Antimicrobial peptide alamethicin insertion into lipid bilayer: A QCM-D exploration

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Alamethicin is a 20-amino-acid, α-helical antimicrobial peptide that is believed to kill bacteria through pore formation in the inner membranes. We used quartz crystal microbalance with dissipation monitoring (QCM-D) to explore the interactions of alamethicin with a supported lipid bilayer. Changes in frequency (∆f) and dissipation (∆D) measured at different overtones as a function of peptide concentration were used to infer peptide-induced changes in the mass and rigidity of the membrane as well as the orientation of the peptide in the bilayer. The measured ∆f were positive, corresponding to a net mass loss from the bilayer, with substantial mass losses at 5 μM and 10 μM alamethicin. The measured ∆f at various overtones were equal, indicating that the mass change in the membrane was homogeneous at all depths consistent with a vertical peptide insertion. Such an orientation coupled to the net mass loss was in agreement with cylindrical pore formation and the negligibly small ∆D suggested that the peptide walls of the pores stabilized the surrounding lipid organization. Dynamics of the interactions examined through ∆f vs. ∆D plots suggested that the peptides initially inserted into the membrane and caused disordering of the lipids. Subsequently, lipids were removed from the bilayer to create pores and alamethicin caused the remaining lipids to reorder and stabilize within the membrane. Based on model calculations, we concluded that the QCM-D data cannot confirm or rule out whether peptide clusters coexist with pores in the bilayer. We have also proposed a way to calculate the peptide-to-lipid ratio (P/L) in the bilayer from QCM-D data and found the calculated P/L as a function of the peptide concentration to be similar to the literature data for vesicle membranes.

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1. Introduction

Antimicrobial peptides (AMPs) are naturally occurring molecules that target and kill a broad spectrum of pathogenic bacteria, fungi, and viruses. All eukaryotic organisms that have been analyzed for the production of AMPs, such as fish, frogs, and moths, have been found to express these molecules [1,2]. AMPs, which are largely cationic and amphiphilic, are believed to kill bacteria by interacting with their negatively charged membranes. Once associated with the bacterial cell surface, AMPs can kill bacteria either by destabilizing the membrane or by translocating through the membrane to interact with intracellular targets. Because of this membrane destabilizing mechanism, the AMPs are less prone to the development of pathogen resistance than antibiotics [3–6]. Their broad spectrum of activity, lower levels of bacterial resistance, and the speed of their action on pathogens, hold promise for AMPs as alternatives to antibacterials, therapeutically and in self-decontaminating surfaces [7,8].

AMPs exist in solution with various secondary structures. When in contact with the membrane, many AMPs assume an α-helical secondary structure [3], with clearly differentiated hydrophilic and hydrophobic surfaces. They attach to and interact with lipid membranes through electrostatic interactions as well as through their hydrophobic and polar domain properties [3]. A fundamental model for AMP-membrane interactions has been advanced by Huang based on the proposition that the peptide exists in two distinct states in the membrane [9–12]. At very low peptide to lipid (P/L) ratios, the peptides are considered to be in an adsorbed mode, referred to as the “surface” or “S” state, with the peptides embedded in the lipid head group region of the bilayer (Fig. 1A). The resulting displacement of lipid head groups causes thinning of the lipid membrane [9,11]. This perturbation of the lipids, at sufficiently high peptide to lipid ratios, drives a phase transition of the peptide to the “inserted” or “I” state wherein the peptides insert into the membrane, giving rise to the barrel-stave or cylindrical pores (Fig. 1B). Some AMPs are presumed to form a different kind of pore structure in which the lipid head groups bend continuously from the outer

Fig. 1. Mechanistic models of AMP action on lipid membranes with α-helical peptides represented as coils and lipids represented with headgroups and attached tails. (A) Peptide adsorption on the membrane surface or the “S” state, (B) peptide insertion into the bilayer resulting in cylindrical pore (“I” state) or toroidal pore formation, and (C) membrane disruption with the formation of lipid-peptide aggregates (“Carpet” model).

membrane leaflet to the inner leaflet, giving rise to toroidal pores in which the peptides are always in contact with the lipid headgroups (Fig. 1B) [13,14]. Once cylindrical or toroidal pores form, the cell is permeabilized, causing the cell to die.

Another mode of interaction known as the carpet model [14] supposes that AMPs first align themselves parallel to the lipid membrane surface (Fig. 1A) and at a large enough surface concentration of the peptide, directly cause membrane disintegration through the formation of peptide–lipid aggregates (micelles or bicelles) that detach from the membrane (Fig. 1C). Some AMPs are also thought to translocate across the cell membrane and destroy intracellular components in addition to their membrane interactions [15]. The specific mechanism of action of the AMP is dependent on the structure and charge of the peptide, as well as the lipid composition of the cell membrane [14].

Alamethicin is a 20-amino-acid, predominantly α-helical peptide that is derived from the fungus Trichoderma viride. It has been widely studied as a model for membrane proteins forming ion channels. Alamethicin is most effective against Gram-positive bacteria and fungi, with known minimum inhibitory concentrations (MICs) between ~1.5 and 25 µM against mollicutes, a class of bacteria that lack cell walls, and which are thought to have developed from Gram-positive bacteria. Alamethicin was also found to deform the helical cell structure of mollicute parasite Spiroplasma melliferum at a lower concentration of 0.1 µM [16–19]. Although alamethicin is less effective against Gram-negative bacteria, possibly due to the lipopolysaccharide barrier present in the bacterium’s outer membrane, it can still inhibit growth at higher concentrations. Alamethicin at 25 µM was found to inhibit the growth of Sinorhizobium meliloti, a Gram-negative bacterium [20].

Alamethicin (Ac-Aib-Pro-Aib-Ala-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Glu-Gln-Phl) contains eight α-aminoisobutyric acid (Aib) residues and one l-phenylalaninol (Phl) residue, which are not commonly found in nature. The alamethicin sequence contains four residues having polar side chain groups: Gln7, Glu18, Gln19 and Phl20. Alamethicin also contains a negative charge associated with the glutamic acid residue (Glu18) located near the peptide’s C-terminus. The helical wheel diagram for alamethicin is shown in Fig. 2. A propensity for the α-helical secondary structure, a clear separation of hydrophobic and hydrophilic regions and significant hydrophobicity of this peptide are indicated by the helical wheel. The X-ray crystallographic structure of alamethicin [21] suggests that the amphipathic α-helical region is about 2.9 nm long. The crystal structure shows small deviations from a continuous α-helical conformation in the form of short (one to two residues) 310 helical regions in the C-terminal domain, with a bend at the Pro14 residue. This conformation allows for the formation of a polar region, consisting of the Gln7 and Glu18 residues and a nonpolar area that includes Val, Aib, Ala, and Leu residues. The Glu18 side chain is typically protonated when the peptide is in a transmembrane state, making alamethicin’s net charge effectively zero. The neutral nature of alamethicin is interesting in that AMPs are typically cationic, which plays a role in their ability to target negatively charged bacterial membranes. However, other factors, such as the hydrophobic side chains on many of alamethicin’s amino acids, may increase alamethicin’s affinity to bacterial membranes.

Multiple techniques have been used to explore how alamethicin interacts with different model membrane systems and a brief

Fig. 2. Helical wheel diagram of alamethicin. The diagram represents the peptide viewed along the axis of the helix, in which the peptide backbone is shown by the inner circle and the spokes represent amino acids. The amino acids are represented by their single letter codes and the number indicates their location in the primary structure starting from the amino end.

Since an α-helix contains 3.6 residues per turn, side-chains adjacent in the linear sequence are separated by 100° of arc on the wheel. Residues are color coded for their functionalities and the helical wheel shows a mostly hydrophobic peptide with a small region of hydrophilicity (see text for details).
survey of literature [22–37] is summarized in Table 1. It is reasonable to conclude that these studies offer evidence for membrane leakage due to pore formation [23,33], surface adsorbed state of the peptide [24,29,30,35,36], membrane thinning [25], and the transition from the surface state of the peptide to the membrane inserted state [24,27,29,36]. Different pore sizes have been proposed with 6 to 12 peptides per pore [22,26,28,31,32,37] and the coexistence of peptide clusters along with water-filled pores has also been suggested [31,32]. The most definitive experimental conclusions have come from studies using hydrated multilayers of stacked bilayers as membrane models rather than simple bilayers typical of cell membranes. In these studies, equilibrium structures generated at pre-determined peptide-to-lipid ratios were characterized and there was no possibility of discerning any information about the dynamics of the process when the peptide encounters the membrane.

Indeed, monitoring the dynamic behavior of AMPs on lipid membranes has typically been difficult, due to the small scale of these interactions. We chose to examine the interactions of alamethicin with lipid membranes using quartz-crystal microbalance with dissipation monitoring (QCM-D), which can monitor these systems in real-time with a mass sensitivity of ~1.8 ng/cm² in liquid. Lipid bilayer supported on the quartz crystal was used as the model membrane. Changes in frequency (Δf) and energy dissipation (ΔD) of an oscillating sensor crystal were measured as mass attaches to and/or removed from the surface of the crystal, during

### Table 1

<table>
<thead>
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<th>Experimental technique</th>
<th>Membrane system/lipids</th>
<th>Experimental observations</th>
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</tr>
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<tr>
<td>Circular dichroism; phenylalaninol fluorescence</td>
<td>Vesicles in aqueous phase</td>
<td>Alamethicin is not aggregated in the aqueous phase but aggregated in vesicle membrane. The P/L ratio in the vesicle was estimated as a function of peptide concentration in aqueous phase.</td>
<td>[23]</td>
</tr>
<tr>
<td>Oriented circular dichroism</td>
<td>Multilayers of oriented bilayers DPPC</td>
<td>If P/L is below a critical value, most of the peptide molecules are on the membrane surface. If P/L is above the critical value, most of the peptide molecules are incorporated in the membrane.</td>
<td>[24]</td>
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<tr>
<td>X-ray lamellar diffraction</td>
<td>Multilayers of oriented bilayers DPPC</td>
<td>Bilayer thickness decreases with increasing peptide concentration in proportion to the P/L ratio.</td>
<td>[25]</td>
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<tr>
<td>Neutron in-plane scattering</td>
<td>Multilayers of oriented bilayers DLPC, DPhPC</td>
<td>In DLPC, the pores are made of 8–9 peptides, with a water pore 1.8 nm in diameter and an effective outside diameter of 4 nm. In DPhPC, the pores are made of 11 peptides, with a water pore of 2.6 nm in diameter and an effective outside diameter of 5 nm.</td>
<td>[22,26]</td>
</tr>
<tr>
<td>Oriented circular dichroism</td>
<td>Multilayers of oriented bilayers DPPC, DPhPC and mixtures</td>
<td>Observed sigmoidal insertion behavior indicating cooperative action in pore formation. Concluded that cooperativity is not associated with a micelle-like aggregation process, but instead, is driven by lipids through membrane-mediated interactions.</td>
<td>[27]</td>
</tr>
<tr>
<td>Multiswavelength anomalous diffraction (MAD)</td>
<td>Multilayers of oriented bilayers Brominated lipid (di 18:0 (9, 10 Br) PC)</td>
<td>Constructed electron density distribution profiles confirmed the formation of barrel-stave pores with eight alamethicin molecules per pore.</td>
<td>[28]</td>
</tr>
<tr>
<td>Calorimetry, sound velocity, atomic force microscopy</td>
<td>Unilamellar vesicles and (for AFM) fused vesicles on mica, DMPC, DPPC</td>
<td>Sound velocity data showed three distinct concentration dependent regions that can be associated with the “S” to “I” transition. AFM images in DPPC system showed peptide-induced defects (&quot;holes&quot;).</td>
<td>[29]</td>
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<tr>
<td>Oriented ¹³N solid-state NMR; Electron Paramagnetic Resonance-Electron Spin Echo Envelope Modulation</td>
<td>Multilayers of oriented bilayers DPPC</td>
<td>Alamethicin was in the surface-oriented “S” state at peptide concentrations of 1 mol % in gel-phase DPPC.</td>
<td>[30]</td>
</tr>
<tr>
<td>X-ray scattering</td>
<td>Multilayers of oriented bilayers DOPC, di C22:1PC</td>
<td>Identified water containing pores with 6 peptides in DOPC and 9 peptides in diC22:1PC. Also proposed the presence of hexagonally packed alamethicin clusters (lacking water) in equilibrium with pores.</td>
<td>[31,32]</td>
</tr>
<tr>
<td>Cryo-TEM and liposome leakage measurements.</td>
<td>Liposomes POPC or POPC/POPG</td>
<td>Leakage from liposomes clearly confirmed the formation of membrane pores, but the pore size was too small for direct detection by the TEM.</td>
<td>[33]</td>
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<tr>
<td>Small-angle neutron scattering with selective deuterium labeling</td>
<td>Vesicles DMPC and chain-perdeuterated DMPC and DMPC mixtures</td>
<td>Alamethicin enriched the outer leaftle of the vesicle with the negatively charged DMPG both in its “S” and “I” states.</td>
<td>[34]</td>
</tr>
<tr>
<td>Sum frequency generation (SFG) vibrational spectroscopy</td>
<td>Supported bilayer DMPC</td>
<td>Alamethicin was able to insert into fluid-phase membranes, but on the gel phase, it was on the surface either as single peptides or as aggregates and did not show significant insertion.</td>
<td>[35]</td>
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<tr>
<td>Sum frequency generation (SFG) vibrational spectroscopy</td>
<td>Supported bilayer POPC</td>
<td>Change in membrane orientation was consistent with “S” to “I” transition. Orientation of the α-helical component of alamethicin changed substantially while that of the 3₃ helical component remained unaffected.</td>
<td>[36]</td>
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<tr>
<td>Electrochemical scanning tunneling microscopy</td>
<td>Langmuir-Blodgett film DMPC + egg PG</td>
<td>Direct imaging showed the formation of cylindrical hexameric alamethicin pore incorporating a water channel.</td>
<td>[37]</td>
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* The lipid molecules are referred to in the Table and the text by abbreviations are as follows:

- DLPC: 1,2-Dilauroyl-sn-Glycero-3-Phosphatidylcholine,
- DMPC: 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine,
- DMPG: 1,2-Dimyristoyl-sn-Glycero-3-Phosphoglycerol,
- DOPC: 1,2-Dioleoyl-sn-Glycero-Phosphatidylcholine,
- DPhPC: 1,2-Diphytanoyl-sn-Glycero-3-Phosphatidylcholine,
- DPPC: 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine,
- DPPG: 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)],
- DSPC: 1,2-Distearyl-sn-Glycero-3-Phosphocholine,
- DSPG: 1,2-Distearyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)],
- PC: Phosphatidylcholine,
- PG: Phosphatidylglycerol,
- POPC: 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine,
- POPG: 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)].
the peptide–membrane interactions. A decrease in frequency can be related to an increase in mass on the crystal surface and vice versa. Changes in dissipation can be related to changes in the viscoelasticity, or “softness,” of the membrane on the sensor. Since disordering of lipids within a membrane can introduce spaces that would weaken the structure, dissipation measurements can reveal information about the level of disruption in a bilayer on the sensor surface. In this study, we examined the action of amaldextrin on an egg phosphatidylcholine (PC) supported bilayer membrane at various peptide concentrations over a period of 1 h. From frequency and dissipation measurements at various overtones of the natural frequency of the crystal we have extracted qualitative and quantitative information on the equilibrium as well as dynamic nature of amaldextrin–egg PC interactions.

2. Materials and methods

2.1. AMP and lipid vesicle preparation

Amaldextrin was purchased from Sigma–Aldrich (St. Louis, MO). Peptide solutions were prepared in Tris–NaCl buffer [100 mM sodium chloride and 10 mM tris(hydroxymethyl) amino methane (Sigma–Aldrich, St. Louis, MO) at pH 7.9]. Experiments were performed at 23°C with peptide concentrations between 0.1 μM and 10 μM, which was within the range of MIC values found for amaldextrin [16–19]. Lyophilized powder egg PC was purchased from Sigma Aldrich (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL). The PC was dissolved in ethanol and stored at −20°C. To prepare small unilamellar vesicles (SUVs), the PC solution was dried with nitrogen gas and placed in a vacuum desiccator overnight. Tris–NaCl buffer was then added to the dried lipids, resulting in a final concentration of 2.5 mg/mL. The mixture was vortexed and homogenized through 5 freeze-thaw cycles. The solution was sonicated using an ultrasonic dismembrator (Model 150T, Thermo Fisher Scientific, Waltham, MA) in pulsed mode for 30 min at 0°C. A 30% duty cycle was used for sonication (pulse on for 3 s, followed by a pause for 7 s) at an amplitude of 60. The vesicle solution was centrifuged at 17,500 rpm (37,000 × g) for 10 min at 4°C to remove probe particles from the ultrasonic dismembrator (J-EI Centrifuge Beckman Coulter, Brea, CA). The supernatant containing SUVs was collected and stored at 4°C under nitrogen for up to 5 weeks [38]. Dynamic light scattering experiments (Zetasizer Nano ZS, Malvern, Worcestershire, UK) determined the diameter of the vesicles to be approximately 37 nm. The stock solution was diluted to 0.1 mg/mL in Tris–NaCl buffer before each QCM-D experiment.

2.2. Quartz crystal microbalance with dissipation monitoring (QCM-D)

The Q-Sense E4 system (Biolin Scientific, Sweden) was used to monitor the real-time mass and viscoelasticity changes to a supported lipid membrane deposited on the quartz crystal during AMP exposure. The supported lipid bilayer was formed using a vesicle deposition method at 23°C, which was above the transition (gel-to-liquid) temperature of egg PC, allowing the lipids to remain in fluid phase during the experiment [39, 40]. The stock solution of PC vesicles injected into the QCM-D chamber at a flow rate of 0.15 mL/min. Changes in frequency and dissipation were monitored as the PC vesicles attached to the sensor’s silica surface and ruptured to form a lipid bilayer. Since the mass and dissipation changes resulting from the attachment and rupturing of lipid vesicles on the crystal surface were consistent in each experiment, the QCM-D data served to confirm the consistent formation of a stable bilayer [39, 40]. This approach to supported bilayer formation was robust as we had confirmed in our previous QCM-D studies on chrysophsin-3 [41].

Following the formation of a stable supported lipid bilayer, the crystals were rinsed with buffer to remove any unattached lipids. After establishing a baseline, the solution of amaldextrin was added for 10 min, at which time the pump was stopped. QCM-D crystals were exposed to a stagnant peptide solution for 1 h, after which the peptide solution was replaced with a final buffer rinse at 0.15 mL/min to remove any unattached particles until the frequency stabilized. For each concentration of amaldextrin, the Δf and ΔD values at different overtones were measured. The Δf was estimated by taking the difference between frequencies at the beginning of peptide exposure and after the subsequent buffer rinse. All the Δf and ΔD data presented in the results refer to the difference between two stages of the interaction process on the crystal surface: the lipid bilayer film after contact with the peptide before contacting the peptide, thus accounting for only the peptide-induced effects on the bilayer. Effects due to liquid properties (such as density and viscosity) are canceled out in this subtraction process. Experiments at each peptide concentration were repeated at least 3 times and the averages of the net changes in frequency and dissipation were reported as the final Δf and ΔD values taken after the buffer rinse. Error bars were determined from the standard deviation of the Δf and ΔD values.

2.3. Analysis of QCM-D data

Methods to relate the measured frequency and dissipation changes to changes in mass and in the viscoelastic properties of the membrane on the surface have been described in detail in the literature [42] and therefore only a brief summary is provided here. For a rigid film of areal mass m_f (mass per unit area) deposited on the crystal surface and exposed to air, the normalized frequency change Δf (with respect to the overtone number) and the areal mass of the film are related by the Sauerbrey equation, while the dissipation change ΔD is zero.

$$\Delta f = -\frac{m_f}{m_q} \Delta D = 0$$

(1)

Here, $f_0$ is the natural frequency of the oscillator and $m_q$ is the areal mass of the quartz crystal. The mass addition due to the film deposited on the crystal surface gives rise to a decrease in the frequency (negative Δf) while net mass loss is indicated by a positive Δf. The dissipation D is related to the loss modulus $G''$ and the storage modulus $G'$ in the form $D = G'/(2\pi G'')$ and the change in dissipation ΔD can be related to the changes in the rigidity or viscoelasticity of the film attached to the crystal surface. Obviously, for the rigid film, the change is dissipation is zero.

If the rigid film is immersed in a Newtonian liquid like water, the frequency and dissipation changes are modified due to the presence of water and are now given by

$$\Delta f = -\frac{\eta_l}{2\pi\delta_l m_q} - \frac{f_0 m_f}{m_q} \Delta D = \frac{\eta_l}{\pi f_0 \delta_l m_q}$$

(2)

where $\eta_l$ is the viscosity of the liquid medium and $\delta_l$ is the decay length of the acoustic wave in the liquid medium. The first term in Δf and the term appearing in ΔD are due to the solvent effect due to the immersion of the crystal in the liquid and they vanish when we consider the changes in the crystal properties after and before the deposition of the rigid film. Effectively, the film mass changes are given just by the Sauerbrey term. If the film is not rigid but
viscoelastic, then the frequency and dissipation changes are given by

\[
\Delta f = \frac{n_l}{2 \pi n_f \delta n_m} - n_f \frac{n_l}{n_f} \left[ 1 - \frac{2}{\rho_l} \left( \frac{n_l}{n_f} \right)^2 \frac{G''}{G^2 + G''^2} \right],
\]

\[
\Delta D = \frac{n_l}{n \pi f_0 \delta n_m} + \frac{m_l}{m_f} \frac{4}{\rho_l} \left( \frac{n_l}{n_f} \right)^2 \frac{G'}{G^2 + G''^2}
\]

where \( \rho_l \) is the density of the film on the crystal surface. As in Eq. (2), the first term in the expressions for \( \Delta f \) and \( \Delta D \) are due to the solvent effect and they vanish when we consider changes in film properties when the film is immersed in the liquid both before and after the change. The film mass change is now given by the Sauerbrey term with a correction factor accounting for the viscoelastic properties of the film.

The contribution of viscoelasticity of the film to the measured frequency change (or equivalently, the measured mass) can be estimated from Eq. (3). For a 5 MHz crystal, taking the density of water and of the bilayer to be 103 kg/m3, the viscosity of water to be 10^{-1} N s/m2, and the viscoelastic ratio \( G'/G'' = 0.1 \), the second term within the brackets, which provides the viscoelastic correction to unity, is approximately 0.03 for \( G'' = 1 \) MPa and 0.3 when \( G'' \) is 0.1 MPa. A viscoelastic ratio of 0.1 and \( G'' = 0.1 \) MPa were used by Voinova et al. for an adsorbed layer of vesicles [42], and for the supported bilayers, one may expect these values to be somewhat different.

There is also a non-vanishing \( \Delta D \) accompanying the film mass change in Eq. (3). An increase in \( \Delta D \) indicates a less rigid, possibly more disordered film, and a decrease in \( \Delta D \) indicates a more rigid film on the crystal surface. In experiments involving supported lipid bilayers (SLBs), \( \Delta D \) can also provide information about changes in the structure and ordering of the lipids. Disruption of the membrane will cause the lipids to become less ordered and potentially allow more water to associate with the membrane, increasing the film’s hydration and \( \Delta D \) values.

As mentioned already, it is possible to measure not only the changes in the fundamental resonant frequency of the quartz crystal, but also changes in its harmonics. Available commercial instruments allow measurements of odd overtones up to the 13th (or even the 15th) multiple of the fundamental frequency. Since higher frequencies dissipate energy faster in a viscous medium, the higher overtones decay faster (the decay length is shorter) and are more confined to the surface region of the crystal. In this study, the 3rd through 11th overtones, or harmonics, were measured and related to processes throughout the film. Due to varying penetration depths of the acoustic waves associated with different overtones, higher overtones are qualitatively more representative of processes occurring closer to the sensor surface while the lower overtones are representative of processes occurring near the water-film interface. Similar \( \Delta f \) and \( \Delta D \) values at all overtones indicate a homogeneous change in mass and viscoelasticity over the depth of the film on the crystal’s surface. On this basis, the overtone analysis has been used by Mechler et al. [43] to differentiate peptides inserted in bilayers with a vertical orientation (with respect to bilayer surface) compared to surface adsorbed peptides. Further, the molecular mass and viscoelasticity changes on the film can be monitored in real time by tracing the relation between \( \Delta f \) and \( \Delta D \) over the entire course of the experiment.

3. Results and discussion

3.1. Changes in \( \Delta f \) and \( \Delta D \) with peptide concentration and overtone number

The measured \( \Delta f \) and \( \Delta D \) at 3rd to 11th overtones are presented in Fig. 3 for aqueous phase peptide concentrations ranging from 0.25 μM to 10 μM. The dissipation changes \( \Delta D \) were small at all peptide concentrations suggesting that the membrane with and without alamethicin was rigid and therefore, the measured frequency changes can be directly related to mass changes through the Sauerbrey equation. Exposure of the supported lipid membrane to alamethicin concentrations above 0.1 μM resulted in positive \( \Delta f \) values implying mass removal from the bilayer. The relative amount of mass lost from the bilayer increased with increasing alamethicin concentration. At 0.25 μM alamethicin, the \( \Delta f \) values were ~1 Hz, and they increased to ~5 Hz at 10 μM alamethicin. Since previous experiments showed that the formation of a complete bilayer corresponded to a \( \Delta f \) value of ~25 Hz, frequency shifts between 1 and 5 Hz were indicative of 4–20% mass loss from the original lipid bilayer [40,41]. Since the data show a net mass loss, any incorporation of alamethicin into the membrane must be overcompensated by depletion of lipid molecules from the bilayer. This would be consistent with the creation of pore structures with water channels in the bilayer, since that would require removal of lipid molecules.

The measured \( \Delta f \) values were uniform across all overtones at each concentration of alamethicin (Fig. 3). This would suggest that mass removal from the membrane was uniform along the depth of the membrane. This mass depletion likely occurred as a result of peptide insertion into the membrane, which was expected to create homogeneous overtone responses. This uniformity in the \( \Delta f \) values for all overtones is consistent with the formation of cylindrical pores since such a pore structure is characterized by uniform behavior along the depth of the bilayer.

The measured dissipation values \( \Delta D \) were small at all peptide concentrations (Fig. 3), suggesting that the ordering of molecules in the membrane before and after exposure to alamethicin resulted in the same membrane “stiffness,” or viscosity. This would be consistent with a cylindrical pore structure where the lipids are allowed to remain unaffected thanks to the creation of peptide wall along the pore boundary. Even though water channels exist, they do not contribute to bilayer viscoelasticity because of the organization of the peptide walls that confine the lipids to a state similar to that in the original lipid bilayer.

One may note that lipid loss from the membrane was observed even at 0.05 μM alamethicin (data not shown here). The \( \Delta f \) and \( \Delta D \) data suggest that even at low peptide concentrations, alamethicin creates cylindrical pores and one may have to go to much lower alamethicin concentrations than what has been considered in this study, to observe a surface adsorbed “S” state.

3.2. Time evolution of \( \Delta f \) vs \( \Delta D \) and dynamics of alamethicin–bilayer interactions

Mechanistic information derived solely from overall \( \Delta f \) and \( \Delta D \) values as in Fig. 3 give no indication of the dynamic processes that would have occurred during the 1 h period in which the PC-bilayer membrane was exposed to the peptide. Therefore, the QCM-D results were also analyzed using \( \Delta D \) vs. \( \Delta f \) plots (Figs. 4 and 5) to infer at least qualitatively the nature of dynamics controlling alamethicin–bilayer interactions. The points shown in these graphs represent \( \Delta f \) and \( \Delta D \) values at evenly spaced time intervals (0.7 s between points). Larger spacing between points indicate that the mass or viscoelasticity changes in the membrane occur at a faster rate. Changes in slopes in these plots generally indicate a change in mechanism [44].

Fig. 4 describes how from the time evolution of \( \Delta D \) and \( \Delta f \) (Fig. 4A) the dynamic \( \Delta D \) vs. \( \Delta f \) plots (Fig. 4B) were constructed. As PC vesicles attached to the QCM-D sensor’s silica surface, the mass on the sensor increased, causing the frequency to sharply decrease (Fig. 4A). This process was shown in the corresponding \( \Delta D \) vs. \( \Delta f \) as the points moved in the north-east direction (shown by the arrow
labeled i), indicating an increase in mass on the surface as well as the viscoelastic nature of adsorbed vesicle layer (Fig. 4B). Arrows pointing east reveal increases in mass while those pointing north reveal an increase in softness or viscoelasticity. Once the vesicles broke up and organized into a planar bilayer, there was a mass loss due to release of water from vesicle interior as well as the loss of excess lipid from the vesicle. Simultaneously there was a decrease in dissipation change due to higher ordering of the planar bilayer compared to the soft water filled vesicles. Correspondingly, the $\Delta D$ vs. $\Delta f$ trace changed in direction (labeled ii), resulting in a south-west trend that indicated a loss in mass and decrease in viscoelasticity. West pointing arrows indicate a decrease in mass while south-pointing arrows indicate an increase in membrane rigidity and therefore more organized molecules. In Fig. 4B, data from the 3rd and 11th overtones showed similar behavior and extended over the same range of $\Delta D$ and $\Delta f$, both being large. This suggested that the bilayer formation process starting from adsorbed vesicles caused significant changes both near the crystal surface (11th overtone) as well as at the interface with bulk water (3rd overtone).

The dynamics of alamethicin–bilayer interactions were explored through the $\Delta D$ vs. $\Delta f$ traces during the time course of alamethicin contact with the bilayer in Fig. 5. At 0.25 μM alamethicin, a very small change to membrane mass and virtually no change to viscoelasticity were observed at both the 3rd and 11th harmonics. At a concentration of 1 μM, the trace (labeled i) showed a similar small mass loss and no change in the lipid membrane ordering during exposure to alamethicin. Since mass was lost at both overtones, all depths of the membrane experienced a loss in lipid mass, possibly due to peptide insertion to create a small number of cylindrical pores. At 5 μM, a much larger change in the membrane was observed. Both overtones revealed overall loss of lipid mass with simultaneous disordering of the membrane that was not captured by the static experimental data in Fig. 3. Initially, the data points traveled in the north-east direction (labeled i),

![Graph showing changes in frequency and dissipation corresponding to various aqueous phase concentrations of alamethicin interacting with the PC membrane.](image-url)
peptides had attached to the surface of the membrane or partially inserted into the membrane. The peptide–membrane interaction mechanism then shifted in the north-west direction (labeled ii), indicating that the membrane began to lose mass, while still experiencing lipid disorder. The traces subsequently changed direction (labeled iii–iv), suggesting that the lipid membrane became more rigid as it continued to lose lipid mass. The decrease in dissipation may have been due to the alamethicin molecules completely inserting into the membrane and reintroducing order to the system as it stabilized the edges of pores in the lipid bilayer. The results for 10 µM alamethicin revealed a similar trend indicating that the dynamic mechanisms did not change with peptide concentration.

In Fig. 5, at both 5 µM and 10 µM alamethicin, the range of Δf is the same for both the 3rd and 11th overtones while the range of ΔD is larger for the 3rd overtone compared to the 11th overtone. This clearly suggests that at the water–bilayer interface (qualitatively indicated by the 3rd overtone behavior) there was considerable lipid disruption during the dynamic process while very close to the quartz surface (indicated by the 11th overtone), the lipid organization was not significantly perturbed. Note that the static data in Fig. 3 shows that the net change in dissipation was negligibly small for both overtones, indicating similar rigidity of the membrane before and after interactions with alamethicin. Evidently, one would have no clues about the process dynamics if only the static data were available.

3.3. Calculation of bilayer properties from QCM-D data

The area per lipid in the bilayer (denoted as \(a_0\)) and the thickness of the bilayer were calculated from the measured frequency change of \(\sim 25\) Hz, accompanying the formation of the supported bilayer. Noting that the proportionality constant \(C\) in the Sauerbrey equation is 17.8 ng/cm\(^2\) for a crystal oscillating at 5 MHz natural frequency, the bilayer areal mass corresponding to a frequency change of 25 Hz is equal to 445 ng/cm\(^2\). It has been shown that this mass includes the mass of a layer of water between the quartz crystal and the supported lipid bilayer and the mass of this water layer has been determined to be \(\sim 102\) ng/cm\(^2\) [45]. Correcting for this

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**Fig. 4.** (A) ΔD and Δf measurements showing stable supported lipid bilayer (SLB) formation on silica, followed by a Tris–NaCl buffer rinse at \(t = 11.5\) min to remove any unattached particles. (B) ΔD vs. Δf plot showing the dynamics of SLB formation. The frequency axis has been reversed to make interpretation more intuitive. As mass increases on the QCM-D sensor, frequency shifts to the right. The initial frequency decrease (i) shows the attachment of vesicles to the surface. The vesicles then rupture, releasing trapped water, and forming a bilayer (ii). The arrows (labeled i and ii) indicate the progression of data points with increasing time. Data points are taken at 0.7 s intervals. The silica surface of the sensor crystal is bare and submerged in Tris–NaCl buffer at \(t = 0\) s.

**Fig. 5.** ΔD vs. Δf plots of alamethicin–bilayer interactions at various concentrations of the peptide in aqueous phase. These plots show changes in viscoelasticity of the membrane in relation to the changes in mass during the time evolution of the interactions. At \(t = 0\) s, a stable bilayer has been formed on the QCM-D crystal. The measurements include 1 h of incubation with the peptide and a subsequent buffer rinse. The 3rd and 11th harmonics are shown to represent the different processes occurring near the surface of the lipid membrane and near the surface of the QCM-D crystal. These graphs show representative data from one experiment based on at least 3 repeated measurements.
water mass, we estimate the areal mass of the lipid bilayer to be 343 ng/cm². The molecular mass \( M_l \) of the lipid is calculated to be \( 1.267 \times 10^{-12} \text{ng/molecule} \) corresponding to an average molecular weight of 760 g/mol for the egg PC lipid. Dividing the areal mass of the lipid bilayer by the mass of a single lipid molecule \( M_l \), we estimate there are 2.7 molecule/nm² on the bilayer or 1.35 molecules/nm², in each monolayer. This corresponds to a lipid area \( a_l \) of 0.739 nm²/molecule.

If the molecular volume \( v_l \) of the hydrophobic tail of the egg PC lipid is 0.96 nm³/molecule (taken as the composition average based on the constituent C16 and C18 chains), then the thickness of the hydrophobic region of the bilayer \( h_l = 2v_l/a_l = 2.6 \text{ nm} \). Alternatively, if we take the lipid density to be about 1 g/cm³, then for a lipid average molecular weight of 760, the corresponding lipid molecular volume \( v_{\text{lipid}} \) is 1.267 nm³. The lipid bilayer thickness will then be \( h_{\text{lipid}} = 2v_{\text{lipid}}/a_l = 3.43 \text{ nm} \). If we consider the lipid head groups on the two layers to take up 1 nm [9], then the hydrophobic region of the bilayer will have a thickness of 2.43 nm. These estimates based on QCM-D data and molecular properties of lipids can be compared to the X-ray measurements of Huang [9] who obtained \( a_l = 0.74 \text{ nm}^2 \) and \( h_l = 2.66 \text{ nm} \) for DOPC, and \( a_l = 0.68 \text{ nm}^2 \) and \( h_l = 2.75 \text{ nm} \) for POPC.

3.4. Differentiating pore formation vs. cluster formation based on the QCM-D data

We have used the QCM-D data along with model calculations to explore whether alamethicin inserts into the bilayer to create water-free clusters as suggested by Nagle et al. [32] in addition to their pore formation. Nagle et al. proposed the formation of hexagonally packed clusters of alamethicin with no water channels (Fig. 6), with the small hydrophilic domains on the peptides facing each other and the larger hydrophobic domains facing other hydrophobic surfaces or the lipid. The cluster could be of any size generated from a trimeric motif as shown in the figure. They also surmised that because the number of peptides in a cluster would be larger than in a pore, the ratio of clusters to bundles would increase as the total peptide concentration increased.

In the case of the peptide clusters, there are no associated lipids or water channels and the area per peptide \( a_p \) is calculated as \( a_p = \pi d_p^2/n \) where \( d_p \) is the diameter of the peptide, visualized as a cylinder. The area per peptide \( a_p \) is estimated to be 0.945 nm²/molecule, taking the diameter \( d_p \) of alamethicin to be 1.1 nm [28]. The molecular mass \( M_p \) of the peptide is calculated to be 3.275 × 10⁻¹² ng/molecule, corresponding to a molecular weight 1965 g/mol of alamethicin.

In the case of a pore, the area per peptide is denoted as \( A_H/n \), where \( A_H \) is the pore area and \( n \) is the number of peptide molecules constituting the pore. If we consider the approximate pore size to be determined by the close packed arrangement of peptides at the pore boundary, then the outer diameter \( D_H \) of a pore with \( n \) peptides and the area of the pore per peptide \( A_H/n \) can both be calculated from

\[
D_H = \frac{nd_p}{\pi} + d_p, \quad \frac{A_H}{n} = \frac{\pi d_p^2}{4} = \frac{\pi d_p^2}{4} \left( \frac{n}{\pi + 1} \right)^2
\]

(4)

For alamethicin, taking the peptide diameter \( d_p \) to be 1.1 nm, the pore diameter \( D_H \) estimated from Eq. (4) will be about 3.9 nm for \( n = 8 \), and 4.6 nm for \( n = 10 \). Indeed, Huang [28] has estimated the number of peptides in the pore, \( n = 8, 10 \) corresponding to which the pore area per peptide \( A_H/n \) will be 1.494 and 1.663 nm²/peptide.

On the addition of peptide to the bilayer, lipid molecules are removed from a fraction \( \lambda \) of the bilayer area and replaced either by peptide clusters or pores. The number of lipid molecules removed will be \( 2\lambda A_l/a_l \), with the factor 2 again accounting for the two layers of the bilayer. We will denote by \( \beta \), the fraction of the affected area where clusters form and by \( (1 - \beta) \), the fraction of the affected area where pores form. Therefore, the \( 2\lambda A_l/a_l \) lipid molecules removed will be replaced by \( \lambda \beta A_l/a_l \) peptide clusters and by \( \lambda(1 - \beta)A_l/A_l/n \) peptides, in the pores. The resulting mass change per unit area can be equated to the areal mass change measured by QCM-D:

\[
\Delta m = -C\Delta f = C \left[ \beta \frac{M_p}{a_p} + (1 - \beta) \frac{M_p}{A_H/n} - \frac{2M_l}{a_l} \right]
\]

(5)

In this equation \( C \) is the proportionality constant between frequency change and mass change in the Sauerbrey equation, and \( M_p \) and \( M_l \) are molecular masses of the peptide and lipid molecule, respectively. The right hand side of Eq. (5) accounts for the lipid leaving the bilayer and the peptide entering the bilayer either as clusters or as pores. With the measured \( \Delta f \), the fractional area \( \lambda \) affected by peptide–lipid interactions can be calculated using Eq. (5) for assumed values of \( n \) and \( \beta \). When \( \beta = 0 \), we have only pore formation and for any value of \( \beta > 0 \), we also have peptides inserted as close-packed clusters.

Having estimated all the molecular constants, and assuming values for \( \beta \) and \( n \), we can calculate the fractional area \( \lambda \) affected by alamethicin–egg PC bilayer interactions using Eq. (5) as a function of the alamethicin concentration in the aqueous phase. The calculated results are shown in Fig. 7 for three assumed values of \( \beta \) and for \( n = 8 \) and 10. As mentioned already, \( \beta = 0 \) corresponds to the case when all inserted peptides are part of pores with water channels. The areal mass change is very small when alamethicin molecules are inserted as clusters since the area vacated by the egg–PC lipids is taken up by the peptides, and the net mass of lipids removed is not too different from the net mass of peptide added. (It should be noted that the areal mass change on cluster insertion could be larger for other choices of peptide–lipid molecules, depending on
their molecular properties. The areal mass change is much larger when peptides are inserted as pores because in this case some of the area from which the lipid has been removed is replaced by the water channel that does not contribute to the film mass. Obviously, the change in areal mass increases with n since that increases the pore and water channel diameters. As a consequence the calculated results in Fig. 7 show that in order to account for a given mass change, the affected area must be larger for \( \beta > 0 \) compared to when \( \beta = 0 \). For the same reasons, the affected area can be smaller when n is larger.

The ratio between the peptide in clusters and that in water-filled pores \( (P_C/P_P) \) can be calculated from Eq. (6).

\[
P_C/P_P = \left[ \frac{\beta}{1 - \beta} \right]^{\frac{nL}{aP}}
\]

For \( n = 8 \), the ratio \( P_C/P_P \) is 0.39 and 1.06 for \( \beta = 0, 0.2 \) and 0.4, respectively. For \( n = 10 \), the ratio \( P_C/P_P \) is 0.44, and 1.17 for \( \beta = 0, 0.2 \) and 0.4, respectively. As \( \beta \) increases, the peptides in clusters become relatively more significant; and for a given \( \beta \), the larger the n, the larger the \( P_C/P_P \) ratio. Nagle et al. have estimated \( P_C/P_P \) values of about 0.6 for DOPC multilayers when P/L values were about 0.05–0.10. From Fig. 7 we note that cluster formation is possible but the QCM-D data cannot confirm or deny the coexistence of clusters. If an alternate technique is available to determine the fractional area affected at a given aqueous phase alamethicin concentration, then the QCM-D data coupled to these model calculations can be used to draw more definitive conclusions.

3.5. Estimation of P/L ratio in the inserted state

The peptide to lipid \( (P/L) \) ratio was a pre-determined experimental value in all of the multilayer membrane experiments. However, in the QCM-D experiments it is an outcome of the dynamic peptide–membrane interaction process. The \( P/L \) ratio in the bilayer was calculated from Eq. (7).

\[
P = \frac{N_{LO}}{N_{LO} - \left( \frac{2A}{L} \right)} \left( \frac{\lambda}{1 - \lambda} \right) \left[ \frac{\beta a_L}{a_P} \left( 1 - \frac{\beta}{1 - \beta} \right) \right]
\]

Here, \( N_{LO} = 2A/a_L \) is the total number of lipid molecules initially present in the bilayer. The numerator accounts for the number of peptide molecules present as clusters and as pores. The denominator represents the number of residual lipid molecules after some of the lipid has been displaced by the peptide. Having already calculated \( \lambda \) from Eq. (5) as a function of the peptide concentration in the aqueous phase for assumed values of \( \beta \) and \( n \), we can use Eq. (7) to calculate the corresponding \( P/L \) ratio. In this manner, the peptide concentration in the aqueous phase can be related to the \( P/L \) ratio in the membrane inserted state. The calculated \( P/L \) ratios for different aqueous phase concentrations of the peptide are plotted in Fig. 8. The calculations show that in order to account for a given mass change, the \( P/L \) ratio would be larger for \( \beta > 0 \) compared to when \( \beta = 0 \); further, \( P/L \) would be larger for smaller \( n \) (e.g., \( n = 8 \) compared to \( n = 10 \)). This is intuitively obvious because replacing lipids by peptides results in a smaller mass change compared to replacing lipids by pores. Also shown in Fig. 8 are experimental estimates of \( P/L \) in DMPC vesicles at 21 °C and DOPC vesicles at 34 °C as a function of the aqueous phase alamethicin concentration, obtained more than two decades ago using circular dichroism (CD) spectroscopy and phenylalaninol fluorescence spectroscopy [23]. Although the lipids are different from egg PC and the membrane system is a vesicle rather than a supported bilayer, the qualitative comparison between the QCM-D estimate for \( P/L \) and these experimental results is interesting to note.

The discrepancies between the calculated and experimental \( P/L \) values may be explained by differences in experimental parameters and assumptions. For instance, the QCM-D results in this study were obtained for egg PC while the experimental data reported were for either DMPC or DOPC. The experimental \( P/L \) values also corresponded to vesicles rather than flat bilayers. In the experiments, the average vesicle size changed and some vesicle fusion was observed during peptide interactions, which may affect the quantitative estimation of the \( P/L \) ratio. Also, the calculation of the \( P/L \) ratio at low peptide concentrations from CD and fluorescence measurements showed a larger intrinsic uncertainty. Finally, the consideration of a polydispersed pore model would modify the results calculated using the QCM-D data. The model assumes all pores to be the same size, which may not be the case in an experimental system. For these reasons, we have only mentioned the qualitative similarity between the QCM-D results and the vesicle experimental data in Fig. 8. The QCM-D studies would have to be complemented with other experiments to obtain additional information needed for more rigorous quantitative comparisons.

4. Conclusions

We have explored to what extent useful information on alamethicin–membrane interactions can be extracted from the application of quartz crystal microbalance with dissipation monitoring (QCM-D) technique. A supported phosphatidylcholine (PC)
bilateral membrane in an aqueous environment was used as the membrane model. The QCM-D responses of changes in frequency ($\Delta f$) and dissipation ($\Delta D$) at different overtones were used to estimate changes in mass and rigidity of the lipid bilayer as well as the orientation of the peptide in the bilayer. The frequency changes at various overtones were equal indicating a homogeneous membrane process suggesting a vertical insertion of the peptide. Such an orientation for the peptide coupled to a net mass loss in the system supports a cylindrical pore formation with enclosed water channel. The very small dissipation change confirming the retention of lipid organization supports the idea that the inserted peptides form the walls of the cylindrical pores retaining the lipid organization. Further, an analysis of the time evolution of $\Delta f$ vs. $\Delta D$ demonstrates that the peptide insertion kinetic process involved significant disordering of lipids, especially in the proximity of the membrane-water interface, even though this disordering was not present in the end state. By developing model calculations we concluded that the QCM-D data cannot confirm or rule out the coexistence of peptide clusters along with pores containing water channels. We also developed a way to calculate the peptide to lipid ratio in the membrane as a function of the aqueous phase peptide concentration and found that to be qualitatively similar to the experimental alamethicin partitioning data reported for vesicles.

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