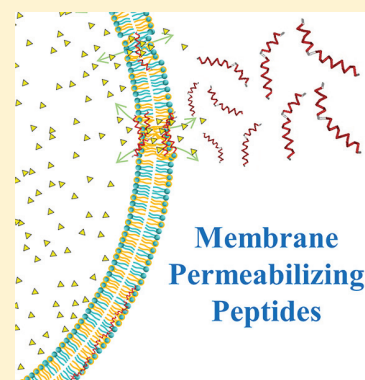


Mechanistic Landscape of Membrane-Permeabilizing Peptides

Shantanu Guha,[†] Jenisha Ghimire,[†] Eric Wu,[†] and William C. Wimley^{*†}

Department of Biochemistry and Molecular Biology Tulane University School of Medicine, New Orleans, Louisiana 70112, United States

ABSTRACT: Membrane permeabilizing peptides (MPPs) are as ubiquitous as the lipid bilayer membranes they act upon. Produced by all forms of life, most membrane permeabilizing peptides are used offensively or defensively against the membranes of other organisms. Just as nature has found many uses for them, translational scientists have worked for decades to design or optimize membrane permeabilizing peptides for applications in the laboratory and in the clinic ranging from antibacterial and antiviral therapy and prophylaxis to anticancer therapeutics and drug delivery. Here, we review the field of membrane permeabilizing peptides. We discuss the diversity of their sources and structures, the systems and methods used to measure their activities, and the behaviors that are observed. We discuss the fact that “mechanism” is not a discrete or a static entity for an MPP but rather the result of a heterogeneous and dynamic ensemble of structural states that vary in response to many different experimental conditions. This has led to an almost complete lack of discrete three-dimensional active structures among the thousands of known MPPs and a lack of useful or predictive sequence-structure–function relationship rules. Ultimately, we discuss how it may be more useful to think of membrane permeabilizing peptides mechanisms as broad regions of a mechanistic landscape rather than discrete molecular processes.



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1. INTRODUCTION

The permeability barrier of the lipid bilayer membrane is a ubiquitous and essential feature of every living organism on earth and thus is also a critical point of vulnerability. For this reason, membrane permeabilizing proteins and peptides are a ubiquitous part of the defensive and offensive biochemical arsenals of all living things. They are also the subject of significant translational research toward development into drugs and molecular tools. Here, we review the field of membrane permeabilizing peptides.

1.1. What Is a Membrane Permeabilizing Peptide?

The hydrocarbon core of a lipid bilayer membrane, made of almost pure hydrocarbon,¹ is one of the most hydrophobic nanoenvironments that can be found in the biosphere. Although the bilayer is a two-dimensional fluid that is only two molecules thick and the hydrocarbon core is only a portion of the total bilayer thickness, the extremely low solubility of polar compounds in the hydrocarbon core effectively prevents them from crossing the bilayer. This barrier function is essential to life and may have been an antecedent of the earliest life forms on earth. For any molecule to breach this barrier, i.e., to permeabilize the membrane, the minimum requirement is that a pathway be created that enables passage of polar molecules across the bilayer hydrocarbon core. A membrane permeabilizing peptide, by definition, is one that creates such a pathway, a process that can happen in a variety of ways. For example, a peptide can self-assemble into an explicit, membrane-spanning structure like a proteinaceous channel. In this review, we specifically refer only to explicit, discrete membrane-spanning structures as “pores” which are created by “pore-forming peptides”. Reliable evidence for such structures in membrane permeabilizing peptides is extremely rare. At a minimum, a membrane permeabilizing peptide must have “interfacial activity”,² which means it must partition into a membrane and be at least somewhat amphipathic^{3–6} in order to drive the rearrangement of membrane lipids,^{3,7,8} disrupting the normally strict segregation between the bilayer hydrocarbon core, the interfacial zone of the bilayer, and the aqueous phase.^{1,9–12} This disruption enables polar lipid and peptide moieties, as well as water molecules, to make incursions into the hydrocarbon core and reduce its hydrophobicity.² A reduction in the hydrophobicity of the core reduces the highest energy barrier to the passage of polar molecules, thus increasing the rate of the limiting step in permeation.

As we discuss in this manuscript, most of the thousands of known MPPs likely act by some form of interfacial activity, rather than by pore formation. Here, we will refer to all such

peptides as “membrane permeabilizing peptides” or MPPs, a generic phrase specifically chosen as a functional description that is not meant to invoke any particular mechanism or structure. In this review, we will discuss membrane permeabilizing peptide sources and structures, and we will also discuss the ways in which one can measure their functions in model systems, in computers, and in living cells. We discuss the behaviors that have been observed in these many systems. Finally, we discuss the concept of a “mechanistic landscape” of membrane permeabilizing peptides (Figure 1) and how an MPP can sample a continuum of behaviors, functions, and structures influenced by a host of experimental conditions. This fundamental behavior must be better understood, and we must learn how to better describe it to enable the design or optimization of membrane permeabilizing peptides that might be useful tools or drugs in the laboratory or in the clinic.

1.2. Why Do We Study Membrane Permeabilizing Peptides?

Membrane-permeabilizing peptides could have utility in a wide variety of applications.^{12–21} Foremost, there has long been promise, but also disappointment, in the possible translational applications of antimicrobial peptides (AMP) to the prevention and treatment of drug resistant bacterial infections.^{7,21,22} Improvement in treatment outcomes may arise from antimicrobial peptides that are engineered or evolved to have improved properties, including selectivity for bacterial membrane permeabilization compared to host-cell membrane permeabilization.^{23,24} Similarly, interfacially active peptides can have potent inhibitory activity against enveloped viruses,^{25–30} a group that includes influenza, HIV, Ebola, Dengue, Zika virus, and many others.^{25,29} In an exciting recent paper,³¹ Cho and colleagues showed that an amphipathic helix called AH peptide, which preferentially lyses both lipid vesicles and enveloped viruses that have high curvature, has potent, protective antiviral activity in living animals. Systemic administration of peptide in Zika virus-infected mice protected against death, and reduced clinical symptoms, including brain damage, and reduced viral propagation. Remarkably, the peptide crossed the blood–brain barrier, without damaging it, and directly reduced viral loads in the brain.

Another potentially far-reaching application of membrane-active peptides is potent membrane permeabilization against host cells. Peptides with this property could be used to kill cancer cells,^{19,32,33} but only if targeting and control of the peptide activity can be optimized to permeabilize only the diseased cells. Along these lines, the bee venom MPP melittin, associated with lipidic “nanocarriers,” targets cancer cells in vitro and in vivo.^{19,34}

Membrane permeabilization can also be used to deliver polar compounds,^{35,36} including drugs, proteins, peptides, oligonucleotides and their analogs, and other molecules,³⁷ to cells. The ability to deliver polar and macromolecule drugs to cells could be very useful in the laboratory and also in the clinic^{38,39} where it would significantly expand the universe of potentially druggable compounds. Although molecular mechanisms and pathways are not always well understood, cell penetrating peptides (CPPs) can sometimes deliver cargo through the plasma membrane in a process that may involve transient local permeabilization,⁴⁰ permeabilization of endosomal membranes following uptake, or both pathways at once.⁴¹ Many CPPs utilize both pathways in a manner that depends on a variety of experimental conditions.⁴¹ Examples of membrane permeabi-

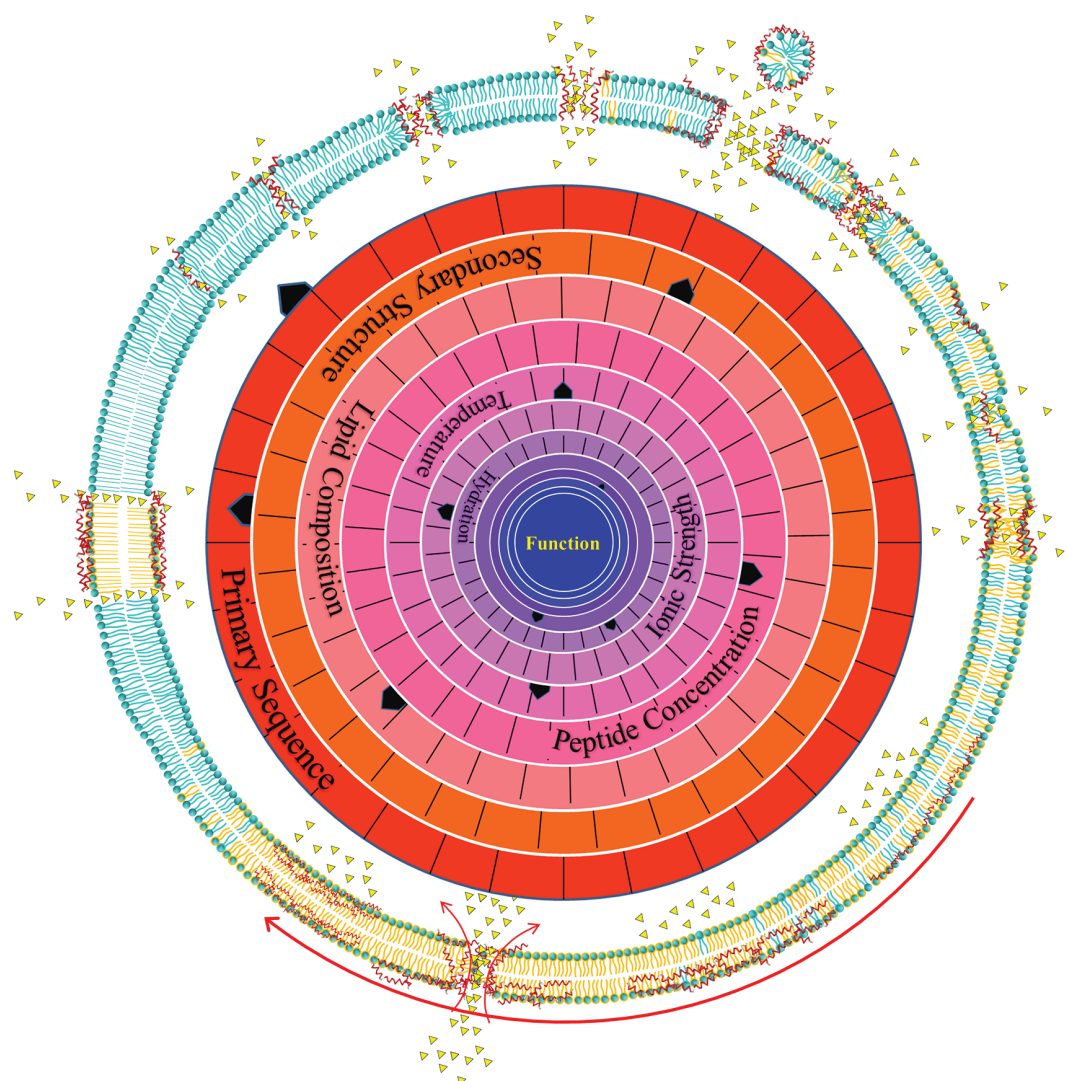


Figure 1. Mechanistic landscape of membrane permeabilizing peptides. The molecular mechanisms of membrane permeabilizing peptides have, for the most part, eluded atomic level description despite decades of intense study. As a result, their active structures and mechanisms are often drawn as cartoons like the imaginary snapshots arrayed in the cartoon bilayer above. Different colored lipids indicate changes in membrane composition, lipid tails, and headgroups. Curvy tails indicate fluid phase bilayers and straight tails indicate more ordered domains. In this review we describe how it might be useful to think of membrane permeabilizing peptides on a mechanistic landscape where molecular mechanism is not a fixed entity but instead depends on the sum of many experimental variables. The image in the center of the cartoon vesicle depicts concentric “dials”, one for each experimental variable (many are not explicitly shown). Each dial can be set to a particular “value” in the parameter space. The combinations of all of the settings give rise to a point on multidimensional mechanistic landscape.

lizing peptides can be found in the relatively young field of “profection”, the delivery of whole proteins to the cell cytosol.^{37,42} Although multiple mechanisms have been proposed, delivery of a protein through the plasma membrane and/or an endosomal membrane requires that a profection reagent must induce a transient and localized but also locally significant permeabilization event at the membrane. An example of a peptide profection reagent has been described by Futaki and colleagues,³⁷ who started with a classical membrane permeabilizing amphipathic α -helix from spider venom and engineered anionic charges into the sequence to make it less lytic to mammalian cell membranes at neutral pH. Ultimately, they obtained a peptide capable of passively delivering some macromolecules to the cytosol of cells in culture.

For all of these important applications, and others not listed, a better understanding of the mechanisms of membrane permeabilization is essential. In fact, growth of the field has been inhibited for decades by the lack of accurate, useful, and predictive rules for membrane permeabilization, forcing the majority of researchers to utilize natural molecules without optimization, or to create novel or optimized sequences by simple trial and error.

1.3. The Sting: Lessons Learned from 60 Years of Studying Melittin

Of all the known MPPs, one of the most studied, and perhaps most enigmatic, is melittin, the 26 amino acid peptide that is the main component of the venom of the European honey bee, *Apis mellifera* (Figure 2). By the 1950s, a proteinaceous, cytolytic, necrotizing component of honey bee venom, which was long recognized, had been named “melittin”.⁴³ The amino

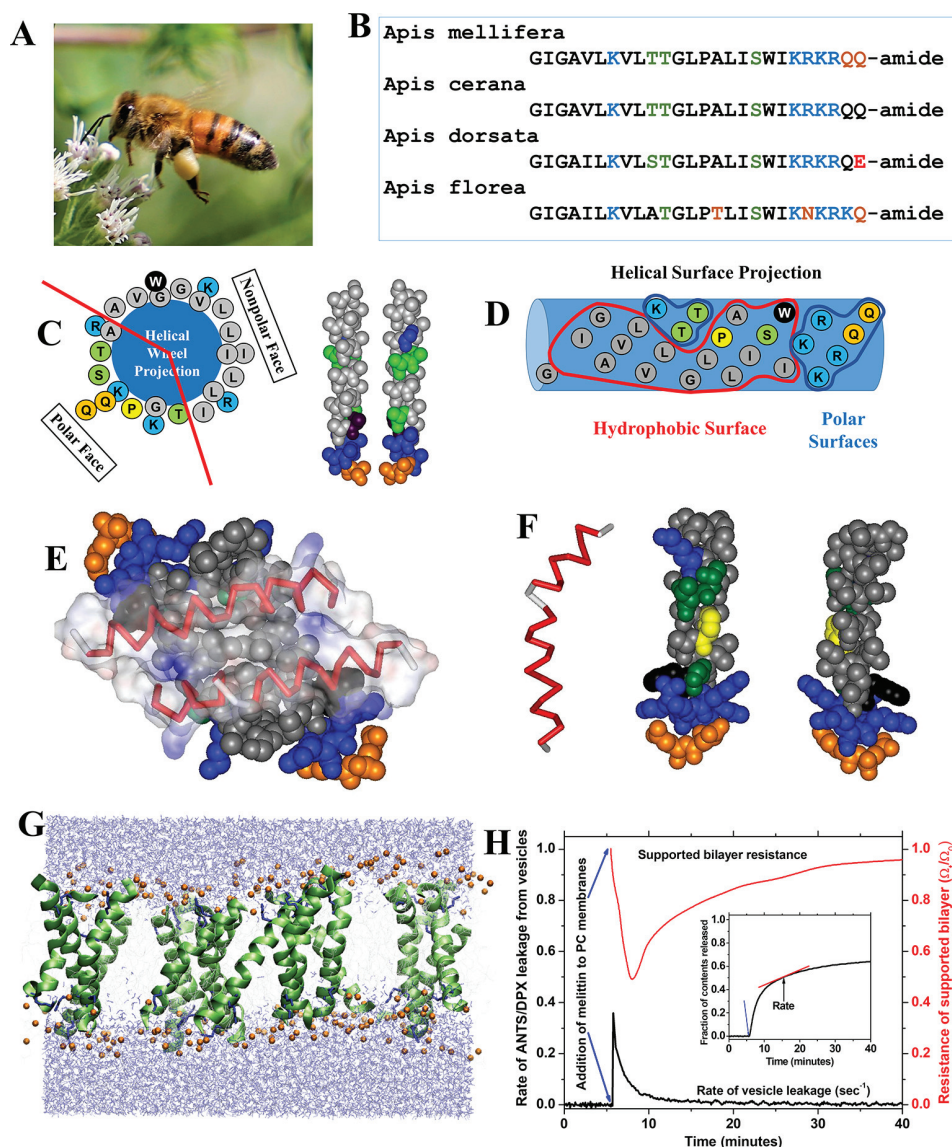


Figure 2. Melittin, the archetypal membrane permeabilizing peptide. **A:** European honey bee (*Apis mellifera*) workers produce a defensive venom that contains many compounds, including peptide and proteins. The most abundant component by weight is melittin, a 26-residue membrane permeabilizing peptide. Photograph by William Wimley, used with permission. **B:** Amino acid sequences of melittin from *Apis mellifera* and several closely related species. Sequences are generally hydrophobic over the first 20 residues, except for lysine at position 7, and are highly polar and basic on the C-terminus. **C:** Helical wheel diagrams show the placement of residues on the surface of an imaginary perfect helix. **D:** On the surface of the ideal melittin helix, hydrophobic residues form a contiguous surface. **E:** In the first three-dimensional structure of melittin in solution⁵² and other structures,⁵⁰ its amphipathicity was apparent in the burial of the hydrophobic surfaces in the core of a tetrameric structure or in the membrane. **F:** The amphipathicity of the melittin monomers in the context of the crystal structure, in which the helices are bent and disrupted at the central Gly-X-Pro. **G:** Some experiments⁵⁰ and biased molecular dynamics simulations^{429,423} demonstrate melittin forming membrane spanning equilibrium pores. However, unbiased simulations of slow insertion equilibrium requires currently unachievably long simulations.⁵²⁸ Images courtesy of Jakob Ulmschneider. **H:** Under other conditions, experiments, such as electrochemical impedance spectroscopy and vesicle permeabilization, show that the permeabilization of membranes by melittin is a transient nonequilibrium process.^{59,60,66}

acid sequence of melittin was determined in 1967 by Haberman,⁴⁴ who also performed some of the first sequence-function relationship studies.⁴³ As early as 1967, antibacterial activity of melittin against drug resistant bacteria was reported.⁴⁵ By 1969, the membrane permeabilizing activity of melittin was well-established^{46,47} and was understood to be the consequence of its structural amphipathicity, which is two-dimensional. Melittin was immediately recognized to be amphipathic along its sequence, mostly hydrophobic from the N-terminus to residue 20, and highly polar on the cationic

C-terminus, residues 21–26.⁴⁷ Later, melittin was also recognized to fold in membranes into an amphipathic α -helix^{48–50} which has polar and nonpolar surfaces that are orthogonal to the helix axis.^{51,52} Like most membrane permeabilizing peptides, melittin, by virtue of its cationic charge and amphipathicity, binds to lipid bilayer membranes and causes permeabilization by creating a pathway through the hydrocarbon core of the bilayer that polar molecules can use to cross the membrane.

But how do we describe the mechanism by which melittin permeabilizes membranes? Early electrophysiology studies by Tosteson provided data indicating that melittin formed voltage-dependent, anion-selective, membrane-spanning pores, which had a concentration dependence that indicated a tetrameric structure.⁵³ Around the same time, however, DeGrado and colleagues showed that melittin releases proteins from erythrocytes⁵⁴ in a transient manner indicating a larger, voltage-independent pathway through the membrane. The concept of explicit pores has also been supported by diffraction and other measurements at low hydration and high concentration which showed melittin to form membrane-spanning helical structures.^{50,55,56} However, other work, including FTIR,⁵⁷ oriented circular dichroism,^{58,59} X-ray diffraction⁵⁸ and EPR⁶⁰ have shown that melittin has its helical axis lying mostly parallel to the membrane surface under many experimental conditions. This is not consistent with equilibrium transmembrane pore formation, unless the equilibrium pores are a minor fraction of the total population of peptide. Further, multiple studies have shown that melittin permeabilizes bilayers to different classes of molecules, sometimes including macromolecules,^{61,62} as lipid composition and peptide concentration are varied. Therefore, melittin can form a range of different structures in the membrane, likely an ensemble of coexisting structures. At a high enough concentration, melittin solubilizes bilayers and forms peptide-lipid micelles, like a detergent.⁶³

Perhaps most surprising, dye leakage studies^{64,65} and electrochemical impedance spectroscopy⁶⁶ have shown that melittin often forms transient, nonequilibrium, pathways through synthetic bilayers. Under many experimental conditions, these permeabilization pathways exist only for a short time after peptide first encounters the membrane surface. Within minutes after leakage begins, it slows or stops completely. As long ago as 1982, DeGrado et al. reported a transient permeabilization process in erythrocyte membranes treated with melittin. They attributed attenuation of permeabilization to peptide translocation,⁵⁴ an idea that is still the leading hypothesis to explain transient permeabilization. In a recent study, Weisshaar and colleagues showed that bacterial permeabilization by melittin proceeds through a complex series of steps that include stochastic membrane permeabilization after a long lag phase, followed by rapid membrane resealing,⁶⁷ observations that are not consistent with equilibrium pores.

Melittin is the archetypal membrane permeabilizing peptide, and also emblematic of the difficulties of the field. We have been able to speak generally of the physical chemical basis of its membrane binding and permeabilizing activity since shortly after its sequence was known. Yet, after five decades of intense study,^{68,69,78–87,70–77} the exact molecular mechanism of melittin continues to be debated and new insights continue to appear in the literature.^{65,67}

1.4. Introduction to the Mechanistic Landscapes of MPPs

Our inability to describe the action of melittin and other MPPs with single or explicit molecular models is not a failure of our techniques, but perhaps is a failure of our vocabulary. This review is inspired by the idea that the molecular mechanism of an MPP is not a fixed entity. We discuss the behaviors of membrane permeabilizing peptides and how they might best be considered to occupy part of a continuous “mechanistic landscape” in which mechanism and behavior are both

heterogeneous and plastic, and also dependent on the contributions of many experimental details. These include peptide sequence and structure, as well as lipid composition, peptide concentration, temperature, ionic strength, pH and many other variables. Experimental “variables” also include the assay(s) used to measure permeabilization, which are numerous, and how the observations are interpreted, which are variable. The detailed shape of the landscape is likely different for each peptide in detail, although they must broadly overlap. The mechanistic landscape is driven by the sum of a large set of physical chemical interactions. For example, important interactions occur between the fluid phase bilayer and peptides that have partitioned into it. These interactions are driven by interfacial hydrophobicity⁸⁸ and electrostatic interactions, which are nonadditive.⁷⁸ There are also interactions between the peptides themselves in the context of the “tumultuous chemical heterogeneity”¹ of the bilayer. These interactions drive peptide self-assembly⁸⁹ and secondary structure formation.^{79,90–92} There are also interactions between the highly amphipathic lipids in the dynamic milieu of the bilayer and how these interactions are altered by interfacially active peptides partitioned into it. Finally, there are important interactions of water molecules with peptides, lipids, and the membrane itself. In fact, penetration of water into the hydrocarbon core may be the most appropriate molecular signature of membrane permeabilization,⁹³ although it is difficult to measure experimentally.

In this review we do not attempt, in most cases, to assign a particular mechanism to any membrane-permeabilizing peptide. Rather, we describe what can be observed and what has been observed in the laboratory as well as what these observations might mean. Ultimately, we hope to begin to understand the mechanistic landscapes occupied by membrane permeabilizing peptides to use this understanding to enable more rational rules for the design, engineering and optimization of their activities, and to generate a more accurate vocabulary to discuss their activity profiles.

2. DIVERSITY OF MEMBRANE PERMEABILIZING PEPTIDES

Given the ubiquity of biomembranes in life on Earth, it is not surprising that membrane permeabilizing peptides are also abundantly found in many living things (Figure 3). There are thousands of known MPPs, many of which are host defense peptides, and there are interesting examples from many sources and structures. Next, we will outline some of the major sources of currently known MPPs.

2.1. Host Defense Peptides

The innate immune systems of plants and animals are the most common source of known membrane permeabilizing peptides. They produce host defense or antimicrobial peptides (AMPs), which were first discovered in the 1980s in insect hemolymph,^{94,95} mammalian neutrophil granules,⁹⁶ and frog skin secretions.⁹⁷ Now, thousands of examples are known⁹⁸ from an immense variety of tissues and cell types in essentially all classes of higher organisms. The number of examples continues to rapidly increase as additional organisms around the globe are examined. AMPs are wildly diverse in structure but are nearly unified by their cationic nature,⁹⁸ interfacial activity,² and by their propensity to permeabilize anionic synthetic bilayers and the anionic cytoplasmic membranes of bacteria (discussed below). Many organisms produce sets of

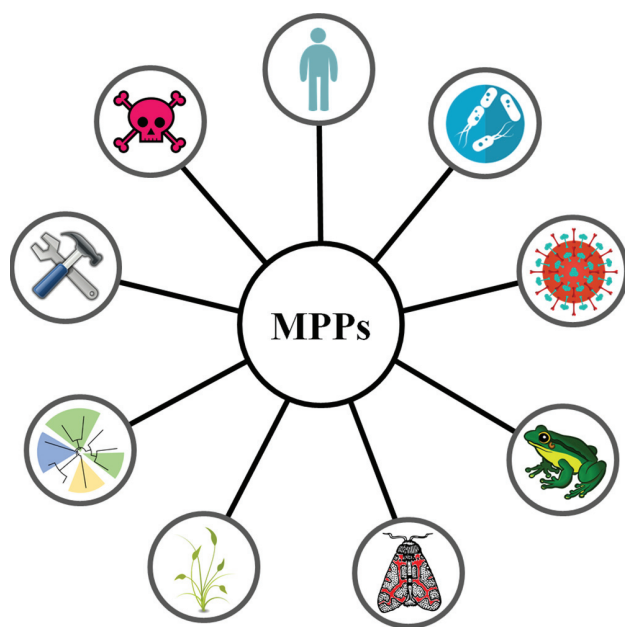


Figure 3. Sources of membrane permeabilizing peptides. There are a multitude of sources for membrane-permeabilizing peptides (MPPs) as shown in the figure above. Clockwise from the top, sources include humans and other mammal host defense, bacteria and fungi, viruses, amphibian and other vertebrate host defense, insect host defense, plant host defense, bioinformatics and computational approaches, engineering and rational design, and venoms and toxins. Host defense peptides are the most ubiquitous, but there are also MPPs which can comprise part of a venom or toxin cocktail, viroporins, de novo designed peptides, synthetically evolved peptides, and other sources which are not listed. Overall, there are thousands of known MPPs and certainly many more to be discovered. Images reproduced with permission under Creative Commons CC0 license.

multiple MPPs that often differ only slightly from one another, such as the multiple α - and β -defensins found in birds and mammals,^{99–101} supporting the idea that different membranes have variable susceptibility to permeabilization by one peptide.

Recently, a novel function for the membrane permeabilizing activity of frog skin MPPs was proposed¹⁰² and then tested by Roelants and colleagues.³⁶ These authors noted that frog skin secretions often contain high concentrations of one or more peptide or protein toxin in addition to multiple MPPs. In *Xenopus laevis* for example, the membrane permeabilizing peptide caerulein precursor fragment (CPF)¹⁰³ and the receptor binding toxin caerulein are both present in skin secretions at millimolar concentrations.³⁶ Roelants and colleagues pointed out that the targets of caerulein are inside the body, and they showed that it cannot spontaneously translocate into cells or permeabilize cells to gain entry. Using cells in culture and live snakes as predator models, these authors showed that CPF transiently permeabilizes eukaryotic cells at physiologically relevant concentrations and that this is both necessary and sufficient to enable entry of the toxin caerulein into the circulation of the snakes. These results suggest that these two molecules comprise a two-part chemical defense system against predators in addition to (or instead of) comprising part of the host defense system against bacteria.

Microbes also produce a vast array of defensive compounds or toxins designed to reduce competition by other microbes. Of course, these include small molecules (e.g., penicillin and

many other conventional antibiotics) and specific protein toxins, such as the colicins.¹⁰⁴ However, bacteria and fungi also produce defensive MPPs, including some of the best studied MPPs such as the alamethicins,^{105–107} the peptaibols produced by the fungus *Trichoderma viride*, and the gramicidins^{108,109} produced by the soil bacterium *Bacillus brevis*. Pathogenic bacteria also produce many offensive toxins, some of which are hemolytic or cytolytic MPPs.¹¹⁰ For example, *Staphylococcus* species produce δ -lysin, a 26-residue helical MPP with potent hemolytic activity^{110–112} that is associated with virulence.¹¹⁰

2.2. Toxins and Venoms

Animal venoms and toxins are complex mixtures of enzymes, peptides, small molecules, and other compounds that aid in prey capture and digestion, as well as defense against other animals. Some venoms are delivered to the body by way of teeth, fangs, stingers, pincers, or spurs, while others are delivered by contact. Some venom components are membrane permeabilizing peptides which contribute to the activity through nonspecific cytolysis and hemolysis, causing cell and tissue damage and exacerbating pain. As described above, the best known example is melittin, the 26-residue MPP that comprises the majority, by weight, of the venom of the European honey bee. Other examples are found in the venoms of bees and wasps, spiders, scorpions, ants, and more.^{113–119}

The α -helical peptide pardaxin is an intriguing MPP that is found in the skin of the Red Sea Moses Sole (*Pardachirus marmoratus*) and related fish.^{120–122} The five nearly identical pardaxin sequences are structurally and functionally similar to melittin. In the laboratory, pardaxin has potent hemolytic and antimicrobial activity^{123,124} and permeabilizes synthetic bilayers.^{121,125,126} It also has activity against cancer cells.^{127,128} However, there is evidence supporting the idea that pardaxin has shark repellent activity in its natural setting.¹²⁹ When introduced directly from the skin of the fish into an attacking shark's mouth and gills, pardaxin causes apparent pain and disorientation.¹²⁹ Unfortunately, tests of the ability of pardaxin as a shark repellent in an open beach setting showed that it was too quickly diluted in open water to be effective.¹³⁰

2.3. Viroporins

Viroporins are virally encoded proteins and peptides that permeabilize internal membranes,¹³¹ viral membranes, or other cell membranes¹³² and “customize host cells for efficient viral propagation”.¹³³ Some viroporins are MPPs. For example, the Ebola virus delta peptide is a 40-residue peptide produced in large quantity by Ebola virus infected cells.^{134,135} The delta peptide permeabilizes both eukaryotic cells and synthetic membranes.¹³⁶ The active portion is a protease resistant, disulfide cross-linked C-terminal hairpin of 15–19 residues.¹³⁶ The influenza M2 channel is a very different sort of viroporin that enables acidification of the core of a virus that has been uptaken into the endosomal pathway.¹³⁷ The essential part of M2 is a membrane spanning α -helix that assembles into a tetrameric channel.¹³⁷ M2 is one of the only MPPs known for which there is strong evidence of stoichiometric self-assembly into a specific membrane-spanning oligomer.¹³⁷

The membrane permeabilizing sequences of viroporin proteins, often amphipathic α -helices, also constitute MPPs in isolated form. For example, the picornavirus 2B viroporin has several amphipathic helices that are potent MPPs^{138,139} in isolated form. Lentiviruses, such as HIV, contain up to three potential amphipathic helix sequences in the intraviral, C-

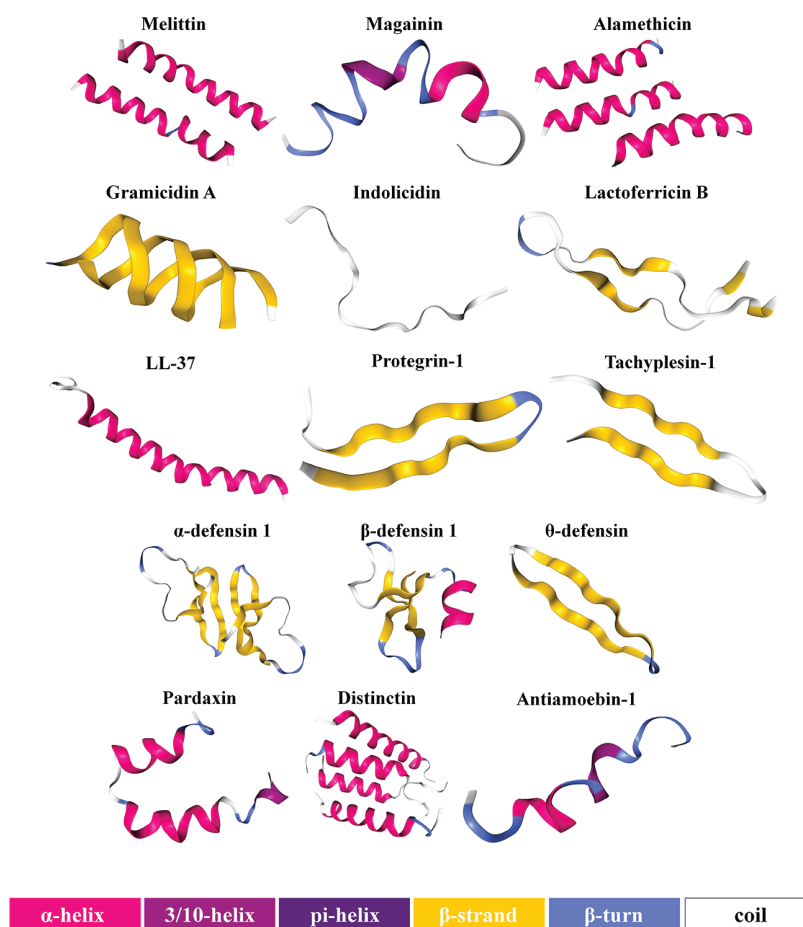


Figure 4. Secondary structures of membrane permeabilizing peptides. Cartoon models for 15 peptides which are known membrane-permeabilizing peptides depicting the wide array of secondary structures. Some peptides are uniformly one secondary structure and other peptides appear to be a hybrid of multiple secondary structure motifs.

terminal domain of their transmembrane fusion proteins.^{140,141} Mutational analysis have shown these sequences to be critical for viral propagation,¹⁴² although their contribution to the viral lifecycle is not well understood.^{143–146} In isolated form, these domains, called lentivirus lytic peptides (LLP), comprise highly potent membrane permeabilizing peptides. In particular, LLP2 of HIV is one of the most potent MPPs known.^{64,143,146–148} HIV and other viruses also have membrane proximal sequences that are interfacially active, such as the membrane proximal external region, MPER, in HIV, which inserts into membranes¹⁴⁹ and permeabilizes them.¹⁵⁰

2.4. De Novo Designed MPPs

Researchers have created new MPPs which bear some relationship to natural sequences. For example, fragments of natural sequences,¹⁵¹ hybrid combinations of known MPPs,^{152,153} or rationally modified variants¹⁵⁴ have been studied. Antimicrobial peptides that permeabilize anionic synthetic lipid bilayers and bacterial membranes have also been designed de novo using first-principles, based on physical chemistry, combined with trial and error testing or screening.^{155–161} They have also been designed using machine learning or data-driven algorithms.^{162–166} In almost all of these cases, the end results are classical interfacially active antimicrobial peptides that are rich in cationic and aromatic residues.

Other types of MPPs, designed de novo from first principles, are very uncommon, especially those that permeabilize zwitterionic bilayers. This is probably because the sequence–structure–function relationship rules are not understood well enough to enable intelligent design of membrane interaction, intermolecular interactions, or oligomer structure of MPPs in membranes. In fact, even if rational modulation of these individual factors were possible, it remains uncertain how they relate, mechanistically, to membrane permeabilization. The amphipathic α -helix is the simplest structure to model and would seem to be amenable to engineering using the regular i to $i + 3$ and i to $i + 4$ spacings¹⁶⁷ to design amphipathic surfaces. Along these lines, there have been a small number of successes. For example, DeGrado and colleagues created and tested a series of de novo designed peptides^{168,169} that were based on the concept of amphipathic α -helical bundles. One active sequence, with 3 repeats of Leu-Ser-Ser-Leu-Leu-Ser-Leu formed ion channels in lipid bilayers¹⁶⁹ that were modeled as small bundles of transmembrane helical peptides.

The peptide GALA is perhaps one of the most well-known examples of de novo design success. It was made by Szoka and colleagues,^{170–173} who wanted to create a pH-triggered MPP. They created an amphipathic α -helix by placing protonatable glutamate residues at positions separated by mostly i to $i + 4$ spacings. “GALA” is named for a repeating unit of glutamate-alanine-leucine-alanine. At pH 7, GALA is inactive, has random

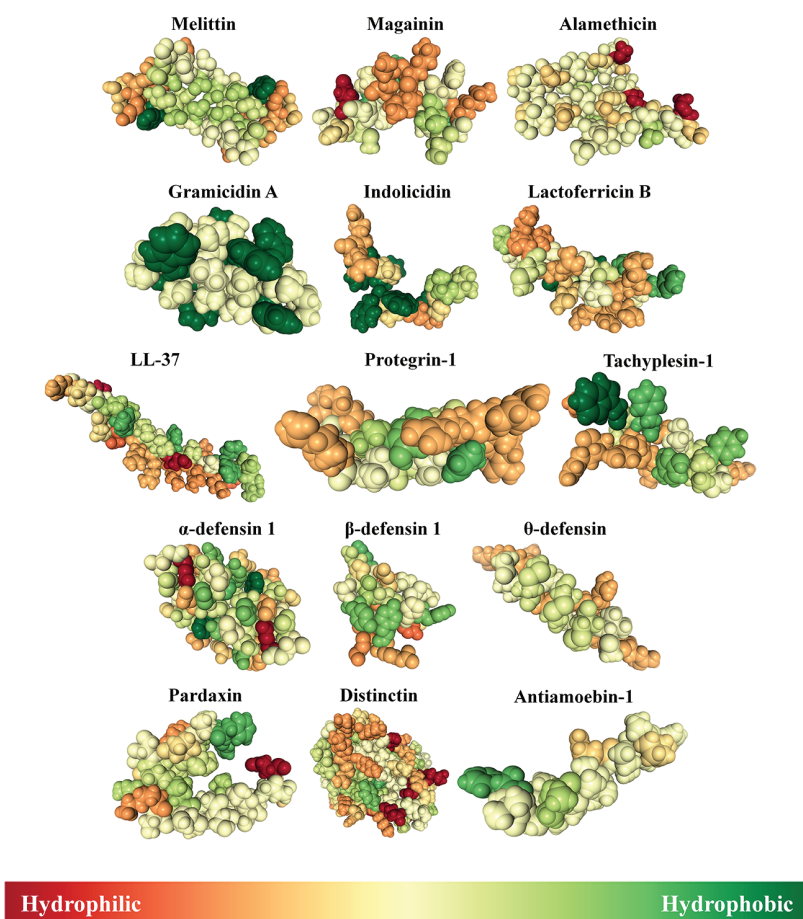


Figure 5. Amphipathic structures of membrane permeabilizing peptides. Space filling models for 15 peptides which are known membrane-permeabilizing peptides depicting hydrophobicity; no peptide here is depicted as singularly hydrophilic or hydrophobic, there are elements of both when considering MPPs.

coil structure, and does not bind strongly to membranes.^{170–173} However, at pH 5 it binds strongly,¹⁷¹ folds into a membrane spanning α -helix,¹⁷⁰ and permeabilizes lipid vesicles^{170,172,173} to small solutes at peptide to lipid ratios (P:L) as low as 1:10 000. This may be the most potent activity ever reported for a membrane permeabilizing peptide.

2.5. Synthetic Molecular Evolution of MPPs

In the absence of explicit sequence–structure–function rules to enable rational design of MPPs, we have turned to synthetic molecular evolution (SME), which we define as the iterative screening of small, rational peptide libraries based on prior knowledge and template sequences. This has proven to be especially powerful for identifying novel membrane activities and gain-of-function peptide sequences. For example, we evolved β -sheet MPPs with potent antimicrobial activity using two de novo libraries that were based on physical chemical first principles. These libraries were screened for peptides that permeabilized somewhat anionic, bacteria-like synthetic lipid vesicles. The “hits” did not reveal singular active sequence motifs but rather showed that active sequences are related by overall physical chemistry.^{5,174,175} A second generation library was designed using one 26-residue first-generation MPP as a template sequence.¹⁷⁶ This “iteration” library was screened for permeabilization of synthetic bilayers made of phosphatidylcholine (PC) lipids. Highly potent, second generation daughter sequences were identified that had previously

unknown properties; β -sheet secondary structure, and highly potent, equilibrium pore forming activity.¹⁷⁶

We have also evolved multiple families of α -helical MPPs with unique functions and properties. First, we created a second generation library based on melittin, which forms transient pores in PC bilayers^{59,64–66,177} and successfully screened for daughter sequences that enable potent, equilibrium permeabilization.⁵⁹ We discovered afterward that at least one of these gain-of-function analogs, called MelPS, enables macromolecule release from lipid vesicles at low P:L.^{62,178} Simultaneously, we screened the same library for loss-of-function analogs, identifying a single amino acid change, Leu 16 to Gly, in melittin which eliminated the membrane permeabilizing activity of melittin against zwitterionic synthetic bilayers and cellular membranes.¹⁷⁹ Subsequently, Hristova and colleagues used MelPS as a template for a third generation library which was successfully screened for more potent macromolecular poration that was triggered by acidic pH.¹⁸⁰ The same library was separately screened for more potent macromolecular poration that was not triggered by pH which led to the discovery of a uniquely potent family of MPPs called macrolittins.¹⁸¹

2.6. Diversity of Structure of MPPs

Membrane-permeabilizing peptides (MPPs) have tremendous diversity in their secondary structures, as there is not a singular secondary structure which best enables membrane permeabi-

lization. In this way, MPPs are unlike integral membrane proteins, which usually have architectures based on helical bundles^{182,183} or beta barrels,^{184,185} and that follow established sequence-structure-function relationships. MPPs do not seem to adhere to a similarly tractable set of rules. Peptides may fall into archetypal designations of secondary structure such as α -helical, β -sheet-like, and random coil, however each of these structures are able to permeabilize lipid bilayers.^{7,75,186–189} Explicit or well-defined tertiary structures or oligomers are generally not found, and are not required, for the observed activity of MPPs. In Figure 4, models of secondary structure for 15 peptides have been outlined to demonstrate the secondary structure variability of MPPs. In Figure 5, spacefilling models of regional hydrophobicity for the same 15 peptides in the same dimensional orientation are outlined.

Circular dichroism (CD) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy^{50,190,191} are widely used techniques to determine secondary structure of peptides in aqueous conditions, solvents, detergents, bicelles, lipid vesicles,^{192,193} and supported bilayers.^{194,195} Linear peptides, for example melittin or the magainins, are often unstructured in an aqueous environment and become structured only when partitioned into lipid bilayers or membrane-like environments.^{7,91,196–198} At the level of secondary structure, helical peptides such as melittin, alamethicin, and LL-37 adhere to the conformational patterns derived from helical hydrogen bonding, using patterns of amino acid hydrophobicity to fold into amphipathic structures.^{58,75,199} The most common helical form is the α -helix with an i to $i + 4$ hydrogen bonding pattern. Arrangements of i to $i + 3$ are found in 3_{10} helices and i to $i + 5$ arrangements are found in pi-helices.^{200–202} These structural patterns can be visualized 2-dimensionally through helical wheel projections (Figure 2). Helical patterns are useful information in rational engineering¹⁷² or synthetic molecular evolution.^{59,180,181} However, for the vast majority of helical MPPs, there is little data to suggest that they assemble into explicit membrane protein-like transmembrane helical bundles (see exceptions below). Rather, the interactions of most MPPs, helical or otherwise, are dynamic. Heterogeneous interactions with the dynamic, fluid phase interface of the lipid bilayer membrane occur where tertiary structure of the peptide is not well-defined. Consequently, knowing how to engineer the amphipathic surfaces of helical peptides is not enough to knowingly engineer MPP activity.

There is also an overarching hydrogen binding pattern that defines β -sheet-like peptides.^{5,176,210,188,203–209} However, there are many more ways to form β -sheet-like structure compared to α -helical structure,^{188,211} including the possibility of both intramolecular and intermolecular hydrogen bonds. Thus, with a few exceptions, the architecture of β -sheet containing MPPs in the active state are especially difficult to describe, predict, or model computationally. For example, the defensins form rigid, globular structures that are amenable to NMR and crystallographic structure determination^{205,208,212,213} yet the molecular mechanism by which these globular, highly cationic structures interact with membranes to cause permeabilization remains only vaguely described.

Multiple Cys residues within an MPP can form internal disulfide linkages and change secondary structure and subsequent peptide function. Many of the β -sheet rich antimicrobial peptides, e.g., protegrin, tachyplesin, α - and β -defensins, Θ -defensins, and others have internal disulfide cross-

links that are sometimes necessary for activity. Wimley et al. showed that the reduction of the disulfide bonds in a human α -defensin greatly increased vesicle permeabilizing activity, while also abrogating biological activity.²¹⁴ He et al. demonstrated that the viroporin activity of the Ebola virus delta peptide, which permeabilizes many cell types in vitro and is entirely dependent on the presence of a disulfide cross-link that forms a short C-terminal hairpin.¹³⁶ This indicates that the secondary structure modification elicited by the disulfide linkage is essential for membrane permeabilization by the delta peptide.

Some AMPs have secondary structures that are influenced by nonproteinogenic amino acids, such as D-amino acids, α -aminoisobutyric acid, ethylnorvaline, isovaline, phenylalanol, hydroxyproline, and others to form peptaibols. Alamethicin is the classical peptaibol.²¹⁵ It has multiple α -aminoisobutyric acid residues which strongly promote α -helical structure. Gramicidin A, on the other hand, has alternating L and D amino acids that enable formation of a unique β -helix structure.^{216,217}

Peptide-membrane interactions are driven by physical chemistry.²¹⁸ Interactions between peptide and membrane are heterogeneous and such peptides often behave as molecules that have partitioned into a fluid. Explicit, discrete three-dimensional structures of MPPs are extremely rare, although many researchers have tried to determine or model them. The ability of peptides to permeabilize membranes is largely dependent on the physical chemical interactions between the membrane and peptide; the amino acid composition plays a role at least as significant as the sequence because the determinants for membrane permeabilization, under some conditions, depends on overall hydrophobicity and amphipathicity of the peptide, along with electrostatic interactions between the peptide and membrane.^{179,219,220}

As an example of the importance of composition, Shai and colleagues made a diastereomer of melittin with four D-amino acids that thoroughly disrupted its helical secondary structure.²²¹ This melittin analog lost its ability to permeabilize red blood cells and synthetic vesicles made of PC, yet retained very high membrane permeabilizing activity against bacteria and against anionic synthetic bilayers. These results, and others,^{78,149,214,222,223} provide an important insight into the mechanism of actions of MPPs; the same peptide can permeabilize different membranes in very different ways. In the case of melittin, permeabilization of synthetic or biological membranes made mostly of PC and sphingomyelin is strongly dependent on the formation of amphipathic α -helical secondary structure, while the permeabilization of anionic synthetic or biological membranes can occur in the absence of α -helical secondary structure. Likely, the molecular mechanism of melittin simultaneously encompasses a broad ensemble of structures and mechanisms. The mechanistic landscape is dynamic, and the dominant mechanisms are strongly influenced by experimental conditions.

As another example of the influence of amino acid composition on function, AMPs with scrambled sequences are sometimes as active as, or more active than, native sequences.^{224,225} Most AMPs are amphipathic and cationic, which provides electrostatic selectivity toward anionic lipids such as phosphatidylglycerol (PG) which is abundant in bacterial membranes or phosphatidylserine (PS) which is found in some eukaryotic internal/organelle membranes, instead of zwitterionic lipids such as PC, which are dominant in the external face of eukaryotic plasma membranes.^{226–230} Aromatic side chains interactions with the interfacial zone of a

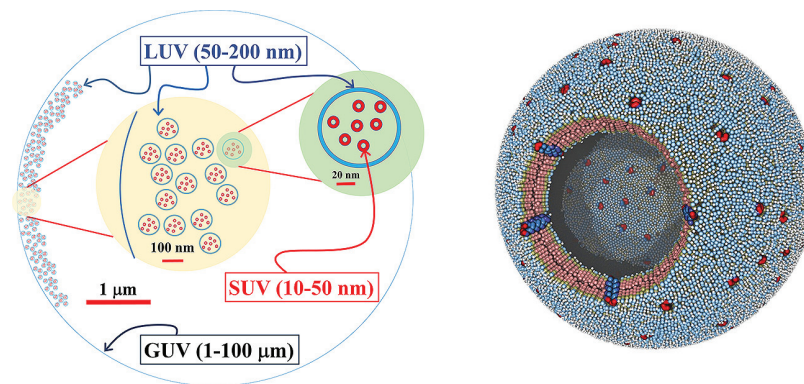


Figure 6. Realistic cartoons of lipid vesicles. In this cartoon, small (SUV), large (LUV), and giant (GUV) unilamellar vesicles are drawn roughly to scale in three different magnifications. Even the membrane thickness is drawn to scale in each magnifications. On the right is a simulation of a vesicle of 34 nm diameter, containing $\sim 40\,000$ lipids. Image courtesy of Andrew Jewett at www.moltemplate.org.

lipid bilayer are also important in peptide interactions with membranes.^{231,232} In keeping with the concept of a mechanistic landscape, MPP-membrane interactions, and thus function, can be dependent on other factors such as peptide concentration, salt concentration, pH, lipid composition, lipid phase state (gel/fluid), temperature, hydration, and many others.

One exception to the usual lack of explicit three-dimensional structures for MPPs is gramicidin-A, an unusual peptide that is made nonribosomally with alternating L- and D-amino acids. Gramicidin assembles into a small number of discrete membrane-spanning structures using a β -helix secondary structure architecture.^{108,109,216,217,233,234} Protegrin, a β -sheet rich, disulfide cross-linked hairpin AMP has also been studied by NMR, and a possible three-dimensional arrangement has been obtained.^{207,235} The M2 channel peptide is one of the only explicit structures ever determined for a helical MPP.¹³⁷ It forms a well-defined membrane spanning tetramer. Trans-membrane helical bundles have also been detected for both alamethicin^{236,237} and melittin,^{53,55,56} although it is possible that such structures are found under a limited range of experimental conditions and are unlikely to be discrete, homogeneous structures. Overall, there is not a singular secondary structure or tertiary structure that best drives peptide-membrane interaction and leads to membrane permeabilization. A multitude of MPP structures can cause membrane permeabilization.

3. SYSTEMS FOR STUDYING MEMBRANE PERMEABILIZING PEPTIDES

In this section, we discuss model systems used to dissect the physical, chemical, and biological principles of membrane permeabilizing peptides. The first model systems we will discuss are synthetic lipid bilayers, including large unilamellar vesicles (LUVs), giant unilamellar vesicles (GUVs), and planar supported bilayers. Their utility is vast as they can easily be created, controlled, and manipulated in the laboratory and yet share many important properties with biological membranes.²³⁸ We will then discuss molecular dynamics simulations which explore the physical chemical basis of peptide-membrane interactions through computer simulations. Finally, we will discuss biological membrane systems and compare membrane permeabilization in biosystems with permeabilization observed in synthetic model systems.

3.1. Large Unilamellar Vesicles

Synthetic lipid vesicles are common and useful model systems for studying membrane permeabilizing peptides. Biological membranes are complex with many components, including an immense variety of lipid species^{239–241} and membrane proteins.²⁴² Because of this complexity, it can be challenging to study peptide-membrane interactions in biomembranes. Synthetic lipid bilayers have only one or a few lipid species with precisely known compositions and yet they can accurately recapitulate the physical chemistry and dynamics of a biological membrane, providing a way to study and interpret peptide-membrane interactions.²⁴³

Synthetic unilamellar vesicles are closed bilayer structures that have an aqueous interior space surrounded by a single lipid bilayer membrane (Figure 6). The first step toward artificial vesicle preparation was made by Bangham et al. in 1965 who showed that a suspension of lipids could encapsulate ions.²⁴⁴ Now we have many tools and methods for making vesicles of a variety of sizes and architectures. Synthetic unilamellar (i.e., one bilayer) vesicles can be categorized by their size: SUVs (small unilamellar vesicles) are ~ 10 – 50 nm, LUVs are ~ 50 – 200 nm, and GUVs are ~ 1 μm in diameter and can be as large as 100 μm . So called oligolamellar vesicles, with a few concentric bilayers,²⁴⁵ can be 400 nm or larger. They are not widely used in MPP research.

In the 1970s, SUVs were the first type of unilamellar vesicles widely utilized. They are prepared by high powered sonication^{246–248} and were utilized in many studies.^{249–251} However, their relatively high curvature created instability and artificially enhanced peptide binding. Later, LUVs were made through ethanol injection²⁵² or detergent dilution or dialysis,^{253,254} but incomplete removal of those chemicals proved problematic.²⁵⁵ In the mid 1980s, Cullis and colleagues^{255–257} described a method of preparing uniform large unilamellar vesicles by high pressure extrusion through Nucleopore polycarbonate filters (Figure 7). This approach had many advantages over other methods, including more stable vesicles and a lack of high curvature effects. This method is applicable, almost without limit, to bilayers of any lipid compositions. Extrusion thus quickly became the method of choice for preparation of uniform LUVs^{258–262} and has proven to be extraordinarily useful in studying the physicochemical interactions and functions of peptides on membranes.

By using different phospholipids, sterols, and other membrane components in a vesicle preparation, many bilayer

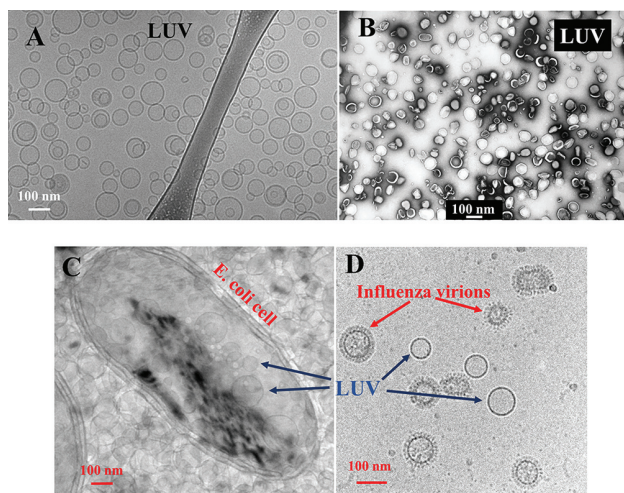


Figure 7. Large unilamellar vesicles. LUVs are the most commonly used synthetic models in the study of peptides in membranes. They are formed by extrusion of multilamellar lipid suspensions through Nucleopore polycarbonate filters at high pressure. A: Cryo transmission electron microscopy of a preparation of LUVs made from fluid phase PC lipids (Jibao He, Tulane University). B: Negative stain electron microscopy of LUVs showing their remarkable size and uniformity (Thomas W. Tillack, University of Virginia). A legacy image of the first extruded LUVs made by author WCW, ca. 1987. C: Comparison of LUVs with the double membrane of *E. coli* bacteria, in the same sample, shows similar size and curvature. D: Comparison of LUVs with influenza virions in the same sample shown similar size and curvature.

properties can be changed. These properties include surface charge, membrane fluidity, phase behavior, and bilayer thickness. Altering these properties gives valuable insight into how peptides interact with membranes. For example, Matsuzaki et al. showed long ago that the antimicrobial peptide tachyplesin is far more active against anionic bilayers made of PG and PS than against zwitterionic bilayers made of PC.²⁶³ This behavior is almost universally observed for cationic antimicrobial peptides.^{264–268} Similarly, Ladokhin and colleagues showed that the interactions, topology, and mechanism of permeabilization of bilayers by MPPs is sensitive to lipid composition, especially headgroup charge.^{78,222,223}

One utility in manipulating vesicle composition is to more closely mimic biological membranes. Including phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) lipids in an LUV preparation, for example, increases vesicle resemblance to bacterial membranes and can be a more relevant model to use for AMP studies.²⁶⁹ Including cholesterol and sphingomyelin increases vesicle resemblance to the outer surface of a eukaryotic cell membrane while including PE, phosphatidylserine (PS), and phosphatidylinositol (PI) increases the resemblance to the inner surface of a eukaryotic plasma membrane.

Another property of biological membranes that has been mimicked in LUVs is their transmembrane asymmetry. In an asymmetric membrane, the inner and outer leaflet compositions are different, allowing for diverse cellular processes to take place.²⁷⁰ The outer leaflet of eukaryotic cells is mainly composed of phosphatidylcholine, sphingomyelin (SM), and cholesterol, while the inner leaflet contains mainly phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS).^{271–273} Membrane asymmetry can

be adjusted in synthetic vesicles, although the field is still developing. London and colleagues developed a method in which LUVs can be made with mainly sphingomyelin and PC on the outer leaflet and PE and PS in the inner leaflet.²⁷⁴ Their method utilizes (2-hydroxypropyl)- α -cyclodextrin (HP α CD) rather than methyl- β -cyclodextrin (M β CD) to mediate lipid exchange. This allowed for the inclusion of cholesterol in the vesicles as HP α CD had much less affinity for it than for M β CD. Through this method, they were able to achieve highly asymmetric vesicles that could be a more accurate model of a biological membrane.

The purpose of using LUVs is to study membranes in a controlled, defined, and reproducible way in vitro while still mimicking the basic physical and chemical properties of biological membranes. These characteristics enable LUVs to be excellent models to study membrane permeabilizing peptides, especially because some physicochemical properties are difficult to study with biological systems or with Molecular Dynamics. Next, we discuss some techniques for studying membrane permeabilizing peptides using large unilamellar vesicles.

3.1.1. Methods of Measuring LUV Permeabilization.

There are many ways in which permeabilization of LUVs can be measured, illustrated in Figure 8. Most share a similar fundamental concept: vesicles are prepared with reporters or reporter-related molecules entrapped within their aqueous space or in the membrane, they are then treated with peptide, and finally the rate or extent of release of the entrapped probe is measured. The readout can be through nuclear magnetic resonance, light scattering, electron spin resonance, or fluorescence spectroscopy, among other methods,²⁷⁵ but fluorescence is used most often because it is versatile, highly sensitive, and widely available. Next, we will discuss a few commonly used types of leakage assays: small molecule and macromolecule release, flip-flop and translocation assays, along with assays that distinguish graded from all-or-none permeabilization and assays that distinguish transient from equilibrium permeabilization.

3.1.1.1. Small Molecule Leakage Assays. The first leakage experiments were done with entrapped carboxyfluorescein in the 1970s.^{276–279} In this assay, liposomes are prepared in a solution with a high concentration, 50–80 mM, of the fluorophore carboxyfluorescein. Then, the vesicles are purified and extra-vesicular dye is removed through gel filtration chromatography. The high concentration of the fluorophore within the vesicles causes self-quenching and low fluorescence. Upon membrane disruption, the fluorophore leaks out of the vesicles and is diluted into the large external volume where its fluorescence is recovered. Permeabilization can be quantified through the increase in fluorescence compared to a sample completely solubilized with detergent, which amounts to 100% release. A difficulty with this assay arises from the fact that the fluorescence of carboxyfluorescein is especially pH sensitive near physiological pH. To overcome this issue, Allen and Cleland replaced the fluorophore with calcein, a pH independent fluorophore (at pH 6–8.5),²⁸⁰ which has since been widely used.

The binary ANTS/DPX assay uses the fluorophore 8-aminonaphthalene-1,3,6 trisulfonic acid (ANTS) and its quencher *p*-xylene-bis-pyridinium bromide (DPX). ANTS does not self-quench and is relatively pH insensitive²⁸¹ but is readily quenched by low mM concentrations of DPX. First described in 1976 by Smolarsky et al., ANTS/DPX was used to

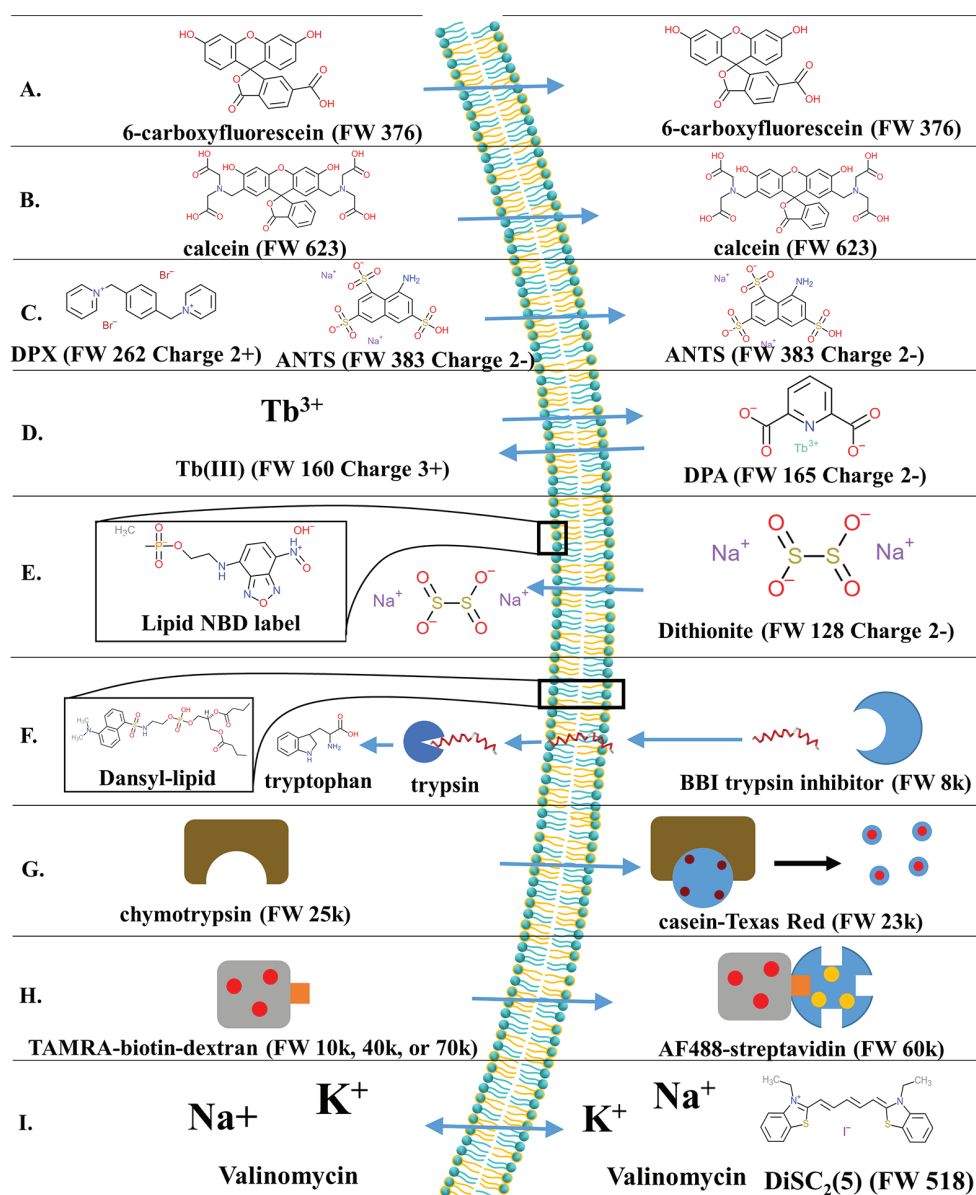


Figure 8. Probes used to measure permeabilization of LUVs. The chemical structures for small molecules and cartoon representations of larger molecules are shown, as well as a general theory of each assay. Only a select few assays are shown. Some assays can be relatively simple and measure leakage of a single fluorophore out of a vesicle, as in (A) and (B). Others are more elaborate, utilizing FRET [(H), (F)], macromolecules [(G), (H)], or even membrane potential [(I)]. The assays are as follows: (A) carboxyfluorescein assay, (B) calcein assay, (C) ANTS/DPX assay, (D) Tb^{3+} /DPA assay, (E) equilibrium permeabilization assay, (F) translocation assay, (G) chymotrypsin release assay, (H) macromolecule release assay, and (I) diffusion potential assay. See text for details.

detect complement-mediated lysis of liposomes.²⁸² It was later developed to its present form by Ellens et al.²⁸¹ and others. In this assay, LUVs are made with coencapsulated fluorophore and quencher at concentrations that cause quenching. The external ANTS and DPX is removed by gel filtration chromatography, filtration, or centrifugation. Upon addition of a membrane permeabilizing peptide, ANTS and DPX escape into the external volume where the increase in ANTS fluorescence can be measured spectroscopically.⁸⁰ This basic form of the ANTS/DPX assay gives valuable insight into the potency of a membrane active peptide to disrupt vesicles in such a way that allows small molecules to escape.⁶²

The terbium (Tb^{3+})/DPA assay is another example of a binary permeabilization assay. In this assay, the lanthanide

metal Tb^{3+} , which is weakly chelated by citrate, is encapsulated in LUVs at mM concentrations. The aromatic strong chelator dipicolinic acid (DPA) is added to the vesicle exterior at μM concentrations. Complexed with citrate, Tb^{3+} is very weakly luminescent, but when chelated by DPA, the complex is brightly luminescent.^{283,284} Leakage of Tb^{3+} out of the vesicle, or the leakage of DPA into the vesicles, enables DPA to replace citrate, creating a very bright complex that can be measured.^{64,284}

The equilibrium permeabilization assay is unique in that it enables the measurement of leakage, as above, but also enables the distinction between equilibrium and transient permeabilization. Krauson et al. developed this assay while studying the mechanism of action of AMPs (LL37 and dermaseptin S1) and

lytic peptides (LLP1, LLP2, melittin, and alamethicin).⁶⁴ In this assay, LUVs with entrapped Tb^{3+} and external DPA are prepared to measure leakage by the standard Tb^{3+} /DPA assay, described above. However, in this assay, these vesicles also contain 1 mol % of diacyl phospholipid which are labeled on the headgroup with the dye nitrobenzoxadiazole (NBD). NBD has fluorescence that can be measured independently from Tb^{3+} /DPA. Its fluorescence can be quenched by the membrane-impermeant reducing agent dithionite, $\text{S}_2\text{O}_2^{2-}$, which can be added at any time during the assay. NBD lipids on the vesicle inner monolayer are protected from quenching unless the membranes are permeable to dithionite at the time of its addition. In the equilibrium permeabilization assay, vesicles are treated with peptide and allowed to equilibrate for up to 8 h; leakage usually ceases within 1 h. Terbium fluorescence is measured to determine the total leakage. Then, dithionite is added and the degree to which the NBD fluorescence is protected is measured. When permeabilization is a transient process, Tb^{3+} /DPA reports on net leakage after peptide addition, while dithionite only quenches external NBD-lipids, showing that the bilayers are sealed at equilibrium despite having been permeabilized initially. When permeabilization is an equilibrium process, both leakage and dithionite quenching of NBD go to completion, because dithionite can access the vesicle interior at equilibrium and quench all NBD lipids. Krauson et al. showed that the AMPs tested were transient membrane permeabilizers²⁸⁵ that induce only short-term membrane permeabilization. On the other hand the peptaibol alamethicin and the lentivirus lytic peptides LLP1 and LLP2 showed highly potent permeabilization to Tb^{3+} /DPA by equilibrium pores that enabled complete quenching of lipid-linked NBD by dithionite at most P:L studied, down to as low as 1:2000. Interestingly, the mechanism of melittin transitioned from transient toward equilibrium behavior over the measured concentration range of P:L of 1:2000 to 1:50.

The diffusion potential assay measures the leakage of small ions in the presence of a transmembrane potential.^{221,286,287} Briefly, LUVs, prepared in the presence of KCl, are diluted into a K^+ free buffer that contains the potential sensitive dye 3,3'-diethylthiadicarbocyanine iodide [DiSC2(S)]. Then, valinomycin, which is a K^+ ionophore, is added to the mixture, creating a negative inside transmembrane potential which causes dye quenching. After equilibrating this solution for a few minutes, the peptide treatment is then added. If there is membrane disruption, there will be a dissipation of diffusion potential as indicated by the increased fluorescence. The fluorescence recovery values can then be tracked over time or at a single time point. Kobayashi et al. employed this assay to characterize the AMP buforin 2. They found that buforin 2 did not permeabilize vesicle membranes as well as magainin, with or without a membrane potential.²⁸⁶ They went on to show that buforin 2 translocates with high efficiency across membranes, while exhibiting low vesicle permeabilization as well as low lipid translocation.

3.1.1.2. Macromolecule Release Assays. Macromolecule release assays provide information on the size of the permeabilization pathway based on measurements of macromolecule permeabilization.^{61,62,78,153,203,285,288} Originally, this measurement was performed with fluorescein-labeled dextrans which self-quench when entrapped at high concentration, in the mg/mL range. Upon release, the fluorescence is recovered. Hristova et al. used this assay to study vesicle permeabilization by the rabbit defensin NP-2. They found that native NP-2

allowed for dextrans from 4 to 70 kDa to leak at the same rate which supported the hypothesis that NP-2 forms large lesions or destroys the vesicle architecture rather than forming size-specific perturbations.²⁸⁹ Ladokhin et al. coentrapped two dextrans of different molecular weights and used gel filtration chromatography to measure their release by melittin, finding that both sizes were released equally well.⁶¹ Similar applications have been used elsewhere.^{214,290} One potential problem with these dextran release assays is that entrapment of concentrated dextrans creates an osmotic imbalance so this should be counteracted by using external, unlabeled dextrans.

A recent adaptation of this assay uses dextrans labeled with biotin and also with the dye tetramethylrhodamine (TAMRA) which are encapsulated in LUVs at very low, $\mu\text{g/mL}$, concentration.⁶² The low concentration used eliminates osmotic stress on the vesicles caused by mg/mL dextrans used previously. The extravesicular solution contains Alexa-Fluor488-labeled streptavidin. If the vesicle membranes are disrupted enough to enable TAMRA-biotin-dextran release, the dextran will complex with the external AF488-streptavidin, leading to FRET-based quenching of the AF488 by TAMRA. Using this assay, Wiedman et al. showed that Melp5, a gain of function variant of melittin,⁵⁹ enables macromolecule release from LUVs at low peptide concentration.⁶² Furthermore, this assay was used in several successful screens for peptides that induce macromolecular release,^{180,181} leading to the identification of highly potent peptides with this activity at P:L = 1:1000, either in a pH-triggered or a pH-insensitive manner.

The chymotrypsin-casein assay is another macromolecular leakage assay described by Wiedman et al. during their characterization of melittin gain-of-function variants.⁶² Here, the vesicles contain entrapped chymotrypsin. Peptide-induced release of chymotrypsin enables the proteolysis of extravesicular casein labeled with Texas Red (EnzChek), which can be monitored by the increase in Texas Red fluorescence.

3.1.1.3. Flip-Flop and Translocation Assays. Lipid and peptide translocation across membranes, sometimes called "flip-flop" is a specialized kind of permeabilization wherein one measures the movement of peptide or lipid polar groups cross a lipid bilayer from one monolayer to the other. Pagano et al. developed a method in 1981 to label the inner or outer leaflet of vesicles with NBD-lipid to measure lipid asymmetry.²⁹¹ Matsuzaki et al. utilized this technique to detect lipid flip-flop in vesicles.²⁹² Here, NBD-lipid is first incorporated into either leaflet of the vesicles. To label the outer leaflet, premade LUVs are incubated in a solution with NBD-lipid. To label the inner leaflet, the NBD-lipid is added to the initial LUV lipid mixture when making the LUVs, and then the outer leaflet is chemically quenched with dithionite reduction. These asymmetrically labeled vesicles can then be treated with peptide to induce translocation. Dithionite is then added to the solution at set times to quench the outer leaflet and NBD fluorescence is measured to determine the degree of protection or exposure by translocation. This assay can only be used for membrane permeabilizing peptides if they cause transient permeabilization because the bilayers must be impermeant to dithionite when it is added. A slightly modified version of this assay has been described for the study of transmembrane peptide sequences on flip-flop behavior.²⁹³ Here, the transmembrane peptide is incorporated into the LUV membrane, the outer leaflet is labeled, and then flip-flop is measured as above. It was found that less hydrophobic peptides induced less flip-flop than more hydrophobic peptides.²⁹³ On the other hand,

Wimley and White showed that alamethicin, even at extremely low concentrations of less than P:L = 1:2000, catalyzed very rapid lipid flip-flop.²⁹⁴ Fuselier and Wimley used a version of this assay to show that translocating peptides also increase lipid translocation.²⁹⁵

Matsuzaki et al. have adapted LUVs to measure peptide translocation rate with a reporter system that involves enzymatic cleavage and FRET.^{286,296,297} In this assay, LUVs containing fluorescent dansyl phosphatidylethanolamine are made with encapsulated trypsin. Peptides are then added to the LUVs. The tryptophan residues of bound peptides come into the vicinity of the dansyl groups and are quenched by FRET. If the peptide translocates across the bilayer into the vesicle, it will be cleaved by the internal trypsin and FRET will be reduced (i.e., the tryptophan fluorescence increases while the dansyl fluorescence decreases). Using this technique, Matsuzaki et al. was able to determine that magainin translocates across vesicle membranes through a permeabilization mechanism that allows small molecule leakage as well.²⁹⁷ Marks et al. developed a variant of the translocation assay to screen for spontaneous membrane-translocating peptides.²⁹⁸ In this assay, the fluorophore aminomethylcoumarin (AMC) is added onto the C-terminal phenylalanine of the peptides. The vesicles contain entrapped chymotrypsin and Tb^{3+} while the extracellular space contains DPA and the $\alpha 1$ -antitrypsin inhibitor. This assay reports independently on permeabilization by Tb^{3+} fluorescence, and translocation, which is reported by the chymotrypsin cleavage of the AMC group. These authors were able to identify spontaneous membrane-translocating peptides that cross PC bilayers and cell membranes without any permeabilization.²⁹⁸ Other methods of measuring peptide translocation rates have also been described.^{38,295}

3.1.1.4. Graded vs All-or-None Leakage Assays. In an ensemble average measurement of leakage, there are multiple ways that leakage can be distributed across the population of vesicles examined. Understanding those mechanisms can help us understand the mechanistic landscape of membrane permeabilizing peptides. For example, leakage from vesicles can be graded, in which all vesicles release a similar portion of their contents before leakage stops. Alternately, leakage can be all-or-none, in which a fraction of the vesicles release all of their contents while the remainder of vesicles release none. In the early 1980s, a modification of the carboxyfluorescein method was used to distinguish these possibilities.²⁹⁹ After leakage measurements were made by the standard method, vesicles in the reaction solution and any entrapped dye molecules were separated from released dye using gel filtration. In the repurified vesicle sample, if there was graded leakage each vesicle would contain only a fraction of the initial fluorophore concentration and there would be less quenching than in the original vesicles. On the other hand, if leakage was all-or-none, then the degree of quenching in the repurified vesicles will be the same as the starting vesicles. Using this method, Weinstein et al. showed that HDL apolipoprotein induces an all-or-none effect at the liquid-crystalline transition temperature of the lipid bilayer.²⁹⁹ Later, similar repurification assays were performed using ANTS/DPX as the leakage reporter.^{172,290,300} Both graded and all-or-none leakage mechanisms have been observed by these techniques.

A simpler method to distinguish between graded and all-or-none leakage was developed by Wimley, Ladokhin, and colleagues.^{80,214,301} This method uses “requenching” of the ANTS fluorophore in situ rather than physical separation of

the vesicles from released molecules (Figure 9). Here, the ANTS/DPX assay is performed as above and fractional leakage

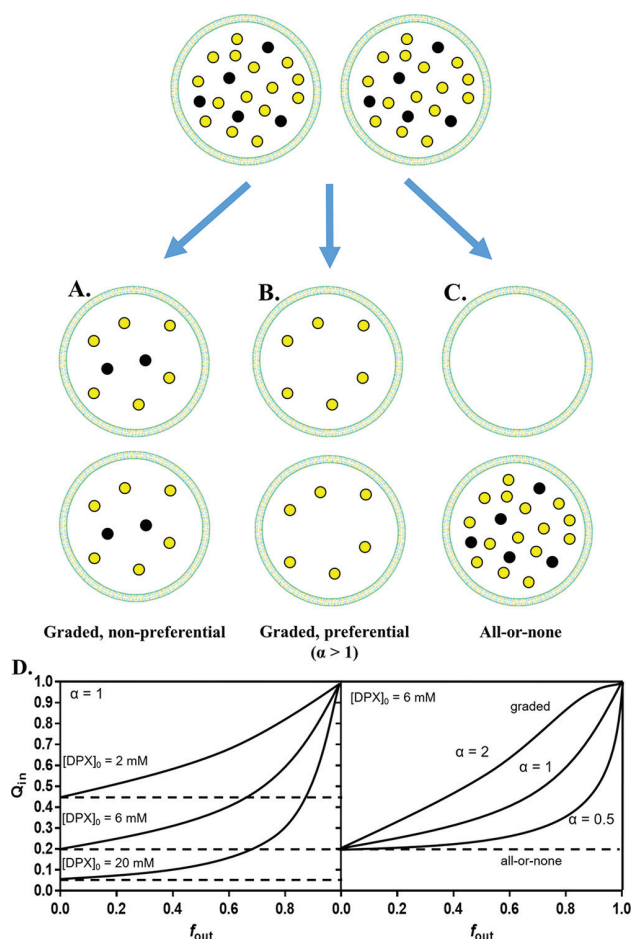


Figure 9. Graded and all-or-none leakage are two distinct peptide-induced leakage mechanisms on vesicles. The ANTS/DPX assay is used as an example here. (A) When all vesicles lose an equal portion of all encapsulated solutes, it is considered graded, nonpreferential leakage. (B) Losing an unequal portion of encapsulated solutes is considered graded and preferential. The equations from ref 72 can determine ANTS or DPX preferential leakage. In this example, $\alpha > 1$ therefore there is preferential leakage of the cationic quencher DPX. (C) Treatment of a sample of vesicles can lead to some vesicles losing all of their contents while the remaining vesicles losing none. This is an all-or-none behavior. Yellow and black dots are ANTS and DPX molecules, respectively. (D) This simulation shows how the two mechanisms can be experimentally distinguished. When plotting Q_{in} (internal quenching) against f_{out} (ANTS released), a steady Q_{in} indicates an all-or-none mechanism; an increasing Q_{in} indicates a graded mechanism. Different encapsulated $[\text{DPX}]$ affects the results (left) such that 4–8 mM is optimal. Here, $\alpha = k_{\text{ANTS}}/k_{\text{DPX}}$ which determines the preferential nature of leakage that is occurring (right). Adapted with permission from ref 301. Copyright 1997 Elsevier, Inc.

is allowed to plateau. Different peptide concentrations can be used to give different fractional permeabilization. Then, additional DPX is titrated into the vesicle solution while fluorescence is monitored. For a control, untreated vesicles are lysed with excess detergent and fluorescence is measured as DPX is added. Through these measurements, the parameters Q_{in} , the degree of quenching of the ANTS remaining inside the vesicles, and f_{out} , the fraction of ANTS released, are

determined. If Q_{in} remains constant with f_{out} then leakage is all-or-none. If Q_{in} increases with f_{out} then leakage is graded.^{203,302–306} Expanding on this, Ladokhin et al. showed that it is possible to calculate the relative preference for leakage of DPX over ANTS, expressed with the variable α .⁸⁰ Using these methods, Hristova et al. showed that for the rabbit defensin NP-5, Q_{in} increased with f_{out} and the α value was 1.6, indicating a graded leakage behavior with preferential DPX leakage.³⁰⁷ Another method of distinguishing between graded and all-or-none leakage has been reported which utilizes time-resolved calcein fluorescence decay measurements as the reporter system.³⁰⁸

Hoernke and colleagues³⁰⁹ used a statistical approach to extend the model of graded and all-or-none leakage to a more realistic continuum of states which can be described by cumulative “leakage events”. In this model, the characteristics of leakage events are a function of the membrane permeabilizing molecule and many other experimental conditions. Each event cumulatively releases a specific fraction of remaining entrapped contents so the total released contents asymptotically approaches 100%. Low release per event ($\leq 10\%$), averaged over many events, gives rise to graded leakage. High leakage per event ($> 75\%$), over a low probability of event occurrence will approach all-or-none leakage. Intermediate values of leakage per event give rise to very broad distributions of entrapped probe concentration, which these authors observed in experimental systems.³⁰⁹

3.1.2. What Behaviors Have Been Observed with LUVs? Many different leakage behaviors can be, and have been, observed in permeabilization assays that utilize LUVs. Leakage is not a singular process, but many possible partially overlapping processes. Understanding the intricacies and expected behaviors of each gives a better understanding of the mechanistic landscape of peptide-induced membrane permeabilization. In leakage assays, the measurement is made either continuously over time or at a single time point. In the literature, measurement of leakage is frequently, but not always, made after leakage is essentially complete. Measuring continuously provides insight into the kinetics of leakage. Since many MPPs cause a burst of leakage that slows or stops soon after it begins, single time point measurements, 30 or 60 min up to several hours after peptide treatment, are generally sufficient for the entire peptide-induced leakage kinetics to take place. However, the cessation of leakage should be empirically verified as some peptides cause slow leakage over many hours.²⁸⁵

In most assays, the fractional leakage at some time point is expressed as a function of peptide concentration, or as a function of the overall ratio of peptide to lipid (P:L) in each sample. It would be much more informative for leakage to be described by the ratio of *bound* peptide to lipid $P_{bound}:L$ as this is the relevant concentration of peptide partitioned into the bilayer. While peptide binding to bilayers can be expressed quantitatively,^{310,311} it is rarely measured. Thus, the overall peptide to lipid ratio (P:L), which denotes the theoretical maximum of $P_{bound}:L$,²⁸⁵ is typically used. Potency of an MPP is commonly displayed as a plot of percent leakage against peptide concentration or P:L. The P:L at which there is 50% leakage, or LIC_{50} (for leakage inducing concentration, 50%) is a good single number representation of potency. LIC_{50} values for MPPs can range from $\sim 1:1$ or $1:10$ for a peptide with barely measurable activity, e.g., melittin L16G,¹⁷⁹ to as high as $\sim 1:10\,000$, for an extremely potent peptide, as reported for

GALA.¹⁷² This latter value is equivalent to about 10 peptides per LUV and may represent the maximum possible potency of an MPP.

3.1.2.1. Transient or Equilibrium Permeabilization. If the peptide-induced pathway through the bilayer that enables permeabilization is an equilibrium structure, or one that continuously fluctuates in and out of existence, then leakage should be a continuous exponential approach toward 100% permeabilization.^{312,313} As peptide concentration is changed, the rate of this approach toward 100% leakage should change accordingly.^{314–316} While equilibrium poration is easy to describe, illustrate, or simulate, it is actually only rarely observed in the laboratory. Most vesicle leakage assays show transient leakage,² which is characterized by a burst of leakage that occurs immediately after addition of peptide, often occurring as fast as the first measurement can be made. Within minutes of the leakage burst, it slows and often stops completely as shown in Figure 2H and discussed below. In this commonly observed scenario, all peptide concentrations show similar rate behaviors, but the plateau level of leakage depends on P:L, such that increasing the peptide concentration will increase the amount of leakage that occurs during the burst. Many membrane permeabilizing peptides exhibit this behavior, including mouse and bovine PrPp,³¹⁴ mastoparan X from hornet venom,³¹⁷ melittin,⁶⁵ and many other families of MPPs.^{5,65,203,223,318–320} This transience is poorly understood but is thought to be due to an initial asymmetry and imbalance (of mass, charge, or surface tension) in the membrane structure that causes a stochastic failure of the membrane, which then resolves itself of the asymmetry²⁸⁵ leading to a cessation of leakage. It is interesting to note that between the burst phase of rapid leakage and the equilibrium phase in which leakage has stopped, there is no change in the concentration of peptide bound to each vesicle and usually no detectable change in peptide secondary structure. Every vesicle has hundreds to thousands of peptides that remain bound to it, yet such systems very quickly transition from rapid leakage to little or no leakage.

In some experiments, melittin has been shown to cause a burst of leakage followed by a slow, almost linear rate of leakage, probably due to a change in the leakage mechanism.^{313,321} In other experiments, melittin causes a burst of leakage with a stable plateau, the classical signature of transient leakage.⁶⁵ In either case, vesicle leakage by melittin can only be explained by transient leakage processes, at least until the concentration is greater than about $P:L \geq 1:200$. At higher concentrations, melittin begins to behave more like an equilibrium pore former.⁶⁴

3.1.2.2. Graded or All-or-None. As discussed above, graded and all-or-none leakage describes incomplete leakage on a per-vesicle basis. Graded leakage describes the case where all vesicles uniformly leak a portion of their contents and all-or-none describes the case where a fraction of the vesicles release all their contents while the remainder release none. Both behaviors have been observed, even for transient leakage. Melittin, long thought to be an equilibrium pore-forming peptide, in fact induces graded, transient leakage in vesicles,³¹² at least at lower P:L conditions in PC bilayers. Both graded leakage and transient leakage imply a mechanism other than equilibrium pore formation.

All-or-none leakage, often observed in conjunction with transient leakage,²⁰³ implies that a rare, stochastic, event is responsible for permeabilization. The event must be

catastrophic in some way as it can lead to complete permeabilization of an individual vesicle and sometimes leads to destruction of the vesicle architecture. However, for transient leakage, the probability of the catastrophic event must decrease rapidly with time to account for the rapid leakage cessation. The AMP cecropin A exhibits all-or-none leakage,³⁰⁴ as do defensins²¹⁴ and other AMPs.^{5,203} Rathinakumar and Wimley posited that transient, all-or-none leakage occurs because there are two ways to dissipate an initial peptide asymmetry: a stochastic, catastrophic leakage event, and a silent peptide translocation process that dissipates the asymmetry without leakage.⁵

3.1.2.3. Dependence on Solute Size and Charge. The size of substances that are allowed through a permeabilized membrane is useful in discussing mechanisms and estimating the dimensions of the path that a peptide forms in a bilayer. Permeability can be selective for small molecules only, such as for protons, ions, ANTS/DPX, and Tb³⁺, or there can be larger membrane disruptions that allow for macromolecules like dextrans of 3, 10, 40, or 70 kDa to pass through. As discussed above, “permeabilization” can also be the result of catastrophic disruption of vesicle architecture, releasing all entrapped contents regardless of size. Methods for measuring macromolecule release can be very informative. For example, the peptides GALA, alamethicin, and MelpS are all highly potent MPPs in PC bilayers,⁶⁴ yet GALA and alamethicin release only small molecules, while MelpS readily releases macromolecules as large as 40 kDa.⁶²

Preferential leakage based on solute charge has also been observed for many peptides, under some condition, including nisin (anion-selective),³²² alamethicin (mildly cation-selective),^{323,324} PG-1 (weakly anion-selective),³²⁵ melittin (anion-selective),⁵³ and the pentapeptide AcWLWLL (cation-selective).⁸⁰ Determining the ionic preference gives insight into the structure of the peptide when it is embedded in the membrane.

3.1.2.4. Dependence on Vesicle Size. The size of an LUV can affect the potency of peptides as line tension and curvature of the membrane contributes to peptide-membrane interactions. Cho and colleagues showed this by using quartz crystal microbalance with dissipation monitoring (QCM-D) to demonstrate that the AH peptide, an amphipathic helix sequence found in the NSSA protein of hepatitis C virus, induced sharply different rupturing capacities for different sized extruded vesicles.³²⁶ The peptide induced complete rupture for vesicles under 67 nm in diameter, incomplete rupture for those under 90 nm, and no vesicle rupture for even larger vesicles. This indicated that the AH peptide only lyses vesicles that have a maximum line tension.³²⁷ They extended this work to confirm a size-dependent activity on smaller Dengue, Zika and Hepatitis C viruses compared to larger vaccinia and vesicular stomatitis virus.³¹

3.1.3. The Problem of Aggregation. Across the field of membrane permeabilizing peptides, there is a pervasive potential artifact that is generally ignored but may influence reported vesicle leakage studies. This is the problem of vesicle aggregation and fusion caused by the addition of polycationic peptides, such as AMPs or CPPs, to liposomes containing anionic lipids. As many researchers in the field have observed, including us,³²⁸ when cationic peptides are added to vesicles containing anionic lipids there is often an instantaneous and dramatic increase in the solution turbidity and a loss of opalescence of the liposomes. These observations are due to

large scale vesicle aggregation, driven by very strong, polyvalent electrostatic interactions. The resulting particles are sometimes so large, $\geq 10\ \mu\text{m}$, that they quickly precipitate from solution. Such large particles may be comprised of thousands or tens of thousands of individual vesicles. Strong adhesion between vesicles can lead to bilayer deformation, vesicle rupture, and membrane fusion,³²⁸ all of which can lead to leakage of entrapped contents. This is a potential problem for the field because these effects report on a special case of peptide-induced bilayer disruption that does not effectively mimic the biological activities that are usually being studied. For example, bacterial membrane permeabilization occurs when peptides interact with isolated bacterial cytoplasmic membranes that cannot undergo aggregation and fusion, thus aggregation and fusion of PE/PG or PG LUVs may skew the observed leakage.

A simple solution to this problem is known but is not widely utilized. The addition of 4–5 mol % PEG lipids establishes a steric barrier between bilayers³²⁹ that prevents aggregation of vesicles. We show the effect of PEG lipids on LUV aggregation in Figure 10. Currently it is not known how much aggregation

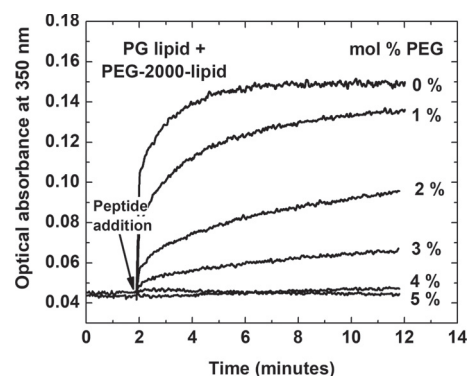


Figure 10. Cationic peptides rapidly aggregate anionic LUVs. Addition of the peptide *ARVA (RRGWALRLVLAY-amide) to POPG vesicles causes immediate and large scale aggregation of vesicles as shown by the increase in light scattering. Incorporation of PEG-2k-POPE lipids decreases aggregation dramatically, completely blocking it at 4–5 mol % PEG-lipids.

and fusion influence the permeabilization reported for MPPs because the reduction in leakage by the prevention of aggregation has not been measured systematically.

3.1.4. Limitations and Caveats of LUVs. A limitation of leakage assays with LUVs is that the measurements are usually an ensemble average of many vesicles so that elementary processes that occur between peptide and individual vesicles cannot be accurately studied.²⁷⁵ The structure of the LUV after permeation is usually not known, so the type of membrane disruption cannot be directly determined.²⁷⁵ Graded or all-or-none leakage assays partly address these questions^{80,214} but only indirectly. The many behaviors that can be observed with LUVs have been very beneficial to our understanding of the mechanisms behind membrane permeabilizing peptides; even so, the use of LUVs is still developing. Over the decades that we have incorporated them into our research, new assays and interpretation of the results have continuously come to light.

3.2. Giant Unilamellar Vesicles

Like LUVs, giant unilamellar vesicles (GUVs) are free-standing lipid bilayer models used to study membranes and membrane permeabilizing peptides. However, the diameter of a GUV is

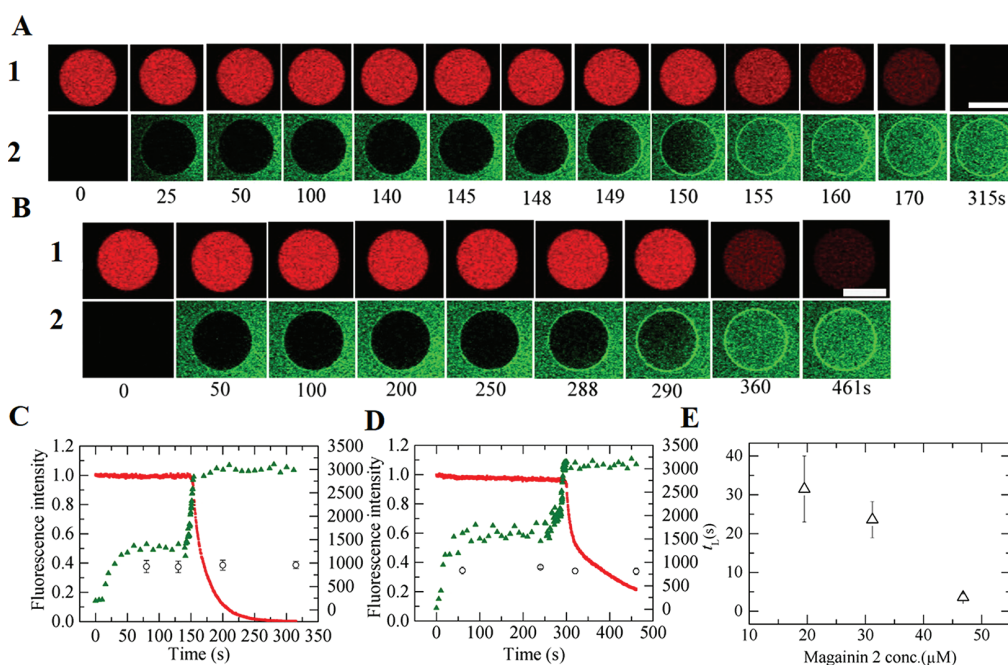


Figure 11. Peptide-induced permeabilization of GUVs. Membrane leakage of Alexa Fluor 647 hydrazide (AF647) and membrane permeabilization of carboxyfluorescein (CF)-labeled magainin 2 (CF-magainin 2) is visualized in single 40%DOPG/60%DOPC-GUVs. Panels A and B show confocal laser scanning microscopy (CLSM) images of (1) AF647 and (2) CF-magainin 2 in a single GUV treated with 31 μM CF-magainin 2/magainin 2 and 20 μM CF-magainin 2/magainin 2, respectively, at a certain time after of CF-magainin 2/magainin 2 addition to GUV denoted in seconds under each image. Panels C and D describe the time course of peptide-induced decrease in fluorescence intensity of AF647 in the GUV and increase in permeation of CF in the rim of GUV following the addition of CF-magainin 2/magainin 2. The solid red line corresponds to fluorescence intensity of AF647 inside the GUV while the green triangles correspond to the fluorescence intensity of CF-magainin 2 in the rim. The circles correspond to the fluorescence intensity of the outside vicinity of the GUV. $FI = I(t)/I(0)$, where $I(t)$ and $I(0)$ are the fluorescence intensity of AF647 inside the GUV at time t . Panel E refers to concentration dependent average lag time between the stochastic permeabilization of magainin 2 into the GUV and the leakage of dye from inside the GUV. These two phenomena can be studied by quantification of increase in the fluorescence intensities of the CF at the rim of the GUV and decrease in the fluorescence intensity of AF647 from inside the GUV. Adapted with permission from ref 358. Copyright 2015 American Chemical Society.

much larger than an LUV, typically 10 μm , ranging from 5 to 100 μm , compared to 0.1 μm for a LUV. This means that the surface area, and number of lipids, for a GUV is roughly 10^4 times larger than an LUV and the volume is roughly 10^6 larger. A GUV bilayer mimics the lipid matrix of cellular membranes and resembles the shape and size of eukaryotic cells. LUVs, on the other hand, mimic the size and shape of bacteria and viruses, as we show in Figure 7.

Giant unilamellar vesicles are commonly produced by hydration of a dried lipidic film. Initially, the gentle hydration method, originally introduced by Reeves and Dowben,³³⁰ was used for preparation of GUVs, where the vesicles spontaneously formed after the addition of an aqueous solution. However, using this method, the yield of GUVs was variable and sometimes low. Even after considerable modification by Akashi et al., this method can be slow and inefficient.³³¹ Angelova and Dimitrov introduced the electroformation technique of GUV preparation which uses an AC electric field on platinum electrodes.³³² Later, this method was modified to work with conducting indium tin oxide (ITO) films and titanium microscope slides that further increased the unilamellarity and yield of GUVs.³³³

Due to their large size, single giant vesicles can be observed with optical microscopy and can be individually manipulated in real time, which is a unique capability. Smaller vesicles such as LUVs and SUVs, on the other hand, require ensemble averaging over many vesicles resulting in loss of information

about individual events.^{334–337} Hence, GUVs are currently intensively utilized in many areas of biomembrane physics and chemistry, including the study of lipid domain formation^{338–340} and dynamics,^{341–343} membrane–protein interaction,^{344–346} membrane growth, budding, and membrane fission and fusion.^{347–349} One very useful application of GUVs is to study the function of membrane permeabilizing peptides or proteins.

3.2.1. Methods of Measuring GUV Permeabilization.

The more traditional method of studying MPPs with GUVs is the GUV suspension method where GUVs are suspended in a solution containing a water-soluble, membrane-impermeant fluorescent probe along with a peptide of interest (Figure 11). The fluorescence inside and outside the vesicle can be measured and analyzed by confocal microscopy. If the peptide permeabilizes the bilayer without rupturing the vesicle, the dye enters the GUV. Ambroggio et al. used this method to visualize and track membrane leakage induced by three antibiotic peptides: maculatin, citropin, and aurein via confocal microscopy.³⁵⁰ Mishra et al. have also used the GUV suspension method to show how the amino acid composition of a CPP affects its membrane translocation mechanism.³⁵¹ Almeida and colleagues studied the translocation of three cationic amphipathic peptides across GUVs and concluded that the Gibbs free energy of peptide insertion into the lipid bilayer may determine the ability of peptide to translocate.³⁵² These authors simultaneously tracked peptide translocation

across the bilayer and dye entry by monitoring the permeabilization of small vesicles that are often found inside larger GUVs.

The suspension method has also been applied by Schön et al. to determine that sphingomyelin and the coexistence of liquid-ordered and -disordered phases are necessary for permeabilization by equinatoxin II, a membrane active protein.³⁵³ The GUV suspension method has also been used to demonstrate graded and all-or-none permeabilization by two different HIV-derived membrane permeabilizing peptides—CpreTM and NpreTM, respectively—via confocal microscopy.³⁵⁴

More recently, an alternate method has been developed called the single GUV method in which the vesicles contain the water-soluble fluorescent probe, and leakage is determined by the loss of fluorescence from inside the vesicle. The structure and fluorescence intensity of the GUVs in this assay are continuously observed throughout the process by phase-contrast and confocal fluorescence microscopy. Hence, interaction of a dye-labeled peptide with a single GUV and the induced leakage of the fluorescent probe from the inside of GUV can both be monitored over time. This provides information on both membrane permeabilization and peptide translocation. Yamazaki and colleagues have used this method to describe the process of membrane permeabilization by two antimicrobial peptides, magainin 2 and lysenin.^{355–358} They used the same method to show that translocation of a cell-penetrating peptide (CPP), transportan 10 (TP10), into a single GUV occurs without any leakage of dye from inside of the vesicle.^{356,359,360}

3.2.2. What Behaviors Have Been Observed with GUVs? Using the improved single GUV method with optical and fluorescence microscopy, Yamazaki and colleagues showed that magainin 2 causes leakage of calcein out of GUVs without the disruption of the vesicle architecture. This leakage occurs without interaction with any other GUVs and without change of its spherical shape indicating that the permeabilization of magainin 2 into the GUV is not due to vesicle fusion or instability of the membrane structure.^{275,357,361} The same group also showed that a six-residue antimicrobial peptide derived from lactoferricin B can translocate into GUVs without damaging the bilayer.³⁶² Domingues et al. studied the effect of gomesin, a potent antimicrobial peptide from a Brazilian spider species, on GUVs composed of mixtures of palmitoylcholine (POPC) and either the negatively charged lipid palmitoylcholinephosphatidylglycerol (POPG) or cholesterol. These lipid mixtures broadly mimic bacterial and mammalian cell membranes, respectively. These authors showed that gomesin caused PG-containing GUVs to suddenly burst, lose the entrapped contents, and collapse into unresolved tubular structures.³⁶³ Although not a peptide, similar bursting activity of GUVs accompanied by decrease in diameter and vesicle transformation into indistinct compact structures was described by Yamazaki's group, when they employed the single GUV method to study the activity of antibacterial flavonoids, e.g., tea catechins or EGCs.^{275,364}

Many groups have been using GUVs to explore mechanistic details of the permeabilization activity of membrane-active molecules including magainin 2, melittin, LL37, and lactoferricin B.^{365–368} In most of the peptide-induced membrane leakage studies described above, membrane permeabilization followed by the leakage of the fluorescent probe from inside of a GUV starts stochastically at different

times for different vesicles. Once it begins, complete leakage occurs rapidly for an individual vesicle. This suggests that the permeabilization event, and not peptide binding or contents leakage, is the rate-determining step in the process.^{356,357,364,368} For example, when magainin 2 was studied, there was a lag time of minutes between the addition of peptide and the sudden, stochastic decrease in fluorescence intensity of GUVs. Once started, the release of contents from the GUV was complete within 30 s.^{356,357} In one experiment, 11 out of 16 GUVs of the same composition examined by the same technique showed sudden rapid leakage of GUV contents after lag phases, while the five remaining vesicles retained their contents.²⁷⁵ This phenomenon of stochastic leakage (discussed in more detail below) is specifically detected through the use of GUVs because they can be observed individually. Similar behaviors have been observed by Cho and colleagues using surface-tethered LUVs³⁶⁹ and by Weisshaar and colleagues who studied the membrane permeabilization of individual bacterial cells by melittin.⁶⁷

3.2.3. Limitations and Caveats of GUVs. In summary, giant unilamellar vesicles constitute a unique and powerful model system that enables the process of membrane permeabilization to be studied at the level of individual cell-sized vesicles. Although one can argue that biophysical measurements of binding and secondary structure are difficult to study using GUVs due to their undefined and usually very low lipid concentration, they nonetheless have revealed the stochastic nature of membrane permeabilization by many peptides and the catastrophic destruction of vesicle architecture by others, all of which are important clues in defining the mechanistic landscape.

3.3. Surface-Supported Planar Bilayers

Surface-supported lipid bilayers have been successfully used for the past 30 years alongside lipid vesicles as biomimetic model systems.^{370–373} In this technique, a supported bilayer lipid membrane (sBLM), which is planar instead of spherical as in vesicles, is attached or adsorbed to a solid support and is generally more stable when compared to unsupported counterparts.^{374,375} They can be designed to replicate cell surfaces of eukaryotes and microbes and are also especially useful for the study of pore and ion channel formation by peptides and proteins.^{375,376} Planar supported lipid bilayers are assembled on substrates such as Teflon, mica, silicon, or gold, the last two of which are conductive and enable membrane electrical properties to be measured.^{375,376} Bilayers of various lipid compositions can be assembled on surfaces by vesicle deposition³⁷⁷ or by Langmuir–Schaefer³⁷⁸ or Langmuir–Blodgett³⁷⁹ deposition techniques. A polymer cushion is sometimes incorporated, allowing for both sides of the membrane to be in contact with the aqueous solution and permitting the flow of ions from one side to the other.³⁷⁶ A polymer cushion also enables integration of membrane proteins which increases cellular biomimetic fidelity.³⁷⁵ Alternatively, tethered bilayer lipid membranes (tBLMs) are similar to sBLMs but are covalently tethered by the lower leaflet of bifunctional phospholipids or alkyl chains, usually to a gold support platform. Tethering increases the stability of the bilayer³⁸⁰ but can potentially limit the degree of biomimetic fidelity as transmembrane cellular proteins cannot be incorporated as readily.

3.3.1. Applications of Supported Bilayers to the Study of MPPs. With respect to the study of membrane

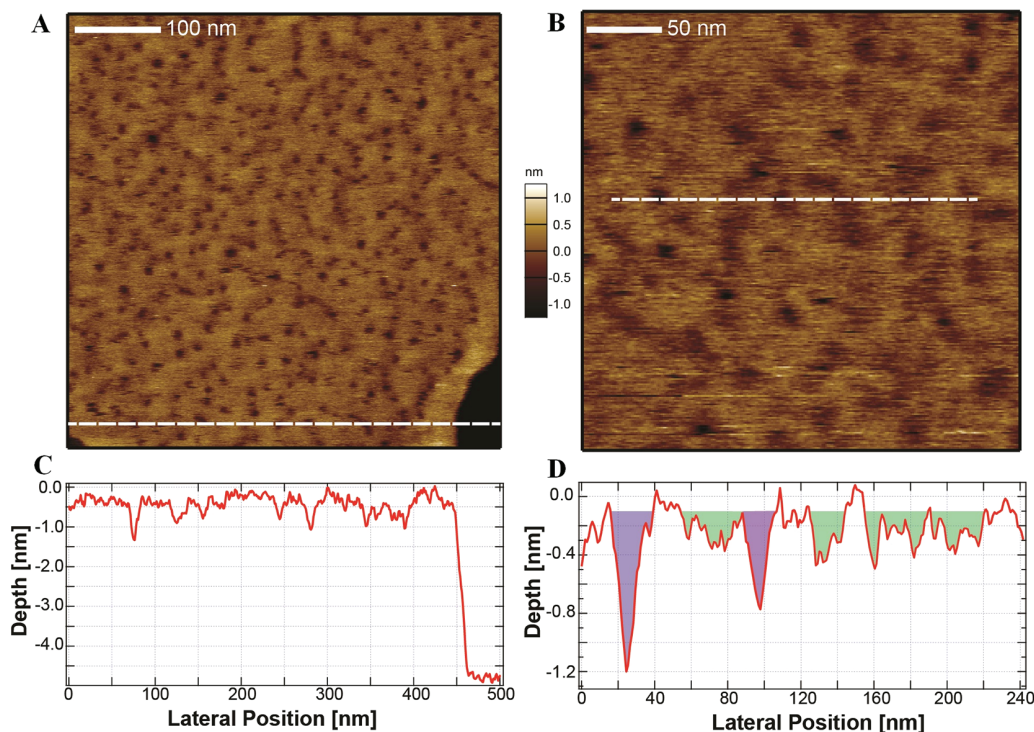


Figure 12. Atomic force microscopy (AFM) demonstrating the effects of MelP5 (a melittin derivative) on POPC (peptide:lipid = 1:1200). A. Punctate perturbations can be visualized in this image and seem to be present across the entire bilayer plane ($500 \times 500 \text{ nm}^2$). B. An image of a MelP5-treated POPC membrane at a higher magnification ($290 \times 290 \text{ nm}^2$). C. A line scan through the image (dashed line in panel A); numerous bilayer perturbations are scanned, and this information can be used to determine the depth of each topological depression. D. A line scan through the image (dashed line in panel B) at increased magnification. In this panel, pore-like features are highlighted in purple and thinned membrane regions are highlighted in green. Adapted with permission from ref 178. Copyright 2018 American Chemical Society.

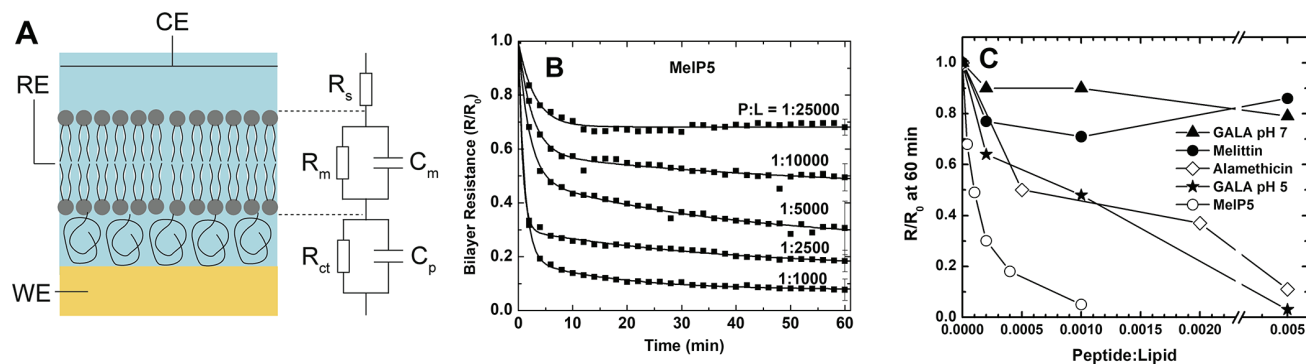


Figure 13. Electrochemical impedance spectroscopy. A. A polymer cushioned planar supported bilayer is adsorbed to the cleaned surface on a silicon crystal and the electrical properties are measured and modeled with an equivalent circuit. B. Resistance changes of a PC bilayer upon addition of the equilibrium pore former, MelP5. Note that the resistance does not recover as it would for a transient permeabilizing peptide.⁶⁶ C. Concentration dependence of the resistance drop for a variety of potent MPPs. Adapted with permission from ref 62. Copyright 2014 American Chemical Society.

permeabilizing peptides, there are numerous techniques that can be used to observe the effects of peptide–membrane interaction with high sensitivity. Atomic force microscopy (AFM) is a widely used probing technique where the surface of a sBLM can be scanned and monitored while interaction forces within the membrane can be recorded by a cantilever tip and piezoelectric scanner³⁸¹ (Figure 12). When membrane-permeabilizing peptides are introduced to sBLMs, AFM images can be taken to observe the subsequent membrane rearrangement in the x , y , z planes with subnanometer resolution in z to show the membrane perturbations and

pores.^{181,381} This can be very useful in helping to determine the mechanism of action for membrane-permeabilizing peptides and has been used in the study of indolicidin, gramicidin A, and other MPPs.^{178,181,382–384} AFM can be used to probe structural changes in the membrane, such as pore formation, as well as membrane thinning.^{381,382,385} Numerous instances of membrane perturbations by peptides using AFM have been reviewed by Morandat et al.³⁸¹ Recently, Pittman and King published an AFM study in which they examined how fluid phase PC bilayers were affected by MelP5,¹⁷⁸ which was shown to have two effects on bilayer structure: localized

bilayer thinning and formation of large pore-like voids in the membrane that form in the thinned areas of the bilayer.¹⁷⁸ The voids were dynamic, with at least some forming and dissipating within a few minutes.¹⁷⁸ The most probable diameter of the MelP5-induced voids was 1.7 nm, but they ranged as high as 4–5 nm diameter, large enough to account for the ability of MelP5 to release macromolecules from lipid vesicles.^{178,180} Recently, King and Pittman with Hristova and colleagues showed how different pore-forming peptides exert variability in the sizes of pores formed in sBLMs by using AFM scanning and comparing the effects of MelP5 and macrolittin 70, a closely related but more potent peptide that is also capable of macromolecular poration.¹⁸¹ Observations through AFM are not limited to these metrics; it can also measure chemical interactive forces and membrane elasticity and can perform protein receptor mapping³⁸¹ as well as map peptide membrane energy landscapes and kinetics.^{381,386,387}

Neutron reflectometry and neutron scattering can also provide information on membrane structural dynamics specifically in an aqueous environment. Properties such as surface architecture, membrane thinning, composition, and hydration state are all studied with these methods.^{388–391} A useful technique that can complement AFM and neutron reflectivity data is electrochemical impedance spectroscopy (EIS; Figure 13). Whereas AFM assesses the topological changes induced by peptide interactions with sBLMs, EIS provides measurements of bilayer electrical properties, resistance, and capacitance.^{392,393} EIS can monitor the permeability of a membrane to small ions (Na^+ , K^+ , Cl^- , etc.) which can be useful in peptide ion channel studies, and it can also be used to determine transiency of pores.^{392,393} This has been demonstrated by Hristova and Searson and colleagues, who compared changes in membrane resistance and capacitance after the introduction of various membrane-permeabilizing peptides.^{62,66,93,136,392–396} EIS is also able to provide information regarding the mode of action of peptides on membranes with respect to established mechanistic models of pore formation.³⁹³ Chang et al. modeled the expected EIS signature of various models of membrane permeabilization. Several related synthetic MPPs, which have different behaviors in vesicle leakage, were then tested. When these MPPs were added to a polymer cushioned bilayer in an EIS cell, resistance decreased within 1 min but to a different degree for the two peptides.³⁹³ The peptide that caused a much larger decrease in resistance in EIS was found to be the one that is more potent in vesicle leakage. Bilayer homogeneity, derived from capacitance, also decreased but only for the more potent peptide not for the other, showing that unique models can be assigned to specific peptides using EIS.³⁹³

Dual polarization interferometry (DPI) can also be utilized to study the effect of peptides on sBLMs. DPI uses dual optical waveguide interferometry to analyze sBLMs by collecting real-time data on membrane thickness, refractive index, mass, and anisotropy in the presence of membrane-active peptides.^{191,397,398} Balhara et al. recently used AFM and DPI to reveal that the AMP GL13K exhibits membrane selectivity when comparing interactivity with liposomes to sBLMs.³⁹⁸ While they found minimal evidence for binding of GL13K to DOPC liposomes, they showed that the peptide does interact with DOPC sBLMs which was confirmed through changes in membrane thickness, birefringence, and membrane mass. Their study exemplifies the ability of AFM and DPI to not only categorize but reconcile differences in leaflet dielectric values

between perturbed and unperturbed membranes. Membrane leaflet dielectric values are affected by membrane potential and architectural membrane defects, both of which may contribute to peptide binding in sBLMs but may not be readily observable in liposomes.³⁹⁸

Furthermore, surface plasmon resonance (SPR) is also used often in conjunction with DPI to determine peptide binding to sBLMs by collecting data on membrane thickness, giving insight into specific peptide-membrane binding dynamics.^{191,399,400} Papo et al. established modes-of-action for melittin and magainin by aligning SPR data to models of membrane interaction with respect to two sequential activities that occurred: (1) peptide association with the membrane and (2) insertion of the peptide into the membrane thereafter.³⁹⁹ In this context, SPR was used to calculate binding constants in real-time after peptides were introduced to a supported lipid membrane and was able to differentiate two different peptide mechanisms of membrane interaction.³⁹⁹ As indicated by the SPR results, in zwitterionic membranes, melittin appeared to bind preferentially and penetrate deeper than two AMPs, magainin and a melittin diastereomer. Against anionic membranes, all three peptides bound strongly, but without deep insertion, indicating a change in both membrane specificity and mechanism for melittin.³⁹⁹

A technique called quartz crystal microbalance with dissipation monitoring (QCM-D) is also used to probe peptide-membrane binding dynamics. QCM-D uses acoustic penetration of sBLMs at different harmonic frequencies to determine changes in membrane mass and elasticity in a leaflet-specific manner when interacting with different peptides.^{383,401,402} Wang et al. used QCM-D signatures to profile four different AMPs (alamethicin, indolicidin, chrysophan-3, and SMAP-29) which have discrete mechanisms of action.³⁸³ By using the change in dissipation (ΔD), they were able to relate the changes in membrane rigidity and viscoelasticity to the four aforementioned peptides, where a small ΔD is indicative of an unperturbed bilayer.³⁸³ To map the mechanistic sequence of events, change in frequency (Δf) was used to provide metrics on peptide insertion into the membrane, the formation of pores, and the adsorption of peptides.³⁸³ Using hybrid ΔD – Δf dynamic plots, each peptide was assigned a distinct mechanistic fingerprint, which outlined the stepwise interactivity of these peptides to a membrane bilayer, effectively giving insight into the modes of action under the conditions studied.³⁸³ Overall, sBLMs are a robust experimental platform for numerous techniques which may elucidate mechanistic details of permeabilization of membranes by peptides and complement information obtained from lipid vesicles.

Secondary structure in supported bilayers can be determined by Fourier transform infrared spectroscopy and circular dichroism spectroscopy. More importantly, the orientation of some secondary structure elements can be assessed in surface supported bilayers by attenuated total reflectance Fourier transform infrared spectroscopy, ATR-FTIR,⁴⁰³ and by oriented circular dichroism (OCD) spectroscopy, which is especially well suited for the determination of helix orientations in membranes, as shown in Figure 14.

3.3.2. Limitations and Caveats of Supported Bilayers.

While surface supported bilayers have been very important to the study of MPPs, there are some limitations to these techniques. A significant limitation in many sBLM experimental models is that the lipid concentration is effectively very

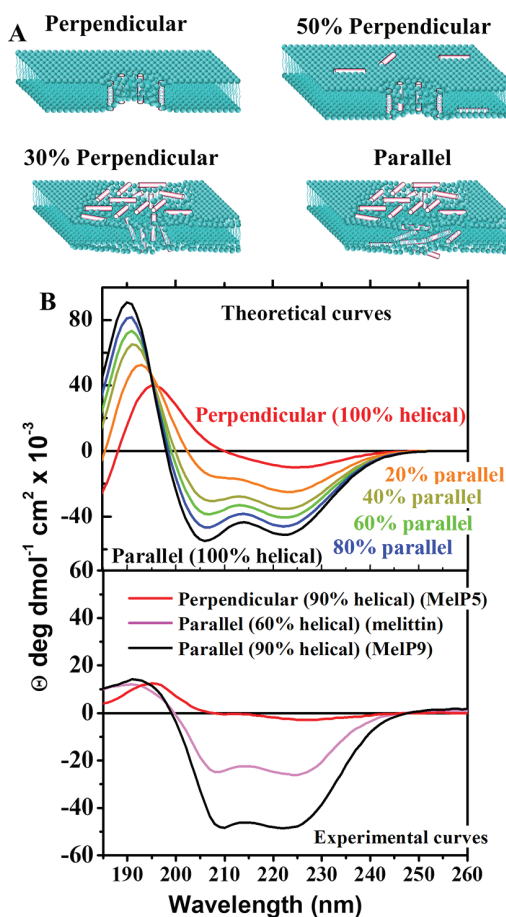


Figure 14. Oriented circular dichroism. In OCD, oriented multibilayers stacks containing a peptide that has mostly α -helical secondary structure are prepared on a quartz disk and hydrated through the vapor phase. Circular dichroism is measured with the bilayer plane oriented perpendicular to the beam axis. A. Bilayers with imaginary helical MPPs that have axes perpendicular or parallel to the bilayer normal, or a combination of the two. B. Theoretical spectra for perpendicular (transmembrane) and parallel (surface-oriented) α -helices are shown, along with linear combinations of the two.¹⁹⁴ These data are scaled to represent residue contributions assuming 100% helicity. C. Real experimental OCD data⁵⁹ for melittin, which is parallel to the bilayer and has a helicity of $\sim 60\%$, and two gain of function analogs, MelP5, which is perpendicular to the bilayer and has 90% helix, and MelP9, which is parallel to the bilayer and has 90% helix. Adapted with permission from ref 181. Copyright 2018 American Chemical Society.

low and is unknown, meaning that the peptide to lipid ratio and the bound peptide to lipid ratio are not known, although they can be indirectly estimated.² This same limitation also decreases the information content of GUV experiments (see above). In some cases, excess lipid vesicles and excess peptide can remain in equilibrium with the supported bilayer, to enable more precise knowledge and control of concentrations.^{66,392,393} In other cases, such as oriented CD and some diffraction techniques, stacked oriented multibilayers are needed, and these require that samples be less than fully hydrated to remain stable.

3.4. Molecular Dynamics Simulations

Molecular dynamics is the simulation of interacting atoms and particles within ensembles of molecules providing predictions

of the energetics and dynamics of the system. These simulations are governed by calculations based on Newton's equations of motion.⁴⁰⁴ There are three general classes of models for performing molecular dynamics on MPPs: all-atom, coarse-grained (CG), and implicit solvent models. These studies give detailed hypothetical structural and kinetic information on possible mechanisms of membrane permeabilization by peptides.

3.4.1. Methods of Simulating Bilayer Permeabilization. The multiple classes of MD simulations arise from the degree to which each utilizes computational resources, which are usually limiting, and from the need to allow a simulated system to reach equilibrium, independent of initial conditions. All-atom models are the most comprehensive as they calculate all of the bonded and nonbonded interactions between individual atoms in molecular ensembles. However, all atom simulations require very significant computer resources. Coarse-grained (CG) models represent molecules using a smaller number of particles, often called "beads", that each mimic groups of connected atoms within a molecule. Thus, CG simulations must simulate fewer particles and require far fewer computer resources to simulate longer trajectories and more extensive systems. Implicit solvent/bilayer models make the most significant deviations from reality by representing water and bilayer with implicit nonmolecular field-like representations. However, implicit solvent models enable much longer trajectories and larger systems to be simulated with much less computer power. Each approach has strengths, weaknesses, and caveats that have been well discussed in the literature. We summarize the techniques below.

All-atom modeling considers all atoms as explicit particles with appropriate properties. Chemistry at Harvard Macromolecular Mechanics (CHARMM) force fields are often used here to simulate membranes, membrane proteins, and peptides. This type of modeling allows for more accurate representations of the peptide, solvent, and the lipid bilayer.⁴⁰⁵ However, questions still exist about the most accurate force field.⁴⁰⁶ A method sometimes employed to address the computational requirements is to increase the temperature of the system. A number of membrane interacting peptides have been found experimentally to be temperature insensitive over a wide range, making them suitable for simulations at up to 90 °C or higher,^{93,407–410} which can decrease the time to reach equilibrium significantly.⁴¹¹

In the CG model, hydrogen atoms are treated implicitly and multiple non-hydrogen atoms are treated as one particle as to simplify the system and extend system size and time scales. The MARTINI force field,⁴¹² which was originally described for lipid and surfactant systems,⁴¹³ is used most often here. It uses 4-to-1 mapping, with four non-hydrogen atoms acting as one "bead" or interaction center. There are only four main types of beads: polar, nonpolar, apolar, and charged. Each of these types are further divided into subtypes.⁴¹⁴ Aggregating atoms in this way and reducing interaction complexity can reduce computing resource needs or increase trajectory times by as much as 3–4 orders of magnitude.⁴¹³

Implicit solvent models further simplify the system by treating solvents, including the bilayer, as a continuum of medium rather than as individual particles. It was originally developed as a necessity when computing power was lacking.⁴⁰⁵ In the implicit membrane model (IMM), the generalized Born theory of solvation is used to simulate the lipid bilayer. This allows 100 $\times$ more efficient simulations as

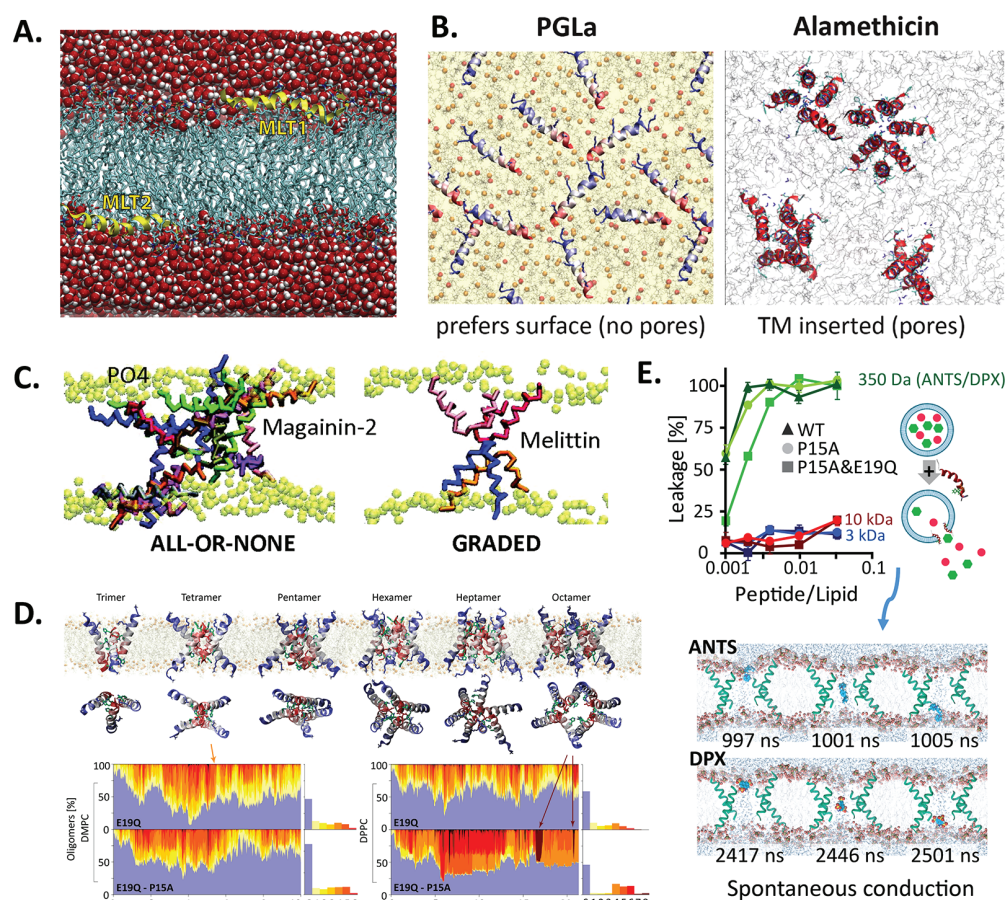


Figure 15. Molecular dynamics simulations of MPPs show binding and structure formation on and in bilayers. (A) A simulation of melittin monomer binding over 17 μ s shows that the peptide settles at a depth near the glycerol groups, which notably is consistent with X-ray diffraction results. Adapted with permission from ref 429. Copyright 2013 Elsevier, Inc. (B) Two different behaviors of multiple peptides binding to a bilayer are shown here. The interfacial S state is preferred by PGLa while a transmembrane configuration is preferred by alamethicin, a known potent pore former. Adapted with permission from ref 405. Copyright 2018 American Chemical Society. (C) Transmembrane structures of an all-or-none and graded peptide. Melittin peptides are in a U-shape that blocks water passage while magainin-2 allows water through more easily. These simulations indicate how all-or-none and graded mechanisms can be structurally different. Adapted with permission from ref 432. Copyright 2012 American Chemical Society. (D) Permeabilization is complex, as shown with the example of maculatin. The peptide does not form just one structure on a bilayer; it forms a variety of structures that assemble and disassemble over time for both DMPC and DPPC bilayers (bottom). However, it should be noted that most peptides are in the surface bound state (S). (E) Unbiased dye-conductance simulations show that P15A-E19Q, a maculatin double mutant that is thermally stable, forms lesions just large enough to allow ANTS and DPX through (bottom). Importantly, this finding was validated with experimental ANTS/DPX and dextran release assays (top). Panels D and E adapted with permission from ref 411. Copyright 2016 Springer Nature Limited (CC) <https://creativecommons.org/licenses/by/4.0/legalcode>.

compared to explicit modeling.⁴¹⁵ In the IMM, factors such as membrane thickness⁴¹⁶ and even poration can be calculated.^{417,418} However, due to the simplicity of the implicit model, complex lipid bilayer composition is difficult to recapitulate.

3.4.2. What Behaviors Have Been Observed in MD Simulations? All-atom simulations have been done with many MPPs including melittin, alamethicin, and magainin and others (Figure 15). In an early monomer binding simulation of melittin, the bilayer showed thinning with water molecule penetration from both faces.⁴¹⁹ The more hydrophobic N-terminus was found to be protonated and it penetrated the bilayer. The setup had a helical melittin molecule that was set parallel in the interfacial region on a DMPC bilayer and simulated for 850 ps. Similar results have been observed in DPPC lipids.⁴²⁰ Alamethicin simulations with POPC over 2 ns found that the peptide interacted with the polar headgroups of

the lipid but not with the hydrophobic core.⁴²¹ A study of magainin indicated that by orienting the peptide with the hydrophobic residues close to the bilayer it will penetrate deeper.⁴²² The same study also showed that the addition of salt to the system reduces the insertion depth of the peptide. However, these early simulations were far too short to reach equilibrium and the trajectories observed reflected mostly just the initial placement of peptide. In more recent all atom simulations, time scales in the microseconds have been observed.^{409,423–428} Using the CHARMM22/36 protein/lipid force fields, Andersson et al. simulated melittin monomer binding to DOPC lipids for over 17 μ s.⁴²⁹ The simulation showed the peptide partitions at the depth of the glycerol groups of the lipids but remains mostly parallel to the surface. Helicity was found to be in line with experimental observations.^{79,430,431} Another study examined the putative transmembrane structure of melittin oligomers.⁴²³ A pre-

formed transmembrane tetramer of the peptide was inserted into DMPC lipids and simulated for 9 μ s, showing formation of local disruption of the lipid bilayer with lipid head groups facing the interior of the pore. This equilibrium pore structure may mimic the structure of melittin under some experimental conditions, although as described earlier (Figure 2), melittin is not an equilibrium pore former under most experimental conditions.⁶⁵

Coarse-grained models of melittin and magainin-2 have suggested possible permeabilization mechanisms. Melittin, which exhibits graded leakage experimentally, exhibited aggregates of 4 to 6 U-shaped peptides in the membrane, forming poorly defined “channels” by 4.2 μ s.⁴³² Magainin-2, which exhibits all-or-none leakage, formed water-filled transmembrane structures by 2.1 μ s at P:L of no less than 24:512. The simulation for both peptides used the MARTINI force field, DPPC lipids, and began with interfacial placement of peptides. These two different structures show how graded and all-or-none leakage could occur in leakage assays, but they fail to explain transient leakage induced by both peptides. Another MARTINI simulation of magainin-2 showed even more dramatic activity; when a layer of peptide was placed on a DPPG/POPG bilayer, the bilayer buckles and forms a quasi-spherical vesicle by 2.4 μ s, indicating yet another possible mechanism of the action of an MPP on a membrane.⁴³³ A potential limitation in the study of MPPs with course-grained simulations is the lack of water in channel-like structures.^{432,434,435} Since some force fields treat four water molecules as one, this can produce water sized cavities that are empty due the artificially large “water” beads.

In a series of AMP studies by Lazaridis and colleagues, an implicit membrane model was modified to include cylindrical or parabolic shaped holes.^{418,436} They showed that alamethicin, melittin, piscidin, and MG-H2 (a magainin analogue) all bind more strongly to the hole region in the membrane than to a flat, intact membrane.^{418,437} These results suggested that the peptides favor a membrane spanning pore-like geometry, a conclusion that is not consistent with all experimental measurements. They also found that alamethicin was the only one that completely embedded in the core of the membrane,⁴¹⁸ consistent with the observation that alamethicin is a very potent equilibrium pore-forming peptide that inserts across membranes under most conditions.⁶⁴ The authors attribute the preferential binding at hole regions to imperfect amphipathicity.²

Ulmschneider and colleagues suggested that, by using experimentally guided simulations, the most correct force field could be determined, based on which produced the most accurate simulation result.⁴³⁸ They also give an example of how setting the right time-scale is critical. They found that when simulating monomer surface binding of PGLa and melittin, the CHARMM/AA-lipids force fields gave much more helical structure compared to the OPLS/UA-lipids force fields. To determine which was more accurate, the authors looked to experimental data, which included CD and ssNMR techniques.^{335,431,439,440} The experimental data matched the more helical conformations of the CHARMM/AA-lipid force fields. They also found that even with 2 μ s simulations, maculatin 1.1 peptides that start from either coiled or partly folded conformations do not converge, indicating that simulations shorter than this time scale may not reach equilibrium and can be unreliable.

Wang et al. also utilized an experiment guided technique in a promising approach to model membrane permeabilization by the AMP maculatin.⁴¹¹ In this study, they used circular dichroism at different temperatures to first show that a thermally stable mutant, P15A, had the same secondary structure as WT and that its structure is temperature insensitive. This enabled a simulation temperature of 90–150 °C with the mutant which substantially decreased the required simulation time. They also performed ANTS/DPX and macromolecule leakage experiments to estimate lesion size; only leakage of the 400 Da ANTS/DPX, and not higher molecular weight dextrans, was observed, consistent with the simulated pore structure. Simulated initial insertion events were shown to occur through the cooperative insertion of two peptides into a transmembrane state, which allowed for more insertion events to take place. Longer simulations of 20 μ s showed that maculatin forms diverse and dynamic structures in the membrane from trimers to octamers that assemble and disassemble continuously. They further note that, while the leakage assays of single and double mutants had similar results, simulations showed they had different ensemble weightings of transmembrane aggregate size. Unbiased dye-conductance simulations showed that the octamers would, in fact, allow leakage of 400 Da molecules, agreeing with the experimental findings. This methodology of experimentally guided simulations bridges molecular dynamics and experimental data in a way that benefits both fields synergistically.

3.4.3. Limitations and Caveats of MD Simulations.

Molecular dynamics simulations of membrane permeabilizing peptides in lipid bilayers is an evolving field. It has shown tremendous growth in capabilities in recent years, a trend that can be expected to continue. The time scales that can be achieved by most researchers remain too short to ensure equilibrium in many cases and are far too short to simulate important slow processes such as peptide and lipid translocation. All atom simulations are currently limited to the nanosecond or microsecond time scale for most researchers. All atom simulations on the millisecond scale with the Anton II supercomputer⁴³⁷ could revolutionize the field. Longer simulations at higher P:L and with larger ensembles are needed to capture important processes that can span the stages from membrane binding to membrane translocation are desirable. Course grained simulations enable equilibration and longer processes to be studied, yet questions remain about the relevance to real molecules. At present, the most significant gap between simulations and experiment is the issue of transient permeabilization, which is currently difficult to simulate because it requires simulation of rare, slow events such as peptide translocation.

3.5. Biological Systems

The study of interactions of MPPs with model membranes and their effects on model membrane permeability has provided many insights into their physical chemistry, structure, and mechanistic landscapes. However, the direct relevance of these model systems to biological membrane systems is uncertain. Relevance varies with each model system and depends on the information provided by the model system and on its biases. No synthetic model system recreates all of the properties of a biological membrane. Ultimately, permeabilization of biological membranes must be measured directly and then carefully compared to model systems to further our understanding of MPPs and to learn how to engineer them for our

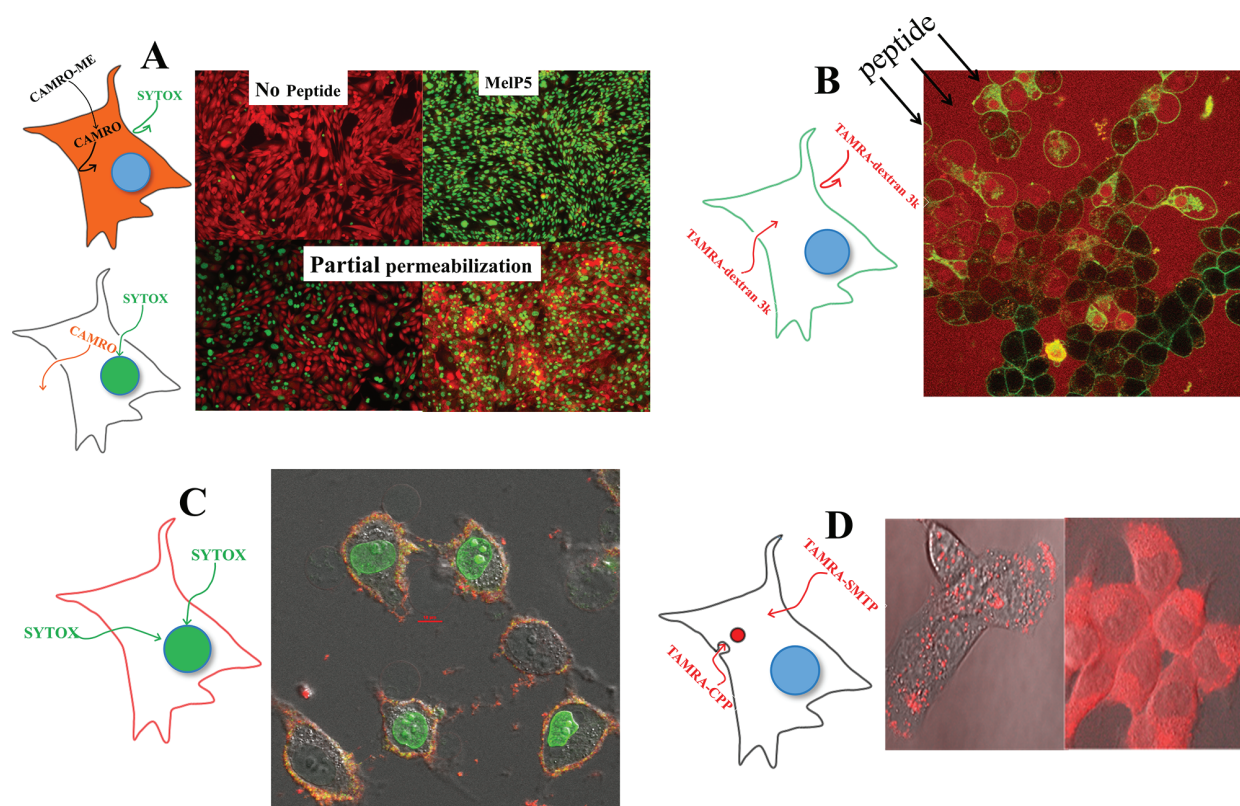


Figure 16. Examples of permeabilization of eukaryotic plasma membranes. A. Cells in culture were treated with calcein red orange acetoxymethyl ester, which freely crosses the cell and is activated by cellular esterases to become entrapped and membrane impermeant (red, upper left). At the same time SYTOX Green, a membrane impermeant DNA binding dye is added to the outside of the cells. Membrane permeabilization, as with MelP5 in the upper right, enables entry of SYTOX Green where it enters the nucleus and becomes fluorescent. B. In this confocal microscopy image, cell membranes are labeled green and a 3000 Da dextran labeled with TAMRA (red) is added outside the cells. Shortly after addition of a membrane permeabilizing peptide in the corner, the first cells exposed are permeabilized to the dextran and they show osmotic swelling. The cells in the opposite corner have not yet been affected by peptide. C. Cell membranes are labeled red and cells are incubated with external SYTOX Green. A low concentration of the Ebola Virus delta peptide, a viroporin,¹³⁶ was added 10 min prior to taking this image. Here cells have been permeabilized to SYTOX Green, which stains the nuclei, but massive water influx and osmotic lysis are not occurring, evidenced by the lack of swelling or cell rounding at this time. D. TAMRA-labeled cell penetrating peptide (Arg₉-TAMRA) and a spontaneous membrane translocating peptide TP2-TAMRA³⁸ are incubated with cells at a low concentration of $\sim 1 \mu\text{M}$. The CPP gets untaken into endosomes, but cannot escape into the cytosol, in this case, because its concentration is too low to disrupt the membrane. The translocating peptide spontaneously crosses the plasma membrane and enters the cytosol. We thank Kalina Hristova for generous access to her confocal microscope.

purposes. In this section, we will discuss MPP studies on biological membranes.

3.5.1. Eukaryotic Plasma Membrane Permeabilization. The plasma membrane of a eukaryotic cell is the essential permeability barrier between the inside of the cell and the rest of the universe. Depending on the application, the plasma membrane can either be the target of an MPP, as in a cancer therapeutic, or an off-target membrane for which activity must be minimized, as in the case of an antimicrobial or antiviral peptide. In either case, permeabilization of the plasma membrane must be measured and we must eventually learn how to predict it. Acute permeabilization can be measured directly, or the effect of permeabilization on cell viability can be measured indirectly, because permeabilization is often cytotoxic. The most commonly used measurement of biomembrane permeabilization is hemolysis, in which the release of protein contents from erythrocytes is measured after peptide treatment.^{47,54} The very high concentration of hemoglobin and its strong optical absorbance provide a simple and sensitive assay measurement. The hemoglobin released into the supernatant is measured after erythrocytes are

incubated with peptides and pelleted by centrifugation. The percent of hemoglobin released as a function of peptide concentration indicates membrane permeabilizing potency, which provides insights into the cytotoxicity of MPPs on erythrocytes.^{441,442}

Permeabilization of nucleated cells in cell culture, perhaps an even more sensitive and informative assay than hemolysis, is measured by assessing the release of proteins or other polar compounds from the cell or by assessing the entry of exogenous polar compounds into the cell (Figure 16). For example, small molecule release assays include the deoxyglucose assay that quantifies efflux of radiolabeled and intracellularly phosphorylated deoxyglucose through the plasma membrane; this allows for the evaluation of the effect of an MPP on the integrity of eukaryotic cells. ATP release has also been used to measure permeabilization. Because fluorescence-based assays are convenient and sensitive, one can preload cells with esterase-activated cytosol trapped dyes, such as calcein-acetoxymethyl ester, which are then released from the cytosol if a cell is permeabilized.¹³⁶ For protein release techniques, biochemical assays that exist for numerous

enzymes can be used to quantitate release. A commonly used permeabilization assay relies on the measurement of the ubiquitous and abundant cytosolic enzyme lactate dehydrogenase that can be measured using a colorimetric detection.^{41,441,443} Permeabilization of the plasma membrane can also be measured by assessing entry of exogenous compounds. Propidium iodide (PI) for example is a widely used plasma membrane-impermeant nucleic acid intercalator that selectively enters compromised cells and stains their nuclei.^{441,444} Similarly, the SYTOX family of DNA intercalating dyes only cross plasma membranes if they have been permeabilized and then become brightly fluorescent when they interact with DNA.^{441,445} Such assays can be performed by counting dye-positive cells, by flow cytometry, or by measuring total fluorescence in a plate reader or microscope. With the use of calcium sensitive dyes, it is also possible to assess the permeabilization of plasma membranes specifically to calcium.⁴⁰

Other live/dead assays, although indirect, are also useful indicators of acute lysis, because lysis kills cells quickly. MTT and Alamar Blue are examples of a colorimetric and a fluorescent assay, respectively, that detect metabolically active live cells. Both assays rely on conversion of a sensor compound by active mitochondrial reductases. For the MTT assay, reduction of MTT creates an insoluble violet-blue formazan that can be quantified by its absorbance.^{441,446} For the Alamar Blue assay, reduction of resazurin to resorufin gives a compound that is red in color and highly fluorescent, indicating live cells.^{441,447}

In all of these cellular leakage assays, unless care is taken to achieve osmotic balance between inside and outside,⁵⁴ which is inconvenient and rarely done, the assays may actually be reporting on osmotic rupture of the membrane due to the influx of water that occurs when membranes are permeabilized. The osmotic imbalance is quite large because of the very high concentration of proteins inside a cell.⁴⁴⁸ In microscopy, this effect is obvious (Figure 16) as eukaryotic cells treated with MPPs swell dramatically before complete destruction of their architecture is observed.

Melittin has been widely studied using many model systems as well as many biological systems. In cells, melittin was first identified as a "direct lytic factor" in bee venom as it induced hemolysis.^{73,449} Different groups have also modified melittin and used hemolysis to better understand its binding and permeabilizing effect in erythrocytes.^{54,73,450–453} The hemolysis and leakage assays have also been used to study potential therapeutic applications of melittin.^{454–459} Unlike the majority of MPPs, which have some selectivity, melittin has remarkably consistent activity against various types of biological membranes. Against eukaryotic cell membranes, including erythrocytes and nucleated cells, melittin causes complete osmotic lysis at concentrations of around 5 μM .^{46–48,54,460–462} Similarly, $\sim 5 \mu\text{M}$ melittin has sterilizing activity against most Gram negative and Gram positive bacteria, due to membrane permeabilization.^{24,45,174,179} These experiments, especially LDH release and hemolysis, have also been used to study another extensively investigated peptide, magainin 2.^{32,33,463} The cytolytic effect of many MPPs including defensins, penetratin, and TAT have also been described using the above assays.^{41,127,464–471}

3.5.2. Mitochondrial Membrane Permeabilization. Mitochondria are an important target for cellular therapeutic drug delivery. The inner membrane of mitochondria limits

diffusive transport, and like all membranes, it inhibits entry of therapeutics into the mitochondrial compartments. Hence, studies have been performed with membrane permeabilizing peptides and model systems mimicking mitochondrial membranes to identify peptides that are able to permeabilize or translocate into mitochondria. Further, the initiation of apoptosis can occur when the mitochondrial outer membrane is permeabilized to release cytochrome c.⁴⁷² This is a process that would be useful to control in some disease states, such as cancer, where apoptosis is dysregulated.^{473–475} The permeabilization of the inner and outer membrane of mitochondria can be analyzed using various experiments.^{476,477} The subcellular redistribution of mitochondrial proteins such as Cyt c, AIF, or adenylate kinase (ADK) to the nucleus or cytosol is used as a measurement of outer-membrane permeabilization or rupture due to matrix swelling.^{476–479} Detection of intermembrane space metabolites or exogenous NADH oxidation, through HPLC or fluorescence respectively, can also provide insight on mitochondrial outer membrane integrity.^{476,477,480,481} Inner mitochondrial membrane permeabilization causes dissipation of the membrane potential across the inner membrane ($\Delta\psi_m$). Different $\Delta\psi_m$ -sensitive fluorochromes may be used to assess the peptide induced loss of $\Delta\psi_m$.^{476,477,482} The permeabilization of the inner mitochondrial membrane is also accompanied by flooding of water and solutes into the matrix resulting in an increase in the mitochondrial matrix volume. This osmotic swelling causes disorganization of cristae and reduction of matrix density. In case of swelling, the integrity of inner mitochondrial membrane can be monitored by measuring absorbance of the suspension in a traditional spectrophotometer.^{476,477,483,484}

Various peptides have been shown to permeabilize mitochondrial membranes. For example, mastoporan, also known as mitoporan, has been shown to be a potent facilitator of mitochondrial permeabilization through various experiments that measure matrix swelling and inner membrane permeabilization.^{485–487} The polycationic peptide BTM-P1 also permeabilizes mitochondria, causing matrix swelling and $\Delta\psi_m$ loss.⁴⁸⁸ Amyloid- β ($A\beta$) is a peptide that contributes to pathogenesis of Alzheimer's disease through apoptosis of cells. Experiments monitoring swelling of matrix and leakage of Cyt c from inner membrane disruption suggests that $A\beta$ could induce apoptosis through mitochondrial membrane permeabilization.⁴⁸⁹ Similarly, many peptides that can enter mitochondria have also been studied for their potential in cancer therapy. For example, BH3 and ABT-737 peptides have been shown to permeabilize the mitochondrial outer membrane through immunoassays for mitochondrial proteins.⁴⁹⁰ In the presence of tumor cells, these peptides can permeabilize inner mitochondrial membrane causing loss of $\Delta\psi_m$ and swelling of matrix, leading to cell apoptosis.⁴⁹⁰

3.5.3. Endosomal Membrane Permeabilization. Many polar therapeutics, molecular tools, and other polar compounds can be endocytosed into cells when conjugated to, or complexed with, peptides and various nanomaterials, including cell penetrating peptides (CPPs) and nanoparticles. However, escape from the endosome requires membrane disruption. Endocytosed cargoes that do not escape endosomes do not reach the cytosol or nucleus where delivery would be beneficial. Failure of endosomal escape is a major bottleneck in cytosolic delivery of bioactive cargoes such as proteins.

Over the past several decades, many peptides, including CPPs and other MPPs have been used to facilitate delivery of

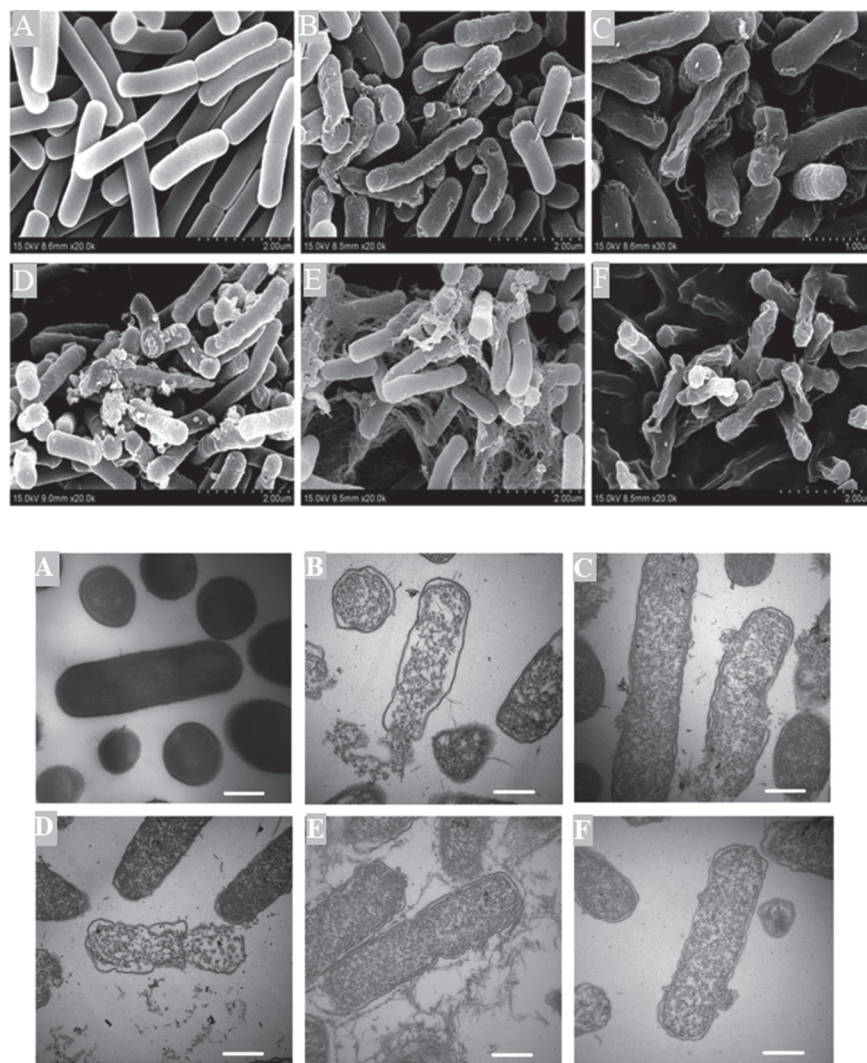


Figure 17. EM images of bacteria treated with AMPs. Top: SEM micrograph of *E. coli* ATCC 25922 (A) control; (B–E) synthetic centrosymmetric α -helical AMP GG2, GG3, AA2, and AA3 treated; (F) MPP melittin treated. Bacterial cells were treated with 1 $\times$ minimum bactericidal concentration (MBC) of peptide for 1 h. Adapted with permission from ref 529. Copyright 2015 Nature Publishing Group. Bottom: TEM micrographs of *E. coli* ATCC 25922. (A) Control; (B–E) synthetic centrosymmetric α -helical AMP GG2, GG3, AA2, and AA3 treated; (F) MPP melittin treated. Bacterial cells were treated with 1 $\times$ minimum bactericidal concentration (MBC) of peptide for 1 h. Scale bar = 500 nm. Adapted with permission from ref 529. Copyright 2015 Nature Publishing Group.

polar compounds to cells via endosomal escape, at least partly through endosomolysis, or endosomal membrane permeabilization. The cargo delivery field is clouded by the fact that cationic CPPs that induce uptake and endosomolysis almost always simultaneously translocate and deliver cargo across the plasma membrane by a parallel process that may include transient permeabilization.^{15,40,41} In either case, membrane permeabilization is required. Direct evidence for endosome lysis is often lacking. In biosystems, endosomal permeabilization has been inferred from cargo delivery characteristics.^{15,41} Endosomal release has been visualized through fluorescence labeling and microscopy.⁴⁹¹ Melittin analogs have been shown to permeabilize endosomes and increase content release.^{73,492–494} Pellois and colleagues used dTAT, a dimerized disulfide-linked CPP, and Salerno et al. used a modified TAT peptide for better cargo delivery showing how MPPs can be modified to increase their endosomal escape efficiency.^{495–497} Some synthetic cationic amphipathic membrane permeabiliz-

ing peptides like KALA and GALA with lysine-alanine-leucine-alanine and glutamic acid-alanine-leucine-alanine (EALA) repeats, respectively, have also been shown to enhance endosomal escape by membrane destabilization.^{172,498,499} Another cationic amphipathic histidine-rich MPP, LAH4, can also efficiently cause endosomal release and permeabilize model membranes in a pH-sensitive manner.^{500–502} Rational variation in arginine topology as well as arginine-rich MPPs have been found to induce endosomal leakage.^{503,504} Hence, MPPs continued to be modified, evolved, or rationally designed to increase the delivery efficiency of therapeutic compounds to cells.

3.5.4. Bacterial Membrane Permeabilization. As discussed above, various model systems have been used to mimic the anionic cytoplasmic membranes of bacteria to study the physical chemical basis for AMP activity. These results roughly compare to what is observed in living bacteria. In bacterial systems, the majority of known AMPs kill bacteria

Vesicle Statistics						
Vesicle type	Size	# lipids	Bilayer/ Radius	Volume (l)	# probes inside (10 mM)	# probes inside (10 μ M)
LUV	0.1 μ M	1x10 ⁵	0.1	5x10 ⁻¹⁹	3000	---
GUV	10 μ M	1x10 ⁹	0.001	5x10 ⁻¹³	---	3x10 ⁶

Typical Experimental Conditions – Vesicle Leakage							
Vesicle Type	Lipid μ M	Peptide	Peptide bound Per Lipid	Peptides bound Per vesicle	# probes (10 mM)	# probes (10 μ M)	Probe per peptide bound
LUV	500	1x10 ⁻⁵	1:60	1600	3000	---	2
GUV	<10	1x10 ⁻⁵	1:12	7x10 ⁷	---	3x10 ⁶	0.04

Typical Experimental Conditions - Bioassays					
Cell Type	Cells/ml	Lipids/cell	[Lipid] μ M	[Peptide] μ M	P:L total
Bacteria	5x10 ⁵	2x10 ⁸	0.2	10	5:1
Erythrocyte	1x10 ⁸	1x10 ⁹	200	10	1:20
Nucleated	5x10 ⁵	1x10 ¹⁰	10	10	1:1

Figure 18. Statistics and stoichiometries in permeabilization experiments. Three tables of statistics and stoichiometries of permeabilization experiments. Top: Vesicle statistics for LUV and GUV, assuming a size of 10 μ m for the GUV. Entrapped probes are assumed to be 10 mM for LUVs which use dye quenching as a probe, and 10 μ M for GUVs which use dye observation in confocal microscopy as a probe. Typical experimental conditions for LUV and GUV experiments assuming 10 μ M peptide concentration a mole fraction partition coefficient⁵⁹ equal to that of melittin, $K_x = 5 \times 10^5$. Lipid concentration in the GIV experiment is assumed to be very low (a few vesicles per μ L). Bottom: Stoichiometries for biosystem permeabilization experiments. Lipids per cell is calculated from surface area using 70 $\text{\AA}^2$ per lipid molecule and multiplying by 2 for each membrane present.

through membrane permeabilization, following accumulation of large amounts of cationic AMP on the anionic membranes of the bacterial cells.^{23,24,174,505} Killing of bacteria by AMPs can be quantitated by broth dilution or agar diffusion assays, described elsewhere.⁵⁰⁶ Rathinakumar and Wimley compared bacterial membrane permeabilization and sterilization by a family of evolved AMPs and found a positive correlation.¹⁷⁴

As with eukaryotic cells, bacterial membrane permeabilization can be measured by leakage of polar compounds or proteins out of the cell or entry of polar compounds into the cell. The permeabilization of the outer and inner membrane of Gram negative bacteria can be quantified by *N*-phenyl-*N*-naphthylamine (NPN) uptake assay and *O*-nitrophenyl-3- β -galactoside (ONPG) influx method, respectively.^{507,508} For the NPN uptake assay, bacterial cells are prepared in the buffer containing NPN. The peptides are then added to the cells and NPN uptake through disrupted outer membrane is quantified using a spectrophotometer. Similarly, ONPG influx assay requires the dilution of cells in buffer containing ONPG followed by incubation with peptides, at which point the influx of ONPG into the cells can be measured and used as indicator of permeability through the inner membrane because β -galactosidase converts OPNG to a colored product.^{509,510} Potential sensitive dyes can also be used to measure disruption of ion gradients across bacterial membranes.¹⁷⁴ Further, DNA intercalating dyes, such as propidium iodide and SYTOX family dyes also report on bacterial permeabilization, although entry of some larger dyes are restricted by the outer membrane of Gram negative bacteria and the cell wall of Gram positive bacteria.⁶⁷ Sani et al. used flow cytometry to show that the AMP maculatin 1.1 enabled efficient entry of a dextran of 4 kDa into bacteria while a 40 kDa dextran entered bacteria less efficiently.⁵¹¹ These authors also showed that release of the two dextrans from bacteria-like anionic lipid vesicles recapitulated the size selectivity observed in bacteria. Eventually, bacteria that are treated with any antimicrobial

peptides are physically damaged to the extent that it can be observed by electron microscopy (Figure 17).

Reller and colleagues performed some of these assays and described the biological activity of 44 antimicrobial agents including conventional antibiotics such as penicillin as well as modified peptides like colistin and polymyxin B.⁵¹² The experiments listed above have also been used to study the antibacterial activity of natural and synthetic peptides. For example, the antibacterial activity of peptides such as LL-37, various defensins, indolicidin, and cecropins have all been studied using broth dilution, radial diffusion, hemolysis, NPN uptake, and ONPG influx assays.^{507,513–521}

3.6. Statistics and Stoichiometries in Typical Experimental Scenarios

To appreciate what “potency” signifies in a membrane permeabilization experiment, it is useful to examine typical experimental statistics as we have done in Figure 18. An LUV of 100 nm diameter contains about 100 000 lipids and entraps a volume of $\sim 5 \times 10^{-19}$ L. The bilayer thickness is about 10% of the vesicle diameter, providing some stiffness to the vesicle. When the bound P:L is 1:50, which is typical, there are about 2000 peptides bound to each vesicle. In a typical probe release experiment, e.g., calcein, ANTS/DPX, or Tb³⁺/DPA, there is roughly 10 mM probe entrapped within the interior space of each vesicle. This amounts to only a few thousand probe molecules inside each vesicle. Therefore, in a representative LUV permeabilization experiment there are the same number of probe molecules inside a vesicle as there are MPPs bound to it. Complete permeabilization, i.e., high potency, requires only that one probe molecule escape from a vesicle for every bound peptide over the course of the whole experiment. That is 1 or 2 probe molecules escape in 30 min, amounting to a flux per peptide of $\sim 10^{-3}$ per second. Compare this to the ion flux through a classical channel protein which can be 10⁷ per second.⁵²² Alternately, a tiny fraction of the bound peptides

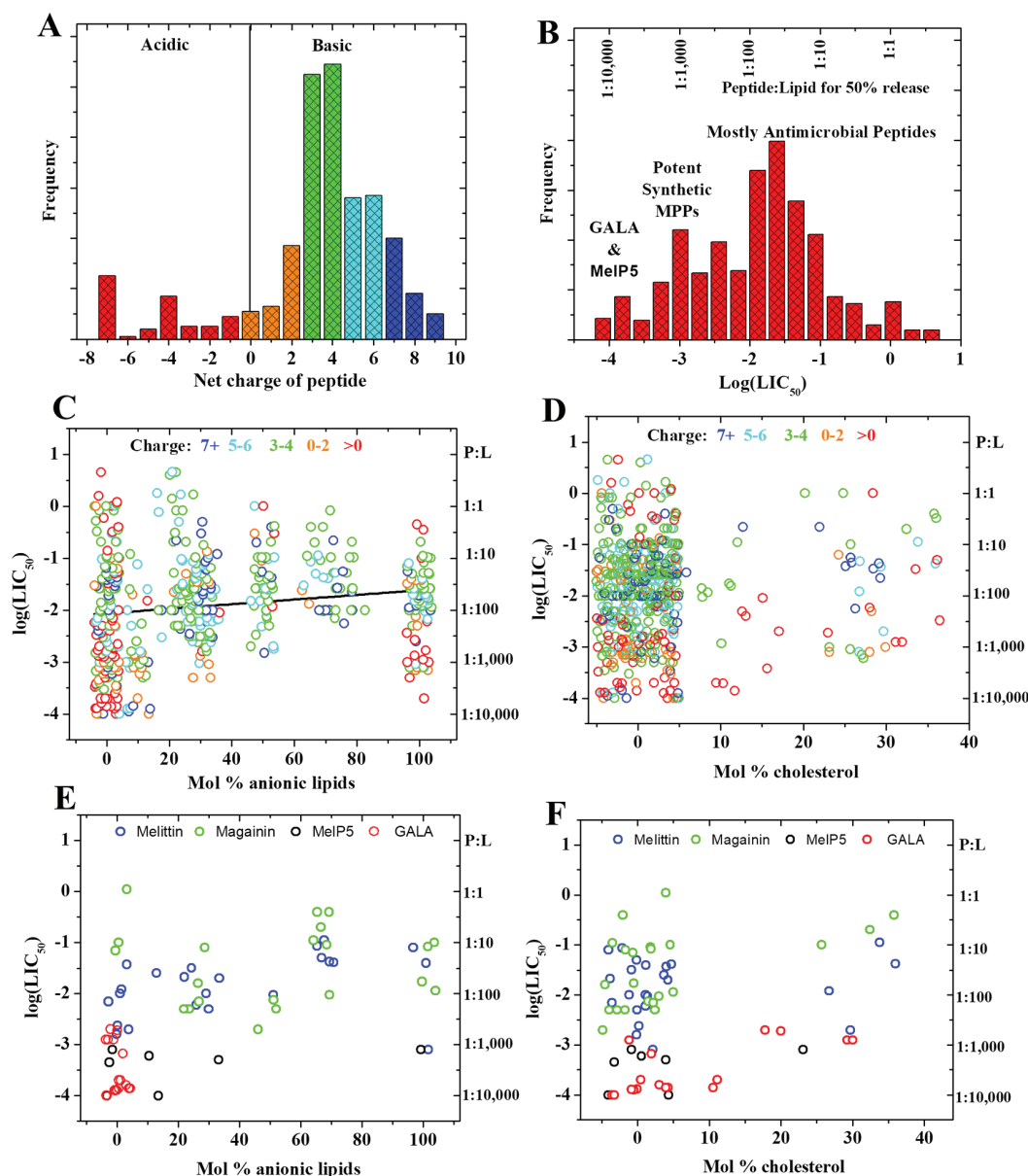


Figure 19. Meta-analysis of membrane permeabilizing peptides. A representative set of high quality published results was aggregated to show the range of behaviors observed for the permeabilization of synthetic vesicles by MPPs. A. Charge distribution in the data set. The colors used here are the same colors used in panels C and D. B. Distribution of the total peptide to lipid ratio required to release 50% of vesicle-entrapped contents, or LIC_{50} . C.: LIC_{50} plotted as a function of acidic lipid content (PG, PS, ganglioside, cardiolipin). The peptide charge states are shown by the color of the points, as shown in panel A. Random noise is added to the X-axis values to spread out the data points. D. LIC_{50} plotted as a function of cholesterol content. The peptide charge states are shown by the color of the points. E. LIC_{50} plotted as a function of acidic lipid content (PG, PS, ganglioside, cardiolipin) for a subset of well-studied peptides. F. LIC_{50} plotted as a function of cholesterol content for a subset of well-studied peptides.

could be responsible for most of the leakage, if local, leakage flux is high but leakage events are rare. Hypothetically, the maximum possible potency of an MPP would be at P:L $\approx$ 1:10 000 where there are only about 10 peptides bound to each vesicle, enough for one or two classical pores. The pH-sensitive peptide GALA is one of the most potent peptides known in LUV experiments with leakage reported at P:L as low as 1:10 000 or 10 peptides per vesicle.^{171,523} Several others, including alamethicin, LLP1, LLP2, and others, are active at P:L $\leq$ 1:2000.^{64,147}

In a GUV the number of lipids is 10 000 times larger than in an LUV, or $\sim 1 \times 10^9$ lipids, assuming a taut $10 \mu\text{m}$ vesicle. GUVs are taut because a moderate osmotic imbalance is created when they are produced, leading to a positive osmotic pressure. The thickness of the bilayer is less than 1% of the vesicle diameter. In a typical experiment, there may be $\sim 7 \times 10^7$ peptides bound to a single GUV. The volume of a GUV is 10^6 times higher than an LUV, but the probe concentration is usually $\sim 10 \mu\text{M}$ because microscopy is used. This means that there are only $\sim 3 \times 10^6$ probes inside, or less than 0.1 probe per bound peptide. It is not known if the stochastic leakage

events observed in GUVs are purely local. However, if it is local, then the permeabilization event has to occur only once, perhaps involving only a handful of peptides, among the 7×10^7 bound to the vesicle. If leakage is a global event, then it can occur when the efficiency of leakage is far less than 1 probe molecule per peptide. Explicit pores are not required to explain leakage efficiencies of ≤ 1 probe per peptide per experiment. In Figure 18, we compare these numbers to typical biomembrane experiments; with typically $\sim 5 \times 10^5$ bacteria/mL (equal to 0.2 μM lipid), $\sim 10^8$ RBC/mL (equal to 200 μM lipid), or $\sim 1 \times 10^5$ adherent nucleated cells in a 200 μL microwell plate, equal to 5×10^5 cells per mL or 10 μM lipid.

Taken together, we can group experiments based on effective lipid concentration. For example, bacterial sterilization, lysis of nucleated cells, and GUV permeabilization are done at very low effective lipid concentration, $\leq 10 \mu\text{M}$. In these experiments P:L is not well-defined, but the bound peptide:lipid ratio will be maximal.² LUV permeabilization and hemolysis are done at effectively much higher lipid concentrations, $\geq 200 \mu\text{M}$, such that the system P:L will mimic the bound P:L if binding is strong.

In biosystems, as in synthetic systems, “potent” membrane permeabilizing activity does not require ion channel-like activity but rather requires only a small number of critical metabolites to escape per peptide to be effective. For example, we showed that about 2×10^8 individual antimicrobial peptides are required to kill an *E. coli* cell. This is more than the number of ATP molecules inside an *E. coli* cell. So, even in the most widely studied model systems of antibacterial activity, high potency means the release of only one critical metabolite molecule per peptide over the entire course of the experiment. In hemolysis or nucleated cell lysis even larger numbers of peptides may be bound to each cell, while biological activity requires only a moderate change in water or ion permeation across the membrane. These stoichiometries mean that it is almost never necessary to propose that an MPP forms an explicit membrane-spanning pore to explain an observed activity.

3.7. Meta-Analysis of Membrane Permeabilizing Peptides

To examine the range of behaviors and potencies observed in the permeabilization of synthetic lipid vesicles, we performed a meta-analysis of published results. We collected several hundred papers describing synthetic vesicle permeabilization published between 1987 and 2018. From this collection, we selected those publications that clearly showed permeabilization data over a range of peptide concentrations that enabled the determination of the concentration that causes 50% leakage, LIC_{50} . Papers for which lipid or peptide concentration or lipid composition could not be ascertained were rejected. We thus identified 71 high quality publications from which we extracted 524 data points. Redundant measurements of the same peptide in different papers were not removed from the database. Several hundred peptides are represented in the database, comprising many different origins. Antimicrobial peptides are common, with synthetic sequences outnumbering natural sequences. As shown in Figure 19, panel A, the database contains peptides with charges that range from -7 to greater than $+9$. All peptides with charges $\geq +9$ are included in the bin at $+9$. The majority of peptides tested in these publications are basic, with a most probable value of $+4$. Anionic MPPs are rare, and the frequency shown in Figure 19, panel A, is exaggerated because the data contain multiple

measurements of a small number of peptides. The highly potent, pH triggered pore-former GALA (see above) which has a charge of -7 (at neutral pH) is the most acidic peptide in the database and has been measured multiple times at many pH values. The assays used to measure permeabilization include most of the assays shown in Figure 8. Carboxyfluorescein and calcein entrapment are frequently used in early papers, but there is a transition toward $\text{Tb}^{3+}/\text{DPA}$ and ANTS/DPX in later work. Vesicles are SUV and LUV in the earliest work, transitioning to only LUV for the bulk of the papers. GUV studies cannot be included here because the lipid concentration is generally not known, and P:L cannot be calculated.

A histogram of MPP potencies is shown in panel B. Activities range across more than 4 orders of magnitude, from LIC_{50} of 1:1 or greater, which is essentially inactive, down to LIC_{50} as low as 1:10 000. The lowest LIC_{50} values observed are $\sim 1:10\,000$. This may be the maximum possible potency because it is equivalent to only 10 peptides per LUV vesicle. The peptide GALA at $\text{pH} \leq 5$, one of the most active MPPs known, dominates the most potent portion of the plot. Also found in this region is MelP5, a gain of function analog of melittin with very high potency.⁶⁴ In the range of $\text{LIC}_{50} = 1:1000$, the database contains data for a number of synthetic MPPs of both α -helical and β -sheet structure but fewer natural sequences. The bulk of the values, overall, fall between LIC_{50} of 1:10 and 1:100. This area contains the values for many AMPs. In panel C we show the dependence of LIC_{50} values on the content of acidic lipids in the vesicles. Panels C and D show the charge of the peptides by the colors of the points. It is well-known that cationic AMPs are more active against anionic bilayers and are often nearly inactive against PC bilayers. However, the data overall do not show this trend, because there are also peptides, such as alamethicin and melittin (and its gain of function analogs) that are highly active against both types of bilayers. In panel D we show the dependence of LIC_{50} values on the content of cholesterol in the bilayers. For many peptides, the increase in bilayer order caused by cholesterol decreased the permeabilizing activity significantly, but again here, as in panel C, there are peptides that are highly active against cholesterol containing bilayers. The main insight gained from the data shown in panels C and D is that while many individual peptides are sensitive to cholesterol content or bilayer charge, and there are at least some MPPs that are highly active against any lipid composition. In panels E and F of Figure 19, we show the dependence of LIC_{50} values on the content of acidic lipids in the vesicles or on the content of cholesterol in the bilayer for some of the most well studied peptides in the data set or for peptides mentioned frequently in this review, including melittin, magainin, and GALA at $\text{pH} \leq 5$, and MelP5.

4. MECHANISTIC LANDSCAPE OF MEMBRANE PERMEABILIZING PEPTIDES

For six decades, researchers have studied membrane permeabilizing peptides in synthetic and biological membranes to learn about the fundamental physical chemical properties of polypeptides that interact with lipid bilayers and alter their permeability. Such information could eventually enable the rational optimization of MPPs for specific translational applications. Yet, the active molecular structures of the thousands of known MPPs in membranes, with only very few well-known exceptions, have not been described at anything resembling atomic level resolution. This is in sharp

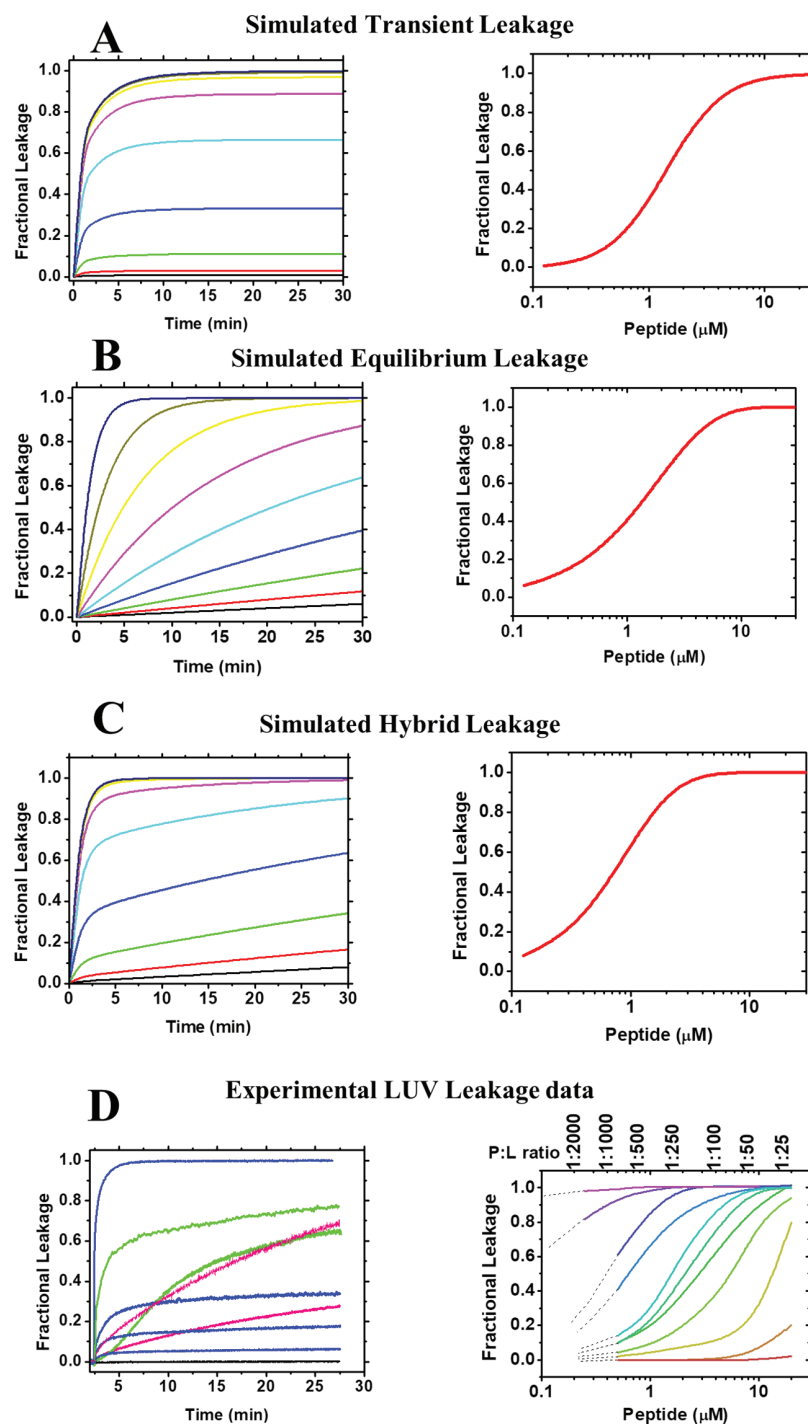


Figure 20. Experimental and simulated permeabilization of LUVs. We simulate vesicle leakage using simple numerical models. A. Transient leakage has a constant exponential rise toward a final value that itself depends on the P:L in the experiment. Many leakage experiments have behavior like this. The exponential rise assumes that the fraction of remaining entrapped contents released per unit time is constant. The plot on the right shows potency profile in a semilog plot. B. Simple equilibrium leakage model assumes an exponential rise toward 100% release where the rate (the fraction of remaining entrapped contents released per unit time) is a function of peptide concentration. This behavior is rarely observed. The plots on the right shows potency profile at 30 min in a semilog plot. C. Hybrid leakage, assumes that there is a major component of transient leakage, followed by a low level steady equilibrium release. Many peptides have this behavior. D. Real experimental leakage curves for various MPPs taken from the authors' manuscripts. Real curves like all three of these simulations are seen in this set. The plots on the right shows various potent curves taken from the authors' manuscripts. They range from high potency MPPs (e.g., alamethicin) that release $\sim 100\%$ of contents at P:L = 1:2000 to some AMPs or analogs that release almost nothing at P:L of 1:10. Peptides, lipid compositions, and other details vary in these curves.

contrast to the closely related field of membrane protein structural biology, which has yielded a large and exponentially growing number of atomic resolution structures.

The idea that has emerged from the long study of MPPs is not a static image of discrete pores. Peptide sequence and secondary structure are rarely enough to constrain a peptide-bilayer system to a single structure or a single mechanism of action under all instances. Driven by the sum of many interactions in the context of the dynamic bilayer milieu, most MPPs form an ensemble of heterogeneous, dynamic structures. The ensemble of structures and mechanisms will shift, sometimes dramatically, with experimental conditions; peptide sequence and secondary structure, bound peptide concentration, bilayer lipid composition (i.e., bilayer charge, fluidity, thickness, and more) and also with physical parameters such as temperature, ionic strength, and pH. Further, the many techniques used to study MPPs each probe a unique part of the functional space. We have referred to this phenomenon as a “mechanistic landscape”.¹⁵ This leads to the concept that each MPP occupies an area across a continuum of structures and mechanisms. This may help explain why there is so little consensus on the detailed molecular mechanisms of MPPs in synthetic membranes. With only a few exceptions, our current state of knowledge provides little predictive power on the activity of MPPs in synthetic membranes and especially in the very heterogeneous environment of the biological membrane. For this reason, researchers in the MPP field still mostly discover new peptides fortuitously or by simple trial and error. To create an image of the properties of the landscape that need to be better understood, next we discuss the MPP behaviors that are commonly observed but are most difficult to explain.

4.1. Transient Permeabilization

Many membrane permeabilizing peptides cause transient permeabilization of synthetic membranes and sometimes of biological membranes (Figure 20). The membranes become permeable shortly after peptides are added, but the leakage slows or stops before all contents have been released. This phenomenon has been noted and discussed in the literature, but its exact cause remains a mystery. It means that many MPPs are actually not membrane permeabilizing peptides at equilibrium or are much less potent at equilibrium than the primary data would suggest. At equilibrium, such MPPs are still bound to membranes and still have the same secondary structure, but once the transient leakage event has taken place the membranes are no longer highly permeable, even in the presence of peptide.

What is the nature of the transient, permeabilization event? The leading hypothesis^{2,5,41,67} is that permeabilization occurs during the sudden dissipation of the initial asymmetric distribution of peptide on the membrane. In other words, upon addition, peptide accumulates on the outer monolayer of a synthetic or biological membrane, creating an imbalance of mass, charge, surface tension, lateral pressure, or some combination of these. After some time, a stochastic, perhaps catastrophic, local dissipation event occurs in which the asymmetry is relieved by peptide, and possibly lipid, translocation. During the dissipation of the peptide asymmetry, the membrane is hypothesized to also become transiently permeable to polar molecules, and a burst of leakage occurs. Ensemble averaging in most vesicle experiments spreads individually rapid events across a broader time range. Although this hypothesis is consistent with what we know about peptides

in membranes and can explain many observations, it has not been directly supported by many experiments or simulations.

As we show in Figure 18, the molecular efficiency of transient permeabilization can be very low and still result in efficient release. In other words, high net permeabilization can occur only when, for every bound peptide, ≤ 1 probe molecule crosses the membrane, during the entire experiment. The observation of transient all-or-none leakage⁵ in which some of the vesicles release all of their contents while others release none, suggests that there is also a “silent” dissipation mechanism, likely to be peptide or lipid translocation²⁹⁸ that can occur without causing leakage. The measured fractional leakage, in this case, denotes the fraction of vesicles that have undergone the permeabilizing dissipation event, rather than the silent one. As peptide concentration is increased, the fraction of permeabilized vesicles increases.

Transient permeabilization, like all other “mechanisms” of MPPs, is only a portion of the mechanistic landscape, although it is widely observed. MPPs that cause transient permeabilization under some conditions, such as low concentration or in the presence of anionic bilayers, may cause equilibrium permeabilization under other conditions, such as high concentration or in zwitterionic bilayers.⁶⁴ For example, Krauson and Wimley showed that melittin causes transient permeabilization of PC bilayers at moderate concentration, P:L < 1:200, but that the mechanism transitions toward one that is more like equilibrium permeabilization at higher P:L.⁶⁴ In anionic bilayers, Ladokhin and White showed that melittin caused transient and catastrophic permeabilization across a wide range of concentrations.⁷⁸

We note that a few MPPs do cause equilibrium permeabilization, rather than transient permeabilization, over a wide range of conditions. For example, Krauson and Wimley showed that alamethicin and the lentivirus lytic peptides LLP1 and LLP2 cause potent equilibrium permeabilization of PC vesicles from P:L of 1:50 to P:L = 1:2000 and below. Similarly, the evolved melittin analog MelPS and its family members also show equilibrium pore formation under a wide range of conditions.^{59,62}

4.2. Stochastic Permeabilization

Most published permeabilization experiments rely on ensemble measurements of many vesicles, often LUVs, where events at the single vesicle level are not known. However, GUV studies and experiments with surface tethered LUVs and bacteria enable individual permeabilization events to be observed. Often, when it is possible to see individual events, researchers observe that permeabilization is a sudden, catastrophic leakage event that occurs after a long lag period. For example, permeabilization of GUVs,^{67,275,356,357} live bacteria,⁶⁷ and tethered LUVs³⁶⁹ have been shown to occur in seconds or less, following a lag phase from tens of seconds to minutes. Again, we note that stochastic permeabilization may only occur on a portion of a mechanistic landscape. A system with this behavior under one set of conditions may behave very differently under another set of conditions.

What is the nature of a stochastic permeabilization event? We note that peptide binding and folding in membranes is typically very fast, occurring in seconds.^{111,315,524–526} Thus, the peptides are bound and structured during the entire lag phase, but there must be a significant energy barrier to the dissipation of the asymmetry of bound peptide. Presumably the hydrocarbon core still effectively blocks peptide translocation. Lipid

cohesion prevents the bilayer architecture from being completely lost. Yet, in the presence of an interfacially active MPP, by definition, the bilayer order is diminished, and the hydrocarbon core is perturbed enough to lower the average energy barrier to the movement of peptides, lipids, water, and polar solutes across the bilayer. We speculate that when the energy barrier is bypassed by a local fluctuation in peptide/bilayer structure and/or peptide concentration, the hydrocarbon core is transiently breached and a locally catastrophic flow of material occurs through the breach toward dissipation of the transbilayer asymmetry. Sometimes this event is accompanied by destruction of the vesicle architecture.^{275,357,363,527} We hypothesize that the amphipathic nature of MPPs may be needed to stabilize the pathway through the membrane, at least briefly, and/or it may help catalyze the formation of an extended breach or additional breaches in the membrane. However, it happens permeabilization is a rare event, such that an individual vesicle may not undergo a permeabilization event over the course of an experimental observation, while other vesicles do. Peptide concentration and other factors determine the probability of the leakage event. Within a few seconds, or a few tens of seconds, the stochastic permeabilization event may be over. As described quantitatively by Hoernke and colleagues,³⁰⁹ if vesicles are empty after a single event, leakage will be all-or-none. If multiple or many individual events are required to empty a vesicle, leakage will be graded.

4.3. Challenge to Computational Scientists

Mathematical modeling and simulations of membrane permeabilization almost universally rely on the assumption that permeabilization is an equilibrium process. This is only occasionally true. Permeabilization is often a transient and stochastic process. In this review, we have described such behavior in detail. We challenge computational scientists and simulation scientists to explore, explain, and recreate these commonly observed behaviors to help create the next generation of testable mechanistic hypotheses.

5. CONCLUSIONS

In terms of detailed molecular mechanisms of membrane permeabilizing peptides, here we have envisioned a mechanistic landscape that includes ensembles of overlapping structures and mechanisms of activity that depend on the sum of many experimental details. For most MPPs, it may not be possible to describe a single unifying description of mechanism. What we have done is define the following: the physical, chemical, and structural commonalities; the many methods used to study MPPs; and the intriguing behaviors that have been observed. While the idea of a mechanistic landscape is not new, we think it is beneficial to begin to use the concept to think and talk about the mechanisms of membrane permeabilizing peptides.

AUTHOR INFORMATION

Corresponding Author

*E-mail: wwimley@tulane.edu.

ORCID 

William C. Wimley: 0000-0003-2967-5186

Author Contributions

[†]S.G., J.G., and E.W. contributed equally.

Notes

The authors declare no competing financial interest.

Biographies

Shantanu Guha was born and raised in Dallas, Texas. He obtained a B.A. in Biology in 2011 from Austin College (Sherman, Texas) and then moved to New Orleans to earn a M.P.H. with a focus on Epidemiology in 2013 from Tulane University (New Orleans, Louisiana). Currently, he is a Ph.D. student in Dr. William C. Wimley's lab at Tulane University in the department of Biochemistry & Molecular Biology. His research is on the topic of the Ebola virus Delta peptide as a potential novel therapeutic target during pathogenesis. The Delta peptide is a viroporin produced by Ebola virus during active infections and may contribute to morbidity and mortality of infected individuals.

Jenisha Ghimire was born and raised in Kathmandu, Nepal. Upon her high school graduation, she moved to New Orleans and obtained her B.S. in Biology from the University of New Orleans with departmental honors. She also obtained her M.Sc. from the University of New Orleans where her work focused on understanding the role of IME4 gene in phosphate metabolism pathway of *S. cerevisiae*. Currently, she is a Ph.D. student in Dr. William C. Wimley's laboratory where her research emphasizes the discovery of novel antimicrobial peptides with high-throughput screening.

Eric Wu is currently a Ph.D. student at Tulane University in William C. Wimley's laboratory. He obtained a B.S. in microbiology in 2010 at San Jose State University and has since worked at the University of Arizona and OncoSynergy, Inc. where he studied the influence of gut commensal microorganisms on the immune system and cancer therapeutics, respectively. Currently, his research focuses on utilizing pH-triggered pore-forming peptides for macromolecule drug delivery into cells.

William C. Wimley is from Monroe, Connecticut. He studied Biophysics at the University of Connecticut (B.S., 1985) and Biochemistry at the University of Virginia (Ph.D., 1990) where his dissertation work focused on the interactions between lipids in synthetic membranes. As a postdoc at the University of California, Irvine, he began a lifelong study of peptides in membranes. He is currently the George A. Adrony Professor of Biochemistry and Molecular Biology at the Tulane University School of Medicine. The current focus of the Wimley laboratory is synthetic molecular evolution of pore-forming, cell penetrating, membrane translocating, antibacterial, and antiviral peptides.

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