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Alexia Prokopik April 6, 2017

Characterization of Influenza Hemagglutinin Fusion Peptide and Transmembrane Domain Membrane Insertion

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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This project aimed to characterize the H1N1 fusion peptide and transmembrane domain in a model membrane environment. Influenza hemagglutinin is a necessary mediator for the membrane fusion step of the viral replication cycle, but many aspects of this mechanism are still unresolved. Though previously thought to be a passive anchor of hemagglutinin, the transmembrane domain is now thought to have a dynamic interaction with the fusion peptide to induce a hemifusion state in the host and viral membranes, ultimately leading to a fusion pore. The work in this thesis sought to characterize, using fluorescence emission, equilibrium Fourier transform infrared, and circular dichroism, each of these peptides individually in a model membrane system to understand how they interact with vesicles, neglecting the rest of the protein. It was demonstrated that the fusion peptide inserts into the vesicles at the melting temperature of the membranes, where the gel to fluid phase transition occurs. As the temperature increased, the peptide exited the membrane, forming aggregates and unfolding from the alpha-helical structure it adopted in the membrane. As for the transmembrane domain, it is still in the early stages of being characterized. However, it was demonstrated that the transmembrane domain could associate with the model membrane system and folds from a beta-hairpin structure to the expected alpha-helical conformation as reported in the literature. In the future, quenching experiments with the transmembrane domain will reveal its orientation within the membrane, and eventually lead to the characterization and dynamic study of the fusion peptide and transmembrane domain together in a model membrane system.

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Chapter 1: An Introduction to Membrane Proteins and Viral Fusion

1.1 Introduction

1.1.1 Membrane Protein Folding and Insertion

Although it has been 45 years since Singer and Nicolson devised the fluid mosaic model in 1972, it still remains the core of modern membrane theory. This model proposes the organization and function of the phospholipid bilayer and associated proteins.[1] These proteins include peripheral membrane proteins, which protrude from the membrane surface, and integral membrane proteins that span across the lipid bilayer. The fluid mosaic model is crucial in establishing the model of a lipid bilayer and giving an introduction to the study of membrane protein folding and interactions. With the passing of time, new layers of complexity and understanding have been added to this model. Rather than the notion that monomers of proteins float along the vast expanse of bilayer surface, it is generally understood today that proteins have preferential associations within the crowded environment, which affect the membrane thickness and fluidity.[2] In addition, lipid rafts and extracellular complexes also influence the structure and dynamics of membranes.[3] Overall since the fluid mosaic model was first proposed, there have been many adaptations and new levels of understanding that have come with the years of studying the membrane-protein interactions and how they affect membrane function and organization. These protein-lipid and protein-protein interactions are an integral part of processes such as cell signaling and membrane trafficking.[4] However, mechanisms of how proteins recruit to and interact with cellular membranes are poorly understood.

Protein folding, insertion, and aggregation in the membrane and membrane-water interface are important processes in cell signaling, protein trafficking, viral infection, etc. In comparison to soluble counterparts, the folding of membrane proteins has been vastly understudied because of the inherent difficulty of studying these interactions. Most membrane

proteins must undergo structural rearrangements within the membrane environment to properly fold and function.[5] One of the first major breakthroughs in this field came from Henderson and colleagues in 1975, who determined the structure of the seven alpha-helix bundle of bacteriorhodopsin. Bacteriorhodopsin is an archaebacterial protein that serves to pump protons across a membrane with captured light energy, and spans the membrane which was resolved via electron microscopy.[6] Later, in 1987, it was discovered this protein's folding patterns could be studied in vitro, with its unfolding and refolding experiments interpreted as a two-state folding mechanism.[7] The two-state model is an early understanding of protein folding, where proteins first fold into individual helices, then there is a stage of intramembrane helix organization.[7] However, the study of bacteriorhodopsin later led to the development that protein folding is associated with membrane order. Booth and colleagues investigated the lipid-based refolding system of bacteriorhodopsin, and found that membrane composition, such as lipid rigidity or length, is correlated with membrane curvature, directly effecting protein folding due to the force the lipids produce.[8]

Protein insertion into a lipid bilayer is closely related to membrane protein folding, and the mechanisms through which these proteins enter a membrane are poorly resolved. However, this protein-membrane interaction is essential for several host-pathogen interactions, including toxins, antimicrobial peptides, and viral fusion proteins.[9, 10, 11] The dynamics and mechanism of how these proteins insert into a membrane are yet to be understood and require further study since viral transmission depends on these types of protein-membrane interactions.

1.1.2 Viral Infection and Membrane Fusion

In the 2015-2016 flu season, there were 5.1 million illnesses, 2.5 million medical visits, and 71,000 hospitalizations due to the influenza virus.[12] Influenza is classified into three types,

influenza A, influenza B, and influenza C. Influenza A virus encompasses 16 hemagglutinin and 9 neuraminidase subtypes, all of which have been found to infect avian species. Certain subtypes have been established in pigs, horses and humans, as well. Currently, the H1N1 and H3N2 subtypes are circulating in the human population.[13] With the widespread illness and pandemics influenza has caused the human population over the years, the study of how viruses propagate and transmit is crucial to lessening the burden of influenza.

During the lifecycle of influenza, hemagglutinin (HA), which is one of the proteins on the viral surface, initiates infection by binding to the host membrane sialic acid receptor, which triggers uptake of the viral particle into an endosome. As the pH of the endosome acidifies, hemagglutinin undergoes a large conformational change and induces membrane fusion, so the viral RNA can be transferred into the cell for protein synthesis and viral budding (Figure 1).[14]

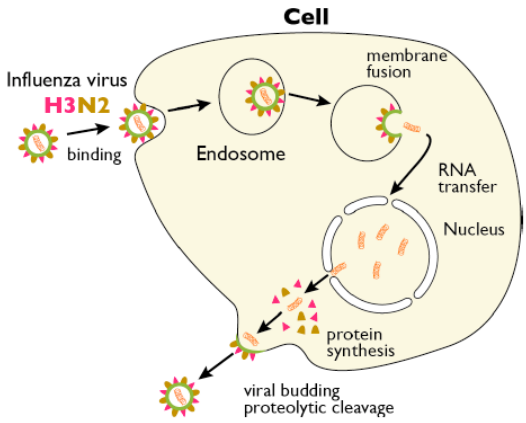

Figure 1.1. H3N2 influenza replication cycle. Reprinted from ref. 14. Copyright 2012.

1.1.3 Influenza Hemagglutinin Structure and Role in Membrane Fusion

A critical step to viral infection is the membrane fusion step in the influenza lifecycle, which is mediated by influenza HA. The structural changes HA undergoes during this process have been determined by NMR and crystallography studies, leading to the distinction between the two

subunits of HA. Upon cleavage between the two subunits, the HA_1 domain is responsible for receptor binding and the HA_2 subunit is considered the fusion domain (Figure 2).

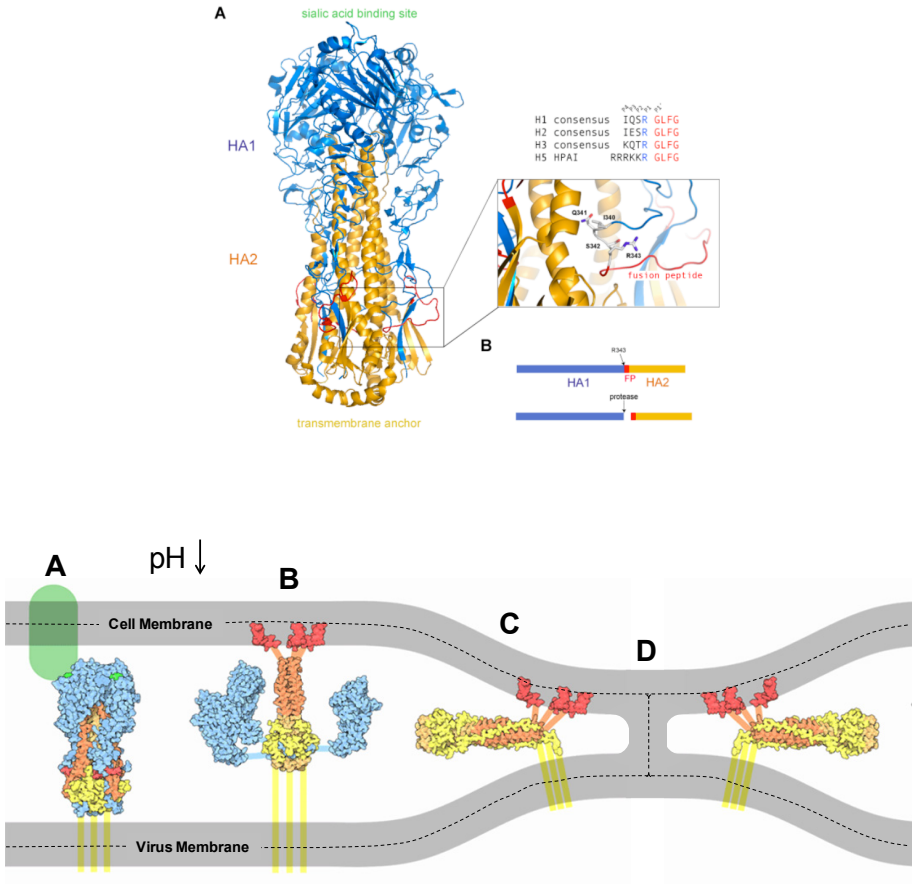

Figure 1.2. Influenza hemagglutinin trimer structure before and after cleavage. Top: Structure of HA precursor before cleavage. Bottom: (A) The moiety that contains the sialic acid group on the cell membrane (green) is where HA_1 (blue) binds. (B) Endosomal pH acidifies and triggers the conformation change of HA_2 that exposes the fusion peptide (red) to the cell membrane. (C) Conformational changes bring the membranes together to form a stalk. (D) It is hypothesized that several HA_2 work together. Reprinted from ref. 14. Copyright 2012.

Cleavage takes place at the C-terminal end, where the newly formed N-terminus of HA_2 begins the fusion peptide (FP). Upon uptake into an endosome, the pH is dropped leading to the extensive conformational change of HA. This exposes the fusion peptides, which insert into the host endosomal membrane. Further irreversible conformational rearrangements bring the transmembrane domain (TMD) and fusion peptide into close proximity and a pore is formed.[14] It is hypothesized that interactions of the fusion peptide and transmembrane domain facilitate pore formation, but little is known about structure and dynamics of this process. Understanding the dynamics and mechanism of fusion pore formation is critical, because it is the last threshold before the virus is able to hijack the cell for replication.

1.1.4 Fusion Peptide

The fusion peptide begins at the newly formed N-terminus of the influenza HA_2 subunit, and is a conserved 23-residue domain. The conformation of the fusion domain is pH dependent, and at high pH the peptide exhibits a helical hairpin arrangement that favors insertion into a membrane environment (Figure 1.3).[15] In the membrane, the large hydrophobic residues on both sides of the kink region of the fusion peptide fix the angle of a boomerang structure and stabilize the fusion activity of this critical domain (Figure 1.4).[16]

The fusion peptide is thought to be a major contributor to the initial stages of membrane fusion in the influenza replication cycle. Its boomerang fusion active structure is hypothesized to stabilize the fusion stalk of a membrane, by enhancing negative membrane curvature with its bulky hydrophobic groups and small hydrophilic head region.[17] Once the endosome acidifies, the soluble ectodomains, the heptad repeat regions, bring the fusion peptide into close proximity to interact with the transmembrane domain.

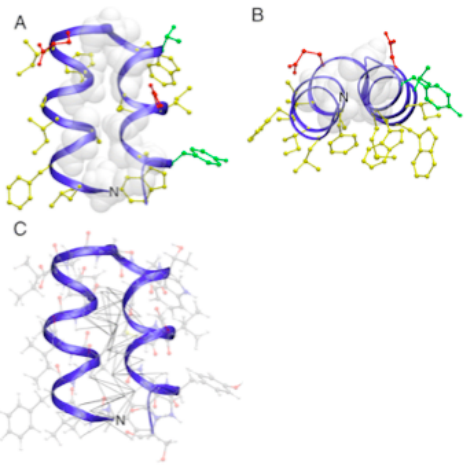

Figure 1.3. H1N1 fusion peptide helical hairpin structure with indicated hydrophobic side chains (yellow), acidic side chains (red), and polar side chains (green). H1N1 sequence: GLFGAIAGFIEGGWTGMIDGWYG Reprinted from ref. 15. Copyright 2010 National Academy of Sciences.

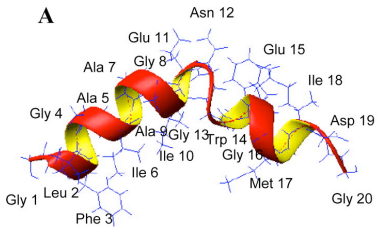

Figure 1.4. H1N1 fusion peptide boomerang structure, fusion active. Reprinted from ref. 16. Copyright 2007.

1.1.5 Transmembrane Domain

Early views of the transmembrane domain consider it as a passive alpha-helical membrane anchor during the hemagglutinin-mediated membrane fusion.[18] The transmembrane domain orients within a membrane, with the C-terminus extending through the membrane and N-terminus connecting to the soluble ectodomain (Figure 1.5).[19] Yao et. al. report the transmembrane domain exhibits membrane-dependent conformations, and alpha-helical structure

is prominent in phosphocholine and phosphoglycerol membranes which is most relevant to actual viral membranes, whereas beta-strand conformation dominate in phosphoethanolamine membranes. The transmembrane domain, like the fusion peptide, effects the membrane curvature and is a crucial mediator in virus entry through changes induced in the membrane topology.[18]

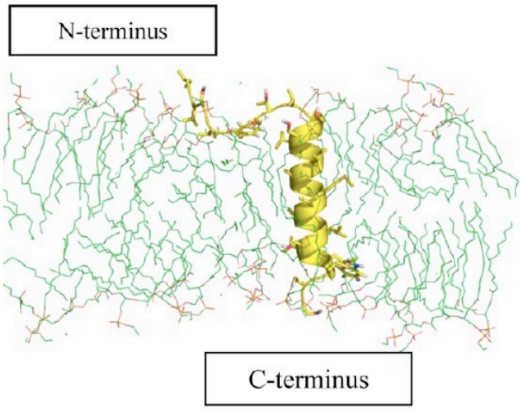

Figure 1.5. Transmembrane domain structure within a membrane. Reprinted from with permission from ref. 19. Copyright 2012 American Chemical Society.

1.1.6 The Interaction Between the Fusion Peptide and Transmembrane Domain

As previously mentioned, the heptad repeat regions bring the fusion peptide into close proximity to interact the transmembrane domain in the fusogenic state. Although the transmembrane domain was once thought to be a passive anchor to the viral membrane, recent studies of hemagglutinin have concluded it actively partakes in membrane fusion and interacts with the fusion peptide within a membrane environment. Chang and colleagues determined that the transmembrane domain forms a complex with multiple fusion peptides, allowing the fusion peptide to bury further within the membrane and aligning the inserted N-terminus of the fusion peptide with the C-terminus of the transmembrane domain. Once the fusion peptide inserts into the endomsomal membrane, the heptad repeat region pulls the viral membrane in proximity for

the hemifusion intermediate to occur and interaction with the transmembrane domain (Figure 1.6).[20]

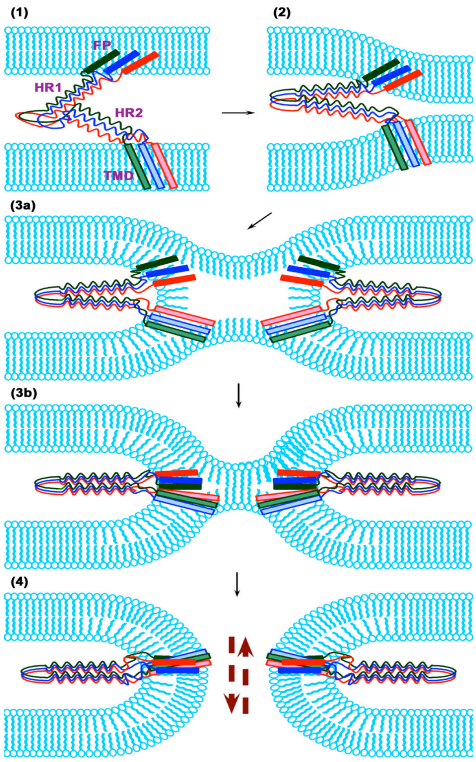

Figure 1.6. Role of FP and TMD in membrane fusion. (1) FP inserts into target membrane. (2) The low endosomal pH prompts HR1 and HR2 to refold and bring the membranes together into close proximity. (3) TMD and FP form a complex causing hemifusion between the membranes and deepening FP insertion. (4)The hemifusion diaphragm transits to a pore, stabilized by the TMD-FP complex. Reprinted from ref. 20. Copyright 2008.

Two main points that are essential for membrane fusion are first, the membranes must be brought together in close proximity and second, they must be destabilized.[21] In the case of hemagglutinin, the fusion peptide and the transmembrane domain are integral parts of mediating this process. The mechanism of fusion, effect on membrane order, and role of the FP-TMD

complex has many unanswered questions that need to be resolved for a better understanding of influenza infection.

1.2 Conclusions and Aims

Although the fusion peptide and transmembrane domain have been extensively studied to determine the interaction and structure, there are still many aspects to their functional relationship yet to be resolved. Specifically, given the dynamic nature of hemagglutinin, there is a lack of time-resolved kinetics studies of fusion peptide insertion and FP-TMD complex formation. This system is particularly difficult to study, based on the hydrophobic nature of both peptides and the optical scattering of the membranes they reside in. In addition, studying the peptides alone brings up a dilemma of what orientation they adopt within the membrane, and if this coincides with previous reports. Though it was originally thought that the transmembrane domain is an inactive anchor, its effects on membrane curvature and interaction with the fusion peptide are critical to membrane fusion and propagation of the influenza virus. These peptide-peptide and peptide-lipid interactions are important to study not only in terms of viral pathogenicity, but also for similar membrane protein systems.

Before investigating the dynamic interaction between the fusion peptide and transmembrane domain, it is necessary to characterize both the fusion peptide and transmembrane and fusion peptide in DPPC vesicles independently to understand how they each behave in a model membrane system. Also, studying these peptides alone raises the question, what orientation do they adopt in the membrane? Determining the characteristics of membrane insertion and orientation are necessary to further studies of the FP-TMD complex and influenza infection.

1.3 References

1. Singer, S. J.; Nicolson, G. L.The fluid mosaic model of the structure of cell membranes. *Science*, **1972**, *175*(4023), 720-31.
2. Engelman, D. M. Introduction Membranes are more mosaic than fluid. *Nature,* **2005**, *438*, 578-580.
3. Nicolson, G. L. The Fluid—Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40 years. *Biochim. Biophys. Acta: biomembranes*, **2014**, *1838* (6), 1451-1466.
4. Cho, W. H.; Stahelin, R. V. Membrane-Protein Interactions in Cell Signaling and Membrane Trafficking. *Annu. Rev. Biophys. Biomol. Struct*, **2005**, *34*, 119−151.
5. Bowie, J. U. Solving the membrane protein folding problem. *Nature,* **2005**, *438*, 581-589.
6. Henderson, R.; Unwin, P. N. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature,* **1975**, *257*, 28–32.
7. Popt, J. L.; Gerchman, S. L.; Engelman, D. M. Refolding of bacteriorhodopsin in lipid bilayers: A thermodynamically controlled two-stage process. *J. Mol. Biol*. **1987**, *198*(4), 655–676.
8. Booth, P. J.; Riley, M. L.; Flitsch, S. L.; Templer, R. H.; Farooq, A.; Curran, A. R.; Chadborn, N.; Wright, P. Evidence that bilayer bending rigidity affects membrane protein folding. *Biochemistry*. **1997**, *36*(1), 197-203.
9. Song L.; Hobaugh M. R.; Shustak C.; Cheley S.; Bayley H.; Gouaux J. E. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science,* **1996**, *274*, 1859-1866.

10. Gouaux, E. Channel-forming toxins: tales of transformation. *Curr. Opin. Struct. Biol.* **1997**, *7*, 566-573.

11. Martens, S.; McMahon, H. T. Mechanisms of Membrane Fusion: Disparate Players and Common Principles. *Nat. Rev. Mol. Cell Biol*. **2008**, *9*, 543−556.

12. Centers for Disease Control and Prevention. Estimated Influenza Illnesses and Hospitalizations Averted by Vaccination — United States, 2014–15 Influenza Season. http://www.cdc.gov/flu/about/disease/2015-16.htm (accessed March 31, 2017).

13. World Health Organization. Influenza. http://www.who.int/biologicals/vaccines/influenza/en/ (accessed March 31, 2017).

14. Hamilton, B. S.; Whittaker, G. R.; Daniel, S. Influenza Virus-Mediated Membrane Fusion: Determinants of Hemagglutinin Fusogenic Activity and Experimental Approaches for Assessing Virus Fusion. *Viruses,* **2012**, *4*, 1144-1168.

15. Lorieau J.L.; Louis J.M.; Bax A. The complete influenza hemagglutinin fusion domain adopts a tight helical hairpin arrangement at the lipid:water interface. *Proc. Nat'l. Acad. Sci. USA*. **2010**, *107*, 11341-11346.

16. Lai, A. L.; Tamm, L. K., Locking the Kink in the Influenza Hemagglutinin Fusion Domain Structure. *J. Biol. Chem.*, **2007**, 282(33), 23946-23956.

17. Smrt, S. T.; Draney, A. W.; Lorieau, J. L. The influenza hemagglutinin fusion domain is an amphipathic helical hairpin that functions by inducing membrane curvature. *J. Biol. Chem.*, **2015**, *290*(1), 228-238.

18. Yao, H.; Lee, M. W.; Waring, A. J.; Wong, G. C.; Hong, M. Viral fusion protein transmembrane domain adopts beta strand structure to facilitate membrane topological changes for virus cell fusion. *Proc. Natl. Acad. Sci. U.S.A.*, **2015**, *112*(35), 10926-10931.

19. Bruno V. L.; Baptista A. M.; Soares C. M. Structural determinants for the membrane insertion of the transmembrane peptide of hemagglutinin from influenza virus. *Journal of Chemical Information and Modeling,* **2012**, *52*(11), 3001-3012.

20. Chang, D. K.; Cheng, S. F.; Kantchev, E. A.; Lin, C. H.; Liu, Y. T. Membrane interaction and structure of the transmembrane domain of influenza hemagglutinin and its fusion peptide complex. *BMC biology*, **2008**, *6*, 2.

21. Martens, S.; McMahon, H. T. Mechanisms of Membrane Fusion: Disparate Players and Common Principles. *Nat. Rev. Mol. Cell Biol*. **2008**, *9*, 543−556.

Chapter 2: Characterization of Fusion Peptide Insertion into Vesicles

2.1 Introduction

The aim of this chapter is to characterize the fusion peptide interaction with model DPPC vesicles using a variety of methods. This characterization is necessary to determine how the FP-TMD complex compares to the fusion peptide or transmembrane domain alone. Also, it is important to determine whether this model system is consistent with previously reported studies of this system as outlined in the introduction.

For the experimental approach, taking advantage of the temperature-dependent lipid fluidity with the gel to fluid phase transition will be a way to control peptide insertion into the membrane. Lipid membranes have four distinct phases increasing in permeability: crystal, gel, ripple, and fluid that transition with increasing temperature. At higher temperatures, there are more kinks in the lipid chains, which causes a more disordered membrane that is more permeable.[1] To study how the fusion peptide will associate with the membrane and closely monitor its insertion, this thermal initiation of gel to fluid phase, ordered to disordered, will be utilized with unilamellar DPPC lipid vesicles. These lipids were chosen because at room temperature they exist in the gel phase, since they have the high melting temperature of 41°C allowing for a trigger for insertion.[2]

2.2 Materials and Methods

2.2.1 Peptide Synthesis and Purification

The H1 serotype of HA_2 fusion peptide with a solubility tag indicated by [] GLFGAIAGFIEGGWTGMIDGWYG[GDGKKKK] was synthesized on a rink amide resin by standard Fmoc solid phase peptide synthesis using an acid-coupling CEM Liberty 1 peptide synthesizer (CEM Matthew, NC). The Fmoc-protected amino acids were obtained from AnaSpec

Inc. (Fremont, CA). The purification of the solubilty tagged fusion peptide was performed via HPLC on a reverse phase C18 column (Phenomenex, Torrance, CA) using a linear gradient of water to acetonitrile, with 0.1% TFA. The purified peptide was confirmed by mass using MALDI mass spectrometry with a 1 mg/mL α-Cyano-4-hydroxycinnamic acid (CHCA) in a 50:50 mixture of water and acetonitrile with 0.1% TFA. MALDI-MS confirmed the mass of the peptide to be 3118 amu.

2.2.2 Lipid Vesicle Preparation

To prepare model membrane lipid vesicles, deuturated 16:0 d62-1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids, Inc. First, lipid cakes were prepared using a gastight syringe to transfer the lipids in chloroform to a 2 mL glass vial and dried with a steady stream of N_2 gas. The lipid cakes were then flash frozen with liquid nitrogen and lyophilized overnight to ensure the chloroform solvent was completely removed. From the lipid cakes, the small unilamellar vesicles (SUVs) were prepared by first hydrating with 20 mM pH 4 sodium acetate buffer, then heating on a hotplate at 54°C for one hour, above the T_m of 41°C. While heating, the sample was shaken and flash frozen in liquid nitrogen every ten minutes and returned to the hotplate. The SUVs were formed upon subsequent sonication when the cloudy lipid solution became transparent.

2.2.3 Fluorescence Emission

Equilibrium fluorescence data was obtained using a Dual-FL spectrophotometer (Horiba Scientific, Edison, New Jersey). To prepare the peptide and lipids, 75 μM of H1N1 fusion peptide was transferred to a 1 cm fluorescence cuvette with a 1:30 peptide:lipid molar ratio from a 30 mM DPPC stock and 20 mM pH 4 sodium acetate buffer. The instrument was set to collect data with the following parameters: an integration time of 0.1 seconds, 100 accumulations, a

total range of 285-550 nm, with a 3 nm excitation and emission slit width, and exciting the Trp at 280 nm.

2.2.4 Circular Dichroism

A JASCO J-8- spectrophotometer equipped with a PFD-425S Jasco temperature controller (Jasco, Inc., Easton, MD) was used to determine the peptide secondary structure. The instrument was set to collet data with a 190-260 nm scanning range, a 100 nm/min scan rate, a 2 second response time, and a 2 nm bandwidth. The peptide:lipid molar ratio was 1:30 with a 75 μM peptide concentration with 20 mM pH 4 sodium acetate buffer in a quartz cuvette with a 1 mm path length.

2.2.5 Equilibrium Fourier Transform Infrared

Temperature-dependent equilibrium fourier transform infrared (FTIR) results were collected on a Varian 3100 Excalibur FTIR spectrophotometer equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector. Instrument parameters were set to 134 scans, 2 cm^{-1} resolution, and 10-80°C temperature range with increments of 2°C. To measure the reference and sample, a split IR cell with CaF_2 windows separated by a 126 μM Teflon spacer in a copper frame was assembled. The reference was composed of 20 mM pH 4 sodium acetate buffer in D_2O, and the sample was composed of a 1 mM fusion peptide concentration with a 1:16 peptide:lipid molar ratio in 20 mM pH 4 sodium acetate buffer in D_2O. The temperature of the cell was controlled by a water bath and monitored via a thermocouple. To determine the absorbance spectra, the negative logarithm of the single beam spectrum was taken for the ratio of the sample side to the reference side of the split IR cell at each temperature.

2.3 Results and Discussion

2.3.1 Fluorescence Emission

In order to study and control fusion peptide insertion into model membranes, DPPC lipid SUVs were utilized for their gel to fluid phase transition at 41°C. Fluorescence emission is able to monitor the insertion of the fusion peptide Trp residues by exciting at 280 nm, because fluorescence spectroscopy can be used to detect changes in solvation of Trp residues elicited from changes in environment due to folding and/or oligomerization.[3] Figures 2.1 and 2.2 show a clear blue shift near the T_m of the lipid vesicles (Figure 2.2.B) and an increase in intensity (Figure 2.2.A), indicated that the Trp entered a more hydrophobic environment. The blue shift is characteristic of Trp sequestering from solvent water, as observed in protein folding.[3] This is attributed to the fusion peptide inserting and burying deep within the membrane. As temperatures increase past the T_m there is a subsequent red shift, indicating after the initial insertion of the fusion peptide at the melting temperature the peptide approaches the membrane surface and perhaps exits the membrane to the hydrophilic environment.

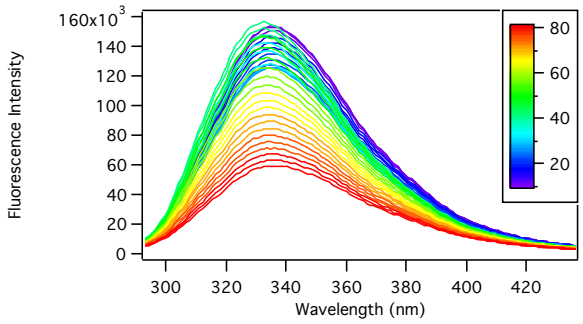

Figure 2.1. Temperature-dependent fluorescence emission of tryptophan excited at 280 nm in H1N1 fusion peptide with DPPC SUVs.

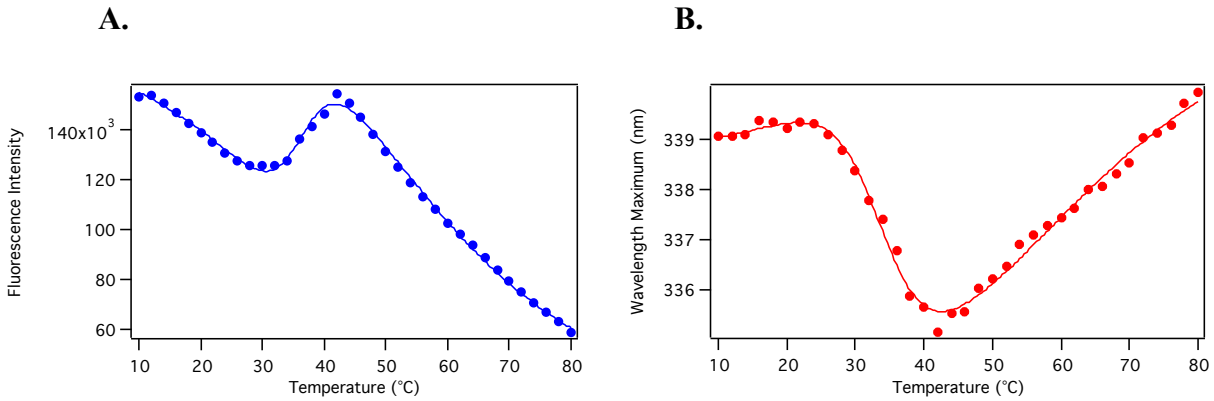

Figure 2.2. Temperature-dependent fluorescence of the fusion peptide in DPPC vesicles (A) Emission intensity maximum and (B) Wavelength shift of maximum emission. Both (A) and (B) are fit to a double sigmoid, showing two-state transitions of insertion and exiting the membrane.

2.3.2. Circular Dichroism

To characterize the secondary structure of the fusion peptide and determine the folding upon insertion into DPPC vesicles, circular dichroism was used. Figure 2.3 depicts the fusion peptide in buffer alone, then in buffer with DPPC vesicles both at 20°C. Surprisingly, in buffer alone, the fusion peptide displays some beta character rather than unfolded random coils. This can be explained by the intra-strand interaction of the bulky hydrophobic domains of the fusion peptide, leading to β-hairpin conformations. With the presence of DPPC vesicles, the fusion

peptide begins to adopt more α-helical structure, indicated by the negative peaks at 208 and 222 nm. Monitoring the 222 nm region which distinguishes the α-helical secondary structure, a temperature dependent melt was performed (Figure 2.4). There is a clear, sharp peak at 41°C, corresponding the melting temperature of the DPPC vesicles. Thus, this indicates when the vesicles transition from the gel to fluid phase, this allows the fusion peptide to insert and fold into the proper, expected α-helical conformation within the hydrophobic membrane environment. However, at higher temperatures the peptide unfolds from the α-helical conformation it adopts at the melting temperature, indicated by the increase of the 222 nm peak. This could indicate that the peptide is unfolded due to aggregate formation at higher temperatures, and that it is no longer deep within the hydrophobic interior at higher temperatures. Exposure to the hydrophilic outer environment near the lipid head groups likely contributes to the unfolding from its conformation in the hydrophobic inner core.

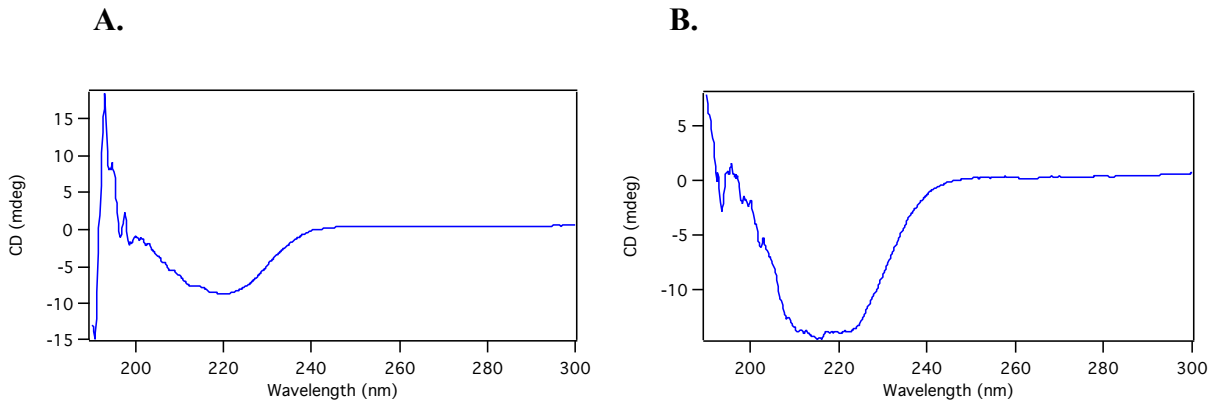

Figure 2.3. Circular dichroism experiments with H1N1 fusion peptide in (A) buffer without DPPC SUVs and (B) buffer with DPPC SUVs.

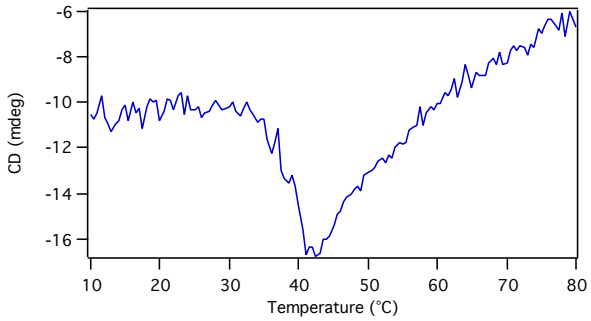

Figure 2.4. Circular dichroism temperature-dependent measurement of H1N1 fusion peptide and DPPC SUVs monitoring at 222 nm.

2.3.3. Equilibrium Fourier Transform Infrared

Infrared absorbance of the amide backbone of peptides is dependent on the dielectric environment. The following data depict the infrared absorption indicating where the fusion peptide transitions from a solvated helix at the more hydrophilic membrane surface to a buried helix upon insertion to the hydrophobic membrane environment. Figure 2.5 presents the raw temperature-dependent absorbance data for the CD_2 symmetric stretch of the hydrophobic core of DPPC membranes and the amide region of the peptide backbone.

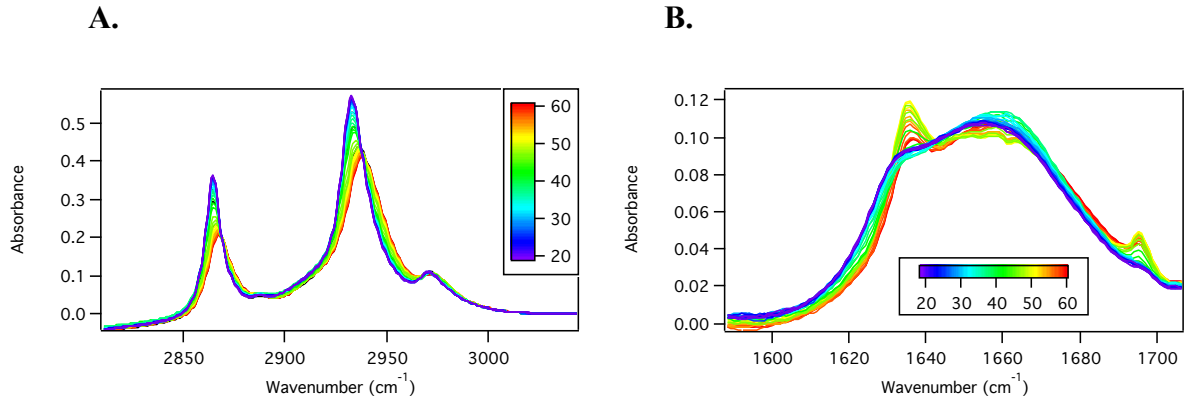

Figure 2.5. Temperature-dependent FTIR absorbance spectrum for H1N1 fusion peptide and DPPC vesicles highlighting the (A) CD_2 symmetric stretch region of the DPPC hydrophobic core and (B) amide region of the peptide backbone.

Figure 2.6 portrays the absorbance shift in both the CD_2 stretch and amide region of the peptide backbone. Looking at the CD_2 stretch region, the inflection point at 41°C indicates the transition from gel to fluid phase of the DPPC vesicles, the reported melting temperature. The absorbance shift for the amide region reveals a two-state transition with a large drop right at the T_m, indicating insertion of the fusion peptide, which will be further discussed in reference to the difference spectra.

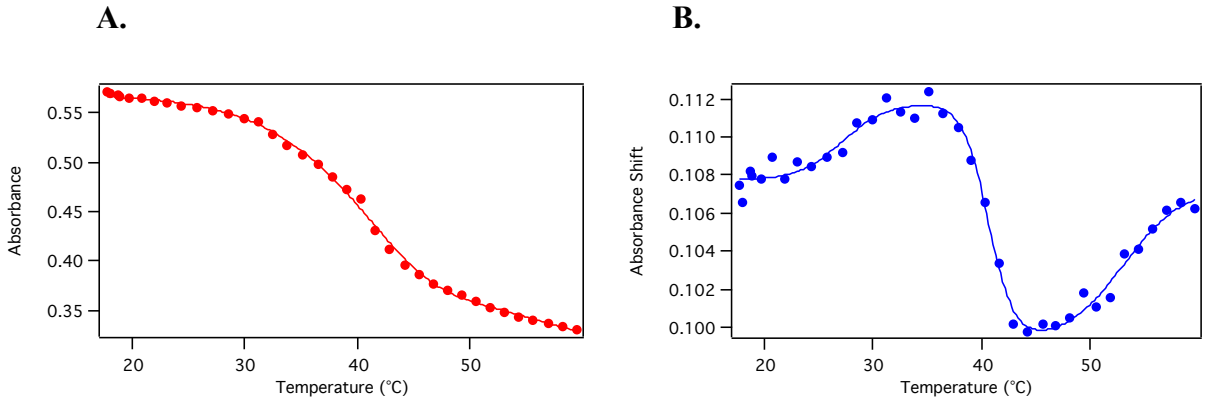

Figure 2.6. Thermal melt curves of (A) CD_2 stretch region and (B) amide region of the data in Figure 2.5. Both (A) and (B) are fit to a double sigmoid, showing a two-state transition.

In order to further explore how the peptide backbone responds to temperature changes in the presence of DPPC vesicles, difference spectra are generated by subtracting the lowest temperature spectrum of 20°C from each higher temperature spectrum. This yields difference spectra that reveal at the higher temperatures there is aggregation between the fusion peptides (Figure 2.7 A). As the temperature increases, the region ~1640 cm^{-1} to 1708 cm^{-1} increases intensity which corresponds to the formation of disordered regions of the peptide.[4] However, at lower temperatures around the melting temperature of the DPPC membranes, there is a bleach with a minimum centered at 1634 cm^{-1} and a maxima at 1655 cm^{-1}.

It has been reported that the 1634 cm^{-1} minimum corresponds to a solvated helix and the

1655 cm^{-1} corresponds to a buried helix, indicating the fusion peptide has inserted into the hydrophobic membrane core around the melting transition from gel to fluid phase.[4] It is important to note that the buried helix become more prominent approaching the melting temperature, consistent with insertion due to increasing membrane fluidity. In addition, this is an equilibrium process, hence there are both indications of the helix burying and solvating by entering and exiting the membrane. The aggregation at higher temperatures is consistent with the results presented in section *2.3.1* where the fusion peptide associates with the membrane at the T_m then exits the membrane or nears the surface of the membrane at high temperatures and forms subsequent peptide aggregates. This is also consistent with the circular dichroism studies, where the fusion peptide unfolds from the α-helix as temperature increases past the phase transition.

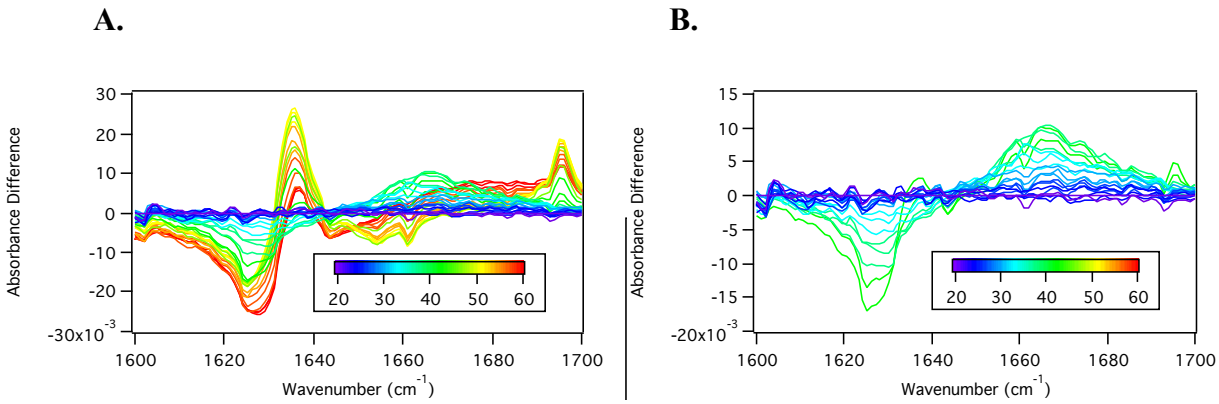

Figure 2.7. FTIR difference spectra for (A) 20-60°C and (B) temperatures around the T_m and below.

2.4 References

1. Nagarajan, S.; Schuler, E. E.; Ma, K.; Kindt, J. T.; Dyer, R. B., Dynamics of the gel to fluid phase transformation in unilamellar DPPC vesicles. *J. Phys. Chem. B,* **2012**, *116* (46), 13749-56.

2. Schubert, T.; Schneck, E.; Tanaka, M., First order melting transitions of highly ordered

dipalmitoyl phosphatidylcholine gel phase membranes in molecular dynamics simulations with atomistic detail. *J. Chem. Phys.*, **2011**, *135* (5), 055105.

3. Eftink, M. R.; Ghiron, C. A. Exposure of Tryptophanyl Residues and Protein Dynamics. *Biochemistr,y* **1977**, *16*, 5546−5551.

4. Brewer, S. H.; Tang, Y.; Vu, D. M.; Gnanakaran, S.; Raleigh, D. P.; Dyer, R. B. Temperature Dependence of Water Interactions with the Amide Carbonyls of Alpha-Helices. *Biochemistry,* **2012**, *51*, 5293−9.

Chapter 3: Transmembrane Domain Characterization Within a Vesicle

3.1 Introduction

Although it is has been previously assumed that the transmembrane domain does not have a role in membrane fusion, it has been proposed a dynamic interaction with the fusion peptide and effects membrane curvature.[1] However, this dynamic role has yet to be proven, hence the need to study the fusion peptide and transmembrane domain to determine their interaction and its effect on a membrane. In order to study this FP-TMD complex that allows for the hemifusion state of the viral and host membrane then subsequent formation of the fusion pore, the TMD's interaction with model DPPC membranes must be characterized, like the fusion peptide characterization in Chapter 3. However, the TMD primarily resides within the viral membrane with the N-terminus coinciding with the soluble ectodomain and the C-terminus within, close to spanning the bilayer.[2] Thus, it is necessary to study how the TMD behaves without the context of the full HA_2 domain and how it orients itself within a model membrane system.

3.2 Materials and Methods

3.2.1 Peptide Synthesis, Purification, and Labeling

The H1 serotype of HA_2 transmembrane domain with a solubility tag indicated by [] [KKKKKKKG]ILAIYSTVASSLVLLVSLGAI/C was synthesized via the same protocol in Section 2.2.1, and Cys modified at the C-terminus to allow for TAMRA-maleimide (Anaspec Inc.) to act as a label through an irreversible maleimide-sulfhydryl thioether coupling reaction specified by the manufacturer. The TAMRA labeled TMD was purified and confirmed with MALDI MS using the same procedure outlined in Section 2.2.1.

3.2.2 Lipid Vesicle Preparation

Lipid cake and vesicle preparation follow the same protocol as Section 2.2.2.

3.2.3 Fluorescence Emission

The equilibrium fluorescence measurements follow the same protocol as Section 2.2.3. In this case vesicles were prepared with 25 mM sodium acetate buffer and to monitor TAMRA coupled TMD emission, 555 nm was set as the excitation wavelength. Also, the vesicles, buffer, and TMD-TAMRA were cooled to 5°C before mixing and beginning measurements.

3.2.4 Circular Dichroism

The same procedure was followed as Section 2.2.4; however, using 25 mM pH 4 sodium acetate buffer.

3.3 Results and Discussion

The aim of this section is to begin to characterize the TMD behavior within a model membrane system to determine how the TMD is oriented within a membrane. Ideally, the TMD will have the solubility tag on the outside of the membrane, and the hydrophobic residues embedding into the membrane with the C-terminus inside the membrane as it naturally occurs in the influenza structure. We expect the insertion of the TMD to leave the solubility tag outside because it is charged and therefore will not penetrate the membrane. The TMD was labeled with TAMRA in order to characterize its behavior and perform quenching experiments using QSY 9, a known TAMRA quencher, in the future to pinpoint this orientation.

The first characterization method was fluorescence emission, because it was unknown how the TMD-TAMRA would behave, especially with the solubility tag. These characterizations will also be an important means of comparison for quenching experiments down the road. Figure 3.1 shows both the temperature-dependent fluorescence emission of TMD-TAMRA in the presence of DPPC vesicles and TMD-TAMRA alone in buffer. In Figure 3.2, the intensity shift

of TMD-TAMRA with DPPC vesicles shows a slight inflection point suggesting membrane association near the T_m. However, this is not as extreme a shift as the blue shift and intensity increase seen with the fusion peptide in Section 2.3.1. The intrinsic temperature-dependence of TMD-TAMRA, obtained from the fluorescence measurements of TMD-TAMRA alone in buffer without vesicles, was then subtracted from the TMD-TAMRA in the presence of vesicles. This yielded (C) in Figure 3.2, which corresponds to the fluorescence of TMD-TAMRA associated with the membrane, not the intrinsic fluorescence. If the peptide and fluorophore were not interacting, it would be expected that the fluorescence intensity would steadily decay. However, there is an intensity increase observed around the T_m of the membranes, indicating the TMD-TAMRA is inserting into the vesicles.

In addition, with the placement of the TAMRA label on the C-terminus, we expect the TAMRA might only transiently insert into the membrane. Once insertion and is complete and the TMD is spanning the membrane, the TAMRA label is likely on the interior of the SUV. This can be tested in the future by encapsulating a TAMRA quencher in the SUV interior.

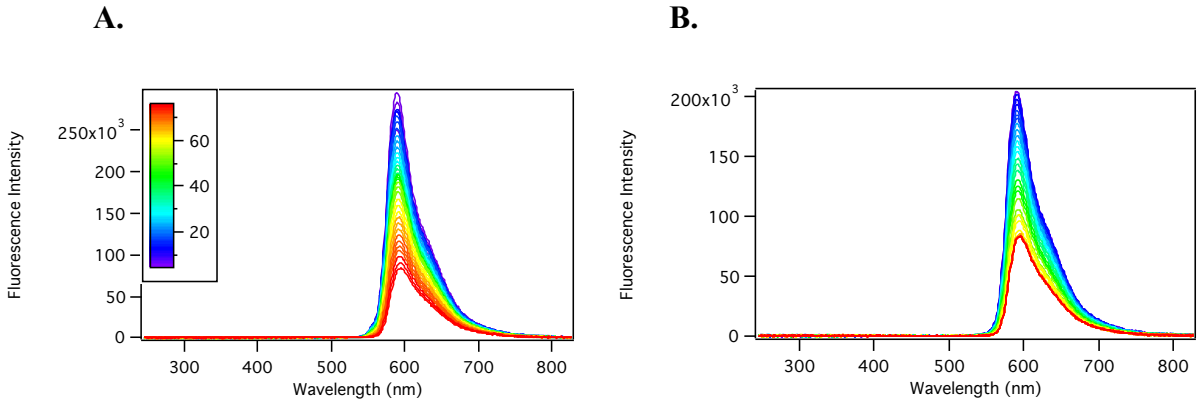

Figure 3.1. Temperature-dependent fluorescence emission of TMD-TAMRA excited at 555 nm (A) with DPPC SUVs and (B) without DPPC SUVs from 5-75°C.

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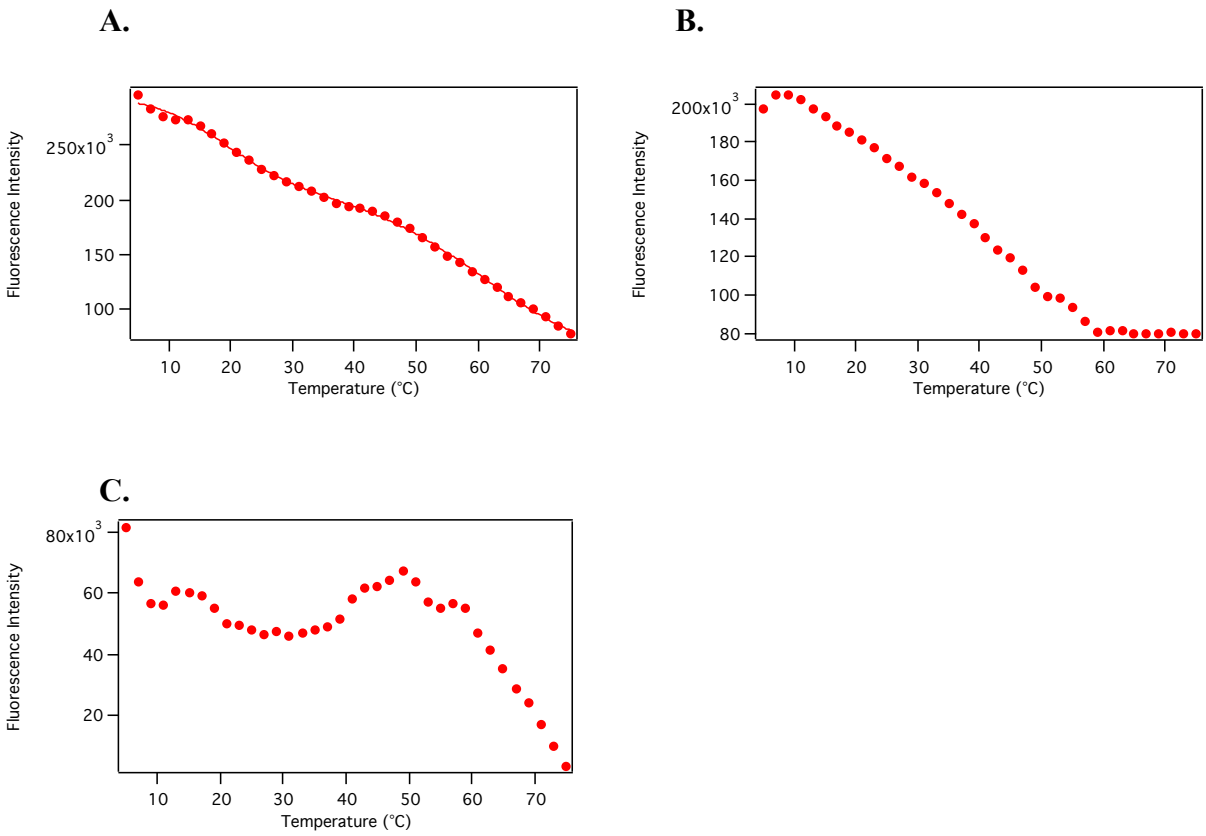

Figure 3.2. Temperature dependent fluorescence intensity shift for H1N1 TMD-TAMRA (A) in the prescence of SUVs and fit to a double sigmoid, (B) without SUVs, and (C) the subtraction of the intrinsic temperature-dependence of TAMRA.

Circular dichroism measurements were also run on TMD-TAMRA in the presence of DPPC vesicles (Figure 3.3). At room temperature, the TMD-TAMRA appears to have a β-hairpin structure. This can be attributed to the interaction of the hydrophobic residues of the TMD stabilizing the β-hairpin conformation, rather than interacting with the vesicles that are highly ordered in the gel phase. Increasing the temperature induced more α-helical character to the TMD-TAMRA, with a relatively sharp transition at the melting temperature. This indicates the TMD-TAMRA is inserting into the membrane and folding into the proper conformation. Unlike the fusion peptide however, it appears the TMD-TAMRA does not unfold from the α-helical structure as seen in Figure 2.4. This could suggest that once the TMD embeds into the

hydrophobic core of the membrane it remains folded within the bilayer, and does not desorb like the fusion peptide.

A. **B.**

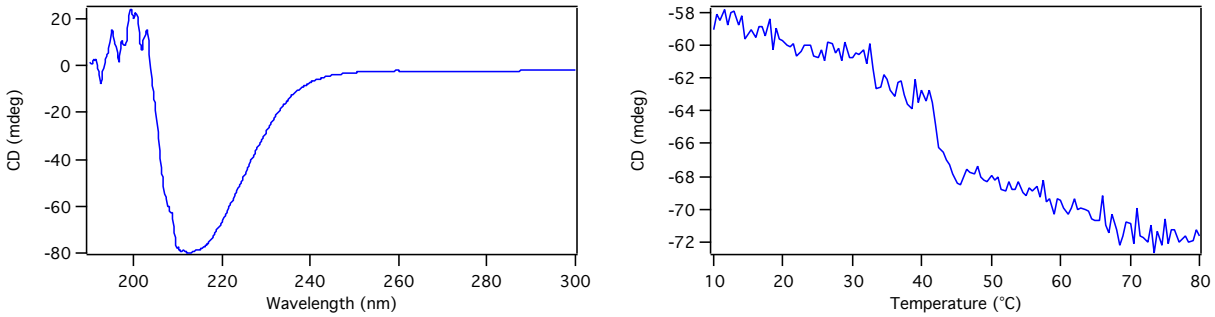

Figure 3.3. Circular dichroism measurements of (A) TMD-TAMRA with DPPC vesicles at 20°C and (B) thermal melt of TMD-TAMRA with DPPC vesicles monitoring at 222 nm.

3.4 References

1. Yao, H.; Lee, M. W.; Waring, A. J.; Wong, G. C.; Hong, M. Viral fusion protein transmembrane domain adopts beta strand structure to facilitate membrane topological changes for virus cell fusion. *Proc. Natl. Acad. Sci. U.S.A.*, **2015**, *112*(35), 10926-10931.

2. Bruno V. L.; Baptista A. M.; Soares C. M. Structural determinants for the membrane insertion of the transmembrane peptide of hemagglutinin from influenza virus. *Journal of Chemical Information and Modeling,* **2012**, *52*(11), 3001-3012.

Chapter 4: Conclusions and Perspectives

This thesis project aimed to show the characterization of H1N1 fusion peptide and transmembrane domain in a model membrane environment. Previous reports in the literature indicate influenza hemagglutinin is an integral mediator for membrane fusion, but many aspects of the mechanism are still unclear. Membrane proteins are vastly understudied compared to their soluble counterparts, due to inherent difficulties with the hydrophobic nature of the peptides and optical scattering of the membrane environment. However, these proteins are crucial for many biological functions, such as viral propagation, and understanding these protein-membrane interactions can elucidate how viruses infect their hosts.

Though previously thought to be a passive anchor of HA_2, the transmembrane domain has a dynamic interaction with the fusion peptide to induce a hemifusion state ultimately leading to a fusion pore. The work in this thesis sought to characterize each of these peptides individually in a model membrane system to understand how they interact with the vesicles out of context with the rest of the protein. For the fusion peptide, it was shown in Chapter 2 that the fusion peptide inserts into the vesicles at the melting temperature of the membranes where the gel to fluid phase transition occurs. As the temperature increased, the characterization methods showed the peptide exiting the membrane and forming aggregates and unfolding from the alpha-helical structure it adopted in the membrane. As for the transmembrane domain, it is still in its early stages of characterization because of its largely hydrophobic nature and difficulty to work with. However, Chapter 3 demonstrated the transmembrane domain can associate with the model membrane system and folds from a beta-hairpin structure to the expected alpha-helix as reported in the literature.

The immediate future directions of this project include further characterization of the transmembrane domain with the TAMRA label. Also, once this is achieved a quenching

experiment using QSY 9 can be performed to determine the orientation the transmembrane domain adopts within the bilayer. The idea behind this experiment is that a quencher will be in solution, or encapsulated within a liposome and the signal and quenching from insertion of the TAMRA labeled C-terminus transmembrane domain can indicate which direction the transmembrane domain associates with the membrane core. In addition, because insertion can be triggered by a temperature-dependent phase transition phase transition of the membrane, temperature-jump experiments can yield time-resolved kinetics of this insertion process. These kinetics studies are necessary to better understand the dynamic nature of the hemagglutinin mechanism. Further down the line, the interaction of the transmembrane domain and fusion peptide can be characterized and studied dynamically using FRET, attaching pairs according to the N-terminus to C-terminus alignment of the fusion peptide and transmembrane, respectively. These experiments will elucidate the dynamic interaction of these peptides critical for membrane fusion and infectivity of the influenza virus.