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Different Interfacial Behaviors of N- and C-Terminus Cysteine-Modified Cecropin P1 Chemically Immobilized onto Polymer Surface

Xiaofeng Han,† Joshua R. Uzarski,‡ Charlene M. Mello,*‡ and Zhan Chen*†

†Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States
‡Bioscience and Technology Team, U.S. Army Natick Soldier Research, Development, & Engineering Center (NSRDEC), Natick, Massachusetts 01760-5020, United States

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1. INTRODUCTION

Biosensors have been widely used to detect pathogens.1–13 A fundamental and key factor for such a device design is the biological recognition element interacting with the target pathogen. These elements can be composed of DNA segments,6,7 peptides,10–17 or proteins (e.g., enzymes or antibodies).18,19 Protein- and peptide-based biological recognition elements can be adsorbed, trapped, or immobilized on a surface. Biosensor performance (e.g., sensitivity, selectivity, detection limit, precision, accuracy, reproducibility, working life, and shelf life) is greatly affected by the structures and orientations of the interfacial sensing biological molecules.20–22 For example, in the case of proteins and peptides, they must be properly oriented so that the binding domains are accessible while preserving their sensor functions. Many current pathogen detection systems use antibodies, which exhibit a multiple-orientation distribution at the PS-MA/peptide solution interface, due to simultaneous physical adsorption and chemical immobilization of CP1c on the PS-MA surface. Differently, in this research, it was found that the interfacial orientation of cCP1 molecules varied from a horizontal orientation to the “tilting” orientation to the “standing up” orientation and then to the “multiple-orientation” distribution as the peptide concentration increased from 0.19 to 3.74 μM. This research shows the different interaction mechanisms between CP1c and PS-MA and between cCP1 and PS-MA.

Numerous surface immobilization methods have been used to improve the performance of protein- and peptide-based biosensors, but the effect of molecular structure and orientation has not yet been elucidated in detail. This is largely due to the lack of appropriate analytical techniques. Recently, sum frequency generation (SFG) vibrational spectroscopy has been applied to investigate peptides and proteins at solid/liquid interfaces in situ.23–40 SFG is a second-order nonlinear optical spectroscopic technique with submonolayer surface sensitivity. In our lab we successfully developed systematic methodologies to determine interfacial orientations of various secondary structures such as the α-helix, 3–10 helix, β-sheet, and complex proteins such as G proteins using polarized SFG spectra.31–33,39 We applied these methods to deduce interfacial orientations of a variety of peptides and proteins at solid/water interfaces.34,35,37–40 In particular, we examined the orientation of physically adsorbed and chemically immobilized α-helical cecropin P1 on polymer surfaces.35,36 To promote chemical immobilization, the C-termini of the cecropin P1 were modified with a cysteine residue (CP1c), which forms covalent thioether bonds with polystyrene maleimide (PS-MA). We found that upon physical adsorption onto a polystyrene (PS) surface CP1c molecules adopt a multiple-orientation distribution which could not be described by a delta or Gaussian distribution.35 CP1c molecules at the PS-MA/peptide solution interface also exhibited a multiple-orientation distribution even at very low peptides concentration; however, after washing off the physisorbed CP1c molecules, the chemically immobilized CP1c molecules at the PS-MA/peptide solution interface exhibited an orientation angle of 35° versus the surface normal.

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This work demonstrated that the physically adsorbed and chemically immobilized CP1c molecules adopt different orientations at the polymer/peptide solution interface. Mello et al. investigated the interactions between CP1c and pathogenic E. coli and showed that when CP1c molecules were deposited on surfaces using different methods, the activities of the surface-immobilized CP1 molecules varied. For example, CP1 molecules physically adsorbed and chemically immobilized via the C-terminus exhibited very different killing efficiencies against E. coli. Furthermore, the overall binding proclivity of cecropin P1 to lipopolysaccharide molecules isolated from the outer membranes of different E. coli serotypes depends on the orientation of the immobilized peptide, with the C-terminal peptide showing higher binding than the N-terminal one. Such observations may be partially interpreted, using our SFG results, to mean that CP1c molecules adopt different orientations/orientation distributions when different surface immobilization/adsorption methods are used. Such binding differences may also be related to the position of the charged residues in the immobilized cecropin P1 relative to the distance from the surface.

In this research, we will investigate the interfacial behavior of N-terminal cysteine-modified cecropin P1 (cCP1). We will compare such results to those previously obtained from CP1c molecules immobilized on the surface and will be able to use the different structures of cecropin P1 immobilized via different termini to interpret their different activities. We believe that this is the first attempt to elucidate the structure-function relationship of the same peptide immobilized via different termini. When cCP1 molecules contact a PS-MA surface, the N-terminal cysteine will react to form covalent thioether bonds with the maleimide group, chemically immobilizing the peptide onto the PS-MA surface. Different from the CP1c peptides, SFG results indicate that the cCP1 molecules at the PS-MA/peptide solution interface exhibit a single orientation distribution at low peptide concentrations (≤0.75 μM); the orientation angle is dependent on the peptide solution concentration. At higher peptide concentrations (≥1.23 μM), the cCP1 molecules adopt a multiple-orientation distribution. ATR-FTIR experiments were performed to measure the relative cCP1 adsorption amount on the PS-MA surface.

2. EXPERIMENTAL SECTION

2.1. Materials and Sample Preparation. All of the chemicals were used as received. (4-Maleimidobutyramidomethyl) polystyrene (PS-MA), potassium phosphate (monobasic and dibasic) solution (1 M), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution (0.5 M), dichloromethane, toluene, and deuterated water (D2O) were used as received. (4-Maleimidobutyramidomethyl) polystyrene was purchased from Aldrich (Milwaukee, WI). The N-terminus cysteine-modified CP1 (cCP1, H2N-GAIAIAIQGGPR-OH, MW = 3442) was ordered from New England Peptide (Gardner, MA). EDTA was obtained from Fisher Biotech. Peptide Solution Interfaces. Our previous SFG studies on E. coli molecules, which ensures that a thiol moiety was present in a cCP1 monobasic potassium phosphate solution (1 M), dibasic potassium phosphate solution (1 M), and DI water (purified by a Millipore system). A specific amount of cCP1 was dissolved into 5 mM phosphate buffer with reducing agent TCEP and EDTA. TCEP was added to prevent disulfide bond formation among individual peptide molecules, which ensures that a thiol moiety was present in a cCP1 molecule. The thiol moiety in the cysteine residue has a strong affinity for the maleimide moieties, which promotes covalent immobilization of the peptide to the PS-MA surface. EDTA was added as a chelating agent to oxidize any metals present in the buffer that may cause formation of unwanted disulfide bonds. The thiol moiety in the cysteine residue can chemically react with a maleimide group on the surface, which promotes covalent immobilization of cCP1 onto the PS-MA surface (Figure 1).

2.2. SFG Measurements. Details regarding SFG theories and equipment have been reported previously and will not be repeated here. In our SFG experiments, right angle CaF2 prisms were used to realize the near total reflection geometry. In this geometry, the incident angles for the input visible and IR beams are 57° and 60° with respect to the Z axis in the lab coordinate system (Z axis is perpendicular to the surface). All of the SFG experiments were carried out at room temperature (23 °C). SFG spectra with different polarization combinations including ssp (s-polarized SF output, s-polarized visible input, and p-polarized infrared input) and spp were collected using the near total internal reflection geometry. When a near total reflection geometry is adopted in the SFG experiment, spp signal probes $X_{ss}$ and will not be repeated here. More details of the SFG data analysis can be found in the Supporting Information of ref 35.

2.3. ATR-FTIR Experiments. A Nicolet 6700 FTIR spectrometer was used to collect ATR-FTIR spectra with a standard 45° ZnSe ATR cell. ZnSe crystal was cleaned using the same procedure as the CaF2 prisms. PS-MA films were prepared by directly depositing the PS-MA solution onto the ATR crystal (ZnSe). Polymer films were equilibrated with D2O buffer solution prior to collection of a background spectrum of the PS-MA polymer film/D2O buffer solution interface. D2O buffer was replaced with the peptide solution (in D2O buffer) and equilibrated for at least 1 h to allow for peptide adsorption to reach equilibrium at the interface. The ATR-FTIR spectrum was then collected from the PS-MA/peptide D2O buffer solution interface. Finally, the amide I and amide II signals of adsorbed/immobilized peptide molecules on the PS-MA surface were obtained by subtracting the background spectrum of the polymer film/D2O buffer solution interface from the later collected spectrum. All ATR-FTIR spectra collected in this work were averages of 128 scans with a 2 cm$^{-1}$ resolution.

3. RESULTS AND DISCUSSIONS

3.1. Different Behaviors of cCP1 and CP1c on PS-MA/Peptide Solution Interfaces. Our previous SFG studies on...
CP1c molecules indicated that when phosphate buffer was used as the solvent the CP1c molecules at the PS-MA/CP1c buffer solution interface adopted a multiple-orientation distribution. The \( \chi_{\text{ppp}}/\chi_{\text{ssp}} \) signal strength ratio was beyond the possible range described by a delta or Gaussian distribution. Therefore, the distribution possibly contained several orientations, like the melittin case we studied previously. However, after washing the interface several times using the buffer solution, the CP1c orientation could be described by a delta or Gaussian distribution. This is because the physiosorbed CP1c molecules can be washed away from the interface, leaving only chemically immobilized CP1c molecules. In this work, cCP1 molecules at the PS-MA/cCP1 buffer solution interfaces have been studied by SFG and ATR-FTIR. Different from the CP1c behavior at the PS-MA/cCP1 solution interfaces, the orientations of cCP1 molecules at the PS-MA/cCP1 solution interface could be described by a delta or Gaussian distribution at low peptide concentrations, which will be presented in more detail below. For some experiments, the peptide solution in contact with the PS-MA was replaced with buffer solution several times to wash away the loosely associated peptides at the PS-MA/peptide solution interface. In this case, chemically immobilized cCP1 molecules on the PS-MA surface should be the only contributor to the SFG signal, which exhibits a similar orientation angle.

SFG ssp and ppp spectra were collected from the interface between the PS-MA surface and the peptide buffer solution. For an \( \alpha \)-helix, we have shown previously that the \( \chi_{\text{ppp}}/\chi_{\text{ssp}} \) signal strength ratio (taken after fitting the ppp and ssp spectra) can be used to determine its orientation. The relationship between the \( \chi_{\text{ppp}}/\chi_{\text{ssp}} \) signal strength ratio and the CP1 orientation described by a delta or Gaussian distribution is shown in Figure 2. The orientation angle \( \theta \) is the angle between the \( \alpha \)-helix principal axis and the surface normal.

\[
\chi_{\text{ppp}}/\chi_{\text{ssp}} = \begin{cases} \delta & \text{distribution (delta)} \\ \sigma & \text{distribution (Gaussian)} \end{cases}
\]

Figure 2. Relations between the SFG susceptibility tensor component ratio and the \( \alpha \)-helix orientation angle with different orientation distributions (assuming Gaussian distributions).

3.2. Peptide Concentration Effect on cCP1 Orientation at the PS-MA/Peptide Solution Interface. 3.2.1. Low Peptide Concentration. To better understand the orientation of cCP1 at the PS-MA/peptide solution interface, SFG spectra were collected from the cCP1 molecules at the PS-MA/peptide solution interface using cCP1 buffer solutions at different concentrations: 0.19, 0.28, 0.37, and 0.75 \( \mu \)M, as shown in Figure 3. All detected SFG spectra are dominated by a single peak centered at \( \sim 1650 \) cm\(^{-1} \), showing that cCP1 molecules adopted an \( \alpha \)-helical conformation at the PS-MA/peptide solution interface, which is similar to previous results from the CP1c molecules. For 0.19 \( \mu \)M cCP1 solution, the fitted \( \chi_{\text{ppp}}/\chi_{\text{ssp}} \) signal strength ratio is about 2.2, as shown in Figure 3a. From the relationship between the \( \chi_{\text{ppp}}/\chi_{\text{ssp}} \) signal strength ratio and the \( \alpha \)-helix orientation, we can see that cCP1 molecules more or less adopt a horizontal orientation at the interface. The SFG signals are quite weak for an accurate angle determination, perhaps due to the low surface coverage (because of the low concentration) of cCP1 molecules as well as the horizontal interfacial orientation of cCP1 molecules. In this case, because of the low surface coverage of cCP1 molecules, there is adequate available surface area to accommodate a horizontal cCP1 orientation. The low peptide surface coverage can be confirmed by later ATR-FTIR experimental data.

When the concentration of the cCP1 buffer solution increased, the cCP1 molecules at the PS-MA/peptide solution interface changed orientation. SFG spectra collected from the PS-MA/peptide solution interface when the cCP1 concentration was increased to 0.28 \( \mu \)M are shown in Figure 3b. The fitted \( \chi_{\text{ppp}}/\chi_{\text{ssp}} \) signal strength ratio leads to an orientation angle of 45° versus the surface normal. Compared to the 0.19 \( \mu \)M case above, when the concentration increased, more cCP1 molecules diffused to the interface and immobilized onto the PS-MA surface. Therefore, each molecule likely occupies a smaller surface area and tilt to accommodate the increased peptide density.

The interfacial cCP1 orientation could still be described by a single delta function when the peptide solution concentration was increased to 0.28 \( \mu \)M. It can be speculated that when more concentrated cCP1 solutions are used in the experiment, interfacial cCP1 molecules will adopt more than one orientation because more peptide molecules can be physisorbed or loosely associated at the interface, creating a multiple-orientation distribution.

We continued to increase the cCP1 buffer solution concentration to 0.37 \( \mu \)M. SFG spectra were collected from the PS-MA/peptide solution interface and are shown in Figure 3c. According to the fitted \( \chi_{\text{ppp}}/\chi_{\text{ssp}} \) signal strength ratio, the cCP1 average orientation was determined to be 15° versus the surface normal. When the peptide concentration reached 0.37 \( \mu \)M, the cCP1 molecules at the PS-MA/peptide solution interface are likely approaching saturation and therefore produce a tightly packed monolayer which adopt a more vertical orientation. Compared to the above two concentrations of 0.19 and 0.28 \( \mu \)M, more cCP1 molecules were adsorbed to the PS-MA surface, which can be confirmed by the ATR-FTIR results presented below. The measurement of 15° assumes that all molecules adopt the same orientation. Even though the cCP1 molecules were chemically immobilized at the PS-MA/peptide solution interface, we believe that these cCP1 molecules must have reached a dynamic equilibrium with those in the peptide solution. We hypothesized that if we removed the cCP1 molecules from the peptide solution, e.g., replaced the peptide solution with buffer, some of the immobilized cCP1 molecules would return to the liquid phase and a new equilibrium might be established. This was confirmed by replacing the peptide solution with buffer several times. SFG spectra were then collected from the PS-MA/buffer interface using the ssp and ppp polarization combinations (Figure 3e). The spectral intensities greatly decreased, and the fitted \( \chi_{\text{ppp}}/\chi_{\text{ssp}} \) signal strength ratio indicated that the cCP1 orientation was around 48°. It could be seen that when some of the molecules desorbed from the PS-MA surface, each cCP1...
molecule had more space and thus could tilt more. It is possible that the optimal orientation for the immobilized cCP1 molecules on the PS-MA surface is around 50°, which is not very different from the previously tested C-terminus-modified CP1c molecules which were chemically immobilized on a PS-MA surface and washed with phosphate buffer (35°).

The cCP1 concentration was increased to 0.75 μM, and ssp and ppp SFG spectra were again recorded to deduce the peptide orientation at the PS-MA interface. In Figure 3d, the fitted χ_{ppp}/χ_{ssp} shows that the peptide orientation is approximately 10°. Despite a 2-fold increase in peptide solution concentration, the orientation difference between 0.75 and 0.37 μM cCP1 was small (10° vs 15°, respectively).

3.2.2. High Peptide Concentration. We then increased the cCP1 buffer solution concentration to 1.12, 1.50, and 3.74 μM and collected SFG ssp and ppp spectra from the cCP1 molecules at the PS-MA/peptide solution interfaces. These are displayed in Figures 4a, 4b, and 4c, respectively. At 1.12, 1.50, and 3.74 μM, the fitted χ_{ppp}/χ_{ssp} ratios were 1.29, 1.22, and 1.24. For all cases the ratio was similar and beyond the possible range for a delta or Gaussian distribution, indicating that the cCP1 molecules adopted a multiple-orientation distribution for these three cases. For the three concentrations, the SFG signal strength did not change substantially, similar to the CP1c case.35

After SFG spectra were collected from the interface between PS-MA and cCP1 solution with a concentration of 1.50 μM, the cCP1 solution was replaced by buffer to wash the interface twice. SFG spectra were then collected from the PS-MA (with cCP1)/buffer interface, and the resulting data is shown in Figure 4d. The two-stage wash should remove the physisorbed cCP1 molecules, thereby allowing the remaining chemically immobilized cCP1 molecules to reach a new dynamic equilibrium. This was indeed the case as the fitted χ_{ppp}/χ_{ssp} ratio leads to a single cCP1 orientation angle of approximately 50°, which agrees with the results of the 0.19 μM cCP1 solution. The result of the higher peptide concentration after washing again shows the optimal orientation of chemically immobilized cCP1 is approximately 50° from the surface normal as shown in Table 1.

3.3. Relative Peptide Absorption Quantified by ATR-FTIR. SFG results showed that the χ_{ppp}/χ_{ssp} ratios measured from the SFG amide I spectra detected from cCP1 molecules at the PS-MA/cCP1 buffer solution interface changed as a function of peptide solution concentration. This suggests that the cCP1 peptide molecules changed orientations when the
peptide concentration was varied. As discussed, the peptide orientation change might be due to the fact that more peptide molecules could adsorb or immobilize on the PS-MA surface at higher peptide concentrations. Differently, such measured $\chi_{ppp}/\chi_{ssp}$ ratios remained more or less the same at higher peptide concentrations (when the solution concentration was at or higher than $\sim 1.12 \, \mu$M). To further demonstrate that the adsorption amount may be varied at different peptide concentrations, ATR-FTIR was applied to measure the relative adsorption amount of cCP1 molecules at interfaces.

Figure 5a shows the ATR-FTIR spectra of cCP1 molecules adsorbed to the PS-MA surfaces at different peptide solution concentrations, varying from 0.19 to 7.5 $\mu$M. To avoid spectral confusion between the H$_2$O bending signal and the peptide amide I signal, peptide D$_2$O buffer solution was used in the experiment. Use of D$_2$O in the peptide solution shifts the amide I peak center to slightly lower than 1650 cm$^{-1}$, showing that the signal is still dominated by the contribution from the $\alpha$-helical component. With the peptide concentration change, no substantial ATR-FTIR spectral feature change was observed in the amide I frequency range, as shown in Figure 5a. This suggests that adsorbed cCP1 molecules at the PS-MA/peptide D$_2$O buffer solution interface adopt similar secondary structures (dominated by $\alpha$-helix) at different peptide solution concentrations.

Table 1. Orientation Angle Deduced for N-Terminus Cysteine-Modified Cecropin P1 at PS-MA/Liquid Interfaces with Different Peptide Solution Concentrations

<table>
<thead>
<tr>
<th>peptide concentration ($\mu$M)</th>
<th>0.19</th>
<th>0.28</th>
<th>0.37</th>
<th>0.75</th>
<th>1.12</th>
<th>1.50</th>
<th>3.74</th>
</tr>
</thead>
<tbody>
<tr>
<td>tilt angle (deg) vs surface normal</td>
<td>lying down</td>
<td>50</td>
<td>15</td>
<td>10</td>
<td>multiple orientations</td>
<td>multiple orientations</td>
<td>multiple orientations</td>
</tr>
<tr>
<td>tilt angle (deg) vs surface normal after washing (replace the peptide solution with PBS for several times)</td>
<td>48</td>
<td>51</td>
<td>54</td>
<td>50</td>
<td>47</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. SFG spectra collected from cCP1 molecules at the PS-MA/peptide solution interface with different cCP1 PB concentrations: (a) 1.12, (b) 1.50, and (c) 3.74 $\mu$M. (d) SFG spectra collected from the cCP1 molecules at the PS-MA/PBS interface after the PS-MA surface is in contact with cCP1 PBS solution with a concentration of 1.50 $\mu$M; then the cCP1 PB solution was replaced by new PB solutions twice.

Figure 5. (a) ATR-FTIR spectra detected from the PS-MA/cCP1 buffer solution interface with different peptide solution concentrations. (b) ATR-FTIR signal intensities of the 1645 cm$^{-1}$ peak at different peptide concentrations for cCP1.
Using ATR/FTIR, we can then deduce the relative adsorption amount in a semiquantitative fashion of cCP1 at the PS-MA/peptide solution interface. Since ATR-FTIR is linear spectroscopy, the adsorption amount should be linearly related to the ATR-FTIR signal intensity. Figure 5b shows that the adsorption amount of cCP1 at the interface when the peptide concentration is 0.37 μM is about three times that at the interface when the peptide solution is 0.19 μM. This can be correlated to the SFG spectral intensity difference detected from these two cases. If we compare the SFG signal intensities observed in the ssp spectra, the intensity detected from the interfacial cCP1 at the peptide concentration of 0.37 μM is about 20 times compared to that detected at the peptide concentration of 0.19 μM. The adsorption amount difference observed by ATR-FTIR is responsible for a factor of 10 difference (SFG intensity is proportional to the square of adsorption amount), and the different orientations may lead to another factor of 2 (the lying-down orientation generates weaker SFG amide I signal from the α-helical structure). The weak SFG signal from the 0.19 μM case prohibits us to perform a quantitative analysis. However, we can see that the ATR-FTIR results can be correlated to SFG data in a semiquantitative fashion.

Figure 5b also shows that the adsorbed amount of cCP1 at the interface when the peptide concentration is 3.7 μM is about 1.3 times of that at the peptide solution of 0.37 μM. The will lead to 1.3 × 1.3 = 1.7 times of the SFG amide I spectral intensity difference detected from these two cases. If we compare the SFG signal intensities observed in the ssp spectra, the one detected from the interfacial cCP1 at the peptide concentration of 3.7 μM is about 1.5 times compared to that detected at the peptide concentration of 0.37 μM. The slight difference between 1.5 and 1.7 might be due to the intensity dependence on the peptide orientation, which is difficult to quantify here. Nevertheless, here again we believe that the ATR-FTIR results are well correlated to the SFG data.

3.4. Further Discussion. It is important to note that after replacing the peptide solution with the buffer, the cCP1 molecules at the interface changed their orientations. For the 0.37 μM solution, the orientation changed from ~15° versus the surface normal to approximately ~50° versus the surface normal. For the 1.50 μM solution, the orientation of interfacial cCP1 molecules changed from a multiple-orientation distribution to a single orientation with approximately a 50° orientation angle versus the surface normal. It is also interesting to see that the final orientation of cCP1 molecules at the PS-MA/buffer interface is the same when the initial concentrations and interfacial orientations are different. It is reasonable for the two orientations to be similar since they are both measured from cCP1 molecules at the PS-MA/PB interface; this seems to be the preferential orientation. This orientation is also similar to that measured from the cCP1 molecules at the PS-MA/peptide solution interface with a peptide concentration of 0.28 μM.

When we investigated the CP1c molecules previously (C-terminus is cysteine modified), we found that a multiple-orientation distribution of CP1c was observed on the PS-MA surface regardless of the peptide concentrations, showing that both physical adsorption and chemical immobilization occurred.16,38 Differently, here when cCP1 is used (N-terminus is cysteine modified), at low peptide concentrations, a single orientation of cCP1 was deduced at the interface. This study indicates clearly that the CP1c and cCP1 molecules interact with the PS-MA surface differently. Figure 6 shows a schematic for such differences. This difference might be due to the differences in the peptide segments near the N-terminus and the C-terminus of CP1. The N-terminus of CP1 is amphiphilic and positively charged, while the C-terminus is hydrophobic. Our further experiments on chemically immobilized CP1c and cCP1 on maleimide-terminated self-assembled monolayers show that they behave very differently. Both SFG experimental studies and molecular dynamics simulations indicate such distinct differences on SAMs. In conjunction with structural studies of CP1 immobilized on SAMs, investigation of the binding of bacterial lipopolysaccharides to both CP1c and cCP1 have shown the effect of immobilization not just on peptide orientation but on interaction with analyte molecules.17 For the polymer system studied here, more details about the different molecular interactions between PS-MA and cCP1 and CP1c are being investigated using molecular dynamics simulations and will be reported in the future.

4. CONCLUSION

In this research, SFG spectra were collected from cCP1 molecules at the PS-MA/peptide buffer solution interface of different peptide solution concentrations using different polarization combinations ssp and ppp. All detected SFG spectra are dominated by a single peak centered at ~1650 cm⁻¹, showing that cCP1 molecules adopted an α-helical conformation at the PS-MA/peptide solution interface, which is similar to previous results from the CP1c molecules. In previous studies, the χ_{ssp}/χ_{ppp} signal strength ratio of CP1c molecules collected from the PS-MA/peptide solution was found to be independent of the peptide solution concentration. The χ_{ssp}/χ_{ppp} ratio remains the same for CP1c concentrations varied from 0.56 to 75 μM in buffer, leading to a multiple-orientation distribution. However, for the cCP1 molecules, the χ_{ssp}/χ_{ppp} ratio changes as a function of the peptide concentration at lower peptide concentrations. cCP1 molecules at the PS-MA/peptide solution interface change orientation from more or less lying down at the interface at 0.19 μM to more or less standing up at the interface at 0.75 μM. When the peptide concentration becomes even higher, e.g., for 1.12, 1.50, and 3.74 μM, the cCP1 molecules at the interface adopt a multiple-orientation distribution because, in addition to the chemically immobilized cCP1 molecules, more physisorbed cCP1 molecules are found at the interface. The orientation angles of cCP1 at the PS-MA/peptide solution interface at different peptides concentration are summarized in Table 1.

We believe that this is the first observation of the different interfacial behavior of the same peptide immobilized on a surface via different termini using SFG. Such differences are
likely due to the different interfacial interactions. We also observed such differences using SFG on a simpler system in which peptides are immobilized on SAMs, and the results can be understood by molecular dynamics simulations, which will be reported in the near future. However, polymers are more widely applicable on various substrates than SAMs; therefore, further studies on interactions between peptides and PS-MA are being carried out using molecular dynamics and will be reported in the future. As we indicated previously, peptides have the potential to supplement antibodies for many biosensing applications. However, before peptide-based applications, such as sensors, can be developed, it is crucial to understand the peptide interfacial molecular structure in order to ascertain the potential effects on the desired analyte interactions. Our observations here that the same peptide immobilized at opposing termini take on different surface orientations is a key first step.

**AUTHOR INFORMATION**

**Corresponding Author**
*E-mail: Charlene.Mello@us.army.mil, zhanc@umich.edu.*

**Notes**
The authors declare no competing financial interest.

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