Observing a Molecular Knife at Work

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Observing a Molecular Knife at Work

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Abstract: Sum frequency generation (SFG) vibrational spectroscopy has been employed to study the molecular interactions between a single substrate supported lipid bilayer and an amphiphilic antibiotic compound **1**, with a design based on the common structural motif of natural antimicrobial peptides. The interfacial sensitivity of SFG allows real-time in situ monitoring of ordering changes in both leaflets of the bilayer and orientation of **1** simultaneously. A critical concentration of about 0.8 μg/mL of **1** is found, above which the inner leaflet of the bilayer is significantly perturbed. This concentration corresponds well to the minimum inhibition concentration of **1** that is obtained from bacterial experiments. Orientation of **1** in the bilayer is shown to be perpendicular to the bilayer surface, in agreement with simulation results. SFG can be developed into a very informative technique for studying the cell membrane and the interactions of membrane-active molecules.

Introduction

After their discovery in the 1940s, antibiotics dramatically reduced illness and death from infectious diseases; however, over the decades, bacteria have developed resistance to many antibiotics. Consequently, antibiotic resistance has been called one of the world's most pressing public health problems, and the search for novel antibiotics is attracting increased attention. Antimicrobial peptides (AMPs) represent a particularly rich opportunity due to their large structural diversity, broad-spectrum activity, and their unique mode of action.[1] Their main target appears to be the lipid bilayer of cell membranes instead of specific protein receptors, effectively minimizing the drug resistance.[1] Nonetheless, the native structures of AMPs remain relatively complex, and to realize their essential biological activity in simpler structures is an important objective.

Recently, we demonstrated that such simple structures can be designed with potent, broad-spectrum activity and these molecules can discriminate between prokaryotic and eukaryotic cells.[2] An example of such a compound, **1**, is shown in Figure 1a. The rationale behind the design of **1** is based on the common structural motif of natural AMPs—the facial distribution of cationic (blue) and nonpolar (green) groups in the folded structure (Figures 1b,c). Our experiments show that **1** is selective with an average minimal inhibitory concentration (MIC) of 0.8 μg/mL for various bacteria but with an $HC_{50} > 75$ μg/mL for human red blood cells. We confirmed that **1** acts on bacteria by disrupting cell membranes through exposing *Staphylococcus aureus*, containing the osmotic potential sensitive dye, diSC3-5, to **1** and detecting concentration-dependent dye leakage (Figure 1d).

At the same time, details about lipid interactions with AMPs, or their simpler mimics, remain poorly understood. This is largely due to the inability of experimental methods to probe the molecular interactions between these molecules and membranes. Molecular dynamics simulations based on a facially amphiphilic structure similar to **1** in a lipid layer suggested that the molecular plane is perpendicular to the bilayer surface. Thus, the molecule functions as a molecular knife by inserting into and disrupting the lipid bilayers.[3] Here, we present direct experimental evidence to elucidate such results using sum frequency generation (SFG) vibrational spectroscopy.

Solid supported planar lipid bilayers have been extensively used as models for cell membranes.[4] However, studying such a single lipid bilayer and its association with other molecules has posed a challenge to researchers due to the small amount of substances involved. In addition, bulky labels are often introduced into either the lipid bilayers or the molecules interacting with the bilayers, which may significantly alter the system studied.[5] Recently, SFG has been used to investigate lipid monolayers and bilayers.[5−13] The structure, transmembrane movement, and transition temperature of solid-supported planar

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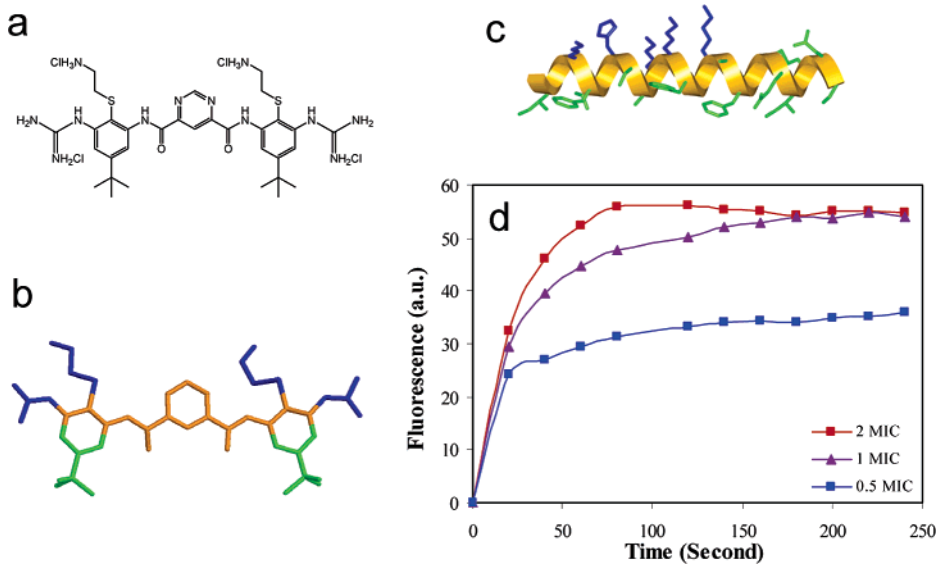

Figure 1. Structure of **1** (a) has a facial amphiphilicity (b), which mimics natural antimicrobial peptides such as magainin (c): green and blue represent hydrophobic and positively charged functional groups, respectively. (d) When *S. aureus* containing the osmotic potential sensitive dye, diSC3-5, was exposed to **1**, leakage of the dye was observed at concentrations very similar to the minimal inhibitory concentration (MIC) of 0.8 μg/mL.

lipid bilayers have been examined using SFG. Such research shows that SFG is a very informative technique for probing a single lipid bilayer, due to its intrinsic surface specificity. In this article, for the first time, we show that the molecular interactions between a single planar lipid bilayer and a novel membrane-active antimicrobial arylamide foldamer, **1**, can be elucidated in situ by SFG at concentrations around its MIC in real time.

Experimental Section

Solid-supported planar lipid bilayers were prepared with the Langmuir−Blodgett−Schaeffer method.[4,6] Hydrogenated and deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG and d-DPPG) were used for the proximal and distal leaflets respectively so that the structure of both leaflets can be monitored with SFG at the C−H and C−D stretching ranges simultaneously. DPPG is examined here because phosphatidylglycerol is the main component of negatively charged lipids in bacterial cell membranes. A neutral CaF_2 prism was used as the substrate instead of the more frequently used negatively charged silica or mica surfaces to avoid interactions between the positively charged **1** and the silica or mica. We ensured that CaF_2 is a valid substrate for supported bilayers. More details about sample preparation are available in the Supporting Information. Details about our SFG setup can be found in our previously published work.[14,15] A total reflection geometry was used for all experiments discussed in this paper.[16] All SFG spectra, unless otherwise specified, were collected with an ssp (s-polarized SFG signal, s-polarized visible, and p-polarized IR beam) polarization combination. A new double resonance SFG spectrometer was used to collect double resonance SFG spectra while varying the input visible frequency, which is discussed in more detail in the Supporting Information.

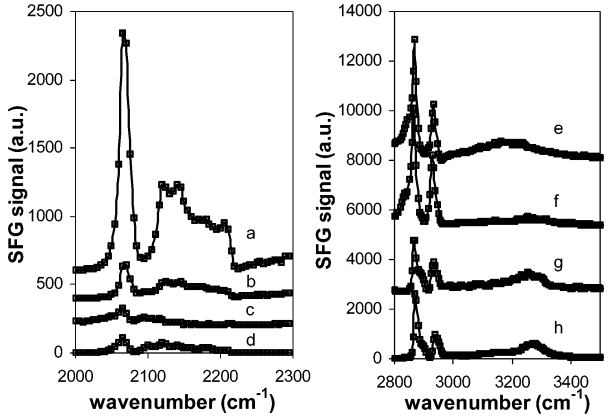

Figure 2. SFG spectra collected from a DPPG/d-DPPG bilayer in the CD stretching range (left panel) and CH/OH stretching range (right panel) before (a), (e) and after contacting **1** at three solution concentrations: (b), (f) 0.1 μg/mL; (c), (g) 0.8 μg/mL; and (d), (h) 4 μg/mL.

Results and Discussion

Shown in Figure 2a is the SFG spectrum from DPPG (proximal leaflet)/d-DPPG (distal leaflet) bilayer in the C−D stretching range before contacting a solution of **1**. The dominant 2070 cm^{-1} peak is from the symmetric stretching mode of the terminal methyl groups of the distal d-DPPG leaflet.[17] Spectra b, c, and d in Figure 2 were collected after the bilayer contacted the solution of **1** with specified concentrations spanning the MIC until no further spectral changes were observed. SFG signals decrease for all three concentrations, although the decrease for 0.1 μg/mL is much smaller compared to that for the other two concentrations. This indicates that **1** partitions to the bilayer and interacts with it. The decrease in CD_3 symmetric stretching

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intensity at 2070 cm^{-1} should be due to the disordering of the terminal CD_3 groups, even though the possibility of displacement of the distal lipid molecules cannot be ruled out from SFG results. Such displacement is unlikely since light scattering experiments conducted in solution (not reported in detail here) indicate that the liposome does not disintegrate upon exposure to **1**, unlike exposure to the control TritonX-100, suggesting that both leaflets still exist after introduction of **1**.

Interactions between the proximal leaflet of the bilayer and **1** were studied using the C−H/O−H stretching bands. Spectra e−h of Figure 2 show the C−H and O−H stretching regions before and after contacting **1**. The C−H signals between 2800 and 3000 cm^{-1} are mainly contributed by the methyl end groups while the O−H stretching signals of water molecules aligned by the charged surface of the bilayer are observed between 3000 and 3500 cm^{-1}.[18] After contacting a solution of **1**, the decrease in the O−H signal indicates that the surface charge is altered by the adsorption of positively charged **1**. Meanwhile a peak centered at 3280 cm^{-1} can be observed in Figure 2, g and h, originating from the N−H stretching mode of **1**, further confirming the interactions between **1** and the bilayer. The decrease in C−H stretching signals in Figure 2, g and h, indicates that the lipids in the proximal leaflet are disturbed. However at the concentration of 0.1 μg/mL, which is lower than the MIC, Figure 2f shows that the C−H signals remain almost unchanged after accounting for the change in the interfering O−H stretching signal. Several other solution concentrations of **1** (e.g., 0.5 and 10 μg/mL) have been examined, and a similar trend, as shown in Figure 2, is observed. This leads us to conclude that for **1** to be effective in inhibiting bacterial growth, it has to disturb not only the extracellular but also the cytoplasmic leaflet of a bacteria membrane, in general agreement with previous studies on AMPs. Our SFG results correlate well with the biological and dye leakage results obtained from **1**.

In addition to the structural perturbation induced by **1**, a transmembrane movement (or flip-flop) of the hydrogenated and deuterated lipids can also lead to a diminished bilayer isotopic asymmetry and consequently a decrease in C−H/C−D SFG signals. We believe that under the experimental conditions used in this paper, **1** does not induce significant transmembrane movement of lipids, as evidenced by the independent signal decrease in the C−H and C−D ranges (Figure 2). We have recently observed antimicrobial peptide-induced lipid flip-flop, and the pattern of signal decrease is dramatically different from that reported here.[19] The DPPG bilayer should be in the gel phase under our experimental conditions, and the intrinsic flip-flop rate of the bilayer alone is negligibly slow. Formation of transmembrane pores has been observed to greatly accelerate the flip-flop process by creating an aqueous channel so that the polar headgroups of lipids do not have to transverse the hydrophobic bilayer core region. We believe that **1**, as a small rigid amphiphilic molecule, is unlikely to form pore-like structures inside a bilayer of DPPG. Therefore, it is most likely that **1** is directly disrupting the bilayer as opposed to forming pores.

To deduce the orientation of **1** inserted in the bilayer, orientational analysis of the methyl symmetric (2907 cm^{-1}) and

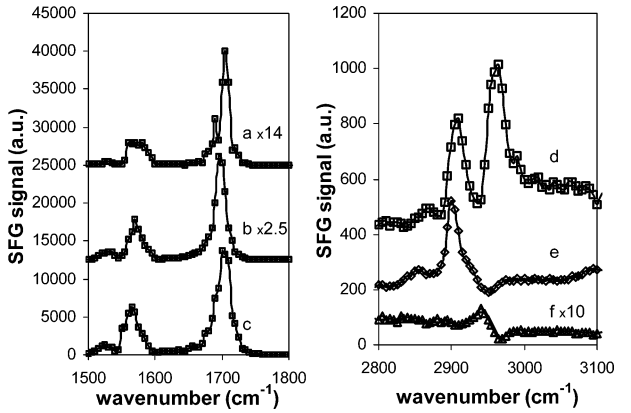

Figure 3. SFG spectra collected from **1** adsorbed onto a bilayer. (Left panel) CO stretching signals collected at three solution concentrations of **1**: (a) 0.1 μg/mL, (b) 0.8 μg/mL, and (c) 4 μg/mL. (Right panel) CH stretching signals from **1** at a solution concentration of 0.8 μg/mL with (d) ppp, (e) ssp and (f) sps polarization combination. A d-DPPG/d-DPPG bilayer was used to avoid spectral confusion. The SFG signals are multiplied by 14, 2.5, and 10 respectively for (a), (b), and (f) for comparison purposes.

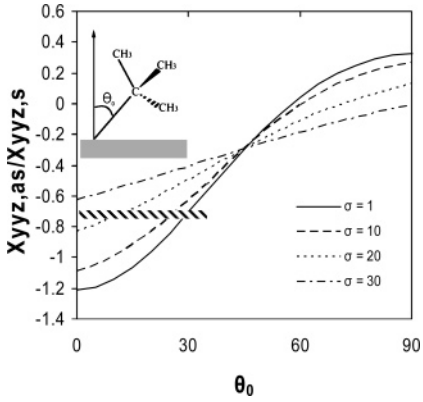

Figure 4. Relation between the $\chi_{yyz,as}/\chi_{yyz,s}$ and the orientation distribution of the *tert*-butyl group. $\chi_{yyz,as}/\chi_{yyz,s}$ is experimentally found to be −0.72, which is marked in the figure by the shaded area.

asymmetric stretching (2957 cm^{-1}) modes of the *tert*-butyl groups adsorbed onto the d-DPPG/d-DPPG bilayer along with the C═O stretching mode (1705 cm^{-1}) of **1** was performed (Figure 3). The above **1** peak assignments have been confirmed by ab initio calculation, which will be reported in detail elsewhere. To quantitatively deduce the orientational distribution of **1** based on C−H stretching signals, we first derive the hyperpolarizibility of the *tert*-butyl group by projecting the methyl hyperpolarizibility based on the *tert*-butyl group geometry. Then we use the ratio of $|\chi_{yyz,as}/\chi_{yyz,s}|$ to determine the possible orientational distribution of *tert*-butyl groups while the relative values of $\chi_{yzy,as}$ and $\chi_{yzy,s}$ are used to cross-check the validity of our conclusion. Figure 4 depicts $|\chi_{yyz,as}/\chi_{yyz,s}|$ for different orientation distributions. The measured ratio is represented by the shaded area, which corresponds to a limited combination of possible orientations ($\theta_0 < 35°$) and orientation distribution width ($\sigma < 30°$). These results indicate a slight tilt of the groups versus the lipid surface normal with a narrow orientational distribution, showing that the molecular plane is more or less perpendicular to the bilayer surface. This is verified by the C═O stretching (1705 cm^{-1}) signal orientation analysis. It is this perpendicular orientation of **1** inserted into the bilayer that is described as a "molecular knife." The structure of **1** allows both vibrational and electronic resonance to be achieved,

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leading to a large enhancement in the C═O stretching signal intensity. Due to such double resonance enhancement, SFG C═O signals of **1** at solution concentrations significantly below the MIC can be detected, demonstrating the superb detection limit of the double resonance SFG technique. More details can be found in the Supporting Information. Furthermore, interference patterns between the methyl groups of **1** and those of the hydrogenated lipids in a DPPG/d-DPPG bilayer are found to be destructive, revealing that the hydrophobic *tert*-butyl groups orient toward the hydrophobic interior of the lipid bilayer. Taken together, the molecular picture deduced from the above analysis indeed matches the rational design of **1** as a planar rigid molecule with the hydrophobic moieties serving as a knife blade, cutting into the bilayer with a perpendicular orientation which leads to membrane disruption.

Conclusions

This paper demonstrates the feasibility of using SFG to elucidate the structure of a single lipid bilayer and its interactions with other molecules in situ. By monitoring the spectral changes in the C−H, C−D, and C═O stretching ranges, we are able to correlate the SFG results to bacteria inhibition and dye-leakage experiments. It is interesting to observe that even below the MIC, the distal leaflet was already disturbed, but the proximal leaflet remained intact. The methods demonstrated in this paper will also be applicable to investigate the interactions between supported bilayers and larger biomolecules such as proteins. The possible substrate influence when studying proteins with large domains on both sides of a membrane can be avoided by using polymer-cushioned bilayers or suspended bilayers.[20,21] We also showed that the double resonance effect can be used to greatly improve the detection limit of the SFG technique. We believe that SFG is a very sensitive method for both fundamental investigation of bilayers and associated biomolecules (e.g., elucidation of membrane protein structure), and for testing the efficacy of antibiotic chemical compounds and antimicrobial peptides for the pharmaceutical industry. The sensitivity and time-resolution of SFG will provide new insight into the molecular action of AMPs and AMP mimics like those reported here. Further studies with various lipid compositions to mimic prokaryotic and eukaryotic cell membranes, other AMPs, and interaction kinetics are being carried out.

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Supporting Information Available: Detailed experimental description, information about **1**, spectral analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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