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Solvent Effect and Time-Dependent Behavior of C-Terminus Cysteine Modified Cecropin P1 Chemically Immobilized onto Polymer Surface

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Abstract

Sum frequency generation (SFG) vibrational spectroscopy has been applied to investigate peptide immobilization to a polymer surface as a function of time and peptide conformation. Surface immobilization of biological molecules is important in many applications such as biosensors, antimicrobial materials, bio-based fuel cells, nanofabrication, and multi-functional materials. Using C-terminus cysteine modified cecropin P1 (CP1c) as a model, we investigated the time-dependent immobilization behavior *in situ* in real time. In addition, potassium phosphate buffer (PB) and mixtures of PB and trifluoroethanol were utilized to examine the effect of peptide secondary structure on CP1c immobilization to polystyrene maleimide (PS-MA). The orientation of immobilized CP1c on PS-MA was determined using polarized SFG spectra. It was found that the peptide solution concentration, solvent composition, and assembly state (monomer vs. dimer) prior to immobilization all influence the orientation of CP1c on a PS-MA surface. The detailed relationship between interfacial peptide orientation and these immobilization conditions is discussed.

1. Introduction

Extensive research has been performed to better understand the physical and chemical properties which control biomolecular structures and orientations on surfaces. Accurate control and detailed characterization of such interfacial phenomena are important to ensure optimization of biological function and performance in many applications including biomedical coatings and implant devices, antimicrobial materials, biosensors and numerous other nanodevices.[1-12] The characterization of interfacial biomolecules, such as peptides and proteins *in situ* at the solid/liquid interface, is challenging due to the lack of appropriate analytical tools. Recently, sum frequency generation (SFG) vibrational spectroscopy has been applied to examine molecular structures of various interfaces, including biological molecules at interfaces.[13-39] Our lab has successfully applied SFG to determine orientation information of various proteins and peptides at interfaces.[40-51] In addition, SFG data analysis methodology has been developed to deduce orientation of various secondary structures such as alpha-helix, 3-10 helix, and anti-parallel beta-sheet at solid/liquid interfaces and in model cell membranes.[40-42] Using such methods, we have successfully determined interfacial orientation of various peptides including magainin 2, melittin, alamethicin, tachyplesin I, G-protein, and fibrinogen.[43-51]

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Our most recent report summarized studies of the alpha-helical peptide cecropin P1 modified with a cysteine amino acid at the C-terminus (CP1c).[51] CP1c molecules are chemically immobilized onto a polystyrene maleimide (PS-MA) surface by forming a thioether bond between the cysteine group in CP1c and the maleimide group of the PS-MA surface (Figure 1). It was found that CP1c molecules adopt a preferred orientation after being chemically immobilized onto the PS-MA surface. In comparison, CP1c molecules that were physically adsorbed onto a polystyrene (PS) surface lacking the maleimide groups do not possess a delta or Gaussian orientation distribution, revealing a distribution with multiple preferred orientations[51] (multiple-orientation distribution[41]).

It is important to understand cecropin orientation on surfaces and interfaces because cecropin and other antimicrobial peptides (AMPs) have been used to capture and sense bacterial pathogens such as *E. coli* O157:H7 and *Salmonella*.[52-58] It was further shown that AMPs are highly stable under adverse conditions and bind selectively to microbial cell surfaces, exerting their antimicrobial activity through membrane disruption.[56] Currently, antibody-based biosensors are widely used to detect a variety of biological molecules and various organisms including bacteria. However, antibody-based biosensors have some disadvantages such as low stability in harsh environments, lack of batch-to-batch consistency, high costs of monoclonal development, low specificity when background interferents are present, or the requirement that there be at least one binding pair for every target detected.[56] Therefore, AMPs have potential to replace antibody-based biosensors in the future.

Mello et al. investigated the interactions between CP1c and pathogenic *E. coli*[59] and showed that when CP1c molecules were deposited on surfaces using different methods, the activities of the surface-immobilized CP1 molecules can be quite varied. For example, CP1 molecules physically adsorbed and chemically immobilized via the C-terminus exhibited very different pathogen binding properties and killing efficiencies against *E. coli*.[59] Clearly, such observations can be interpreted using our SFG results which showed that CP1c molecules adopted different orientations/orientation distributions when different surface immobilization/adsorption methods were used.

In this research, we followed the chemical immobilization process of CP1c molecules onto PS-MA surface as a function of time. Solvent composition was also investigated to determine how the conformation of CP1c in solution will influence immobilization kinetics to a PS-MA surface as well as their orientation on the surface. In addition, adsorption behavior of CP1c dimers, prepared in the CP1c solution without the addition of reducing agent TCEP, onto a PS-MA surface will be investigated using SFG. We demonstrated that factors such as the CP1c solution concentration, solvent composition, and presence of the reducing agent in the solution all influenced the final interfacial orientation of CP1c.

2. Experimental

2.1 Materials and Sample Preparation

Polystyrene (PS) (standard, narrow distribution, Mw = 393,400) was ordered from Scientific Polymer Products, Inc. (4-Maleimidobutyramidomethyl) polystyrene (PS-MA, molecular formula shown in Figure 1), potassium phosphate (monobasic and dibasic) solution (1 M), tris (2-carboxyethyl) phosphine (TCEP) hydrochloride solution (0.5 M, pH 7.0), dichloromethane, toluene and 2, 2, 2-trifluoroethanol (TFE) were all purchased from Aldrich (Milwaukee, WI, USA). Cysteine-terminated cecropin P1 (CP1c, sequence H_2N-SWLSKTAKKLENSAKKRISEGIAIAIQGGPRC-OH, MW = 3,442) was synthesized using standard FMOC-solid phase methods by New England Peptide (Gardner, MA, USA). EDTA was obtained from Fisher Biotech.

The substrates and right-angle CaF_2 prisms were purchased from Altos (Bozeman, MT). CaF_2 prisms were soaked in toluene for 24 hours, and then sonicated in Contrex AP solution from Decon Labs (King of Prussia, PA) for 1 hour. The prisms were thoroughly rinsed with DI water and placed into a glow discharge plasma chamber to be cleaned for 4 minutes immediately before being coated with polymers. PS-MA films were coated on CaF_2 prisms by using a spin coater from Specialty Coating Systems from a 0.01% PS-MA/ dichloromethane solution at 1500 rpm. The PS films were spin coated from a 1 wt % PS/ toluene solution at 2500 rpm. The polymer films were kept at room temperature for 24 h prior to performing SFG experiments.

A 50 mM phosphate buffer solution (PB), pH 6.5, was prepared by mixing monobasic potassium phosphate solution (1 M) and dibasic potassium phosphate solution (1 M) and DI water (purified by a Millipore system). CP1c was dissolved in PB, PB/TFE, or PB/ethanol mixtures. TCEP and EDTA were added to reduce the disulfide bond among individual peptide molecules and prevent chelating between thiol groups and metals present in the buffer. The reduction conditions were 3.0 mM TCEP and 1.0 mM EDTA.

The thiol moiety in the cysteine residue has a strong affinity for the maleimide moieties, which promotes the covalent immobilization of the peptide to the PS-MA surface, as shown in Figure 1.

2.2 Circular Dichroism Experiments

Circular Dichroism (CD) spectra were taken on a Jasco circular dichroism spectrometer. Experiments were carried out at a controlled temperature of 25 °C. Wavelengths were scanned from 200 to 260 nm. Monochromator scan rate was 50 nm/minute, with a bandpass of 1 nm and acquisition time of 4 seconds per data point. Each measurement consisted of 10 scans averaged together. A strain-free quartz cell with a path length of 1 mm was used. Solutions were made up in 50 mM pH 6.5 potassium phosphate buffer with 1.0 mM EDTA mixed with TFE. Peptide solutions were reduced with the addition of 3.0 mM TCEP and overnight incubation at room temperature.

2.3 SFG measurements

Details regarding SFG theories and equipment have been reported previously[60-65] and will not be repeated here. Some SFG data analysis methods used in this research are presented in the previous study on CP1c using SFG.[51] The authors' SFG system has been described in detail previously as well.[66] All of the SFG experiments were carried out at room temperature. SFG spectra with different polarization combinations including ssp (s-polarized SF output, s-polarized visible input, and p-polarized infrared input) and ppp were collected using the near total internal reflection geometry.[51,66] SFG time-dependent signal was collected by fixing the IR frequency at a peak center in the SFG spectrum and detecting SFG signal as a function of time.

3. Results and Discussions

3.1 CP1c in PB Buffer

In our previous study, we collected SFG spectra at one hour after contacting the PS-MA surface with a CP1c solution concentration of 75 μM.[51] The spectra are dominated by a single peak centered at 1650 cm^{-1}, contributed by the CP1c amide I mode. One hour is sufficient time for the adsorbed/immobilized CP1c molecules to reach equilibrium.[51] This concentration is too high to follow detailed immobilization kinetics, therefore in this present study we used CP1c solutions with much lower concentrations: 5.6, 2.3, and 1.1 μM and monitored the SFG signal at 1650 cm^{-1} immediately after contacting the PS-MA surface to

follow the immobilization process (Figure 2). Figure 2 clearly demonstrates that the rate of immobilization and time required to reach equilibrium (evidenced by no further time-dependent SFG signal change) are dependent upon the CP1c solution concentration. It can be seen from Figure 2 that it took about 200, 250, and 1000 seconds for CP1c immobilization onto the PS-MA surface to reach equilibrium when solutions of 5.6, 2.3, and 1.1 μM CP1c in PB were used respectively.

SFG spectra were collected from the PS-MA/CP1c solution interface after the time-dependent SFG signal reached the plateau using ssp and ppp polarization combinations (Figure 3). The SFG spectra had very similar features to those collected in the previous research, even though the CP1c solution concentration was much lower. The dominating peak in all the spectra was centered at 1650 cm^{-1}, and was contributed by the alpha-helical secondary structure. The SFG spectra can be fitted quite well by using only one peak, thus showing that the interfacial CP1c molecules adopt an alpha-helical conformation. Their signal strength ratio (χ_{ppp}/χ_{ssp}) can be calculated from the fitted parameters of the amide I band in the ppp and ssp spectra. For the three CP1c solutions studied here, the χ_{ppp}/χ_{ssp} ratios are measured to be 1.11 (for 5.6 μM), 1.15 (for 2.3 μM), and 1.07 (for 1.1 μM). These measured ratios are very similar to the one published previously, which is 1.05.[51] Using this measured ratio, the orientation of alpha-helical CP1c at the PS-MA/CP1c interface may be deduced.[51]

Figure 4 displays the relationship between the orientation angle of an interfacial alpha-helical CP1c molecule and the measured χ_{ppp}/χ_{ssp} ratio, assuming a delta orientation distribution or Gaussian distributions with various angle distributions.[40,51] The measured ratios here do not fall in the range for alpha-helical molecules with a delta or a Gaussian distribution. As we discussed previously, the deposited CP1c molecules can have a multiple-orientation because some molecules are chemically immobilized, but others are physically deposited.[51]

It has previously been reported (also supported by our CD data herein) that in a solution of PB, CP1c possesses a very low alpha-helical content,[59] with a predominance of random structure. Our SFG spectra collected from interfacial CP1c molecules (Figure 3) indicated that they adopted an alpha-helical conformation at the PS-MA/peptide solution interface. Therefore, when CP1c molecules were deposited onto the PS-MA surface a significant structural change is observed. We believe that this process can be quite complicated, and thus individual CP1c molecules may experience a different interaction with the PS-MA surface. Some of them can be immobilized chemically, while others are loosely associated with the surface, leading to multiple orientations.

3.2 CP1c in 2,2,2-triflouroethanol/PB Mixtures

Hydrophobic solvents such as 2,2,2-triflouroethanol (TFE)- with PB are well known to induce a conformational transformation from random coil (buffer alone) to alpha helical.[59] CD spectra of CP1c were taken in TFE-PB solutions with a range of TFE content. Figure 5 (A) shows these CD spectra of CP1c with a final concentration of 3.7 uM in TFE-PB solutions with 0%, 25%, 50%, and 75% v/v of TFE. Alpha helical content of CP1c can be estimated from mean residue ellipticity at 222 nm.[67] Figure 5 (B) indicates that 25% TFE in the solution is insufficient to produce substantial helical content of CP1c in solution, but 50% or higher TFE is sufficient to do so. A sharp change of CD spectrum is visible between 25% and 50%, while changes between 0% and 25% TFE and 50% and 75% TFE are relatively smaller.

Therefore in the 50% TFE-PB solution, a substantial portion of CP1c molecules are adopting an alpha-helical conformation. Thus their adsorption/immobilization process to the

PS-MA surface from the TFE-PB solution may be simpler than that from the PB solution alone. It is not necessary for a conformational transition to occur at the interface while adsorbing to the PS-MA/solution interface. We hypothesized that a simple immobilization which circumvents this conformational change may result in peptides which adopt a preferential orientation rather than multiple orientations. This will be confirmed experimentally below.

SFG spectra were collected from CP1c molecules at the PS-MA/CP1c 50%TFE-50%PB solution interface after contact for one hour using ssp and ppp polarization combinations (Figure 6). It can be seen in Figure 6 that the SFG spectra are still dominated by a single peak at 1650 cm^{-1}, indicating that the interfacial Cp1c molecules adopt an alpha-helical conformation. Unlike the above PB solution case, the χ_{ppp}/χ_{ssp} ratios obtained by fitting the ppp and ssp spectra are relatively high. The measured χ_{ppp}/χ_{ssp} ratios for CP1c solutions with 6.6 and 1.1 χM concentrations are 1.37 and 1.80 respectively. According to Figure 4, assuming a delta distribution, the orientations of CP1c in 50%TFE-50%PB at a PS-MA/CP1 solution interface vary with the solution concentration. The orientations at 6.6 and 1.1 μM CP1c are 15 and 55 degrees relative to the surface normal, respectively. Presumably, when the CP1c concentration is high (e.g., 6.6 μM) a larger number of CP1c molecules can be immobilized onto the PS-MA surface at equilibrium. This may result in molecular crowding and therefore to accommodate this crowding the peptides stand more upright at the interface, producing a smaller orientation angle versus the surface normal. When the concentration is low (e.g., 1.1 μM), fewer molecules can be immobilized onto the PS-MA surface, thus each molecule has more space and can tilt more at the interface. As expected, when alpha helical CP1c molecules were immobilized to the PS-MA surface negating the need for conformation change at the interface, they adopt a preferred orientation, rather than multiple orientations.

When the TFE percentage in PB was varied (0-75%), the immobilized CP1c molecules at the PS-MA/CP1c solution interface also adopted different orientations. In these studies, the CP1c concentration is 3.7 μM. These SFG spectra displayed in Figure 7 are still dominated by a single peak centered at 1650 cm^{-1}, confirming that interfacial CP1c molecules still adopt an alpha-helical structure. When the peptides are exposed to 50 and 75% TFE in-PB the fitted χ_{ppp}/χ_{ssp} ratios of interfacial CP1c molecules are 1.49 and 1.39, respectively. Both ratios can be correlated to a delta distribution, and had orientation angles of 30 and 17 degrees versus the surface normal, respectively. In both cases, CP1c molecules tend to stand up at the interface. However, in a 25% TFE in PB solution, the χ_{ppp}/χ_{ssp} ratio of interfacial CP1c molecules was measured to be 1.26, which is beyond the possible range for a delta or Gaussian orientation distribution. This is similar to the orientation of CP1c molecules at the PS-MA/peptide PB solution interface, suggesting that the orientation angles of CP1c molecules are dependent on the solution TFE concentration and corresponding alpha helical content, as revealed by CD experiments above. When the TFE concentration is low, many CP1c molecules possess a random coil structure in the solution, therefore, they need to transition to the alpha-helical structure during deposition onto the PS-MA surface. Clearly, 25% TFE solvent composition is not sufficient to ensure a delta or Gaussian distribution for CP1c molecules immobilized onto a PS-MA surface, while 50% TFE (or more) in the solvent is sufficient.

It is worth mentioning that a delta or Gaussian distribution with a small deduced orientation angle will have a narrow orientation distribution according to the curve in Figure 4. In the above determination, there were several cases in which the orientation angle was small, e.g., 15, 17, or 30 degrees. Thus, in these cases, the orientation distribution is narrow. Previously, we have extensively studied orientation of peptides, especially helical peptides, in a lipid bilayer. Since a lipid bilayer is a well defined environment, the orientation distribution of the

peptides is usually narrow, as we demonstrated.[40,41.48,49] We also studied orientations of peptides and proteins physically adsorbed on various polymer surfaces.[23,42,44,68,69] Since polymer surfaces can have quite complicated structures and the physically adsorbed peptides and proteins can have many different orientations, the deduced orientation distribution can be quite broad.[42] It is interesting to see that the orientation of a peptide chemically immobilized on a polymer surface can be narrow in this research. This is because the c-terminus of the peptide is bound to the surface, resulting in a similar orientation for all the immobilized peptides.

Figure 8 displays the time-dependent SFG signals at 1650 cm^{-1} collected from the CP1c molecules in various TFE-PB solvent compositions immobilized on the PS-MA surface. All three time-dependent changes are different from those shown in Figure 2. The time-dependent SFG signal collected from the 25% TFE solvent is most similar to those shown in Figure 2. As discussed above, it is possible that some CP1c molecules were adsorbed to the PS-MA surface, and at the same time underwent a conformation change from random structures to alpha helical structures. Therefore, the time-dependent SFG signal is similar to the PB case. However, when the solvent only contains PB, the signal remains stable after reaching a plateau (Figure 2). This is slightly different from that observed with 25% TFE in which the SFG signal decreased slightly after reaching a maximum. The small decrease may be due to the immobilized molecules changing their orientations slightly as a function of time. The time-dependent SFG signal decrease was more pronounced when a higher percentage of TFE was used. For example, the time-dependent SFG signal of a 75% TFE in PB substantially decreased after reaching the maximum. In this case, CP1c molecules had already adopted an alpha-helical structure in the solution. They were quickly immobilized onto the PS-MA surface, therefore, the SFG signal increased substantially within the first tens of seconds. Then the immobilized CP1c molecules change orientation gradually, so the signal decreased and then reached the equilibrium at 500 seconds. Figure 8 shows that the SFG intensity decreased as a function of time, indicating that the CP1c molecules titled more at the interface as time went by. This is further confirmed by the time-dependent intensity ratio of the ppp and ssp signal below. The time-dependent SFG signal detected from the CP1c molecules at the PS-MA/solution (with 50%TFE-50%PB) interface exhibits an intermediate trend compared to the two previous solvent composition extremes.

Time-dependent SFG ssp and ppp signals detected from CP1c molecules at the PS-MA/ solution (75%TFE in PB) interface were shown in Figure 9. Both SFG signals decreased as a function of time after reaching the maximum, showing that the immobilized CP1c molecules tilted more at the interface. The ratio of the ppp/ssp spectral intensities was also shown in Figure 9, which increased between 100 and 500 seconds. According to Figure 4, this indicates that CP1c molecules tilted more at the interface. This ratio has substantial changes before 100 seconds. Since the spectral intensity in both ssp and ppp spectra changed substantially within the first 100 seconds, we may not be able to reliably analyze such an intensity ratio change over such a short time. Thus we will not discuss it further. After 500 seconds, the ratio remains approximately constant, showing that the CP1c molecules would not change orientations any more.

It is interesting to observe the immobilization behaviors of CP1c molecules onto the PS-MA surface when different solvents, PB or TFE-PB, were used. It is believed that the variations in CP1c behavior were caused by the hydrophobic TFE in the solution, which enabled CP1c molecules to form alpha helices in the solution. Therefore, it is the perflouro group in TFE that was significant. This can be further confirmed by using a mixture of regular ethanol and PB as the solvent to prepare the CP1c solution. Time-dependent SFG signal collected from the CP1c molecules at the PS-MA/CP1c solution (75% ethanol in PB) interface is shown in Figure 10. For comparison purposes, time-dependent SFG signal from the PS-MA/CP1c PB

solution interface is shown in the same figure. The two curves are almost the same, and are very different from the time-dependent SFG signal when 75% TFE was used in the solvent. Therefore, we believe that when ethanol is used, CP1c molecules still adopt a random structure. When they are immobilized onto the PS-MA surface, they change their conformation to an alpha helical structure, exhibiting the same time-dependent behavior as the PB case shown in Figure 2.

3.3 CP1c Solution without Reducing Agent

A remaining question is whether the conformation change of CP1c molecules at the PS-MA/PB solution interface is completed. If some random structured CP1c molecules remain at the interface, and if they could contribute to amide I signals centered at 1650 cm^{-1}, then their existence would affect the ppp/ssp signal strength ratio. This would lead to errors in orientation determination because the orientation analysis is based on the alpha-helical structure. Is it possible that this is the reason that some measured ppp/ssp signal strength ratios were beyond the possible range for a delta or Gaussian distribution? We believe that this is highly unlikely because the SFG spectral features of CP1c molecules at the PS-MA/peptide PB solution interface are identical to those detected from CP1c molecules in lipid bilayers.[51] It is well known that CP1 molecules adopt an alpha-helical structure in lipid bilayers.[70,71] We designed an experiment to further confirm that the ppp/ssp signal strength ratios that were beyond the range for a delta and a Gaussian distribution were not due to some of the CP1c molecules having random structures.

As we demonstrated in the experimental section, all the SFG spectra and time-dependent SFG signals were collected from CP1c molecules at the PS-MA/peptide solution interfaces while enough reducing agent TCEP/EDTA was added to the solutions. The addition of the reducing agent into the CP1c solution ensures that the CP1c molecules do not form intermolecular disulfide bonds. Therefore the cysteine residues at the CP1c C-terminus can react with the maleimide group on the PS-MA surface, resulting in chemical immobilization of CP1c molecules onto the PS-MA surface. In the following experiment, we prepared the CP1c solution by dissolving CP1c molecules in a 75%TFE in PB without the addition of TCEP. In this case, CP1c molecules may form dimers, but the dimers can still adopt alpha-helical conformation. When the PS-MA surface is in contact with this CP1c solution, CP1c dimers should be physically adsorbed onto the PS-MA surface because there are not free cysteine groups to react with the PS-MA surface. Since most CP1c molecules form dimers, they would not be able to adopt a delta or Gaussian distribution, but multiple distributions. Therefore, the measured ppp/ssp signal strength ratio should be beyond the range of a delta or Gaussian distribution. The SFG spectra collected from the CP1c molecules at the PS-MA/solution (75%TFE in PB without TCEP) interface using ssp and ppp polarization combinations are shown in Figure 11. The fitted ppp/ssp signal strength ratio of 1.23 is beyond the possible range for a delta or a Gaussian distribution, which is as expected. A schematic of a CP1c dimer is also shown in Figure 11. Apparently, when such dimers are immobilized onto a surface, the orientation distribution of alpha helices cannot be a delta or Gaussian distribution. Time-dependent SFG signal from CP1c dimers adsorbed onto a PS-MA surface (Figure 10b) exhibited a different behavior in comparison to those shown in Figure 2. We believe that there is no substantial conformational change for the dimer case with 75% TFE in the solvent because the signal decreases after reaching the maximum, which is due to an orientation change. After 600 seconds, SFG signal remains approximately the same.

Since the solvent used for the above experiment contains 75% TFE, the dimers should approximately adopt an alpha helical structure in the solution and at the interface. Therefore, the reason that the ppp/ssp signal strength ratio is beyond the possible range for a delta or a Gaussian distribution is not because CP1c molecules adopt a random structure, instead, it is

because the different orientations of the two monomer alpha helices in dimers at the interface (leading to multiple distributions). For comparison purposes, time-dependent SFG signal was also collected from CP1c dimers at the PS-MA/CP1c PB solution interface without the addition of TCEP (not shown). In this case, the time-dependent SFG signal is similar to those shown in Figure 2. The dimers were adsorbed to the PS-MA surface while simultaneously changing conformation.

In our previous study, we compared the chemical immobilization of CP1c onto a PS-MA surface and physical adsorption of CP1c onto a PS surface.[51] The above studies in this research were all based on the PS-MA surface. We then decided to study CP1c molecules at the PS/solution (with 50% TFE and 50% PB) interface. Unlike the previous study, CP1c molecules in the solution had already adopted an alpha helical structure. As we showed above, when CP1c dimers were adsorbed onto the PS-MA surface, a multiple-orientation was determined. For this dimer case, due to the formation of inter-molecular disulfide bonds, there is no free cysteine group in CP1c which can react with the maleimide groups on the PS-MA surface. Therefore, CP1c dimers should be physically adsorbed onto the PS-MA surface, which may be similar to the physical adsorption of CP1c molecules onto a PS surface shown previously. Surprisingly, no discernable SFG signal can be observed from the PS/CP1c solution (with 50% TFE) interface. This shows that the alpha-helical CP1c molecules either do not segregate to the PS/solution interface, or have a random orientation at the PS/solution interface. The difference between the CP1c dimer/PS-MA and CP1c/PS cases when TFE is used indicates that the interaction between the CP1c dimers and the PS-MA surface is different from that between the CP1c molecules and the PS surface. Also, this is different from the previous observed case when only PB is used as the solvent. This shows that the interaction between the random structured CP1c molecules and the PS surface is different than the interaction between alpha-helical CP1c molecules and the PS surface.

Conclusion

In this research, we studied the time-dependent behavior of immobilization of CP1c molecules onto PS-MA surfaces in different CP1c solutions *in situ* in real time using SFG. When a CP1c PB solution was used, it was found that CP1c immobilization reaches equilibrium faster when the CP1c solution concentration is high. Regardless of the solution concentration, the deposited CP1c molecules adopted a multiple-orientation distribution, perhaps due to the deposition and conformational change taking place simultaneously. When 50% or more TFE was used in the CP1c solution, immobilized CP1c molecules on PS-MA adopted a single orientation. This is because CP1c molecules formed an alpha-helical structure in the solution. The immobilization process onto the PS-MA surface did not involve conformational change of the peptide. Time-dependent SFG studies indicated that, in this case, CP1c molecules were immobilized onto the PS-MA surface and then orientation changes occurred. SFG was also applied to investigate CP1c dimers at the PS-MA/peptide solution interface. Results indicated that the CP1c dimers exhibit a multiple-orientation distribution at the interface as expected. As we discussed in the introduction, peptide molecules have great potential to replace antibodies in order to develop robust biosensors. However, characterization of peptide structure after surface immobilization is difficult. We successfully demonstrated that SFG can be applied to examine peptide conformation and orientation at the solid/liquid interface *in situ*. Time-dependent behaviors of interfacial peptides can be followed in real time. It was found that the solvent composition, reducing agent, peptide concentration, and surface structure can greatly affect the immobilized peptide structure, including orientation.

In order to optimize the interfacial structure and orientation of surface immobilized peptides or proteins to achieve the best activity, immobilization of the same molecule through

different amino acids should be studied and compared. Currently, we are studying N-terminus cysteine modified cecropin P1 immobilized onto the PS-MA surface. We found that it has markedly different immobilization behavior compared to CP1c, which will be reported in a forthcoming article.

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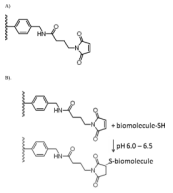

Figure 1.
A). (4-Maleimidobutyramidomethyl) polystyrene; B). Schematic showing S-H groups linking to maleimide groups.

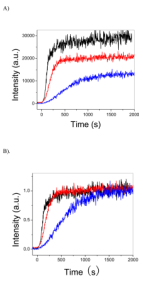

Figure 2.

A). Time-dependent SFG signal (ppp) at 1650 cm^{-1} collected from CP1c molecules at the PS-MA/CP1c PB solution interface B). Same signals as those shown in (A) but normalized according to the final equilibrium value. The black, red and blue curves are for 5.6, 2.3, and 1.1 μM CP1c solutions respectively. The results clearly demonstrate that the rate of immobilization is faster and time required to reach equilibrium (evidenced by no further time-dependent SFG signal change) is shorter for the CP1c solution with a higher concentration.

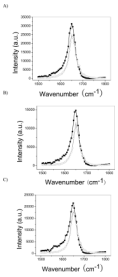

Figure 3.

SFG ssp (open dots) and ppp (filled dots) spectra collected from the PS-MA/CP1c PB solution interface after equilibrium is reached. Concentrations for CP1c solutions are A) 5.6, B) 2.3, and C) 1.1 μM respectively. The dominating peak in all the spectra centered at 1650 cm^{-1} indicates that the peptides adopt an alpha-helical secondary structure.

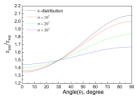

Figure 4.
Relations between the SFG susceptibility tensor component ratio and the α-helix orientation angle with different orientation distributions (assuming Gaussian distributions).

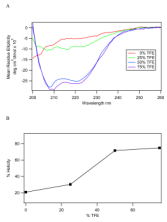

Figure 5.

A). Circular dichroism spectra of 3.7 uM CP1c in 50 mM pH 6.5 potassium phosphate buffer with various amounts of TFE. A sharp change of CD spectrum is visible between 25% and 50%, while changes between 0% and 25% TFE and 50% and 75% TFE are relatively smaller. B). Alpha helical content of CP1c in 50 mM potassium phosphate pH 6.5 solution with various amounts of TFE, estimated from mean residue ellipticity at 222 nm.[67] The alpha helical content is low in solutions with 0% and 25% TFE, but is high in solutions with 50% and 75% TFE

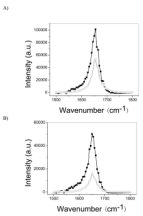

Figure 6.

SFG ssp (open dots) and ppp (filled dots) spectra collected from: A). PS-MA/CP1c solution (with 50%TFE-50%PB, CP1c concentration 6.6 μM) interface; b). PS-MA/CP1c solution (with 50%TFE-50%PB, CP1c concentration 1.1 μM) interface. Different ppp/ssp intensity ratios indicate that the peptide orientations are different for different peptide solution concentrations.

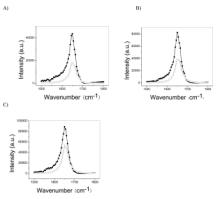

Figure 7.
SFG ssp (open dots) and ppp (filled dots) spectra collected from CP1c at the PS-MA/CP1c solution interfaces with different solvent compositions: A) 75%TFE-25% PB; B) 50%TFE-50%PB; C) 25%TFE-75%PB. The ppp/ssp intensity ratios indicate that for solutions with 50% and 75% TFE, peptides adopt a single orientation at interfaces. For solutions with 25% TFE, peptides adopt a multiple-orientation distribution.

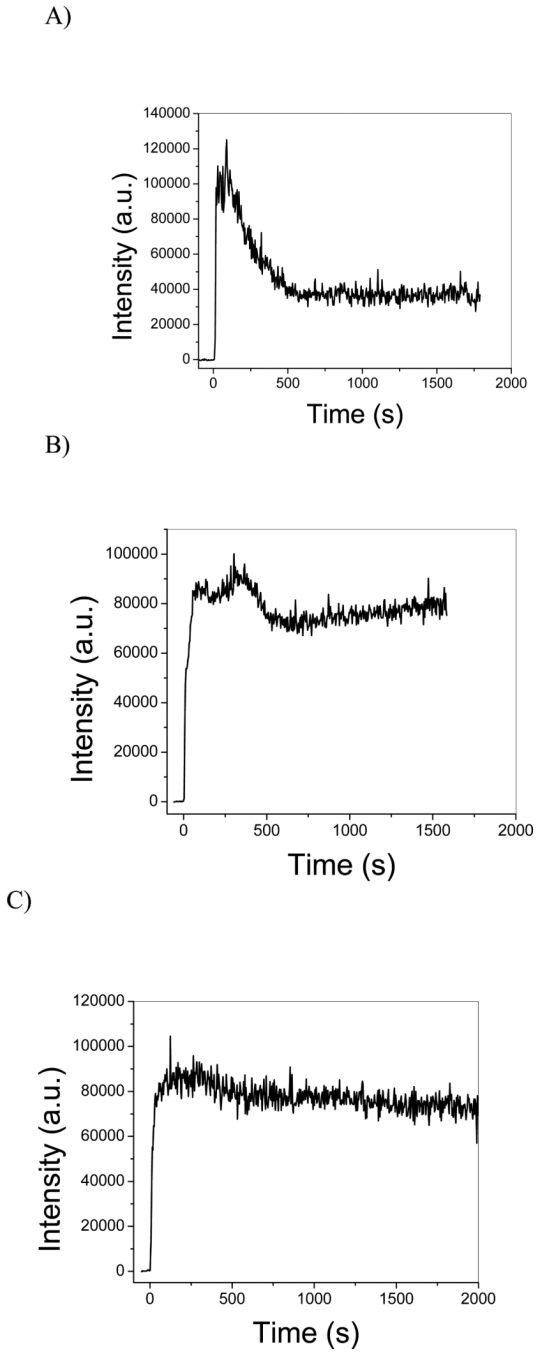

Figure 8.

Time-dependent SFG ppp signal collected from the CP1c molecules at the PS-MA/CP1c solution interfaces with different solvent compositions: A) 75%TFE-25%PB; B) 50%TFE-50%PB; C) 25%TFE-75%PB. The time-dependent signals are different from that collected from the PS-MA/CP1c PB solution interface.

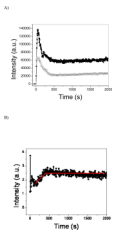

Figure 9.

A) Time-dependent SFG signal collected from the CP1c molecules at the PS-MA/CP1c solution interfaces with solvent composition of 75%TFE-25% PB; ppp: filled dots, ssp: open dots. Both SFG signals decrease as a function of time after reaching the maximum, showing that the immobilized CP1c molecules tilte more at the interface. B) Time-dependent intensity ratio between the ppp and ssp amide I signal at 1650 cm^{-1} collected in A), two red lines are used to guide eyes. The ratio of the ppp/ssp spectral intensities increases between 100 and 500 seconds, showing that CP1c molecules tilte more at the interface.

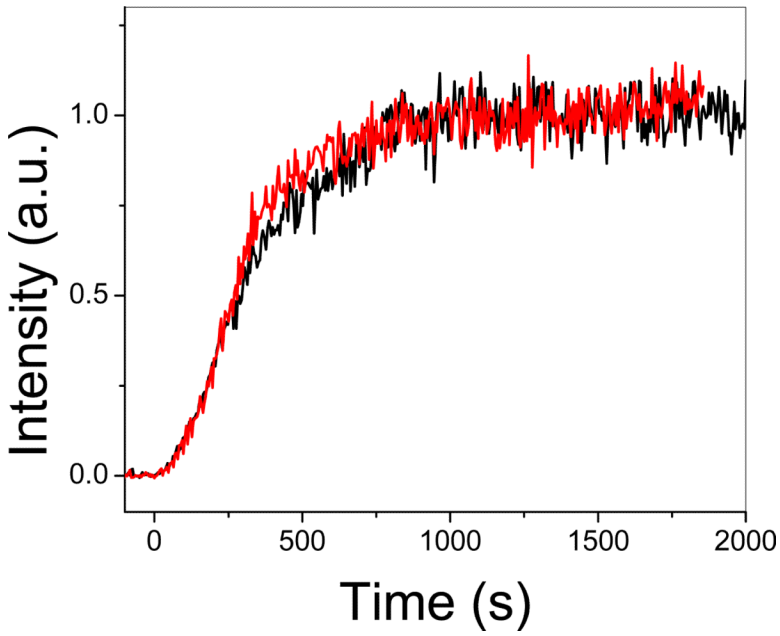

Figure 10.

Time-dependent SFG ppp signal (red) at 1650 cm^{-1} collected from the interface between PS-MA and CP1c solution with 75% ethanol and 25% PB. Time-dependent SFG ppp signal (black) at 1650 cm^{-1} collected from the interface between PS-MA and CP1c solution in PB is also shown. They are very similar, but different from that detected from the interface between PS-MA and CP1c solution with 75% TFE and 25% PB.

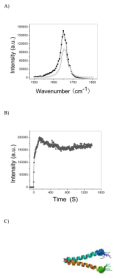

Figure 11.

A) SFG ssp (open dots) and ppp (filled dots) spectra collected from the interface between PS-MA and CP1c solution without the addition of TCEP. The CP1c molecules should form dimers. The ppp/ssp intensity ratio indicates that peptides adopt a multiple-orientation distribution. B) Time-dependent SFG ppp signal at 1650 cm^{-1} collected from the interface between PS-MA and CP1c solution without the addition of TCEP. C) Schematic of a CP1c dimer.