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Simulation of Membrane Proteins and Lipids:
Dynamic Behavior of a Small Antimicrobial Peptide

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Simulation of Membrane Proteins and Lipids: Dynamic Behavior of a Small Antimicrobial Peptide

By

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Abstract

Simulation of Membrane Proteins and Lipids: Dynamic Behavior of a Small Antimicrobial Peptide

By Keon A. Reid

Antimicrobial peptides (AMPs) are ubiquitous across multicellular systems. These small peptides are highly functional and can be easily modified. In recent decades, AMPs have been on the frontier of therapeutic advances. Particularly, these small proteins have now been used in studies that target cancer. Namely, SVS-1 is an antimicrobial hairpin peptide that was engineered to preferentially bind, fold and disrupt cancer membranes. SVS-1 is of particular interest because of the relatively high efficacy in which the peptide disrupts cancer cells in vitro. This thesis expands the understanding of this novel peptide design to decipher the ingredients necessary for its mode of action.

Atomistic molecular simulation conducted on the small peptide reveal the complex role electrostatics and lipid packing play on its mode of action. SVS-1 is a beta-hairpin AMP designed to selectively adsorb to cancer cells, fold at the surface and consequentially disrupt the cell membrane. *In vitro* studies conducted by Sinthuvanich et al. [*JACS*, 2012] demonstrate the selectivity of SVS-1 towards anionic cancerous cells versus normal mammalian cells. SVS-1 has 18 residues consisting of alternating Lys-Val sidechains on each arm and maintains a fairly rigid turn resulting from a ^{L}Pro-^{D}Pro motif. These lysine residues dominate the initial contact and sustained binding atop charged surfaces. On the contrary, the AMP will only undergo transient binding in the presence of zwitterionic surfaces. Simulations reveal binding to the surface of the charged surface promotes folding at the surface and reorganization of the sidechains such that hydrophobic residues face solution and hydrophilic ones face the surface of the membrane. We found that at sufficiently long timescales or with an applied surface tension, SVS-1 can undergo a “flip and dip” mechanism via the turn or the tail of a folded structure. Provided with sufficient area to embed beneath the headgroups, the peptide rotates in a cascading motion to bury the valine residues below the headgroups. This buried state is possibly a precursor to a deeper insertion state that could lead to membrane disruption of charged membranes similar to that of cancer cells. It is also possible that a cooperative effect of multiple peptides could lead to insertion events that precedes subsequent break down of the membrane integrity. The dynamic behavior of this insertion mechanism elucidates the efficacy of AMPs and the need for further experimental and theoretical work. Future studies on sidechain modifications and free energy of insertion will provide more insight on improving peptide design and function.

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Acknowledgement

“Heights by great men reach and kept were not attained by sudden flight, but they, while they’re companions slept, were toiling upwards through the night.”
- Henry Wadsworth Longfellow

Longfellow let me into a little life secret as a teenager—there will be sacrifices and discomfort. Life is interesting and comes with its fair share of challenges and hiccups. For whatever reason, I’ve learned to embrace the different trajectories that are available while understanding my journey is unique. Grad school was an interesting time for me, but one that I think will be memorable and far more valuable for my future. Over the past four years, I’ve cried, doubted myself, gotten married and subsequently put on some weight. Thanks, Kish! But through it all, I’ve learned to trust in Jesus, I’ve learned to trust in God. Ha! So cliché!

On a more serious note, writing this acknowledgement allowed me to reflect deeply on my shortcomings and achievements at Emory. Oft I think to look on both sides of the coin, but it was during the decision to leave with my Master’s that I could only see tails everywhere I looked or when I thought about my next steps; truly, I had an eye-opening experience. The sound words of comfort and encouragement from my wife, advisors, mentors, pastors, friends and family were awe-inspiring. They allowed me to look on the other side of the coin—head. The one thing I hear echoing in this time is success, that I was successful to make it this far and that my future is bright. Though I have my reservations, my phenomenal support system keeps me pushing forward. WOW

As a child, I looked at my life as a template and unwittingly put such a huge burden on my shoulders to be a role model and succeed in EVERYTHING. Instead, I should have tried to be the best me, learn more and let my life speak for itself. Moving on to the next chapter of my life is so exciting. I can truly feel the burden lifting and looking at this transition as my call to greatness.

But… There’s this one little thing that bothers me, that feeling that I haven’t unlocked my potential. Whether it be through my work ethic, discipline or some unknown factor, I am determined to become Keon Andre Reid. I want my story to encourage my children and all the youth I’ll come in touch with, not to mention all the old heads. Persevere. Have courage. Never give up. Fight to the last. Give it your best and God will do the rest.

Lord, thank you for taking me through this amazing journey. I appreciate that there are so many shoulders that I could lean on and hands outstretched to help me. Dr. Kindt, thank you for believing in me and standing in my corner. I don’t think words will go far enough to express my gratitude. To Dr. Dyer and Dr. Evangelista, thanks for your kind words and guidance. To the chemistry department faculty and staff, it was great sharing such a wonderful time with you. To my cohort and colleagues, may the wind of life blow you to your destiny. To my parents Henrick and Carline, I love you both and thanks for everything —Don’t worry, I’m going to retire you soon. Kish, mi love you babes. I’ll make this up to you, alright? ;-)

In the words of the great NBA philosopher, Kobe Bryant “Reidy Out!” LOL *drops mic*

Reidy '18

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Reference to Published Work

Chapters 2 and 3 include excerpts from:
Reid, K. A.; Davis, C. M.; Dyer, R. B.; Kindt, J. T., Binding, folding and insertion of a β-hairpin peptide at a lipid bilayer surface: Influence of electrostatics and lipid tail packing. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2018,** *1860* (3), 792-800.

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Chapter 1

Introduction

1.1 Overview

Decades of detailed studies on protein-lipid interactions by both theoreticians and experimentalists have contributed to our basic understanding of life. Understanding the molecular principles that govern the behavior of these biomolecules first starts with their basic properties. A crucial component of understanding these phenomena lie within how these systems cooperate between themselves.

This thesis aims to address the complement of two biomolecules, proteins and lipids, with particular interest in a therapeutic application—cancer. This work will focus on SVS-1, an anti-cancer peptide, and its mode of action making it an effective cancer treatment in vitro. Simulation studies highlight important electrostatic interactions that are a precursor for the insertion mechanism witnessed in our studies. My detailed study involving different membrane mimics contribute understanding to how tuning AMPs improves efficacy of these systems. This overview will survey proteins and lipids, while focusing on the interactions of the two classes of molecules. I will further give a brief introduction to experimental and theoretical methods used to investigate the mode of action of antimicrobial peptides, and extend the discussion as it relates to cancer studies.

Chapters 2 and 3 describe the behavior of a novel hairpin peptide designed to selectively disrupt cancer membranes. This report focuses on the key contributions electrostatic interactions play in folding dynamics in solution and at a membrane surface. Furthermore, we describe the importance of lipid tail packing that assist in membrane insertion. This body of work expresses the need for further investigation of AMPs as novel cancer targets.

1.2 Protein and Lipid Interactions

Proteins (such as enzymes), lipids (such as fats), nucleic acids (such as DNA) and carbohydrates (such as sugars) are macromolecules created through polymerization of smaller subunites called monomers. Together they all coexist and work in organisms to cover a broad range of functions essential for life. The scope of this work focuses on proteins and lipids along with their structural and functional features.

Proteins can be both simple or complex systems made up of amino acids. They function as enzymes that catalyze many biological process in the body, not limited to building muscles and tissues or improving ones metabolism. Proteins can be an extended or unstructured chain of amino acid sequences or they can adopt well-defined secondary structures as hairpins or helices. Combinations of smaller secondary subunits give rise to tertiary and quaternary structures that drive signaling pathways, transport and storage, among other biological processes. Whereas proteins regulate many living systems, their misfolds can lead to interruption of functionality or neurodegenerative diseases such as Alzheimer's or Parkinson's disease[1]. The cumbersome goal to tune protein structure for desired behavior has led to what Dill et al. describe as 'the protein folding problem' [2]. Probing the thermodynamics, kinetics and forecasting structural motifs are the driving force to understanding proteins and their viability as therapeutic agents [2].

Lipids on the other hand are usually water-insoluble and serve to store energy, build membranes and protect the cell [3]. Packing of individual lipids and structural components give rise to different structures, such as micelles, bilayers, liposomes, fat droplets, or waxes. For the purposes of this thesis, phospholipids that compose membranes will be the

main focus of discussion. A phospholipid is amphiphatic (having both hydrophobic and hydrophillic portions) with a simple composition: a phosphate-containing headgroup, a glycerol backbone and two fatty acyl chains (tail-group) predominantly made of hydrocarbons. Altogether, lipids and proteins work to regulate transport and other biological processes in the body, ones that would otherwise be impossible individually. The field of protein-lipid or peptide-lipid interactions remains an area of intense research and debate.

To understand the interplay of roles between membrane proteins and lipids, it is important to study what drives binding and dynamic behavior and to learn how structure affects function. These interactions can be classified in two broad groups: molecular and physical. At the molecular level, studies focus on bonding, binding, hydrophobicity and electrostatics, whereas physical characters are found in properties such as induced peptide folding or bilayer changes in the form of its composition, fluidity, curvature, thickness or line tension. A clear picture of molecular resolution can establish the link between molecular underpinnings and physical characteristics that elucidate induced mechanisms between the two aforementioned bodies. Protein function is controlled by lipids in cell transduction, transportation and varying signaling pathways [4]. The wide range of possible functions drive investigation into activies of integral membrane protein behavior.

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1.3 Roles of Peptides in Anticancer Studies

Cancer remains a global issue with the mortality rate of cancer projected to increase beyond 70% by 2030 [1]. Therefore, exploring molecular targets for cancer therapies and translational approaches for clinical applications is an urgent need. Cancer therapies continue to face significant challenges. The goal for these new strategies is to create a potent tool while limiting the adverse side effects and drug resistance [2]. Novel therapies to target cancer cells surround the fundamental differences between membrane charges of cancer and normal mammalian cells. The role of new drugs will be purely driven by specificity. The function of integral membrane protein have made peptides a suitable molecular targets for membrane-lipid cancer therapies [3,4]. It is well understood that most marketed drugs have physical contact of incorporated proteins through oral or topical application [5]. Therefore, translational studies for future applications are likely to rely heavily on improving design of novel peptides or the molecular scaffold to directly interact with apoptotic and cancer cells.

Targeting membranes is driven by peptide charged sidechains. Therefore, methods are derived to target the negatively charged lipids in the exterior surface of cancer cell membranes, versus a net neutral charge of zwitterionic lipids in the outer leaflets of normal healthy cells [6]. The charge difference a result of change of lipid composition and structure. Non-cancerous cells maintain phosphatidylcholine (PC) and sphingomyelin lipids on the outer membrane, while the negatively charged phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the inner leaflet. In cancerous cells, PS and PE lipids are exposed at the outer membrane leaflet due to inability to maintain native location at the inner membrane. As a consequence, PE or PS

binding peptides have been used to untangle biological function of the negatively charged lipids. For example, PE lipids exist normally at the inner leaflet of normal cells, but are overexpressed on the outer leaflet in cancer cells. The emergence of PE lipids at the outer leaflet that disrupt membrane symmetry provide a strategy to tune peptide for interactions with PE headgroups [7]. Two short peptides, duramycin and cinnamycin, are known to bind tightly to PE-phospholipids and disrupt gram-positive bacteria and cancer cells [8,9]. Recent studies with cyclic peptides also show binding and insertion that leads to membrane disruption and death of cancer cells [10-12]. Although, they show a lesser extent of toxicity to non-cancerous cells. Cancer cells are known to contain lipids with negatively charged serine headgroups on the outer leaflet also. In 2012, Sinthuvanich et al. engineered a beta-hairpin peptide to bind, fold, insert and destroy cancer cells in vitro [13,14]. Here, the peptides are tuned with positive lysine sidechains to interact with the negatively charged serine headgroups. A number of these antimicrobial and defense peptides have been stored in the CancerPPD database [15]. It stores experimental data for activies targeting different cancer cell lines.

There has been notable advances in anti-cancer peptide therapies that led to drug approvals in 2012 [16,17]. Though there has been renewed interest in the field, there still remains limitations to production cost, viability of the drug and safety for oral applications [16]. With further improvements in research methodology and drug development, it is possible to overcome these challenge.

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1.4 Approaches for Studying Membrane-Active Peptides

1.4.1 Membrane-Active Peptides

In this section, I describe the classes of membrane-active peptides as well as cover general experimental and theoretical approaches to studying membrane-active or integral membrane proteins. Membrane-active peptides are studied and re-engineered to tune biological functionality for therapeutic applications. Examples of these membrane-interacting peptides are cell-penetrating peptides (CPP), antimicrobial peptides (AMPs), viral peptides and amyloidogenic peptides. CPPs are small peptides that have have membrane-translocating properties to deliver cargo. They carry positive charges and are characterized by their amphiphatic nature. CPPs have been applied in a number of areas, including transport of proteins, oligonucleotides, quantum dots, polysaccharides, nanoparticles, chemotherapeutics, polymers, and liposomes [1–10]. Viral fusion peptides undergo structural modifications to promote membrane fusion. Three classes of fusion proteins are known to promote fusion based on their structural motifs, through some role of membrane destabilization [25,26]. The molecular details of the process are still under scrutiny. A peptides that aggregates into fibrils is considered ~~as~~ an amyloidogenic peptide. They are usually unfolded and are triggers for amyloid formation once interacting with membranes [27]. Aggregation of these peptide units correlate with amyloid diseases [28]. The remainder of the section now focuses on antimicrobial peptides.

Antimicrobial peptides (AMP's) are short, charged membrane proteins that are ubiquitous in the tissues of plants, animals, and invertebrates and are typically comprised of 50 or fewer amino acids [11]. They are amphiphiles composed of hydrophilic and

hydrophobic amino acid residues adopting different structures (α-helical, β-stranded, β-hairpin, extended) [12,13]. In addition, they act as a host defense against microbial pathogens. AMPs display cytotoxic activity towards gram-positive and gram-negative bacteria, fungi and cancerous cells [14–21] and are selective towards negatively charged membranes, which allows AMPs to kill microorganisms without harming normal mammalian cells [22]. Cancer cells, in common with some bacteria, display a net negative charge on their surface [23]. Selectivity for disrupting anionic membranes therefore gives AMPs anticancer activity in addition to antimicrobial activity. However, the exact mechanisms by which AMPs disrupt these cells are still elusive.

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1.4.2 Experimental Techniques

In recent years, there have been great strides in improving methodologies to elucidate peptide-lipid interactions. Experimental studies of model systems not only reveal key interactions highlighting how peptides are stabilized in membranes, but they contribute a wealth of insight to improve design or research landscape. A brief survey of some experimental techniques are described below.

X-ray crystallography is used to identify the 3D positions of atoms in a protein [1,2]. To date, over 100,000 protein structures have been characterized in the Protein Data Bank [3]. Arguably, crystallography is one of the most direct ways to observe the true nature of protein-lipid interactions. Solution preparation and inadequate refinement of the technique are known limitations [4]. One can probe peptide-lipid binding properties through surface plasmon resonance [5] and quartz crystal microbalance [6,7]. Both techniques measure association constants between peptide and membranes. Along with atomic force microscopy (AFM), these experimental approaches thoroughly probe the energetic contributions of the interactions, a characteristic of lipid and peptide structure. Additionally, AFM provides high resolution images at the sub-nm scale and is able to measure forces between individual biomolecules [8-10].

Lipid phase changes are analyzed the calorimetry measurements. Once can gather sensitive measurements that comment on a system's thermodynamic parameters, namely the free energy, enthalpy and entropy [11]. By using scanning probe microscopy tools, researchers are now able to visualize accurately peptide- or protein-membrane complexes [12,13], with a distinct advantage with aqueous membrane systems.

Spectroscopic methods such as NMR [14], linear and circular dichroism [15,16], x-ray

scattering [17], and total attenuated total reflection FTIR [18], all probe the structural features of peptide-lipid interfaces. These tools comment on the peptide orientation and location with respect to a membrane normal [19]. Peptide-lipid interactions have also been investigated through Raman spectroscopy [20,21]. Protein folding dynamics have been studied using time-resolved techniques such as fluoresence, resonance raman and IR which indicate helical, beta-sheet or fast-folding methods on the nanosecond to millisecond timescale [22,23].

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1.4.3 Simulation Methods

Interaction at the atomistic level is necessary to characterize structure, function and dynamics of peptides interacting with lipid bilayers. In 1959, Alder et al. developed a molecular computation simulation coined Molecular Dynamics (MD) to describe the trajectories of atoms or molecules, numerically solved using Newton's equations of motion [1]. Molecular simulation is an important tool that complements experimental approaches for studying peptide-lipid systems and provides a detailed description of interactions at the molecular level. The force between these interactions are defined through interatomic potentials, commonly referred to as a molecular mechanics 'force field.' Typically, simulation studies will work with a simple model to extrapolate trends that extend to larger systems. Though MD simulation is a powerful tool, factors such as (1) imperfect or overly simplified force fields (2) inaccurate simulation methodology (3) slow biological processes (4) system size and (5) insufficient computational resources limit the accuracy of results. Nevertheless, simulation studies can allow comparisons between data measured in experimental studies and molecular observation from atomistic dynamics. MD studies can

also elucidate processes that experiments are unable to probe.

The biomolecular simulation community has an array of popular atomistic force fields, namely AMBER [2], CHARMM [3,4], GROMOS [5], and OPLS [6], to conduct simulation on the femtosecond to microsecond timescale. Marrink et al. developed a less detailed Martini or 'coarse-grained' model [7]. Whereas the aforesaid atomistic models explicitly consider each individual atoms, the Martini model treats for heavy atoms or non-polar hydrogen atoms by bundling groups of atoms in a single molecule. In doing so, the Martini model sacrifices detail or resultion for increasing the sampling time of the simulation. Among the class of atomistic models, some have been optimized to treat for specific systems based on the potential energy functions of their systems. For example, the AMBER force field has been tuned to treat protein folding, whereas CHARMM and GROMOS best mimics fluid membranes properties such as area per lipid or acyl tail order parameters [8]. Interestingly, the GROMOS model is deploys a united-atom approach (near atomistic) to represent non-polar CH, CH_2 and CH_3 bonded atoms as a single group. Together, atomistic and coarse-grained methods provide an enriched depiction of biological phenomena at the molecular level, allowing visualization at different degrees of resolution. Collectively, both strategies simplify the biological description of molecular systems through overcoming their individual limitations.

Several simulation models have been developed to address membrane behavior [9-12], peptide-lipid interactions [13] and antimicrobial peptides [14,15]. Im et al. developed an implicit membrane model to study structure and stability of integral membrane proteins [9,10]. Ulmschneider et al. later improves on the generalized Born implicit model to map for insertion free energies [12]. Long scale studies on peptide-lipid interaction has shown

the complexity of pore formation [15,16], peptide translocation [17,18] and lipid 'flip flops' [19]. Several improvements to standard MD approaches have also contributed to understanding of peptide-lipid interactions. A simple development by McCammon et al. allowed for faster MD simulations by raising energy level thresholds that allowed easier sampling across the free energy landscape [20,21]. They coined the method 'accelerated MD' (aMD) because of the more efficient and faster simulation speeds. Torrie et al. developed the umbrella sampling technique that is used to improve sampling through a biasing potential to overcome free energy barriers using normal [22]. This method was used to investigate peptide insertion and pore formation by Awasthi et al. [23]. Another approach to increase sampling is Replica Exchange MD (REMD) developed by Sugita et al. [24]. The algorithm to sample system states with similar potential energies at different temperatures. REMD has proved advantageous for sampling minimas and barriers in the free energy landscape in addition to sampling different conformational states. In a recent development, Shelley et al. developed a hybrid all-atom/coarse-grained (AACG) approach to model peptide folding in acqueous solution and aggregation of the alpha-helical peptide melittin [25]. Over decades of simulation studies, refinements to methodology and force fields have drastically improved detail and understanding at molecular level. Furthermore, the emergence of supercomputers such as Anton [26] have extended the timescales on which molecular simulation can explore. A collected effort by simulation and experimental approaches is necessary for refinement of current thereapeutic agents and discovery of the next novel target.

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1.5 Thesis Outline

In **Chapter 2**, we report our findings on the folding and binding dynamics of an engineered antimicrobial peptide, SVS-1, in the presence of different membrane mimics. The chapter focuses on the influences electrostatics play in initial adsorption of the peptide atop an anionic membrane (A 1:1 ratio of 70 zwitterionic POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine lipids and 70 negatively charged POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol)) and subsequent folding. Simulations reveal the hairpin peptide will only interact transiently with 100%

zwitterionic POPC bilayer. Formation of spurious structures are also observed, pointing out weaknesses associated with using different protein force fields. **Chapter 3** describes a novel insertion mechanism for a hairpin antimicrobial peptide. This "flip and dip" mechanism demonstrates the propensity for the peptide, in part, to first associate into an amphiphilic structure, afterwards, insert by rotating its hydrophobic sidechains below the headgroups of the anionic lipid bilayer. Application of a surface tension further promotes insertion of the peptide by increasing the accessible area per lipid of the leaflet where the peptide is presented. We also report on the contribution lipid rearrangement and packing play when our simulated systems are placed under an applied surface tension. **Chapter 4** sumises the major conclusions for this work and future outlook.

Chapter 2

Investigating Binding and Folding Behavior of an Anti-Cancer Peptide

Chapter Abstract

Simulations using the CHARMM force field offer complementary insight into timescales and mechanisms of folding and binding. Folding dynamics are validated using different CHARMM force fields. We highlight the need for accurate force fields due to spurious structures that can be produced, resulting from incorrect parameters. Studies of the peptide in both solution and at the surface of different membrane mimics reveal the peptide will retain a rigid hairpin, but would modulate its degree of folding based on the environment. The CHARMM 36m force field reproduces folding dynamics closest to experimentally observed states. In the presence of the zwitterionic surface, the peptide interacts transiently and does not stick to the surface. On the other hand, SVS-1 simulated at an anionic mixed POPC/POPG bilayer folded into a hairpin over a hundreds of nanoseconds.

2.1 Introduction

Naturally occurring AMPs display a wide variety of sequences and structures. Characteristically, they are less than 50 amino acids and have charged sidechains. Furthermore, the small peptides can display amphiphilic character. AMPs are generally disordered in solution, but fold into an ordered secondary structure when associated with lipid bilayers [1]. Sidechain interactions with lipid headgroups, typically electrostatic charges, drive binding and folding to different membrane mimics. Studies of these peptides surround membrane-mediated folding, membrane stability, modifying peptide sidechains, in addition to there corresponding kinetic and thermodynamic parameters.

Here we have investigated the bilayer interactions of SVS-1, a cationic AMP engineered to disrupt cancer cell membranes with low cytotoxicity towards noncancerous cells [2,3]. Specifically, spectroscopy studies and computer simulations examined the binding, folding and insertion of SVS-1 in the presence of zwitterionic or negatively charged lipid bilayers. According to Sinthuvanich et al. [2], SVS-1 is the first example of an anticancer β-hairpin whose action is dependent on membrane-induced folding. Lu et. al. also showed preferential folding of a hairpin in the presence of an anionic bilayer using NMR relaxation studies [4]. In the design of SVS-1, the unnatural D-enantiomer of proline (Pro) was used to create the β-turn in the secondary structure of the peptide sequence. The SVS-1 peptide (sequence KVKVKVKV^{D}P^{L}PTKVKVKVK) contains a D-Pro/L-Pro motif that folds into a structured Type II' β-turn [5] as shown in the Figure 1 [2]. The alternating valine and lysine residues allow SVS-1 to adopt an amphiphilic structure, which is a common feature of AMPs and aids in aggregation and electrostatic interactions on the surface of anionic membranes. Though the peptide remains unfolded in solution or in the presence of

zwitterionic membranes due to the positively charge lysine repulsions, the turn is structured because of the D-Pro and L-Pro isomers. SVS-1 is hypothesized to fold once it binds to a charged membrane due to complementary electrostatic interactions. We report Molecular Dynamics (MD) results on dynamic binding and folding behavior of SVS-1 in solution and at different lipid bilayer surfaces.

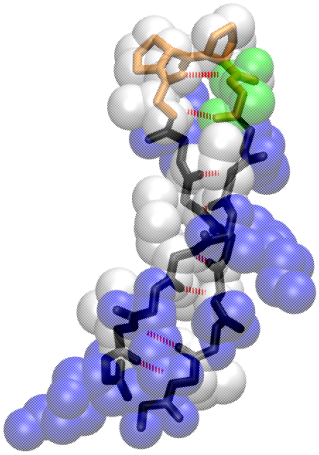

Figure 2-1: Model of folded SVS-1 structure created using Visual Molecular Dynamics (VMD) [6]. Peptide backbone (black) is represented as a licorice, and $V^{D}P^{L}PT$ turn motif colored in orange. Peptide sidechains are presented as VDW beads – hydrophobic valines and prolines (white), basic lysine (blue) and polar threonine (green). The intermolecular backbone hydrogen bonds are drawn in red.

2.2 Computational Methods

SVS-1 Structure

The SVS-1 hairpin structures were generated using the Molefacture Protein Builder tool of VMD (version 1.9) [6]. The peptide sequence was added into the Protein Builder to generate an unfolded SVS-1 structure, with an amidated $-NH_2$ C-terminus end used in experiment producing a +9 overall charge on the peptide. The phi/psi angles were selected to generate a Turn conformation for the folded peptide.

Bilayer Creation

The CHARMM-GUI membrane builder online software was used to generate a negatively charged POPC/POPG bilayer structure and a pure POPC bilayer [7-9]. The charged bilayer consists of 70 zwitterionic POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine**)** lipids and 70 negatively charged POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol). The pure POPC bilayer was composed of 140 lipids.

Molecular Dynamics Simulations

All simulations were performed using CHARMM 36 (C36) [10,11] or CHARMM 36m (C36m) [12] all-atom force field with the MD package Gromacs [13,14]. The simulation box size was subject to constant particle number, pressure, temperature (NPT) dynamics. Periodic boundary conditions were applied in the X and Y dimensions with semi-isotropic coupling using the Berendsen barostat [15] at 1 bar and a coupling constant of 1.0 ps. The compressibility factor used in both directions was 4.5×10^{-5} bar^{-1}. Simulations were conducted at 323 Kelvin (50 °C) using Velocity Rescaling [16]. TIPS3P waters [13], K^+ and Cl^- ions were added to neutralize the charge across the simulation box. Minimization to relax the structure of the system was conducted using the steepest descent method prior to conducting MD simulations. A MD integration timestep of 2 fs was used. VMD [6] was used to visualize trajectories and Gromacs analysis tools were utilized for reporting data. Simulations were carried out locally and via a XSEDE allocation [17].

We conducted 0.5-1.5 microsecond simulations with a single unfolded SVS-1 molecule in pure solution and in the presence of zwitterionic and charged bilayers (See Table 1). The peptide was initiated in solution approximately 2 nm from the surface of the zwitterionic or anionic lipid bilayer, starting with different peptide orientations.

Table 2-1: System components of long MD Simulations of SVS-1 in solution and presence of lipid bilayers.

Simulation	Simulation length (ns)	no. of atoms	no. of water molecules	POPC lipids	POPG lipids	SVS-1 peptides	K^+ ions	Cl^- Ions
C36								
1	1030	21488	7049	0	0	1	0	8
2	1500	40455	7417	140	0	1	0	8
3	1500	40497	7633	70	70	1	70	8
4	1500	40452	7607	70	70	1	70	8
5	1500	40437	7603	70	70	1	70	8
C36dmap								
6	1000	21488	7049	0	0	1	0	8
C36m								
7	500	42456	7784	140	0	1	0	9
8	1500	40470	7262	70	70	1	70	9
9	1000	21470	7042	0	0	1	0	9

Data Analysis

Distance fluctuations were tracked between portions of the peptide and hydrophobic tails at long timescales. The terminal amine nitrogen (residue 1-LYS) and terminal carbonyl carbon (residue 18-LYS), along with the alpha carbon atom (residue 9-PRO) were selected to track the distance fluctuations to the nearest acyl tail carbon atom. Hydrogen bond formations were calculated for the eight correct backbone nitrogen donor and oxygen acceptor pairs of the peptide using Gromacs analysis tools. Cutoffs for hydrogen bond criterion are 3 Å and 20º. The valine contacts were tracked by counting the number of beta carbon valine CH side chain sites within 0.5 nm of any lipid acyl CH_2 or CH_3 tail site. Plots of H-bond and valine contacts are smoothed, showing averages over 1 ns. Area per lipid (APL) changes in bilayer was calculated by multiplying the X and Y

box dimensions and dividing by 70, the number of lipids in a monolayer.

2.3 Results and Discussion

2.3.1 Characterizing folding dynamics with different force fields

Variants of the CHARMM forcefield were used to probe the folding dynamics of SVS-1 in solution and in the presence of zwitterionic and anionic lipid bilayers. In our initial simulations with a carboxylate –COO- C-terminus (net +8 charge), we found the C36 forcefield produced spurious alpha-helical structures. Older versions of the CHARMM force field had the propensity to overestimate the stability of helical structures in small peptides [18,19], although this tendency was supposedly corrected in C36. The cmap parameters in the C36 force field were updated based on the C36m improvements to treat the D-isomer proline (we term this update C36dmap). The C36dmap improvements did a poor job replicating experiment and was inconsistent with C36 folding behavior while producing helical structures. Our most recent trajectories with the full C36m forcefield (with an amidated $–NH_2$ C-terminus (net +9 charge)) provide more consistent results with experiment. We report our findings and comparisons between the forcefields in Figure 2-2.

The C36 forcefield overstabilized the hairpin in solution and produced helical structures, contrary to ~~our~~ experiment, whereas the C36dmap switched between helical and hairpin states frequently over the course of the simulation. The C36m forcefield folds at the turn predominantly over 1000 ns, but no helices are formed (See snapshots, Fig. 2-3). We believe the updated C36m cmap parameters for d-isomer proteins correct for erroneous helices but could overstabilize the hairpin as well. In a 1 μs simulation of the peptide in

solution using the C36-m force field, dynamic folding and unfolding are observed (Figure 2-3) and 1-2 of the native hairpin hydrogen bonds are formed on average. These hydrogen bonds are found close to the peptide turn where the hairpin structure is strongly influenced by the ^{D}Pro-^{L}Pro motif [2,20]. Figure 2-3 shows snapshots of the different folded and extended states witnessed in solution.

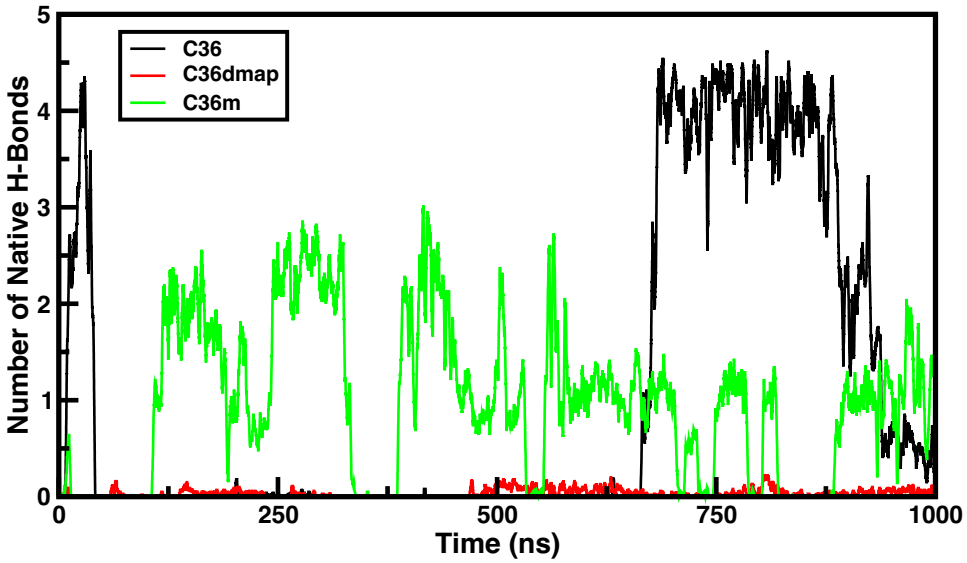

Figure 2-2: Comparison of protein folding in solution using different CHARMM force fields. The trajectory frames are smoothed, showing averages over 1 ns.

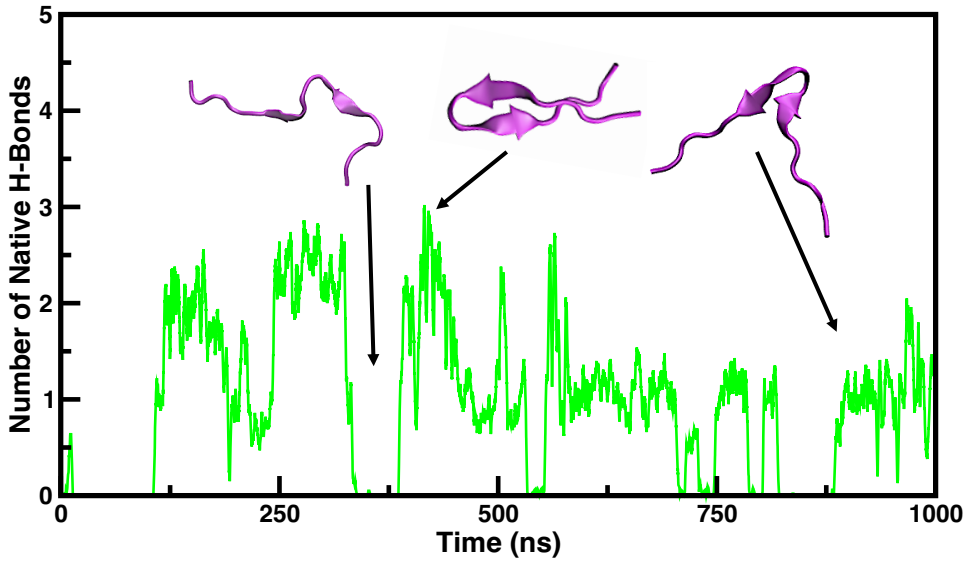

Figure 2-3: Number of native hairpin SVS-1 hydrogen bonds in 1000 ns solution-phase simulation using C36m, with corresponding peptide snapshots. Folded peptides are drawn in New Cartoon method and colored magenta using VMD. The plot has been smoothed, showing averages over 1 ns.

2.3.2 Characterizing binding and folding peptide-lipid interactions

Simulations of SVS-1 interaction with lipid bilayers were conducted on a pure zwitterionic POPC bilayer and a 1:1 POPC/POPG mixture. By using the anionic POPG lipid with one monounsaturated tail, we mimic the PG headgroups of the saturated DPPG lipids used in our experiments, avoid complications arising from unpredictable phase behavior in the simulation model encountered using saturated lipid tails (See further discussion in section 3-4), and eliminate the possibility of hydrogen bonding with serine POPS headgroups [21] used in earlier [2] experiments.

Simulations modeled the initial stage of contact of the unfolded peptide with the bilayer. During a 500 ns trajectory, SVS-1 makes only transient contact with the bilayer via its N-terminus (Figure 2-4, upper left panel). Its folding behavior is similar to that seen in solution, switching between completely unfolded and partially folded states (Figure 2-

4, lower left panel). In contrast, an initially unfolded SVS-1 interacting with the anionic POPC/POPG mixed bilayer maintains close contact with the bilayer (through its N-terminus or turn region) throughout a 1.5 μs simulation (Figure 2-4, upper right panel). Four pairs of H-bonding sites approach each other within the first 100 ns of contact (Figure 2-4, lower right panel) and the peptide maintains a partially folded structure with 3 to 5 bonds in contact, for over 1 microsecond. The partially folded structure presents the lysine side-chains towards the charged lipid headgroups, leaving the hydrophobic valine side-chains facing the solvent (Figure 2-5A).

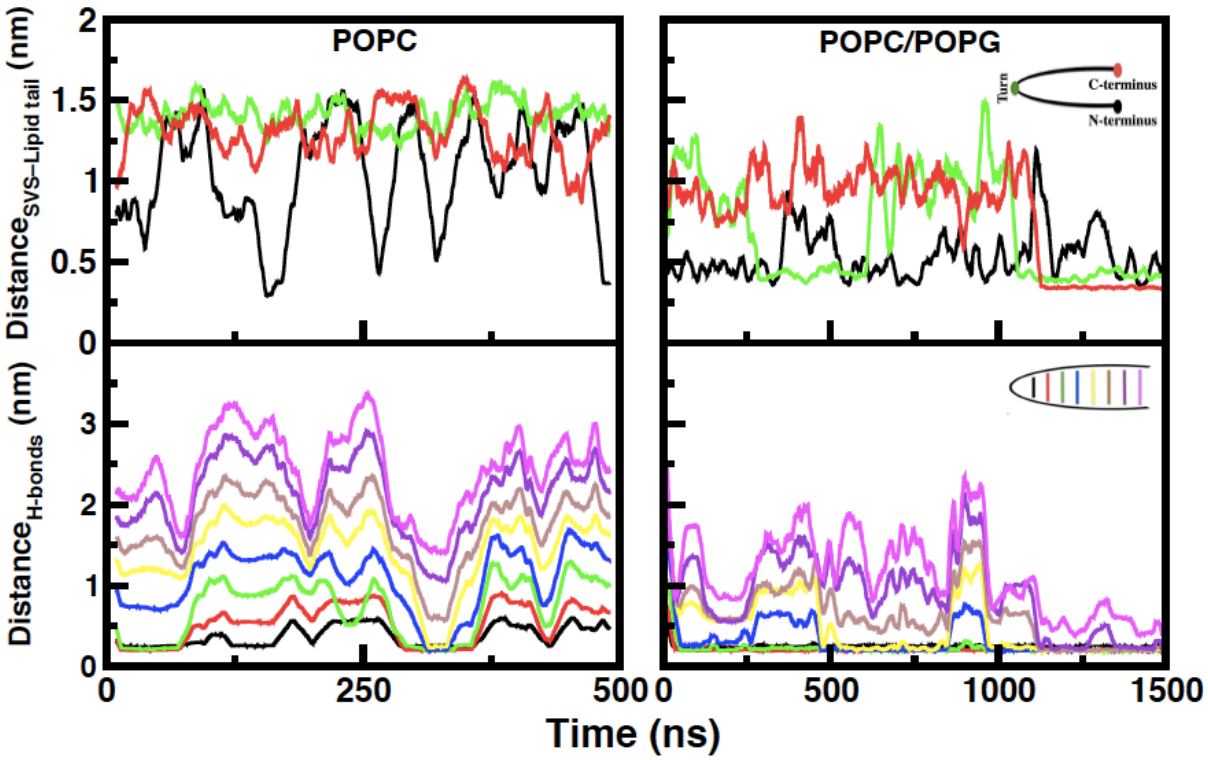

Figure 2-4: Bilayer contact fluctuations and hydrogen bond formation (folding dynamics) of SVS-1 in presence of a zwitterionic POPC bilayer (500 ns) and an anionic POPC/POPG bilayer (1500 ns). Fluctuations track distances of terminal sites and center of turn to the closest acyl tail carbon. The atoms are colored based on description in Methods (See cartoon in upper right panel). Hydrogen bond order from 1-8 (starting from turn to the tail-end) are represented in order by the colors: black, red, green, blue, yellow, brown, purple and magenta (See cartoon in lower right panel). The curves represent averages smoothed over 1 ns.

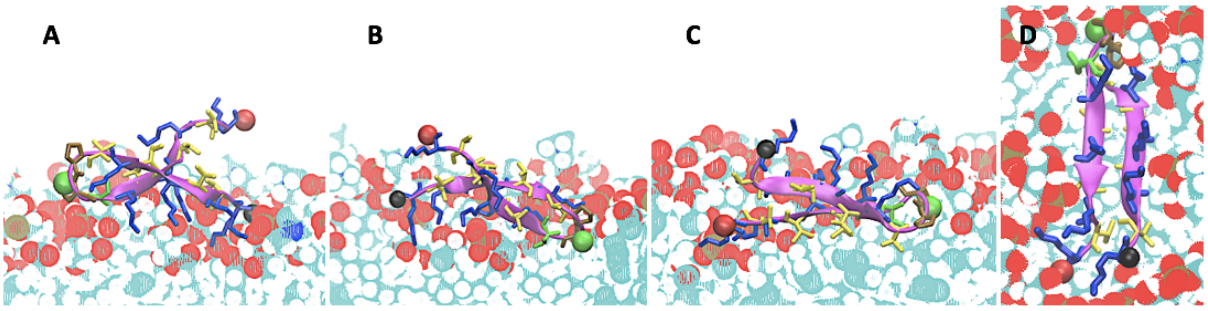

Figure 2-5: Snapshots from the 1500 ns simulation of SVS-1 interaction with 1:1 POPC/POPG bilayer. Figure A shows partially folded structure with strong interactions of lysine sidechains with headgroups at 1040 ns. Figure B highlights the initiation of the "flip and dip" mechanism, where the turn buries beneath the headgroups (at 1075 ns). Figure C depicts endpoint of the "flip and dip" mechanism after 50 ns (1130 ns). Figure D (top-down view) represents SVS-1 with valines inserted and lysines facing upwards into solution (end of 1500 ns simulation). Red, green, and black sites as defined in Figure 8 are shown as spheres. SVS-1 sidechains are depicted as a licorice: valine(blue), lysine(green), proline(orange) and threonine(yellow). Lipids are represented as dotted in VMD drawing method. Some lipids are removed for Figures A-C for clarity of mechanism. Water and ions removed for clarity.

Simulation reveals a novel insertion mechanism we coin the "flip and dip" mechanism. After a microsecond, the ^{D}Pro-^{L}Pro turn region dips into the bilayer interior (1075 ns, Figure 2-5B) while at the same time the hairpin twists (1080-1110 ns, Figure 2-5C) and brings the Val sidechains into contact with the bilayer interior (Figure 2-4, upper right). As the turn buries, the N-terminus (black curve) which had stayed close to the surface pops off while the C-terminus (red curve) moves in towards the lipid tails. Finally, the N-terminus joins the turn and the C-terminus in contact with the lipid tails, while the sixth and seventh pairs of hydrogen bonding sites along the hairpin come into close contact, effectively completing the folding process (Figure 2-4, lower right). A jump in the counts of valine side-chain contacts with the lipid tails to an average near 7 confirms that the peptide has made full hydrophobic contact (Figure 2-6). Once buried beneath the headgroups, the peptide retains a high degree of folding until the end of the simulation

(Figure 2-5D). Interactions between most Lys sidechains and bilayer headgroups can be maintained as the sidechains rotate around from pointing inward toward the bilayer to pointing outward toward the solvent. In previously reported coarse-grained simulations [22], the valine sidechains folded under the headgroups from a similar on-top, valine-out state within about 50 ns. This mechanism is much slower within the atomistic model due to the greater constraints on backbone dihedrals.

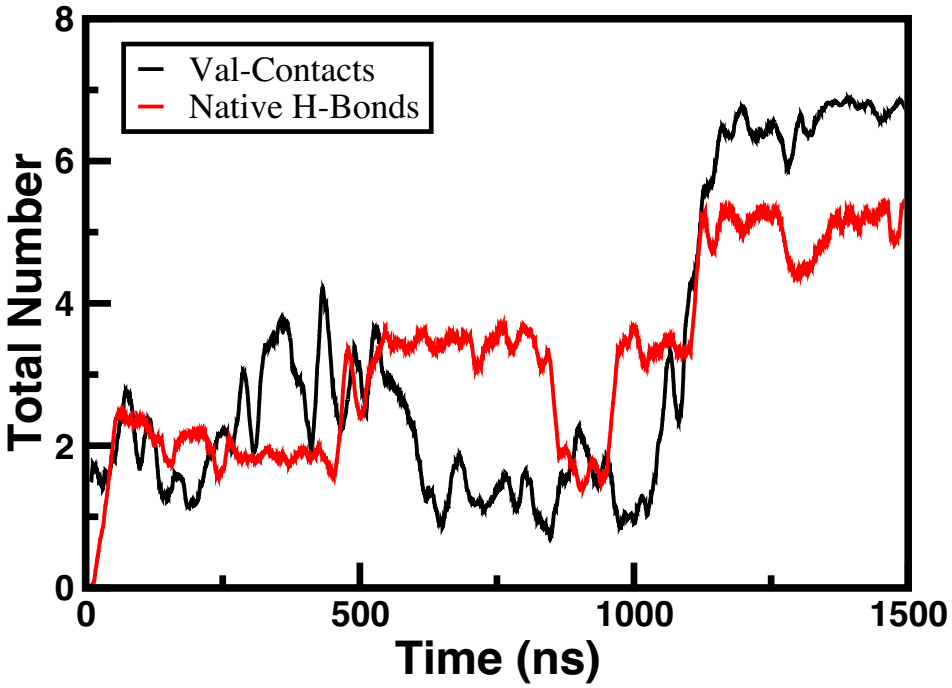

Figure 2-6: Timeseries of hydrogen bond formation and number of valine-to-tail contacts for 1500 ns simulation of SVS-1 peptide in the presence of a 1:1 POPC/POPG anionic bilayer (at 323 K) using C36m. The trajectory frames are smoothed, showing averages over 1 ns.

2.4 Conclusions

It is important to note that theoretical studies are important for extending our understanding of experimental observables, however detailed treatment of the force fields for MD simulations take particular care. The new C36m force field reports the correct hairpin structures while avoiding spurrious helices formed using the older C36 and

modified C36dmap force fields. Simulations of SVS-1 interaction with charged and zwitterionic lipid surfaces in the fluid phase are broadly consistent with the observed experimental behavior and suggest a pathway for preliminary stages in surface binding and folding. We found two key behaviors of the peptide: (1) Unfolded SVS-1 interacts only transiently and superficially with the POPC surface but binds tightly to the POPC/POPG surface. (2) In the latter case, folding was seen to proceed gradually over 100's of nanoseconds with the peptide's cationic lysine side-chains largely facing towards the lipid head-groups and most of the hydrophobic valine side-chains facing the solvent. Full contact between the hydrophobic valine side-chains and the lipid tails was made upon a "flip and dip" transition, which coincided with a jump in the degree of folding. The "flip and dip" mechanism of the on-top folded state, a cascading rotation of the peptide backbone promoted by valine insertion, can be contrasted with the rapid insertion seen in CG simulation under tension-free conditions [22], where the side chains can just invert due to much lower torsional barriers. Ample sampling of the peptide atop the anionic surface saw the peptide insert its hydrophic groups beneath the surface. The strong electrostatics witnessed between the complementary positive side-chains and negative lipid headgroups promote binding and help facilitate the folding.

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Chapter 3

The Influence of Surface Tension

Chapter Abstract

SVS-1 simulated at an anionic mixed POPC/POPG bilayer folded into a hairpin over a microsecond, the final stage in folding coinciding with the establishment of contact between the peptide's valine sidechains and the lipid tails through a "flip and dip" mechanism. Partial, transient folding and superficial bilayer contact are seen in simulation of the peptide at a zwitterionic POPC bilayer. Only when external surface tension is applied does the peptide establish lasting contact with the POPC bilayer. Our findings reveal the influence of disruption to lipid headgroup packing (via induced curvature or surface tension) on the pathway of binding and insertion, highlighting the collaborative effort of electrostatic and hydrophobic interactions on interaction of SVS-1 with lipid bilayers.

3.1 Introduction

Several membrane disruption mechanisms have been proposed but none of them is applicable to all classes of AMPs [1,2,3]. The most prominent peptide-mediated membrane pore formation mechanisms are: the barrel stave pore, the carpet mechanism, the toroidal pore, the disordered toroidal pore, and electrostatic effects [1,2,4,5]. Factors such as peptide composition, secondary structure, peptide crowding and membrane composition all contribute to the overall function of AMPs in the presence of different biological membranes. High-resolution experimental techniques (such as NMR and X-ray crystallography) are unable to fully resolve AMP/lipid interactions and lytic mechanisms, thus computational techniques are needed to further molecular insight [1,6-8]. MD studies have been applied in this field to optimize the conformation of AMPs in the presence of membrane-mimicking solvent mixtures [4], to observe the adsorption process of the peptide into membrane mimics [9,10], and to study the spontaneous nature of peptide assembly that leads to membrane disruption [11–16].

In chapter 2, we witnessed a novel mechanism where the valine residues inserted by rotating downwards below the lipid headgroup. Here, we probe the effect of a applying a surface tension to investigate the influence it plays on insertion. Through a surface tension application, the bilayer undergoes an expansion in the area per lipid. From prior work, we know binding of the peptide to one leaflet of the bilayer induces stress on the monolayer, resulting in more accessible area for the peptide sidechains to insert. We utilize this strategy to promote insertion events of the SVS-1 peptide.

3.2 Computational Details

We probed the effects of stress by applying a surface tension to the bilayer in the X and Y dimensions. A series of simulations were conducted at 323 K and at applied surface tensions factors of 10 or 30 mN/m for 250 ns. Two types of trials were conducted for the negatively charged POPC/POPG bilayer where a pre-folded or unfolded SVS-1 peptide was placed in a random orientation approximately 1 nm above the membrane. Only pre-folded peptides were used for the POPC bilayer.

3.3 Results and Discussion

3.3.1 Effects of Surface Tension

To facilitate interaction of the valine sidechains with lipid tails, we perform simulations under applied surface tension. Increasing surface tension on the bilayer increases the bilayer surface area per lipid, exposing more of the hydrophobic interior to the solvent and the adsorbed peptide. This added exposure may be present in the highly curved external leaflets of SUVs [17], in fluid domains embedded in gel phase near T_m [18], in domain boundaries in SUV's below the bulk transition temperature [19], or under crowded conditions in which lateral pressure among surface adsorbates leads to expansion of the underlying membrane [20].

Simulations of modest length (250 ns) were performed under varying degrees of surface tension with either zwitterionic (POPC) or anionic (POPC/POPG) lipid bilayers and with the peptide either initiated in a folded or an unfolded state. The peptide's behavior observed in any of these single trajectories may just be a sample of multiple possible outcomes that would appear in repeated trajectories. As mentioned above, an ideal fully-

folded SVS-1 hairpin structure contains eight characteristic hydrogen bonds; we never see the eighth h-bond formed consistently. Taking into account fluctuations across the H-bond cut-off criteria, we consider averaged hydrogen bond content of 3-4 to be partially folded and five or above to be fully folded. The N-terminal arm contains 4 valines while the C-terminal arm contains 3, so structures with 2-4 valines in contact with lipid tails likely have one arm embedded in the bilayer while contact by 5 or more valines indicates that both arms are embedded.

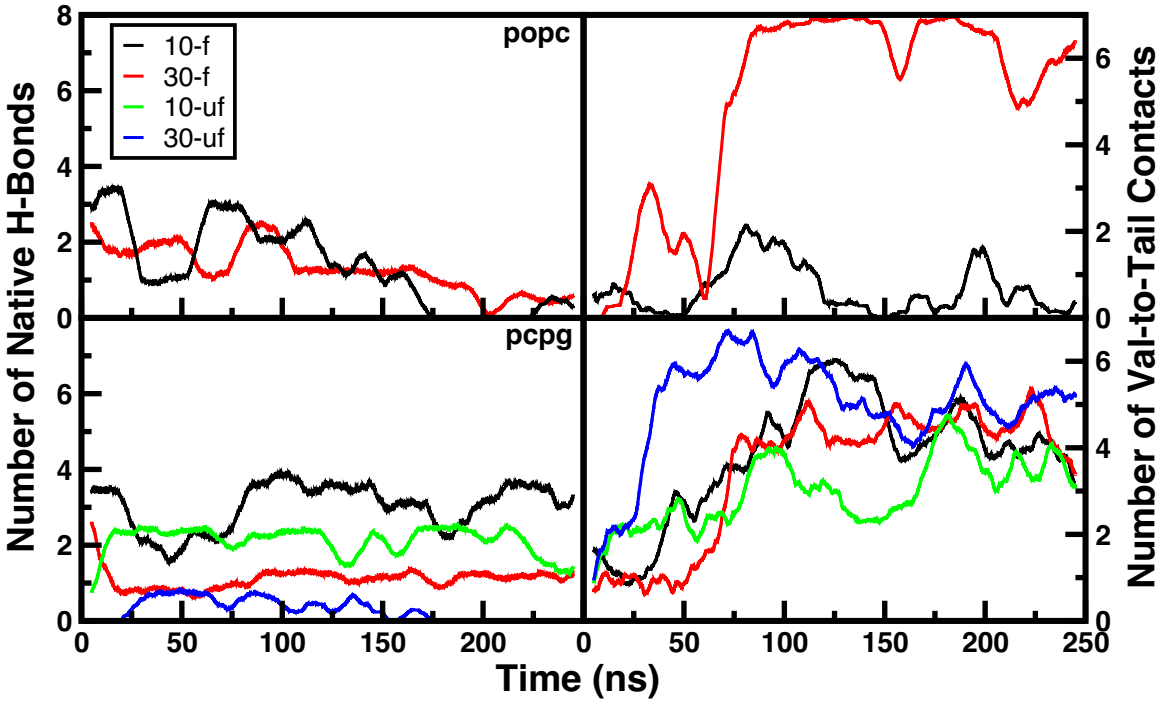

Figure 3-1: Number of native hydrogen bonds formed (left) and number of valine-to-tail contacts (right) for a folded or unfolded SVS-1 peptide in the presence of an anionic or zwitterionic bilayer (at 323 K) using C36m, as defined in section 2.2.4. Simulations for each of the starting configurations, folded (f) or unfolded (uf), were performed under applied surface tension factors of 10 or 30 mN/m, respectively. Curves represent averages smoothed over 1 ns.

A pre-folded peptide was introduced to a zwitterionic membrane experiencing surface tensions of 10 mN/m and 30 mN/m, whereas both an unfolded and a pre-folded

peptide were placed in the presence of the anionic membrane under the same conditions. Figure 3-1 depicts the valine contacts and hydrogen bond formation for results. In each folded peptide trajectory, the plots show 3-4 hydrogen bonds formed at the earliest timepoint plotted because the peptide partially unfolded within the first nanosecond, in solution or during initial contact with the bilayer. Interactions of the peptide with the surfaces under 10 mN/m surface tension were qualitatively similar from those seen in the longer trajectories in the absence of surface tension, with somewhat increased levels of hydrophobic contact; the peptide unfolded and made only sporadic contact with POPC, and maintained partial folding and partial hydrophobic contact with the POPG/POPC surface over 250 ns.

Increase of the surface tension to 30 mN/m changes the interactions between the peptide and bilayer significantly. In the case of SVS-1 interacting with POPC, by 75 ns the peptide has made full hydrophobic contact with the bilayer interior, inserting via the turn by a pathway similar to that seen in the 1.5 μs tension-free POPG/POPC simulation, and remains in strong hydrophobic contact for the remainder of the trajectory. The degree of folding increases somewhat during or directly after this insertion, but the peptide then gradually loses its folded structure. The anionic surface under 30 mN/m tension promotes rapid insertion of the initially unfolded peptide, apparently preventing or slowing further folding.

Figure 3-2 captures a detailed picture of our finding. In these simulations, SVS-1 peptides do not stick to the zwitterionic POPC surface unless hydrophobic contact is made; without exposed hydrophobic surface (which we produce using applied surface tension, but whose presence might be expected for highly curved small unilamellar vesicles)

desorption is rapid. At the PC/PG surface, electrostatics bind the peptide to the bilayer, so that initially unfolded SVS-1 can either begin to fold atop the bilayer surface before making full hydrophobic contact with the lipid tails (as seen in the absence of surface tension or at 10 mN/m) or, when the bilayer's hydrophobic interior is exposed under high surface stress, can rapidly engage in hydrophobic contact with the bilayer without folding.

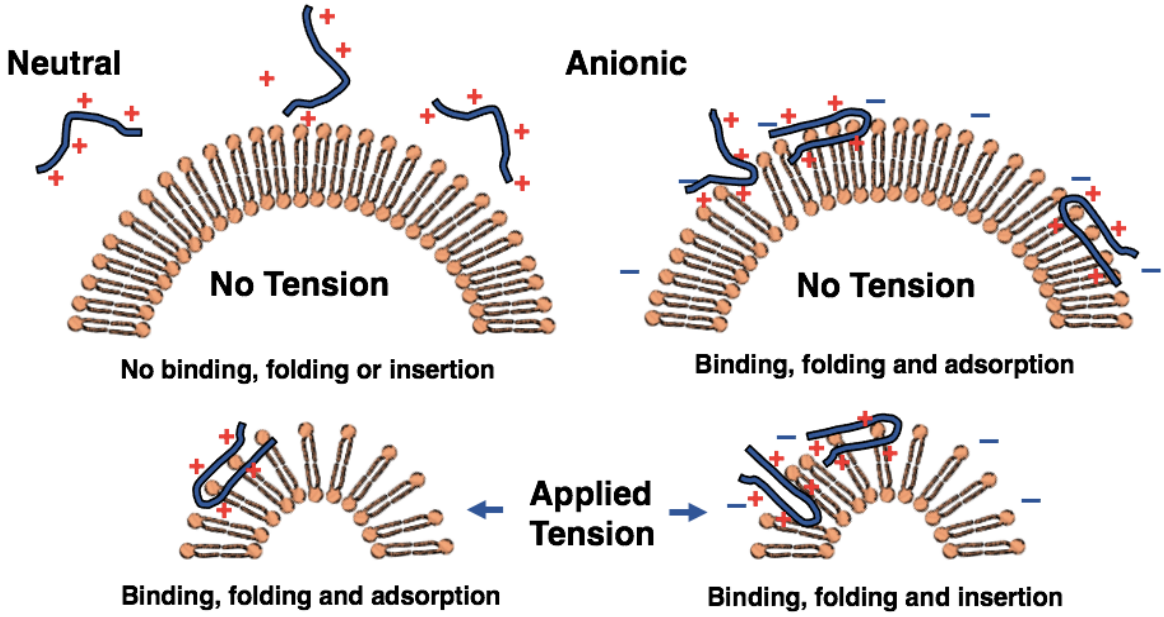

Figure 3-2: Summary of simulation results with and without tension.

3.3.2 Lipid Tail Packing

Simulations in the presence of lipid bilayers were initially performed using a lipid composition that matched experiments, with an eye toward investigating binding of SVS-1 to different lipid phases. The phase behavior of the 1:1 DPPC/DPPG mixtures simulated using the CHARMM 36 force field under Gromacs shows a significant over-stabilization of ordered (gel or ripple-like) structures, with ordering occurring spontaneously even at temperatures 10 K above the main phase transition, 323 K, complicating data interpretation. Similar behavior has been noted [21] and attributed to cut-off schemes.

Ordered structures appear to be thermodynamically stable up to T=328 K as indicated by growth of an ordered domain in a "stripe" multiphase arrangement, even using the cut-off scheme suggested by Lee et al. [21] and provided by the current CHARMM-GUI output. Because the phase behavior is unpredictable and depends on the box dimensions, systematic analysis could not be undertaken. Qualitatively, under conditions where the bilayer remains in the disordered fluid phase, SVS-1 folding and interaction with the bilayer is consistent with its behavior in POPC as reported below. An example of how SVS-1 might interact with a bilayer in a low-temperature ripple phase was observed in one trajectory, in which the (pre-folded) peptide is situated in a disordered region in the narrow part of the ripple, with several of its hydrophobic valine sidechains making contact with lipid tails (Figure 3-3).

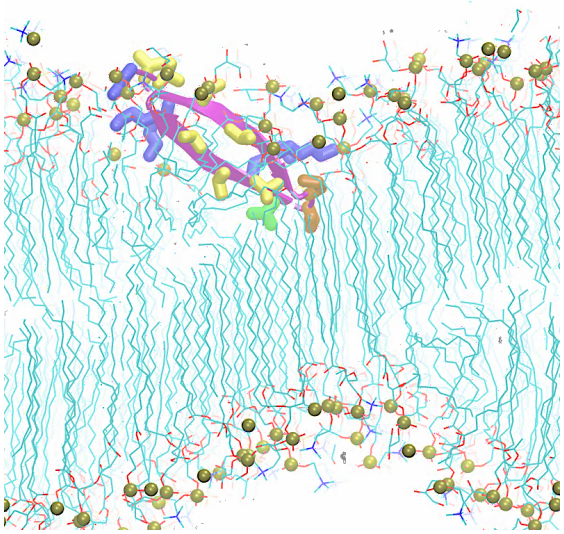

Figure 3-3: SVS-1 adsorption in ripple phase 1:1 DPPC DPPG mixture. SVS-1 hairpin is colored magenta as a new cartoon. Peptide side chains are represented as lysine (blue), valine (yellow), proline (brown) and threonine (green). Lipids are represented as lines and phosphorus headgroups are colored as tan beads. Water and ions removed for clarity.

Redistribution of lipids partition the top leaflet in distinct domains, evident by a higher concentration of POPG lipids clustered closer to the lysine nitrogens (See Figure 3-4. We carried out a simulation on a pre-folded SVS-1 at pure POPG bilayer and observed no insertion even after 500 ns under 30 mN/m tension (See Figure 3-5). The barrier to the 'flip and dip' mechanism is apparently enhanced in a pure POPG bilayer, due to the strong electrostatic attractions between lysine sidechains and headgroups.

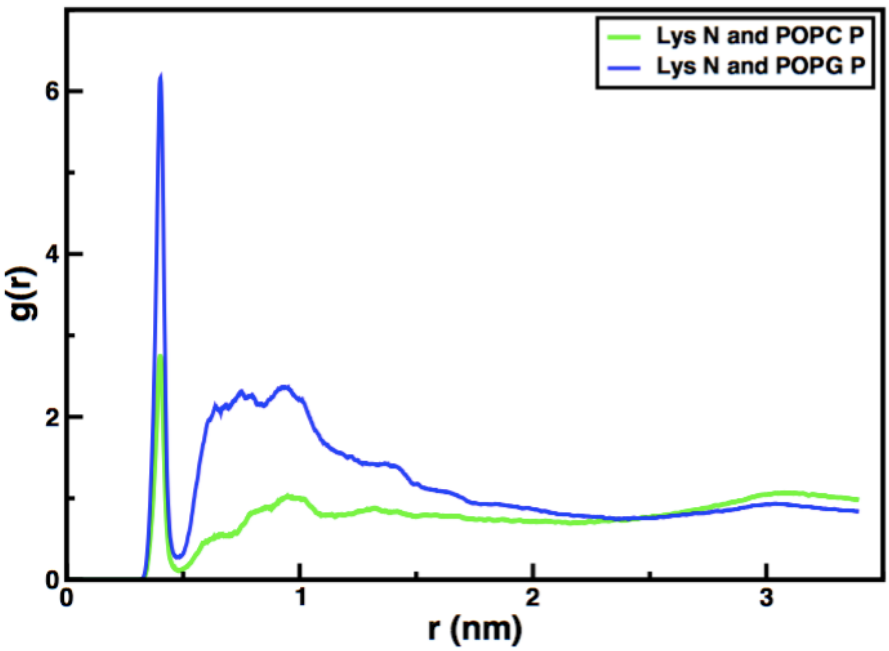

Figure 3-4: The radial distribution function (RDF) for 250-ns trajectory of "flip and dip" mechanism. g(r) is calculated between lysine nitrogens of SVS-1 and phosphorus atoms in respective POPG and POPC lipids after 50-ns.

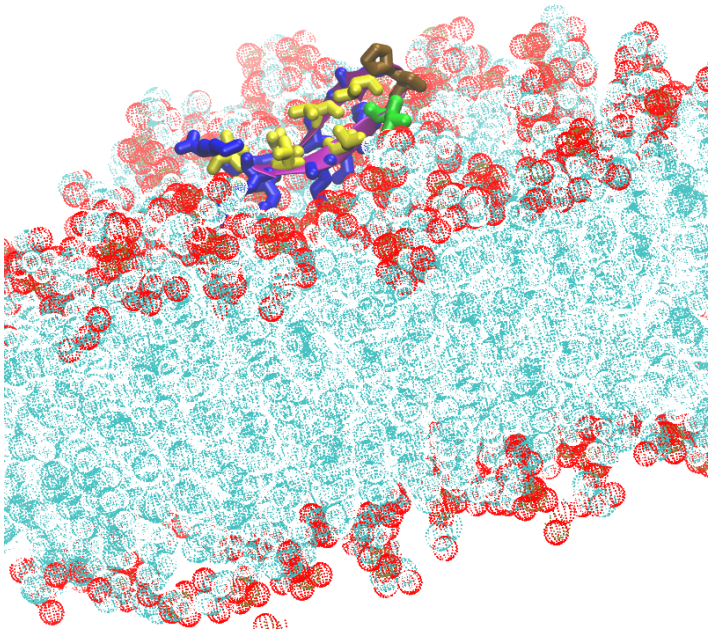

Figure 3-5: SVS-1 adsorption to pure POPG bilayer under 30 mN/m surface tension. Protein is folded and represented as a new cartoon in magenta. SVS-1 sidechains are depicted as a licorice: valine(blue), lysine(green), proline(orange) and threonine(yellow). Lipids are represented as dotted in VMD drawing method.

3.4 Conclusions

In Chapter 3 we explored the influence tension plays on the role of peptide insertion. The most unexpected simulation observation, given previous reports of the inability of SVS-1 to bind to or fold in the presence of zwitterionic bilayer surfaces, is that the SVS-V8W binds and folds in the presence of the zwitterionic bilayer under an applied tension. In simulations SVS-1 was observed to fold and insert its valine sidechains into contact with the lipid tails of an anionic bilayer but to form few and transient contacts with an all-zwitterionic lipid bilayer. Only when the stress-free zwitterionic bilayer was placed under surface tension, increasing its area per lipid, did the peptide establish and maintain close contact with the bilayer plus partially folding.

Simulations of SVS-1 showed that it can bind to a POPC surface that is significantly

expanded in its area per headgroup through application of 30 mN/m surface tension, and follow a similar "flip and dip" transition marked by a (transient) jump in the degree of folding. It seems that the zwitterionic surface under tension is able to stabilize the hairpin temporarily, in the absence of the electrostatic screening provided by the anionic headgroups, by providing an environment favorable to the amphiphilic arrangement of Lys and Val side-chains in the folded state.

We observe this shallow, folded subsurface state in simulations with the POPC/POPG bilayer (e.g. Figure 3-4d) or forming at a DPPC/DPPG bilayer surface in a ripple phase environment in simulation (See Figure 3-3). Formation of the deeper state, presumably a lytically active state that we have not seen in simulations, thus appears to require a combination of charged headgroups and a sufficiently fluid lipid environment or a cooperative mechanism. These requirements suggest that the headgroups may be an integral part of the deeply inserted state, perhaps as part of a toroidal structure with the headgroup charges interacting with the lysine sidechains. This would contrast with the beta-barrel structures modeled by Gupta et al. [3] in a study of the closely related MAX1 peptide, structures which do not provide for interactions between the anionic headgroups and the lysines in the peptide pore. In fact, some instability of the simulated beta-barrel structures in that study was attributed to Lys-Lys repulsions. In a toroidal structure, these repulsions would be screened by the anionic headgroups, albeit at the cost of disrupting intermolecular hydrogen bonds between peptide backbones. The relative stabilities of these different structures (or perhaps some intermediate state, with the beta-barrel only disrupted at one or more locations) could be a topic for future simulation studies. Together the results indicate that the fate of the peptide encountering the surface and inserting will

depend on a combination of surface charge and solvent exposure of the hydrophobic tails.

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Chapter 4

Concluding Remarks and Outlook

This body of work presents new findings on the influence electrostatics and lipid packing play on binding, folding and insertion dynamics of a small cationic AMP. SVS-1 is an engineered peptide shown to have efficacy against cancer cells as opposed to normal healthy cells. The susceptibility of cancer cells to SVS-1 is an intriguing advancement and addition to the field of anti-cancer studies using small peptides. Though SVS-1 has shown proclivity to disrupting cancer membranes, the underpinning mechanism lacked understanding. Therefore, molecular simulation was deployed to survey the atomistic scale for insight.

Simulation results suggest that SVS-1 binds strongly to anionic lipids at the surface, adopting an initial configuration with its valine sidechains facing away from the bilayer surface. (SVS-1 associates only transiently with the surface of the zwitterionic POPC membrane, unless sufficient surface tension is applied to produce enhanced binding and insertion through hydrophobic exposure.) At the charged bilayer surface, SVS-1 gradually and reversibly adopts a hairpin structure over 100's of nanoseconds, with most of its valines remaining far from the hydrophobic lipid tails. A transition to a state with significant contact between the hydrophobic face of the peptide and the lipid tails was observed to involve an initial dip of the ^{D}Pro-^{L}Pro turn accompanied by a flip of the valine side-chains beneath the lipid head-groups. We have no simulation evidence for pore-like structures, which probably require cooperative behavior of multiple peptides. We speculate, however, that the different degrees of insertion (inferred from fluorescence spectra of SVS-V8W) observed depending on temperature and lipid charge may reflect the presence or absence

of toroidal pores, whose stability would be influenced by the bilayer's flexibility and ability to screen the electrostatic repulsions among embedded peptides.

Added surface tension accelerates the partial insertion of folded SVS-1 peptide, facilating simulation studies of many different conditions. Interestingly, replacement of the 50/50 POPG/POPC mixed bilayer with a pure anionic POPG bilayer resulted in significantly slower insertion, suggesting that insertion is aided by the boundaries between charged and zwitterionic lipid domains.

This thesis establishes the need for further work on antimicrobial peptides as targets for anti-cancer studies, but also highlights the importance of accurate simulation tools for extending experimental research. Numerous studies have shown how readily AMPs can be modified due to its small size and intrinsic nature for selectivity. Ideally, our simulation protocol should map the four phases presented by Sinthuvanich et al.: (1) free dynamics in solution (2) binding to the charged membrane surface (3) folding atop the membrane (4) insertion and disruption of anionic membrane. While the work presented on SVS-1 gives some idea of the timescale for binding and folding, we still lack insight into deep insertion and membrane poration events. Presumably, advanced sampling techniques or biased systems can provide answers to the important questions surrounding lipid tail rearrangement and peptide-lipid interactions necessary for disrupting the bilayer integrity. In an effort to extend the work on SVS-1, a first step for better understanding the peptide-lipid interactions is to conduct Umbrella Sampling. Through US, we can extrapolate the free energy of insertion for the peptide from the solution phase to deep within the bilayer midplane. Here, the influence of electrostatics can be better understood as the peptide binds and must undergo the 'flip and dip' mechanism in order to bury further in the membrane. The quantitative data from the free energy profile would map to the effects of electrostatics necessary for binding and those which facilitate the insertion. An alternative method is simulating the folded or unfolded peptides in a preformed pore. A number of

questions could be answered: How stable is the poor in the presence of the charged peptide? What peptide-lipid interactions predominate this structure? Do water molecules inhibit or promote folding within the pore? If the pore is unstable, will it push the peptide outside the cavity or will it envelope the peptide like a micelle? By answering these questions, experimentalists and theoreticians can take the next steps in engineering new peptide structures and improving the efficacies of anti-cancer peptides. It is important to note that computational resources and timescales of these biological phenomena are the limiting step to this cancer disruption study. Therefore, collaboration and improving on current tools will be key in unlocking the potential of AMPs.