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Differentiating antimicrobial peptides interacting with lipid bilayer: Molecular signatures derived from quartz crystal microbalance with dissipation monitoring

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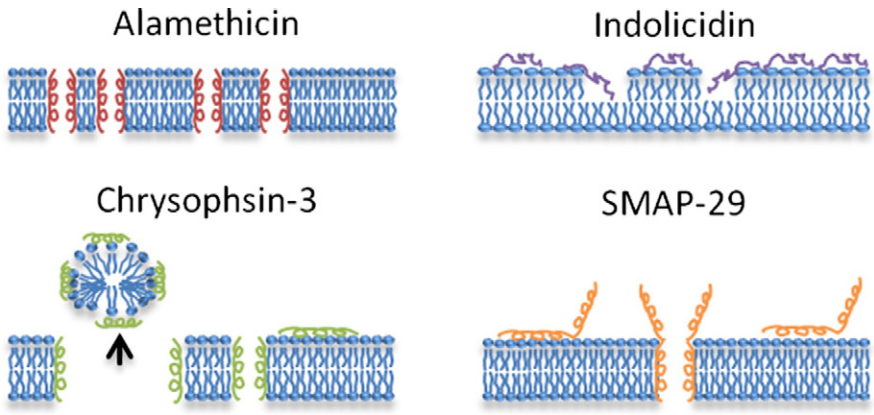
HIGHLIGHTS

- Interactions between lipid bilayers and 4 structurally diverse AMPs were examined.
- Each peptide's interaction mechanism produces a unique molecular QCM-D signature.
- QCM-D signatures give information about the dynamics of AMP–membrane interactions.
- Mechanistic variations were related to AMP structural properties (e.g. hydrophobicity).

GRAPHICAL ABSTRACT



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ABSTRACT

Many antimicrobial peptides (AMPs) kill bacteria by disrupting the lipid bilayer structure of their inner membrane. However, there is only limited quantitative information in the literature to differentiate between AMPs of differing molecular properties, in terms of how they interact with the membrane. In this study, we have used quartz crystal microbalance with dissipation monitoring (QCM-D) to probe the interactions between a supported bilayer membrane of egg phosphatidylcholine (egg PC) and four structurally different AMPs: alamethicin, chrysophsin-3, indolicidin, and sheep myeloid antimicrobial peptide (SMAP-29). Multiple signatures from the QCM-D measurements were extracted, differentiating the AMPs, that provide information on peptide addition to and lipid removal from the membrane, the dynamics of peptide–membrane interactions and the rates at which the peptide actions are initiated. The mechanistic variations in peptide action were related to the fundamental structural properties of the peptides including the hydrophobicity, hydrophobic moment, and the probability of α-helical secondary structures.
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1. Introduction

Antimicrobial peptides (AMPs) are pathogen-killing molecules that were originally derived from various organisms, including frogs and moths [1,2]. They are known to kill a broad spectrum of pathogenic bacteria, fungi, and viruses. AMPs are believed to kill bacteria by destabilizing bacterial membranes or translocating through the membranes to interact with intracellular targets. Because of the nature of these interactions, pathogenic bacteria are less able to develop resistance against the membrane-active AMPs, in contrast to the ease of

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developing antibiotic resistance. A bacterium must substantially change the characteristics of its membrane if it has to succeed in developing resistance to AMPs, but because the lipids are highly conserved in microorganisms, this occurrence is unlikely [3–5]. This unusual property of low susceptibility to development of AMP resistance by the microorganisms has stimulated major research efforts to chemically synthesize AMPs replicating some of the structural features of the naturally occurring AMPs, with the expectation of reproducing their mechanism of action in killing bacteria for practical applications.

The membrane-destabilizing mechanisms exhibited by AMPs are thought to fall into several categories. Many membrane-active peptides have been shown to insert into lipid bilayers and create pores using a mechanism described by the barrel-stave model [6]. These pores have been detected by studying voltage-dependent conductance that occurs via transmembrane channels that are created as a result of peptide insertion [7,8]. The AMPs may also disrupt cell membranes by first attaching to the surface and forming lipid–peptide aggregates, which then leave the membrane causing lysis, in a mechanism described by the carpet model. Variations of these models have also been developed. For instance, the barrel-stave cylindrical pores, in which the edges are lined by perpendicularly-oriented peptides, may be distinguished from toroidal pores, in which the pore edges consist of peptides and lipid head groups that bend continuously from the top bilayer leaflet to the bottom bilayer [9]. The mechanism of interaction between a peptide and a cell membrane may follow any one or a combination of these molecular events, depending on the type of peptide and lipid present in the system.

Peptide characteristics of secondary structure, charge, and hydrophobicity are usually thought to play substantial roles in determining AMPs' mechanisms of action on a cell membrane [10]. Studies have shown that increased helicity in AMPs can be correlated with increased antibacterial activity [11,12]. The cationic nature of AMPs is also thought to play a large role in their ability to target negatively charged bacterial cell membranes. Electrostatic interactions are largely responsible for drawing cationic AMPs to anionic bacterial cell membranes. If the membrane is primarily made up of zwitterionic lipids, then the strong ionic attractions that exist between the anionic lipid membranes and the AMP are replaced by relatively weaker attractive interactions between the dipoles of the neutral membrane and the charges on the AMP. In this case, the hydrophobicity of the peptide may become a more significant factor in determining antimicrobial activity. Hydrophobicity has been shown to affect the antibacterial and hemolytic activity of AMPs, but the correlation with antibacterial activity may not be strong [10,13–17]. Although numerous studies in the literature have examined the relationship between AMP structure and antibacterial or hemolytic activity (represented by experimentally determined minimum inhibitory concentrations), less is known about how the structure of the AMP and its resulting physicochemical properties determine the specific mechanism of interaction with cell membranes.

To discover the mechanistic variations between different AMPs, four molecules, alamethicin, chrysophsin-3, indolicidin, and sheep myeloid antimicrobial peptide (SMAP-29), with varying secondary structures, charges, and hydrophobicities were chosen for this study (Table 1). The helical wheel diagrams for these four peptides are shown in Fig. 1. This diagram provides a projection of amino acids perpendicular to the helix long axis assuming that the peptide exists in an α-helical secondary structure. Since the α-helix contains 3.6 residues per turn, adjacent residues on the peptide are separated by 100° on the helical wheel.

The first AMP, alamethicin, is a 20-amino-acid, α-helical peptide that is derived from the fungus *Trichoderma viride* and is known to insert into membranes at higher concentrations, forming well-defined cylindrical pores [18–20]. The structure of alamethicin includes two amino acids that are rarely found in nature, aminoisobutyric acid and L-phenylalaninol. Alamethicin contains a negative charge associated with the glutamic acid residue near the C-terminus. However, this side chain is typically protonated when the peptide is oriented in a transmembrane state, making alamethicin's net charge effectively zero in a peptide–lipid membrane system [21]. The helical wheel diagram shows a clear separation between the dominant hydrophobic face and a smaller polar face in the alpha-helical structure for this peptide.

Chrysophsin-3, another 20-amino-acid AMP, is derived from the gills of the red sea bream, *Chyrsophrys major*. It also assumes an α-helical structure when in contact with a biological membrane and is amphipathic [22,23]. Chrysophsin-3 exhibits a positive net charge of +5 (fractional charge 3.2 at pH 7), which differentiates it from alamethicin. Again in this case, the helical wheel diagram shows a clear separation between the dominant hydrophobic face and a polar charged face in the alpha-helical structure for this peptide.

Indolicidin, a 13-residue AMP derived from bovine neutrophils, is one of the smallest of the known naturally occurring linear peptides [24]. Indolicidin's amino acid content is quite remarkable because of its five tryptophan and three proline residues. Indolicidin carries a net charge of +4 at pH 7 and assumes a specific coiled and folded conformation when in contact with a cell membrane, unlike the α-helical or β-sheet conformations formed by most other AMPs [25,26]. Intramolecular cation–π electron interactions allow it to assume a folded, boat-shaped conformation with positive charges at the peptide termini and a hydrophobic core [27–29]. Studies have shown that indolicidin does not cause hemolytic lysis at concentrations below 30 μM [30]. The helical wheel diagram shows a separation of hydrophobic and hydrophilic residues but the large presence of prolines in this small peptide prevents it from assuming an α-helical secondary structure.

Sheep myeloid antimicrobial peptide (SMAP-29) is a cationic AMP composed of 29 amino acids that carries a +11 net charge at pH 7 [31]. Its structure is predominantly α-helical, with the hydrophobic residues aligned along one side and the polar residues along the other [32–34] as can be seen from the helical wheel diagram. The SMAP-29 used in our study contains a C-terminal cysteine residue, which was introduced to support other research where the AMP was attached to fluorescent dyes or other surfaces through the sulfhydryl functionality of cysteine [35,36]. The presence of the terminal cysteine could possibly give rise to the formation of SMAP dimers in solution through disulfide bonding.

In addition to the selection of peptides, it is necessary to specify the membrane model, the type of lipids and the experimental technique to explore the AMP–membrane interactions. In the literature, different membrane models and experimental techniques have been reported, each technique providing useful observations about one or another aspect of the AMP interactions, making it necessary for multiple techniques to be applied to piece together a comprehensive molecular scale picture of the AMP action. For example, the AMP alamethicin has been studied using black lipid membranes, vesicles, liposomes, multilayers, and Langmuir–Blodgett film as membrane models, prepared with different choices of lipid molecules and employing experimental techniques including electrical conductance [37], crystallographic analysis [38], circular dichroism [39], phenylalaninol fluorescence [39], oriented circular dichroism (OCD) [19], neutron in-plane scattering [40], X-ray diffraction [41], cryo transmission electron microscopy (cryoTEM) [42], liposome leakage measurements [42], electrochemical scanning tunneling microscopy [43] and also computer simulations [43-45].

Because of its real time monitoring capability, we employed quartz-crystal microbalance with dissipation monitoring (QCM-D) as the technique on the membrane model of solid supported lipid bilayer (SLB), to monitor the dynamics of AMP–membrane interactions and to search for molecular signatures specific to each peptide. With QCM-D, changes in mass and viscoelasticity of a supported lipid bilayer can be quantitatively determined by monitoring the changes in frequency (Δf) and energy dissipation (ΔD) of a quartz crystal sensor on which the bilayer is assembled [46–52]. Due to varying acoustic penetration depths of the different overtones [46], higher overtones are correlated with processes occurring closer to the sensor surface [46] while the lower overtones