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Lipid Fluid–Gel Phase Transition Induced Alamethicin Orientational Change Probed by Sum Frequency Generation Vibrational Spectroscopy

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Abstract

Alamethicin has been extensively studied as an antimicrobial peptide (AMP) and is widely used as a simple model for ion channel proteins. It has been shown that the antimicrobial activity of AMPs is related to their cell membrane orientation, which may be influenced by the phase of the lipid molecules in the cell membrane. The “healthy” cell membranes contain fluid phase lipids, while gel phase lipids can be found in injured or aged cells or in some phase separated membrane regions. Thus, investigations on how the phase of the lipids influences the membrane orientation of AMPs are important to understand more details regarding the AMP’s action on cell membranes. In this study, we determined the orientational changes of alamethicin molecules associated with planar substrate supported single lipid bilayers (serving as model cell membranes) with different phases (fluid or gel) as a function of peptide concentration using sum frequency generation (SFG) vibrational spectroscopy. The phase changes of the lipid bilayers were realized by varying the sample temperature. Our SFG results indicated that alamethicin lies down on the surface of fluid and gel phase 1,2-dimyristoyl(d54)-sn-glycero-3-phosphocholine (d-DMPC) lipid bilayers when the lipid bilayers are in contact with a peptide solution with a low concentration of 0.84 μM . However, at a medium peptide concentration of 10.80 μM , alamethicin inserts into the fluid phase lipid bilayer. Its orientation switches from a transmembrane to an in-plane (or lying down) orientation when the phase of the lipid bilayer changes from a fluid state to a gel state. At a high peptide concentration of 21.60 μM , alamethicin adopts a transmembrane orientation while associated with both fluid and gel phase lipid bilayers. We also studied the structural changes of the fluid and gel phase lipid bilayers upon their interactions with alamethicin molecules at different peptide concentrations.

Keywords

sum frequency generation; alamethicin; supported lipid bilayers; membrane orientation; lipid phase

1. Introduction

The increased bacterial drug-resistance against traditional antibiotics becomes a major challenge in healthcare and creates an urgent need to develop novel compounds to treat infectious diseases. It has been shown that antimicrobial peptides (AMPs) have potential to be developed into new generation antibiotic drugs.^{1–3} Many AMPs are naturally occurring

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host defense molecules that exist in different life forms. Extensive research has been performed to examine the structure-activity relations of naturally occurring and synthesized AMPs to develop new antibiotics with improved properties to overcome the bacterial drug-resistance.

Alamethicin is an AMP with 20 amino acid residues extracted from the fungus *Trichoderma viride*; it has been extensively studied as an AMP and also frequently used as a model for large channel proteins to study the ion channel gating mechanism.⁴⁻⁶ It is currently believed that alamethicin interacts with cell membranes through the barrel-stave mode, in which the molecules form parallel bundles of the helical monomers surrounding a central, water-filled pore.⁷⁻¹⁰

It has been shown that alamethicin membrane orientation is related to its antimicrobial activity and the capability of forming ion-channels.^{11,12} The study of alamethicin orientation on lipid bilayers under different environments is important to understand the molecular mechanisms of diseases and develop effective treatments.¹⁰⁻¹² Such an orientation depends on the alamethicin solution concentration. At low peptide concentrations, alamethicin molecules are oriented parallel to the cell membrane plane (or lying down on the membrane). No pores can be formed in the membrane by the peptides and therefore the peptides are in an “inactive state”. On the other hand, at high peptide concentrations, the peptides are nearly perpendicular to the membrane plane. Transmembrane pores can be formed by the peptides and the peptides are in an “active” state.^{11, 12} The membrane associated alamethicin orientation also depends on the lipid bilayer phase.¹³

In our previous research, we have demonstrated that sum frequency generation (SFG) vibrational spectroscopy can be used to determine interfacial membrane orientation of peptides that adopt various secondary structures.¹⁴⁻¹⁸ SFG is a surface sensitive second-order nonlinear optical technique and is capable of investigating interfacial structures of peptides and proteins in real time *in situ*.¹⁹⁻²⁵ SFG experiment only requires a very small amount of peptide and protein samples.²⁶⁻³¹

Alamethicin contains an N-terminal α -helical structure (residues 1-13) and a C-terminal 3_{10} -helical component (residues 14-20). The Pro 14 residue separates the two segments and acts as a bend in the helix.¹³ The two different secondary structures generate SFG amide I signals with different peak centers, which enables us to measure the orientation of each segment.¹³⁻¹⁵ Recently, we applied SFG to investigate the membrane orientation of alamethicin with different peptide concentrations on fluid phase lipid bilayers.¹⁵ Our previous SFG studies indicated that alamethicin molecules lie down on the fluid phase lipid bilayer surface at low peptide concentrations while insert into the fluid phase lipid bilayer at high peptide concentrations.¹⁵ We also studied alamethicin interacting with lipid bilayers with different phases using SFG. We hardly detected any SFG amide I signal of alamethicin when the peptide solution is in contact with a gel phase lipid bilayer, due to the “lying down” orientation of alamethicin on the gel phase lipid bilayer surface.¹³

Healthy cell membranes are usually composed of fluid phase lipid bilayers. Maintaining the fluidity of the cell membranes is vital for ensuring the normal properties and functions of the membrane. However, in some injured or damaged cells (e.g., cells with chilling injury) a transition from a fluid phase to a gel phase for the lipids in the cellular membranes can occur. Such a phase transition will result in alterations in the metabolism of injured cells and eventually lead to the cell death. Moreover, the transition from the normal fluid state to the gel phase can also occur during senescence. In this case, lipids in the ordered gel phase will gradually appear in the otherwise disordered fluid phase lipids of the membranes, leading to the biochemical and physiological changes of the cell. Besides, most natural cell membranes

have a complex mixture of different lipid molecules, and in such mixtures fluid and gel phase lipids may coexist in spatially separated populations. Nevertheless the fluid and gel phase lipid bilayers in the cell membranes are related to the different activities of cell membranes. We believe that alamethicin interacts differently with fluid and gel phase lipids, and the conformation and membrane orientation of alamethicin in fluid and gel state membranes may also be quite different. Since the orientation of alamethicin is related to its activity, it is important to measure the membrane orientation of alamethicin associated with lipid bilayers with different phases.

Previous NMR experiments show that alamethicin molecule adopts a transmembrane orientation in fluid phase lipid bilayers, and it switches from a transmembrane orientation to an in-plane orientation when the lipids change to gel phase.⁹ In this study, we applied SFG to investigate the change of membrane orientation of alamethicin when the phase of the lipid bilayer changes from fluid to gel. SFG has several advantages over other techniques: unlike NMR, which uses multilamellar liposomes, SFG experiment uses a planar supported single lipid bilayer, which is believed to more closely resemble the real cell membrane.¹⁵⁻¹⁸ In NMR, premixed lipid-peptide mixtures were used; while in our SFG experiment, different amounts of alamethicin solution were added to the subphase in contact with the lipid bilayer and we can use SFG to monitor the interaction process between the peptide and the lipid bilayers in real time. More importantly, SFG is very sensitive therefore we can probe alamethicin-lipid bilayer interactions at low peptide concentrations. By using deuterated lipids to prepare the lipid bilayers, we can observe the structural changes of the lipid bilayers upon their interactions with alamethicin molecules at various peptide concentrations by collecting the SFG signal in the C-D stretching frequency region. In summary, SFG can provide unique information regarding alamethicin-cell membrane interactions, which can be very hard to be deduced by using other techniques.¹⁵⁻²¹

In this work, 1,2-dimyristoyl(d54)-sn-glycero-3-phosphocholine (d-DMPC) was used to build lipid bilayers (as model cell membranes). The d-DMPC/d-DMPC lipid bilayers were chosen because the gel-fluid phase transition temperature for d-DMPC is 20 °C,³² we can therefore easily change the lipid phase by varying the temperature. In this research, SFG was applied to characterize the interactions between alamethicin and fluid or gel phase supported lipid bilayers *in situ* as a function of peptide concentration.

2. Materials and Methods

2.1. Materials

Alamethicin from *Trichoderma viride* was purchased from Sigma-Aldrich (St. Louis, MO) with a minimum purity of 90%. The deuterated d-DMPC lipid was purchased from Avanti Polar Lipids (Alabaster, AL), and its molecular structure is shown in scheme 1. Right-angle CaF₂ prisms were purchased from Altos (Bozeman, MT). The CaF₂ prisms were cleaned in toluene, soap, methanol, and then rinsed thoroughly with deionized water before treated in a glow discharge plasma chamber immediately before the deposition of lipid monolayer. A more detailed cleaning procedure was reported previously.¹⁶ The Langmuir-Blodgett and Langmuir-Schaefer (LB/LS) method was used to deposit the proximal and distal leaflets of single lipid bilayers onto CaF₂ prism. A KSV2000 LB system and ultrapure water from Millipore system (Millipore, Bedford, MA) were used for lipid bilayer preparation, as described previously.¹⁶ The lipid bilayers were then contact with ultrapure water or peptide solution inside a 1.6 mL reservoir throughout the entire experiment. The transition temperature between the gel and fluid phase for d-DMPC lipid is 20°C,³² and thus d-DMPC/d-DMPC bilayers are in the fluid phase at room temperature (~24°C). Because water in the reservoir was in contact with the supported lipid bilayers, we can change the lipid phase by varying the temperature of the subphase water. The temperature-controlled experiment was

done using an Isotemp stirring hot plate (Fisher Scientific, PA) and a thermal couple sensor which measures the temperature of the sample. First, the hot plate was used to heat the subphase water in contact with the lipid bilayer and keep the temperature of water at 30 °C. For alamethicin–lipid bilayer interaction experiments, an appropriate amount of alamethicin solution (in methanol with a concentration of 2.5 mg/mL) was injected into the reservoir filled with 1.6 mL of ultrapure water at 30 °C (the lipids were in fluid phase at this temperature) to achieve the desired peptide solution concentration, and allowed alamethicin molecules to diffuse to and interact with the lipid bilayer over 1 hour. A magnetic micro stirrer was used to ensure a homogeneous concentration distribution of alamethicin. The SFG spectra were then collected after the alamethicin–lipid bilayer interaction reached equilibrium and SFG signal became stable. After that we used ice water to surround the reservoir (the level of the ice-water was below the top of the reservoir so that the ice-water would not contaminate the peptide solution in the reservoir), which decreased the temperature of water in the reservoir to 10 °C. In such a condition, the temperature of lipid bilayers should also be around 10 °C and the lipids changed from fluid to gel phase. SFG spectra were then collected after the signal from alamethicin became stable.

2.2. SFG and Orientation Analysis

SFG is a second-order nonlinear optical spectroscopic technique that has proven powerful in investigating interfacial structures of peptides and proteins.³³⁻³⁷ SFG is a surface specific technique and it is capable of detecting the conformation and orientation of peptides/proteins on a model cell membrane surface in a sub- μM concentration.³⁸⁻⁴⁵ The details regarding SFG theories and measurements have been published previously, and will not be repeated here.⁴⁶⁻⁵¹ For IR-visible SFG experiments conducted in this research, SFG signal is generated as the frequency sum of the two input beams (with different frequencies): a frequency tunable infrared (IR) beam and a 532 nm visible beam. SFG spectra from interfacial alamethicin in different polarization combinations including ssp (s-polarized output SFG signal, s-polarized input visible beam, and p-polarized input IR beam) and ppp were collected using the near total internal reflection geometry.

The average orientation of alamethicin was deduced by analyzing the polarized ssp and ppp SFG amide I signal. The error bars generated in the fitting procedures were included in the fitting results. The 1670 cm^{-1} signal is contributed by the alamethicin α -helical component, and the signal centered at 1635 cm^{-1} is generated by the 3_{10} -helical structure.¹³ We have developed an orientation analysis method to determine orientation angles of α -helical and 3_{10} -helical structures by using SFG amide I spectra collected with ssp and ppp polarization combinations, which details have been introduced in our previous papers.¹⁹ This orientation analysis method has been successfully applied to examine the membrane orientation of peptides and proteins with α -helical and 3_{10} -helical structures such as alamethicin,¹³⁻¹⁵ magainin 2,²⁰ cecropin P1,²⁴ MSI-78,¹⁶ Pep-1,²² melittin,²⁶ cytochrome b5,²¹ and G-proteins.^{17,18}

In addition to the peptide, we studied lipid bilayer structural changes during the peptide–bilayer interaction using SFG. SFG only detects signals when inversion symmetry in the sample is broken. After the d-DMPC/d-DMPC bilayer was prepared by the LB/LS method, the SFG C-D stretching signal from monolayer d-DMPC lipids disappeared immediately due to the inversion symmetry of the lipid after the formation of bilayer. If alamethicin interacts with the two leaflets of the bilayer differently, the bilayer inversion symmetry will be broken and SFG signal from the d-DMPC lipid will be detected. Investigating this SFG signal coming from the d-DMPC lipids should provide us information about structural changes of the lipid bilayers.

3. Results and Discussion

3.1 Orientation of Alamethicin on Fluid and Gel Phase d-DMPC/d-DMPC Bilayers

The d-DMPC/d-DMPC lipid bilayers were prepared by the LB/LS method and were placed in contact with subphase water. A hot plate was used and carefully adjusted to control the temperature of water at 30 °C, measured by a thermocouple sensor. At this temperature the d-DMPC/d-DMPC lipid bilayer is in the fluid phase. SFG signals were collected from the bilayer/water interface and no signal was detected in the amide I frequency range (between 1600 and 1700 cm^{-1}). Certain amount of alamethicin stock solution was then injected into the subphase, resulting in a final alamethicin concentration of 0.84 μM . At the same time, SFG signal at 1670 cm^{-1} (from alamethicin α -helical structure) was monitored as a function of time.¹⁵ The intensity of this signal increased gradually and became stable at about 1 h after the introduction of the alamethicin stock solution to the subphase. After that, ppp and ssp polarized SFG spectra in the amide I frequency range were collected (Figure 1A).

After the collection of SFG spectra of alamethicin in the fluid phase lipid bilayer, we changed the lipid bilayer phase to gel phase by lowering the subphase water temperature from 30 °C to 10 °C. The phase transition temperature from the gel to fluid phase for d-DMPC lipid is 20 °C, therefore at 10 °C, the d-DMPC bilayer is in the gel phase. SFG ppp and ssp spectra in the amide I frequency region were collected from the bilayer/peptide solution interface again (Figure 1B).

In both Figures 1A and 1B, we observed two peaks in the SFG spectra: a dominant peak centered at 1670 cm^{-1} originated from the alamethicin α -helical structure, and a 1635 cm^{-1} peak contributed by the 3_{10} -helix segment. The SFG spectra were fitted and the solid lines in Figures 1A and 1B are the fitting results. More details of the amide I peak assignments and SFG spectral fitting methods have been described in previous publications and will not be reiterated here.¹³⁻¹⁵ According to the fitting results, we obtained the measured SFG ppp and ssp signal strength ratio (or ppp/ssp) of 2.25 ± 0.10 for (alamethicin associated with the) fluid phase lipid and 2.23 ± 0.10 for gel phase lipid at 1670 cm^{-1} , and 1.65 ± 0.10 for fluid phase lipid and 1.64 ± 0.10 for gel phase lipid at 1635 cm^{-1} . By using these ratios, we can determine the orientation angles of different secondary structural segments of alamethicin associated with the fluid and gel phase d-DMPC/d-DMPC bilayers.

Alamethicin consists of two helical segments due to the presence of the helix-breaking Pro 14 residue. According to the previous research on alamethicin, the α -helical structure which contains residues 1-13 contributes to the signal centered at 1670 cm^{-1} ; the 3_{10} -helical structure formed by residues 14-20 contributes to the signal centered at 1635 cm^{-1} .¹³ Here, we define that the orientation angle χ_1 represents the tilt angle between the principal axis of the α -helical structure (with residues 1-13) and the d-DMPC/d-DMPC bilayer surface normal, while angle χ_2 represents the tilt angle between the principal axis of the 3_{10} -helix (with residues 14-20) and the d-DMPC/d-DMPC bilayer surface normal.¹⁵

For α -helical and 3_{10} -helical structures, the details of orientation determination using polarized SFG spectra have been published previously.^{15,19} Using the relation between the measured ppp/ssp ratio for the peak at 1670 cm^{-1} and the α -helical orientation angle χ_1 for an α -helix with thirteen amino acids, the orientation angle can be deduced assuming a α -orientation distribution (Figure 2A). Using Figure 2A and the experimentally measured ppp/ssp ratios of the amide I signal of alamethicin associated with fluid ($\text{ppp}/\text{ssp} = 2.25 \pm 0.10$) and gel ($\text{ppp}/\text{ssp} = 2.23 \pm 0.10$) phase lipid bilayers at 1670 cm^{-1} , the orientation angles (χ_1) were determined to be around 77° (between 67° to 90°) and 75° (between 65° to 90°) for alamethicin associated with fluid and gel phase lipid bilayers, respectively.

The relation between the measured $\rho_{\text{ppp}}/\rho_{\text{ssp}}$ ratio at 1635 cm^{-1} and the 3_{10} -helix orientation angle χ_2 for a 3_{10} -helix with seven amino acids can be deduced using the developed method assuming a β -orientation distribution (Figure 2B).^{15,19} The orientation angle (χ_2) is determined to be 47° (between 43° to 50°) for alamethicin associated with both the fluid and gel phase lipid bilayers. Table 1 summarizes the above deduced orientation angles of β -helical (χ_1) and 3_{10} -helical (χ_2) segments of alamethicin associated with the fluid and gel phase d-DMPC/d-DMPC bilayers at the peptide concentration of $0.84\text{ }\mu\text{M}$.

Figure 2C shows a schematic for membrane orientations of alamethicin on the fluid (left) and gel (right) phase lipid bilayers. On the fluid phase lipid bilayer, the orientation angle of β -helical (χ_1) segment is 77° and 3_{10} -helical (χ_2) segment is 47° . The result suggests that the β -helical component is more or less lying down on the distal leaflet of bilayers. For 3_{10} -helix segment, because it is more hydrophilic, we believe that it orients outside of the hydrophobic core of the lipid bilayer, as shown in the left panel of Figure 2C.^{10,15} As the phase of the d-DMPC/d-DMPC bilayer changed from fluid to gel, both orientation angles of the β -helical (χ_1) segment and the 3_{10} -helical (χ_2) segment were similar, indicating no substantial alamethicin orientation change occurred. At the low peptide concentration of $0.84\text{ }\mu\text{M}$, the alamethicin molecule lies down on both fluid and gel phase d-DMPC/d-DMPC bilayers.

We also detected the SFG amide I signal intensity change as a function of time when the lipid phase changed from fluid to gel. Figure 1C shows the time-dependent ssp SFG intensity changes at 1670 cm^{-1} (from the β -helix structure) at $0.84\text{ }\mu\text{M}$ peptide concentration when the temperature of the subphase changed from $30\text{ }^\circ\text{C}$ to $10\text{ }^\circ\text{C}$, which corresponds to the lipid phase change from fluid (0 s) to gel (~ 4000 s). As shown in Figure 1C, the SFG intensity from β -helical segment of alamethicin decreased substantially when the phase of lipid changed from fluid to gel. SFG intensity change may be caused by (1) a change of peptide density associated with the membrane, or (2) the reorientation of the membrane associated peptides, or (3) a combination of both.¹⁵ Since the orientation angle of alamethicin is almost the same when associated with the fluid or gel phase lipid bilayer (Table 1), this SFG intensity decrease must be due to the decrease of the peptide adsorption amount associated with the membrane. Therefore some of the alamethicin molecules originally associated with the fluid phase d-DMPC/d-DMPC bilayer were dissociated from the bilayer and returned back to the solution subphase when the lipid phase changed from fluid to gel, resulting in fewer peptides associated with the gel phase lipid bilayer. Compared to the fluid phase lipid bilayer, the lipid molecules in the gel phase are more tightly packed, which may push the originally associated peptides away from the lipid bilayer. Another possibility is that the hydrophobic interaction is stronger at higher temperatures, the lower temperature in gel phase can decrease the interaction between the hydrophobic peptide and the hydrophobic lipid tails.⁵² The outcome is that the interaction between alamethicin and gel phase lipid bilayers is weaker than that between alamethicin and fluid phase lipid bilayers.

Figure 3A shows the SFG spectra collected from the fluid d-DMPC bilayer/subphase interface at about 1 h after a certain amount of the alamethicin stock solution was injected into the subphase to make a $10.80\text{ }\mu\text{M}$ peptide solution at $30\text{ }^\circ\text{C}$. After lowering the subphase temperature to $10\text{ }^\circ\text{C}$, we believe that the d-DMPC bilayer changed into gel phase. SFG spectra were then collected from the gel phase d-DMPC bilayer/peptide solution interface (Figure 3B). Orientation angles of the β -helical and the 3_{10} -helical segments of alamethicin associated with the fluid and gel phase lipid bilayers were deduced and listed in Table 1. At this peptide concentration of $10.80\text{ }\mu\text{M}$, the orientation angles of the β -helical segment (χ_1) were measured to be 45° (between 37° to 51°) and 75° (between 65° to 90°) on the fluid and gel phase lipid bilayer, respectively (Figure 4A). The orientation angles of the

3_{10} -helical segment (α_2) were deduced to be 44° (between 41° to 48°) and 48° (between 45° to 51°) on fluid and gel phase lipid bilayer, respectively (Figure 4B). Figure 4C shows the schematic of the membrane orientations of alamethicin on the fluid (left) and gel (right) phase lipid bilayers at the peptide concentration of $10.80 \mu\text{M}$. With the fluid phase lipid bilayer, the orientation angle of alamethicin α -helical (α_1) segment is 45° vs. the surface normal, while the 3_{10} -helical (α_2) segment is 44° vs. the surface normal. Therefore both the alamethicin α -helical and 3_{10} -helical segments insert into the fluid phase lipid bilayers adopting the similar orientation, as shown in Figure 4C (left).

Compared to the membrane orientation of alamethicin associated with the lipid bilayer at a lower peptide concentration of $0.84 \mu\text{M}$, the orientation of lipid bilayer associated alamethicin changed at the medium peptide concentration of $10.80 \mu\text{M}$ for fluid lipid bilayer, which agrees with our previous conclusion.¹⁵ As the phase of the d-DMPC/d-DMPC bilayer changed from fluid to gel, the orientation angle of alamethicin α -helical (α_1) segment changed from 45° to 75° while the 3_{10} -helical (α_2) segment changed from 44° to 48° (Figure 2C, right) at the peptide concentration of $10.80 \mu\text{M}$, which indicates that the membrane orientation of bilayer associated alamethicin molecules switched from transmembrane to more or less lying down.

Concerning the lipid bilayer phase effect, our results show that changing the lipid bilayer phase from fluid to gel can induce the alamethicin α -helical (α_1) segment to change its orientation from the “inserting or tilting in the bilayer” (45°) to the more or less “lying down orientation” (75°). However, the tilt angle of 3_{10} -helix (α_2) only varied slightly, from 44° (between 41° to 48°) to 48° (between 45° to 51°) when the lipid phase changed from fluid to gel (Table 1). In fact, such a change is within the experimental error. We believe that at the peptide concentration of $10.80 \mu\text{M}$, the alamethicin molecule inserts into the fluid phase lipid bilayer; while it lies down on the gel phase bilayer surface.

It was reported in the literature that the orientation of membrane associated alamethicin is related to its antimicrobial activity: when the peptides are oriented parallel to the cell membrane surface, no pores can be formed in the membrane by the peptides and therefore the peptides are in an inactive state which cannot kill bacteria. This is the case when the peptide concentration is $0.84 \mu\text{M}$. On the other hand, when the peptide solution concentration is $10.80 \mu\text{M}$, the peptides are perpendicular to the fluid phase lipid bilayer surface, they may form transmembrane pores and therefore they may adopt an active state and can kill bacteria;^{11,12} after the lipid changed from fluid to gel phase, alamethicin molecules lie down on the lipid bilayer surface and may adopt an inactive state. It shows that we may control the activity of alamethicin by changing the lipid phase. By using the planar supported lipid bilayers and SFG technique, we are able to reveal the effect of lipid phase on alamethicin orientation.

Figure 3C shows the time-dependent ssp SFG intensity change at 1670 cm^{-1} (contributed from the α -helix structure) at $10.80 \mu\text{M}$ peptide solution concentration when the lipid phase changed from fluid (0 s) to gel (~ 4000 s) by lowering the subphase temperature from 30°C to 10°C . The ssp SFG intensity from the α -helical segment in alamethicin decreased substantially when the lipid phase changed from fluid to gel. It is well known that SFG signal intensity is related to the number of molecules detected and their orientation.¹⁵ As shown in Figure 4C, the membrane orientation of alamethicin is different in fluid and gel phase lipid bilayers. Our previous research indicated that an α -helix generates the strongest SFG signal when it stands up on the surface, while it generates weakest SFG signal when it lies down.^{16,19,20} This observed SFG intensity decrease may come from the membrane orientational change from membrane insertion to lying down. In addition, this SFG intensity

decrease may also come from the number change of the adsorbed alamethicin molecules on the lipid bilayers.

Figure 4D displays the relation between the ssp SFG signal strength (I_{ssp}) and the α -helical segment orientation angle (θ_1). As presented above, changing the lipid bilayer phase from fluid to gel can cause the change of θ_1 from 45° to 75° (Table 1). From Figure 4D, we can see that the ssp SFG signal strength of alamethicin on gel phase bilayer is about ~34% of that on fluid phase, assuming that the number density of adsorbed alamethicin is the same for the two lipid phases. Our fitting results show that the SFG signal strength of the α -helical segment of alamethicin associated with gel phase bilayer is ~43% of that from the alamethicin with fluid phase bilayer, which is larger than 34% calculated above. Possibly this is because after the initially inserted peptides changed to the lying down orientation (due to the lipid phase change), they can interact with more peptides in solution to form aggregates on the lipid bilayer surface, generating strong SFG signals. This is just opposite to the low peptide concentration case discussed above, where the peptides returned back to the solution upon phase changing from fluid to gel.

Figure 5A shows the SFG spectra collected from the fluid lipid bilayer/subphase interface at about 1 h after a certain amount of alamethicin stock solution was injected into the subphase to reach a peptide concentration of $21.60 \mu\text{M}$ at 30°C . Figure 5B shows the SFG spectra collected from the above lipid bilayer/alamethicin solution interface after the lipid phase changed from fluid to gel by lowering the subphase temperature to 10°C . The above SFG spectra were fitted and the alamethicin orientation angles were deduced. The orientation angles of the alamethicin α -helical segment (θ_1) were found to be 35° (between 26° to 43°) vs. the lipid bilayer surface normal for fluid phase lipid bilayer and 34° (between 25° to 42°) for the gel phase lipid case (Figure 6A). The orientation angle of the alamethicin 3_{10} -helical segment (θ_2) was deduced to be 37° (between 33° to 41°) for alamethicin associated with both the fluid and gel phase lipid bilayers (Figure 6B). Figure 6C shows a schematic for membrane orientations of alamethicin associated with the fluid (left) and gel (right) phase lipid bilayers at the peptide concentration of $21.60 \mu\text{M}$. Since at this peptide solution concentration for alamethicin associated with the lipid bilayer, the orientation angles for the α -helical (θ_1) segment and the 3_{10} -helical segment are similar, the alamethicin molecule is more or less linear. Also, the orientation of alamethicin is almost the same when associated with the fluid and gel phase lipid bilayer at the high peptide concentration of $21.60 \mu\text{M}$. The alamethicin molecule inserts into both fluid and gel phase d-DMPC/d-DMPC bilayers.

Figure 5C shows the time-dependent ssp SFG intensity change at 1670 cm^{-1} (from the α -helix structure of alamethicin) at $21.60 \mu\text{M}$ peptide concentration when the lipid bilayer phase changed from fluid (0 s) to gel (~ 4000 s). The SFG intensity from the α -helical segment in alamethicin did not exhibit substantial changes when the lipid phase changed from fluid to gel. Since the orientation of alamethicin is the same associated with the fluid and gel phases (Figure 6C), this similar SFG intensity indicates that the alamethicin density in lipid bilayers remained the same when the lipid phase changed from fluid to gel. At the high peptide concentration of $21.60 \mu\text{M}$, alamethicin inserts into both the fluid and gel phase lipid bilayers, and the change of the lipid fluid phase to gel phase cannot “push” inserted alamethicin molecules to the bilayer surface. Neither membrane orientation nor surface coverage shows substantial difference in fluid and gel phase lipid bilayers.

In our previous studies on the interactions between lipid bilayers and various AMPs such as melittin²⁶ and MSI-78,¹⁶ multiple-orientation distribution of peptides was observed due to the toroidal pore formation. In the present work, a single orientation angle distribution can satisfy all the observed SFG data for alamethicin associated with both fluid and gel phase

lipid bilayers. We believe that for high alamethicin concentration, peptides act via a barrel-stave mode, in which all the peptides more or less stand up in the membrane.⁸

The previous NMR experimental results indicated that alamethicin switches from a transmembrane (fluid phase) to an in-plane (gel phase) orientation in DPPC membrane when the phase of the membrane changes from fluid to gel,⁹ which agrees with our SFG results presented above at the medium peptide concentration of 10.80 μM . Such an orientational change was explained by an increase in the membrane hydrophobic thickness and the resulting hydrophobic mismatch.⁹ In such NMR experiments, premixed multilamellar lipid vesicles and peptides were used as model systems.⁹ In our SFG experiments, we used planar single lipid bilayers and injected peptide stock solution to the subphase water to make peptide solutions with different concentrations, which enabled us to probe the interactions between alamethicin and lipid bilayers *in situ* in real time. Thus we believe our SFG experiments may be more closely related to the physiologically relevant conditions.

Previously, we used other types of lipids, such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DPPG) to prepare lipid bilayers to study their interactions with alamethicin. All these lipids are in gel phase at the room temperature.¹³ In those studies, at the low peptide concentration of 3.6 μM , we observed very weak SFG signals in the amide I frequency range on those gel phase lipid bilayers. We were unable to fit such weak SFG spectra to deduce the accurate orientation angles of alamethicin but believed that the alamethicin molecules more or less lie down on the gel phase lipid bilayers. Here, we successfully collected and fitted SFG spectra from alamethicin in the amide I frequency range on gel phase d-DMPC/d-DMPC lipid bilayers, and determined the alamethicin orientation angles. Our results demonstrated that the membrane orientation of alamethicin on gel phase lipid bilayer depends on the subphase peptide concentration: the peptide more or less lies down on the gel phase lipid bilayer surface when the bilayer is in contact with the subphase alamethicin solution of low (0.84 μM) and medium (10.80 μM) concentrations, while inserts into the gel phase lipid bilayer at a high (21.60 μM) peptide concentration.

3.2. SFG Signal from Fluid and Gel Phase d-DMPC/d-DMPC Lipid Bilayer

After we studied the membrane orientation of alamethicin in fluid and gel phase lipid bilayers, we continued to examine the peptide induced structural change of lipid bilayer. Before the addition of alamethicin solution to the subphase, no discernible SFG signal was detected in the C-D stretching frequency range (between 2000 and 2250 cm^{-1}) from the lipid bilayer/subphase interface due to the symmetry of the bilayer (data not shown). However, discernible ssp SFG signal in the C-D stretching frequency region was detected from the interface when either fluid or gel phase d-DMPC/d-DMPC bilayer was in contact with the 0.84 μM alamethicin solution (Figure 7A). The detected C-D signals are dominated by the contributions from the CD_3 symmetric stretching mode of the d-DMPC lipid ($\sim 2065 \text{ cm}^{-1}$). Because alamethicin itself has no contribution to this CD_3 signal, we believe that this signal must come from the asymmetric d-DMPC/d-DMPC bilayer after interacting with alamethicin, indicating that the two leaflets of the lipid bilayer were not disrupted in the same degree and therefore the bilayer was not symmetric anymore. As shown in Figure 2C (left), for the fluid phase lipid bilayer, alamethicin lies down on the surface of the distal leaflet and may be able to disrupt the packing order of the lipid molecules in this distal leaflet at this low peptide concentration. After we decreased the temperature to change the lipid phase to gel state, this CD_3 signal still could be detected. This is because the associated alamethicin molecules are also lying down (Figure 2C, right) on the gel phase lipid bilayer, and the observed SFG CD_3 signal is due to the asymmetry of the gel phase lipid bilayer.

At the medium peptide concentration of 10.80 μM , no SFG signal could be observed in the C-D stretching frequency region from the fluid phase lipid bilayer/alamethicin solution interface (Figure 7B). Based on our calculation, the alamethicin adopts a transmembrane orientation when associated with the fluid phase lipid bilayer at this peptide concentration, as shown in Figure 4C (left). It is reasonable that the lipid bilayer is still symmetric and generates no SFG signal because the alamethicin disrupts the two leaflets in the same degree. This also agrees with the observation that alamethicin interacts with cell membrane through the barrel-stave mode.⁸ After decreasing the temperature to change the fluid phase lipid bilayer to gel phase, the associated alamethicin molecules changed the orientation from “inserting into the bilayer” to “lying down on the surface of the distal leaflet”. In this case only the distal leaflet was disturbed (Figure 4C, right). Strong SFG CD_3 signals from the gel phase d-DMPC lipids were observed now because the lipid bilayers were not symmetric due to the interaction between the alamethicin and the distal leaflet. The markedly different SFG signals from the interfaces between the alamethicin solution and gel as well as fluid phase d-DMPC/d-DMPC lipid bilayers show another strong evidence that alamethicin interacts with gel and fluid phase lipid bilayers differently. Besides, this SFG CD_3 signal detected from the deuterated lipids also confirms that the phase change of the lipid bilayer can cause the re-orientation of alamethicin molecules.

At the high peptide concentration of 21.60 μM , no SFG signal in the C-D stretching frequency region was observed from the interfaces between the peptide solution and both the fluid and gel phase lipid bilayers (Figure 7C). This indicates that the lipid bilayers are always symmetric at this high peptide concentration, regardless of the lipid phase. This result supports our conclusion that alamethicin adopts a transmembrane orientation through the barrel-stave mode at this high peptide concentration,^{8,15} and its orientation is not changed when the phase of the lipid bilayer changes from fluid to gel, as shown in Figure 6C.

AMP's mode of action is thought to involve the transient perturbation of bacterial membranes but the molecular mechanism underlying the rearrangement of the lipid molecules to explain the interaction mechanism is still poorly understood.¹⁻³ The characterization of the lipid bilayer structure affected by the membrane associated AMP molecules can shed light onto understanding such interaction mechanisms. Besides, our SFG studies on the C-D stretching modes are well correlated to the SFG amide I signal reported above, which further confirms that alamethicin interacts with cell membrane through the barrel-stave mode.

Although previous publications have reported that the addition of peptides may induce changes of membrane phase or membrane curvature,⁵³⁻⁵⁵ the present work which applies the unique surface-specific technique, SFG, can unveil the similar or different disruption effect of peptide on the inner and outer leaflets of the bilayers.

4. Conclusion

SFG has been developed into a powerful tool to study peptide and protein structures on surfaces and at interfaces. In this work, we applied SFG to characterize the peptide concentration- and temperature-dependent structural and orientation changes of alamethicin in d-DMPC/d-DMPC bilayers *in situ* in real time. Our results demonstrate that, depending on the peptide concentration, alamethicin may adopt different membrane orientations on a fluid or gel phase lipid bilayer, and accordingly, the structures of the fluid and gel phase bilayer may exhibit different changes. At a low peptide concentration of 0.84 μM , alamethicin lies down on both fluid and gel phase lipid bilayers, and the outer layer of the d-DMPC/d-DMPC bilayer is disrupted more. Changing the lipid phase from fluid to gel leads

to a decrease in the number of alamethicin molecules associated with the bilayer, with some of the alamethicin molecules returning to the subphase. At a medium peptide concentration of 10.80 μM , alamethicin inserts into the fluid phase lipid bilayer, and its orientation changes from “membrane insertion” to “lying down” when the lipid phase changes from fluid to gel, while the outer layer of the gel phase lipid bilayer is disrupted more. This observed orientational change from “membrane insertion” to “lying down” is related to the active/inactive state of alamethicin - an active state usually requires an inserting orientation of alamethicin, while in an inactive state the peptide is usually lying down. At a high peptide concentration of 21.60 μM , alamethicin adopts a transmembrane orientation on both fluid and gel phase lipid bilayers. The bilayers are always symmetric in both phases, and the associated peptides have a similar density. Our results further reveal that at this high peptide concentration, the observed SFG data can be interpreted by all alamethicin molecules adopting a nearly identical orientation and the lipid bilayers being always symmetric, both indicating that the alamethicin has the barrel-stave mode of action.

It is worth mentioning that usually zwitterionic PC bilayers serve as model membranes for mammalian cells, not bacterial cells. However, our previous study indicates that alamethicin interact with zwitterionic bilayers and negatively charge bilayers similarly if they have the same phase.¹³ Therefore we believe that the results obtained from this study shed light on antimicrobial activity of alamethicin. We choose d-DMPC to study here because it is easy for us to vary the lipid bilayer phase. As we discussed above, the “healthy” cell membranes contain fluid phase lipids, while gel phase lipids can be found in injured or aged cells or in some phase separated membrane regions. Thus, investigations on how the phase of the lipids influences the membrane orientation of AMPs are important to understand more details regarding the AMP’s action on cell membranes, especially for details on the differences of interactions between AMPs and the membranes of healthy cells or injured/aged cells.

This work revealed that the membrane lipid phase is related to the structure and orientation of the associated AMPs, which provides a more comprehensive and biologically-relevant picture on the peptide–membrane interactions. The results and conclusions obtained from this work may have profound implications for our understanding towards the peptides’ function as antimicrobial agents. It is worth mentioning that in order to understand detailed interactions between antimicrobial agents and bacteria, in addition to investigate the antimicrobial peptide – cell membrane interactions, it is necessary to examine molecular interactions between antimicrobial peptides and bacterial cell walls (outer membranes), which are under the current investigation in our lab.

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Figure 1.

SFG ppp and ssp amide I spectra collected from a (A) fluid phase and (B) gel phase d-DMPC/d-DMPC bilayer in contact with a 0.84 μM alamethicin solution. (C) Time dependent ssp SFG spectral intensity monitored at 1670 cm^{-1} (from α -helical segment of alamethicin) when the phase of lipid bilayer changes from fluid (0 s) to gel (4000 s).

Figure 2. Relation between the χ_{ppp}/χ_{ssp} ratio and the (A) α -helical and (B) 3_{10} -helical segment orientation angle in a d-DMPC/d-DMPC bilayer (assuming a delta angle distribution) in contact with a 0.84 μM alamethicin solution. The dotted lines are experimental data for fluid or gel phase lipid bilayer. (C) Schematic illustrating the orientation of alamethicin in fluid (left) or gel (right) phase d-DMPC/d-DMPC bilayer at 0.84 μM peptide solution concentration.

Figure 3.

SFG ppp and ssp amide I spectra collected from a (A) fluid phase and (B) gel phase d-DMPC/d-DMPC bilayer in contact with a 10.80 μM alamethicin solution. (C) Time dependent ssp SFG spectral intensity monitored at 1670 cm^{-1} (from α -helical segment of alamethicin) when the phase of lipid bilayer changes from fluid (0 s) to gel (4000 s).

Figure 4.

Relation between the $\chi_{\text{ppp}}/\chi_{\text{ssp}}$ ratio and the (A) α -helical and (B) 3_{10} -helical segment orientation angle in a d-DMPC/d-DMPC bilayer (assuming a delta angle distribution) in contact with a 10.80 μM alamethicin solution. The dotted lines are experimental data on fluid or gel phase lipid bilayer. (C) Schematic illustrating the orientation of alamethicin in fluid (left) or gel (right) phase d-DMPC/d-DMPC bilayer at 10.80 μM peptide solution concentration. (D) Calculated relation between χ_{ssp} and α -helical segment orientation angle. The dotted lines are experimental data of alamethicin associated with fluid or gel phase lipid bilayer.

Figure 5.

SFG ppp and ssp amide I spectra collected from a (A) fluid phase and (B) gel phase d-DMPC/d-DMPC bilayer in contact with a 21.60 μM alamethicin solution. (C) Time dependent ssp SFG spectral intensity monitored at 1670 cm^{-1} (from α -helical segment of alamethicin) when the phase of lipid bilayer changes from fluid (0 s) to gel (4000 s).

Figure 6. Relation between the χ_{ppp}/χ_{ssp} ratio and the (A) α -helical and (B) 3_{10} -helical segment orientation angle in a d-DMPC/d-DMPC bilayer (assuming a delta angle distribution) in contact with a 21.60 μM alamethicin solution. The dotted lines are experimental data on fluid or gel phase lipid bilayer. (C) Schematic illustrating the orientation of alamethicin in fluid (left) or gel (right) phase d-DMPC/d-DMPC bilayer at 21.60 μM peptide solution concentration.

Figure 7.

SFG ssp spectra in C-D stretching frequency region collected from a fluid (open squares) and gel (solid circles) phase d-DMPC/d-DMPC lipid bilayer in contact with (A) 0.84 μM, (B) 10.80 μM, and (C) 21.60 μM alamethicin solution concentrations.

Scheme 1.
Molecular structure of lipid d-DMPC:

Table 1

Deduced orientation angles from SFG data for α -helical segment (χ_1) and 3_{10} -helical segment (χ_2) in alamethicin associated with the fluid or gel phase d-DMPC/d-DMPC bilayer with different peptide solution concentrations.

Concentrations (μ M)	χ_1 (deg), α -helix	χ_2 (deg), 3_{10} -helix
0.84 (Fluid phase)	77 (between 67 to 90)	47 (between 43 to 50)
0.84 (Gel phase)	75 (between 65 to 90)	47 (between 43 to 50)
10.80 (Fluid phase)	45 (between 37 to 51)	44 (between 41 to 48)
10.80 (Gel phase)	75 (between 65 to 90)	48 (between 45 to 51)
21.60 (Fluid phase)	35 (between 26 to 43)	37 (between 33 to 41)
21.60 (Gel phase)	34 (between 25 to 42)	37 (between 33 to 41)