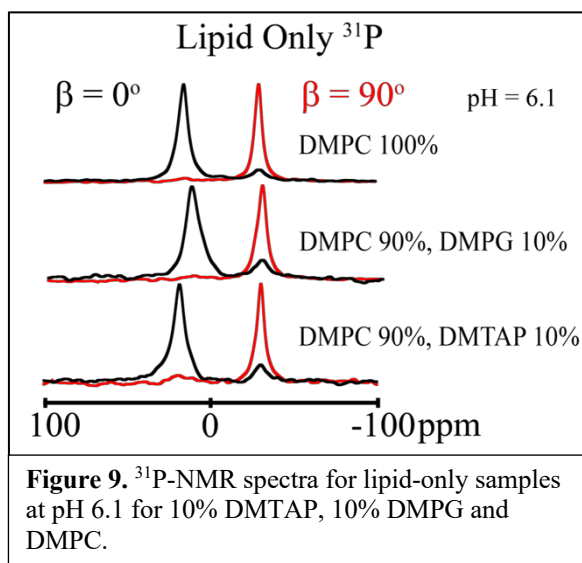
Figure 8. Trp Fluorescence spectra for GWALP23-E14 and GWALP23-Q14 in 10% DMTAP, 10% DMPG and DMPC lipid bilayers. Maxima at or near 333 nm suggest that the Trp residues are situated at the lipid bilayer interface.

Fluorescence Maxima			
	DMPC	DMPG	DMTAP
Peptide	Q14	Q14	Q14
Wavelength (nm)	333	333	330
Peptide	E14	E14	E14
Wavelength (nm)	333	333	330

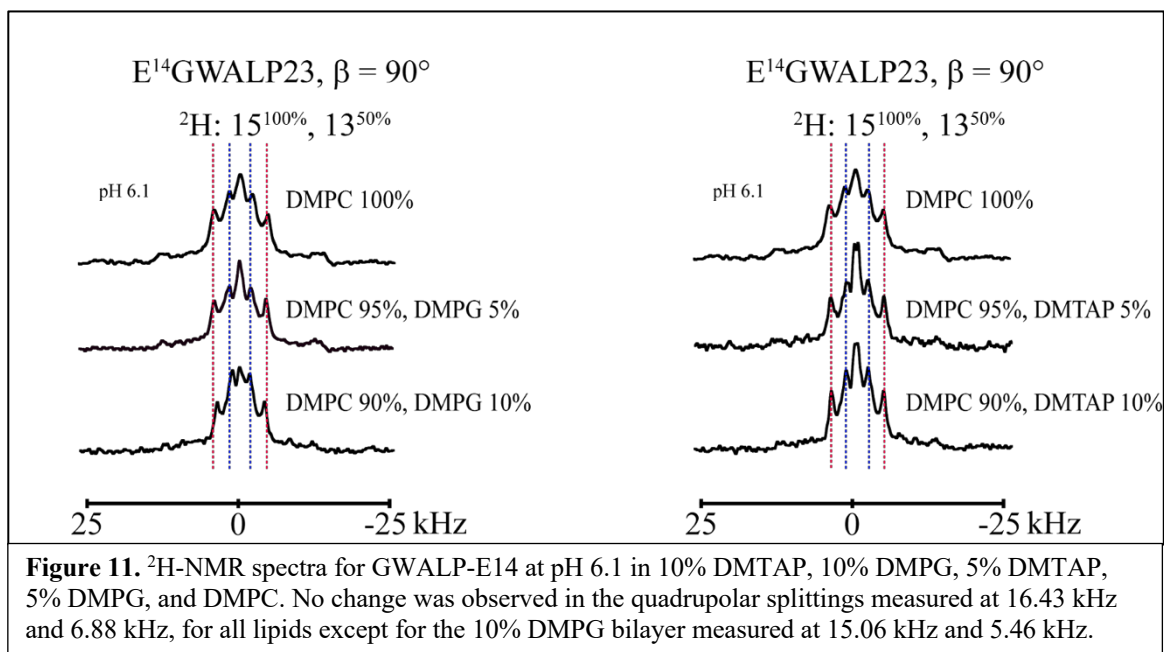
Table 3. Numerical data for Fluorescence Maxima for GWALP23-E14 and GWALP23-Q14 in 10% DMTAP, 10% DMPG and DMPC lipid bilayers. The maxima at or near 333 nm suggest the Trp residues are situated at the lipid bilayer interface.

^{31}P -NMR Spectroscopy was used to examine the lipid bilayer alignment of the oriented samples in various lipid concentrations. An initial experiment, I did with 20% charged lipid and 80% neutral lipid did not form adequately oriented bilayers, so the focus was shifted to bilayers containing 10% or less charged lipid. Lipid-only samples as well as samples containing

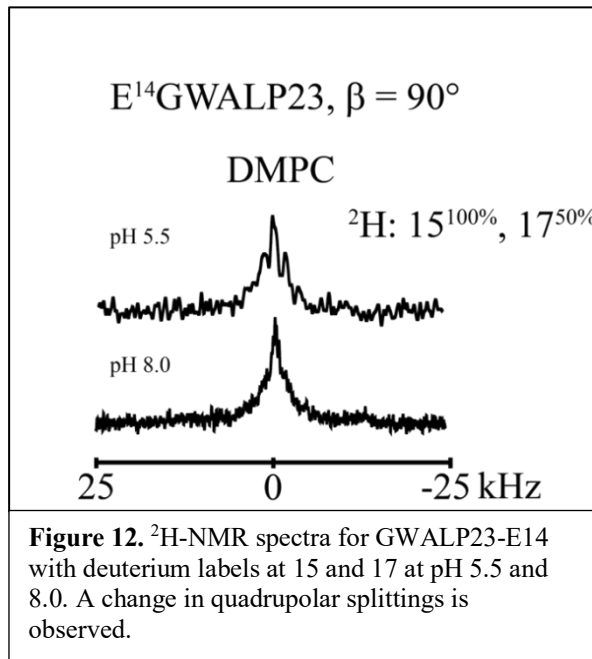
GWALP23-E14 were analyzed through this method, the lipid-only samples as 10% DMTAP, 10% DMPG and DMPC and the lipid-peptide samples as 10% DMTAP, 10% DMPG, 5% DMTAP, 5% DMPG and DMPC. These were conducted at a pH of 6.1. The lipid-only samples are demonstrated in Figure 9 and the lipid-peptide samples in Figure 10. Each spectrum demonstrates proper bilayer alignment, with major well-defined peaks for the major DMPC component, along with small shoulders for the minor lipid components.



^2H -NMR Spectroscopy was also used to examine the oriented samples. This data reveals peaks resulting from the 50% and 100% deuterium labelled alanines in the peptide that can be measured to determine the quadrupolar splittings. This data can be used to determine if there is a change in position and orientation in the lipid bilayer based on the lipids and pH variation. Experiments were focused on characterizing GWALP23-E14 at a pH of 6.1 with deuterium labels at 13 and 15 so that further work could be carried out across a range of pH levels. It was found that there was no change in quadrupolar splittings for all lipids, at 16.43 kHz and 6.88 kHz, except for 10% DMPG samples, at 15.06 kHz and 5.46 kHz. The results are depicted in Figure 11. The spectra for these samples using DMPC bilayers showed much improvement from the noisy spectra obtained in previous experimentation of GWALP23-E14 in DLPC, another neutral lipid, shown in Figure 1⁸.



Additional characterization of GWALP23-E14 was conducted across a range of pH levels and analyzed using ^2H -NMR spectroscopy. Analysis of GWALP23-E14 with 15 and 17 deuterium labels and 13 and 15 deuterium labels was conducted. Preliminary experiments focused on the 15 and 17 labels at pH 5.5 and 8.0 in 100% DMPC bilayers. A clear



change in quadrupolar splittings was observed, as shown in Figure 12. Further experimentation

was focused on characterizing these changes in 10% charged lipid bilayers, of DMTAP and

DMPG, and carried out from pH 3.0 to 8.0.

This revealed that no change was observed

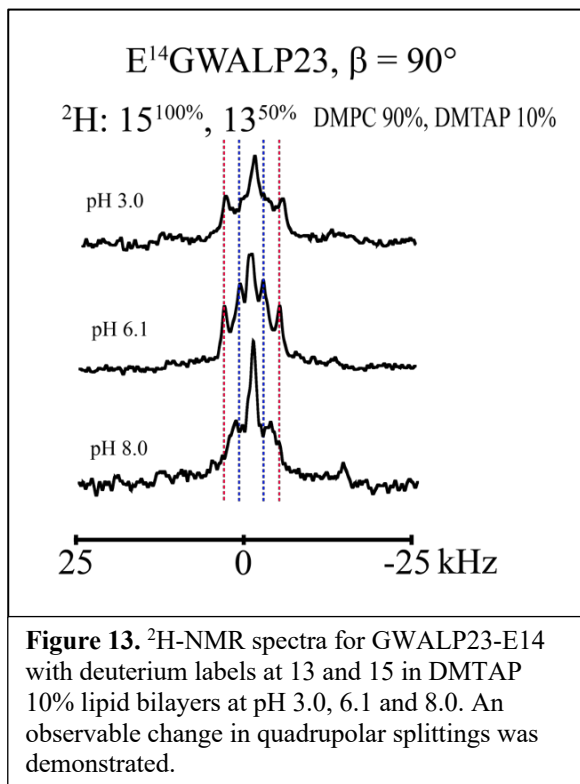
for DMPG bilayers across a range of pH,

but there was a change in quadrupolar

splittings, indicating titration, for DMTAP

bilayers. This change is highlighted in

Figure 13.



Final experiments were underway using GWALP23-E14 with deuterium labels at 3 and 21 as well as 9 and 11, when the laboratory was shut down due to COVID-19 outbreak. These samples have been preserved, awaiting hydration, and analysis in solid-state ^2H -NMR and ^{31}P -NMR spectroscopy.

Discussion

The secondary structure of GWALP23-E14 was determined to be α -helical based on results of Circular Dichroism (CD) spectroscopy. CD is a method of absorption spectroscopy that measures the difference between right and left circularly polarized light¹⁵. Different structures give characteristic spectral patterns. A key feature of α -helical structure is two minimum peaks at around 208 nm and 222 nm. These peaks were observed for the peptide of interest in all bilayers; therefore, the conclusion can be made that the peptide adopts an α -helical formation in these bilayers as expected. Additionally, the positioning of tryptophan residues was analyzed using fluorescence spectroscopy. The residues were confirmed to be at the peptide-lipid interface based on their maxima located at or near 333 nm in all bilayers. This wavelength is characteristic of this interface. Maxima above 333 nm indicate a more hydrophilic position, and below 333 nm a more hydrophobic position in the membrane¹⁶.

Upon confirmation of these characteristics, the peptide was analyzed using ³¹P-NMR spectroscopy, to confirm bilayer alignment. The results produced confirm this alignment through the presentation of single, established peaks, as shown in Figures 9 and 10. With the lipid-peptide samples containing 5% DMPG, 10% DMPG and 100% DMPC, a shoulder was introduced. While a shoulder is expected from a minor component in a lipid mixture, it is not so clear why there is a shoulder for the 100% DMPC, unless perhaps the peptide is not distributed evenly within the mixture in some samples. After this determination and results of initial ²H-NMR spectra, all experiments were conducted using 10% of charged lipid and 90% DMPC or 100% DMPC. This percentage was selected because experiments with 20% charged lipid did not form proper bilayers. Charged lipids in the amount of 10% was selected over 5% for further

experimentation because it more closely resembles the complex composition of cellular lipid bilayers which contain many charged lipids.

Several experiments were carried out to characterize peptide behavior in response to change in lipid charge, charge lipid concentration and pH variation by ^2H -NMR spectroscopy. The resulting spectra for 10% DMTAP, 5% DMTAP, 5% DMPG and 100% DMPC showed no change in quadrupolar splittings based on change in charged lipid concentration and charge type. The splittings for these spectra were measured at peak distances of 16.4 kHz, representing 50% deuterated alanine at position 13 and 6.9 kHz, representing 100% deuterated alanine at position 15. The pH in this analysis was held constant for all experiments at 6.1. Interestingly, the 10% DMPG spectra demonstrated a change in the peak distances with splittings measured at 15.1 kHz, representing 50% deuterated alanine at position 13, and 5.5 kHz, representing 100% deuterated alanine at position 15. These changes can be attributed to changes in the helix orientation when DMPG is present.

An additional focus in the characterization of GWALP23-E14, was the determination of titratability of the glutamic acid side chain in the charged lipid bilayers. Glutamic acid alone, has a side chain pK_a of around 4.25 and in GWALP23-E14 the pK_a has been determined to be high based on the effects of neighboring residues and insertion in a bilayer system. The titration potential of glutamic acid is the reason for a glutamine residue to be used for comparison in preliminary experimentation in GWALP23-Q14, as glutamine does not contain a titratable R-group⁸. ^2H -NMR spectra of GWALP23-E14 in DMPC only shows a change in quadrupolar splittings from pH 5.5 to 8.0. ^2H -NMR spectra of GWALP23-E14 in 10% DMPG bilayers demonstrated no change in splittings and in 10% DMTAP a change was observed in splittings from experiments conducted in a pH range of 3.0 to 8.0. These changes indicate that the peptide

is titrating. More experimentation was conducted to gather spectra for pH values above 8.0 and below 3.0 in an effort to examine the titration curve of the peptide; however, no clearly resolved spectra were obtained from the initial samples. Additional experiments will be needed. Notably, it is known and documented to be difficult to prepare oriented lipid samples with peptides that contain glutamic acid⁸.

Final analyses of GWALP23-E14 were to be conducted through experiments at pH 6.1 in 10% DMTAP and 10% DMPG using deuterated alanine labels at 9 and 11 as well as 3 and 21. These peptides were synthesized and partially prepared according to the outlined oriented sample preparation. The prepared samples were under vacuum for 48 hours awaiting hydration, when due to the outbreak of COVID-19, experimentation had to be halted as the laboratory was shut down. The samples were preserved for future use and are only a few days of experimentation away from achieving results. These results would have served to perform a GALA data analysis, geometric analysis of labeled alanines, in order to examine the peptide helix orientation in these bilayers and the end fraying of alanines 3 and 21. From such analysis, helical dynamics, tilt and rotation would have been able to be examined to view a full picture of peptide behavior, based on the quadrupolar splittings to form a quadrupolar wave plot. The plan from this point will be to complete this study in future work¹⁷.

Conclusions

The experiment was successful in synthesizing the proper peptide, GWALP23-E14, as confirmed by CD spectroscopy, Fluorescence spectroscopy and MALDI-TOF mass spectrometry, and successful in the analysis of the peptide in various charged lipid bilayers and across a range of pH values. GWALP23-E14 was confirmed to be α -helical and was verified that its insertion in a lipid membrane is anchored by tryptophan residues at positions 5 and 19. GWALP23-E14 does not show any change in behavior in response to pH variation in 10% DMPG but does show titratability in 10% DMTAP and 100% DMPC.

The peaks in the ^2H NMR spectra for labeled GWALP23-E14 in DMPC are quite broad and poorly resolved. Inclusion of 10% DMPG alters the ^2H quadrupolar splittings only slightly. Inclusion of 10% DMTAP notably improves the ^2H spectral resolution without changing the quadrupolar splittings at pH 6.1. The spectral improvement observed with DMTAP will offer technical advantages for future experiments.

It was also determined that the peptide GWALP23-E14 introduces a shoulder in the ^{31}P NMR spectra of 100% DMPC and DMPG bilayers, but not when DMTAP is present. The minor shoulders in some of the spectra are not yet completely understood.

Finally, experimentation to complete a GALA analysis of peptide orientation was close to completion, before laboratory shutdown¹⁷. This analysis would present data about the rotation and tilt of the peptide in response to these variables, offering a complete view of its behavior in the membrane system created. Of particular interest will be the complete results when 10% DMTAP is present with the peptide helix in bilayer membranes of DMPC.

Future work should aim to complete the characterization of this glutamic acid-containing peptide through a GALA analysis and investigate the use of other lipid systems in order to

characterize the titration of this peptide at more extreme pH levels. Additionally, an approach to further characterize tryptophan position in the bilayer would be to add quenchers in order to specifically measure the Trp depth in the membrane¹⁸. This could be performed to further understand peptide helix behavior, orientation in the bilayer and the influence of glutamic acid. The overall results will be important for understanding glutamic acid residues in membrane proteins.

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