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Orientation Difference of Chemically Immobilized and Physically Adsorbed Biological Molecules on Polymers Detected at the Solid/Liquid Interfaces *in Situ*

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Abstract

A surface sensitive second order nonlinear optical technique, sum frequency generation (SFG) vibrational spectroscopy, was applied to study peptide orientation on polymer surfaces, supplemented by a linear vibrational spectroscopy, attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Using the antimicrobial peptide Cecropin P1 as a model system, we have quantitatively demonstrated that chemically immobilized peptides on polymers adopt a more ordered orientation than less tightly bound physically adsorbed peptides. These differences were also observed in different chemical environments, e.g., air versus water. Although numerous studies have reported a direct correlation between the choice of immobilization method and the performance of an attached biological molecule, the lack of direct biomolecular structure and orientation data has made it difficult to elucidate the relationship between structure, orientation and function at a surface. In this work, we directly studied the effect of chemical immobilization method on biomolecular orientation/ordering, an important step for future studies of biomolecular activity. The methods for orientation analysis described within are also of relevance to understanding biosensors, biocompatibility, marine-antifouling, membrane protein functions, and antimicrobial peptide activities.

1. Introduction

Significant research efforts have been directed towards the development of methods to control the structure and orientation of biological molecules such as peptides and proteins on surfaces, as well as efforts to understand the impact of such control on biological functionality. Effective, molecular-level control of surface structure has the potential to advance the development of novel synthetic methods for metal oxide catalysts and composite materials, design of biomedical coatings and implant devices, robust antimicrobial materials, biosensors with improved performance and numerous other nanodevices. For example, the performance of a biosensor or biochip (e.g., sensitivity, selectivity, detection limit, precision, accuracy, reproducibility, working life, and shelf life) is greatly affected by the structure and activity of the interfacial proteins and peptides used

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Supporting Information Available: The SFG spectra of CP1c bound in lipid bilayer and data analysis details. This information is available free of charge via the Internet at <http://pubs.acs.org>.

for biological recognition.^{1–12} If the protein binding domains are randomly oriented or deformed, the selectivity and sensitivity of the sensor will be significantly reduced; uniformly orienting the molecules for optimal binding is thus expected to improve sensor performance.^{1,11–19}

Towards this end, numerous surface “anchoring” methods have been studied in an effort to produce “optimized” surface orientations.^{1,11,12,18–25} In particular, it has been suggested that site-specific immobilization strategies cause the immobilized proteins or peptides to adopt certain preferred orientation, reflected in improved performance and stability of biosensors or biochips.^{19–25} For example, Holland-Nell et al. observed a distinct difference in the performance of site-specifically vs. randomly immobilized enzymes (aldo/keto reductase AKR1A1); they found that site-specifically immobilized enzymes show a dramatic increase in activity, and greater long-term stability than randomly immobilized enzymes.²⁰ Likewise, Lee et al. stated that immobilizing cysteine-tagged protein G on a gold surface in the “proper orientation” leads to substantially enhanced antigen detection.²⁶ However, in both examples, the claim of a relationship between orientation and performance was not supported by “direct” experimental evidence that specifically probed the orientation of biomolecules immobilized by different methods. Various analytical techniques such as fluorescence imaging and surface plasmon resonance have been applied to characterize a range of biosensors and biochips;^{1,11,12,27–29} yet these techniques are unable to directly explore the link between sensor performance and biomolecular structure or orientation. In this research, we have applied Sum Frequency Generation vibrational spectroscopy (SFG) to directly observe the effect of various immobilization methods on peptide orientation.

In this work, the orientation of the α -helical peptide cecropin P1 (CP1) deposited on polymer surfaces using both physical adsorption and chemical immobilization was determined. Recently, CP1 and other antimicrobial peptides (AMPs) have been used to capture and sense bacterial pathogens such as *E. coli* O157:H7 and *Salmonella*.^{30–36} AMPs have immunomodulatory functions, and are found widely in host defense systems of species as diverse as plants, insects, amphibians, and mammals (including humans); these peptides exhibit significant antibiotic properties and enable rapid defense against the invasion of bacteria, fungi and viruses.^{37–41} Most importantly, AMPs are highly stable under adverse conditions and bind selectively to microbial cell surfaces, exerting their antimicrobial activity through membrane disruption.³⁴ Thus, AMPs have the potential to improve upon existing antibody-based detection methods by overcoming shortcomings 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.³⁴

Taitt et al. investigated the role that peptide immobilization chemistry has on the pathogen binding behavior of the antimicrobial peptide, magainin I.³⁴ Magainin I was immobilized onto silanized glass slides by direct covalent attachment (interaction of succinimidyl ester with the primary amines on the peptide) and an indirect avidin-biotin interaction whereby covalently attached avidin captured the biotinylated peptide. They reported that magainin I which was directly attached to the slide yielded a significant improvement in the detection limits of both *Salmonella* and *E. coli*.³⁴ In a separate study, Mello, et al. explored the interactions between CP1 and pathogenic *E. coli*.⁴² It was found that the activity of the surface-immobilized CP1 molecules was dependent on the method of peptide immobilization used. For example, CP1 molecules physically adsorbed and chemically immobilized via the C-terminus exhibited very different killing efficiencies against *E. coli*.⁴² This difference in activity was believed to be due to differences in peptide structure and/or orientation related to the particular deposition methods used, but no direct structural study was carried out to characterize these differences. Such information is important for an

understanding of the relationship between peptide structure and function in a biosensor. Furthermore, studying the orientation of peptides adsorbed or immobilized on a surface will provide a fundamental basis for the study of larger surface adsorbed and immobilized proteins, as well as other biological molecules such as DNA, RNA, and aptamers.

In this research, we examined the orientation of covalently immobilized and physically adsorbed CP1 peptides on surfaces *in situ*, using Sum Frequency Generation (SFG) vibrational spectroscopy and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). SFG is a nonlinear optical laser technique which provides vibrational spectra of surfaces and interfaces. It has several advantages over other techniques: it is intrinsically surface-sensitive, requires small amounts of sample, and can probe surfaces and interfaces *in situ* in real-time. As a polarized vibrational spectroscopy, SFG permits the identification of interfacial molecular species (or chemical groups), and also provides information about the interfacial structure, such as the orientation and the orientation distribution of functional groups on the surface.^{43–53} SFG has been applied to study the structure and orientation of various biomolecules (including peptides and proteins) at interfacial environments,^{54–61} but it has not been applied to characterize differences in the structure/orientation of biological molecules caused by the use of different surface adsorption/immobilization methods. In this study, we successfully demonstrated that such differences could in fact be observed between CP1 peptides that were physically adsorbed onto a polystyrene (PS) surface versus those which were chemically immobilized onto a polystyrene maleimide (PS-MA) surface at the solid/liquid interface *in situ*.

2. Experimental

2.1 Materials and Sample Preparation

Polystyrene (PS) (standard, narrow distribution, Mw = 393,400) was purchased from Scientific Polymer Products, Inc. (4-Maleimidobutyramidomethyl) polystyrene (PS-MA, molecular formula shown in Figure 1), potassium phosphate (monobasic and dibasic) solution (1 M), dithiothreitol (DTT) solution (1 M), dichloromethane, toluene, and deuterated water (D₂O) were all ordered from Aldrich (Milwaukee, WI, USA). Cysteine-terminated cecropin P1 (CP1c, sequence H₂N-SWLSKTAKKLENSAKKRISGIAIAIQGGPRC-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. Right-angle CaF₂ prisms were purchased from Altos (Bozeman, MT).

All of the chemicals were used as received. CaF₂ prisms were thoroughly cleaned using a multi-step procedure: soaking in toluene for 24 hours; sonicating in Contrex AP solution from Decon Labs (King of Prussia, PA) for 1 hour; rinsing with deionized (DI) water (purified by a Millipore system); and soaking in methanol for 10 minutes. All of the prisms were finally rinsed thoroughly with DI water and cleaned inside a glow discharge plasma chamber for 4 minutes immediately before depositing polymers on them. No SFG signal was observed from the CaF₂ substrates prior to polymer deposition.

A spin coater from Specialty Coating Systems was used to prepare polymer films on CaF₂ prisms from a 1 wt % PS/toluene solution at 2500 rpm. PS-MA films were prepared by directly depositing 0.01% PS-MA/dichloromethane solution onto CaF₂ prisms. The polymer films were kept at room temperature for 24 h prior to performing SFG experiments.

A 50 mM phosphate buffer, pH 6.3, 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). Approximately 250 µg of cecropin P1 was dissolved into 1

mL of phosphate buffer with 2 μ L of EDTA disodium salt (1 M) and 2 μ L of dithiothreitol (DTT, 10 mM) to produce a 75 μ M peptide solution in 50 mM phosphate buffer, pH 6.3, containing 2 μ M EDTA and 20 nM DTT. DTT was added to prevent disulfide bond formation among individual peptide molecules. EDTA was added as a chelating agent to oxidize any metals present in the buffer that may cause formation of unwanted disulfide bonds.

By adding DTT into the peptide solution, inter-peptide disulfide bonds in the c-terminal cysteine residue of CP1c were reduced to ensure that a thiol moiety was present. 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 Polarized ATR-FTIR Experiments

A Nicolet Magna-IR 550 spectrometer was used to collect ATR-FTIR spectra with a standard 45° ZnSe ATR cell and a ZnSe grating polarizer (Optometrics LLC). The ZnSe crystal was cleaned using the same procedures as the CaF₂ prisms. The PS and PS-MA films were prepared by directly depositing polymer solutions onto the ATR crystal (ZnSe). The polymer films were equilibrated with D₂O prior to the collection of a background spectrum of the polymer film/D₂O interface. The D₂O was replaced with CP1c solution and equilibrated for at least 1 h to allow for peptide adsorption at the surface to occur. Loosely adsorbed peptides were removed by rinsing with 50 mM phosphate buffer, pH 6.3 containing 2 μ M EDTA and 20 nM DTT and then D₂O before spectra were collected from the polymer (with adsorbed peptide)/D₂O interface. Finally, the Amide I and II signals of strongly adsorbed/immobilized peptide on the surface in D₂O was obtained by subtracting the background spectrum of the polymer film/D₂O interface. All spectra collected were averages of 256 scans with a 2 cm⁻¹ resolution.

2.3 SFG measurements

Details regarding SFG theories and equipment have been reported previously^{62–67} and will not be repeated here. Some SFG data analysis methods used in this research are presented in the supporting information. The authors' SFG system has been described in detail previously as well.⁶⁸ Here, 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 ppp were collected using the near total internal reflection geometry.⁶⁹

3. Results and Discussions

Before contacting polymers with CP1c peptide solutions, SFG spectra were collected from PS and PS-MA surfaces in air (not shown). SFG spectra collected from the PS surface are dominated by aromatic C-H stretching signals, identical to those published.⁷⁰ Aliphatic C-H stretching signals are dominated in the spectra from PS-MA, confirming that the MA groups segregate to the surface.

SFG spectra in the amide I frequency region were collected from the PS-MA/CP1c solution interface after the peptide solution had been in contact with the PS-MA surface for at least one hour (to equilibrate and ensure that no further time-dependent changes occurred). SFG spectra were collected in both ssp and ppp polarization combinations of the input and output beams (Fig. 2a). Both ssp and ppp spectra are dominated by a single resonance peak centered at 1650 cm⁻¹, which is the typical peak center for amide I signals contributed from an α -helical structure. It has previously been shown that CP1 adopts an α -helical structure in

cell membranes,^{71,72} and SFG spectra were collected from CP1 in a lipid bilayer (model cell membrane) for comparison to spectra from the polymer surface. It was found that the SFG spectra collected from the CP1 molecules in a lipid bilayer have the same peak center and the same spectral features as those collected from the polymer/CP1c solution interfaces, supporting the assumption of α -helical CP1c structure at the polymer/solution interfaces. Details regarding SFG spectra collected from CP1c in the lipid bilayer can be found in the supporting information. Verification of the assignment of secondary structure will be further discussed in conjunction with the orientation analysis of CP1c at the polymer/solution and polymer/water interfaces (below).

We have developed a methodology for the analysis of interfacial α -helix orientation that uses the fitted spectral strength ratio between the amide I signals in ppp and ssp spectra.^{73–75} Figure 3 shows the relationship between the measured signal strength ratio, $\chi_{\text{ppp}}/\chi_{\text{ssp}}$, and the orientation angle θ of the α -helix, where θ is the angle between the α -helix principal axis and the surface normal. Figure 3 also shows the effect of different orientation distributions. These relations are described in further detail in the supporting information. For CP1c peptides at the PS-MA/peptide solution interface, the fitted SFG signal strength ratio, $\chi_{\text{ppp}}/\chi_{\text{ssp}}$, is 1.05. Using the relations shown in Figure 3, we found that the orientation of CP1c cannot be described using either a δ -orientation distribution or a Gaussian distribution. In this case, the orientation distribution of CP1c molecules may be quite complex- for example, multiple distinct orientations being present simultaneously.^{73–75}

The PS-MA surface was removed from the CP1c solution and exposed to air. SFG spectra collected from such a surface in air (Fig. 2b) compared to those collected at the solid/liquid interface are much weaker. This reduction in SFG signal is not due to the loss of adsorbed peptides, because strong SFG intensities could be detected by returning the PS-MA surface to DI water or buffer. The Fresnel factor in water (or solution) is only about 2.3 times compared to that in air for our experimental geometry, which would result in roughly a factor of five SFG intensity difference if peptides in the two environments adopted the same structure and orientation. In fact, the SFG signals detected from CP1c at the polymer/solution interface were more than fifty times stronger than those from peptides in air, suggesting that CP1c molecules at the PS-MA/air interface adopt a more horizontal or randomized orientation. This result is in agreement with a recent study in which CP1c on gold was also found to adopt a predominately flat, random orientation in the absence of a hydrogen bonding solvent.⁷⁶

After removing the PS-MA surface from the peptide solution, rinsing with DI water numerous times, and re-contacting with buffer, SFG spectra were collected from the washed PS-MA/buffer interface (Fig. 2c). Weaker SFG spectral intensities were observed, suggesting that rinsing washes away loosely deposited peptides, leaving only chemically immobilized CP1c molecules on the surface. Using the relation between the measured ppp and ssp intensity ratio, we should be able to deduce the chemically immobilized peptide orientation. Here the fitted $\chi_{\text{ppp}}/\chi_{\text{ssp}}$ ratio is about 1.55, corresponding to an orientation angle of about 35° versus the surface normal for chemically immobilized CP1c (assuming a Gaussian orientation distribution of width $\sigma \leq 10^\circ$) (Figure 3). There exists some concern that the ratio of ppp to ssp signal intensity in a given orientation will depend on the length of the helix, deviating from the ideal ratios when the number of the amino acids in the helix deviates from multiples of 18 (the smallest integer number of residues that produces a perfect α -helix at 3.6 residues per turn). For example, a molecule of CP1c contains 31 amino acids, and thus the α -helical symmetry is slightly broken in the CP1c case. To address the above concern, we employed a bond additivity model to assess the effect of helix length on the relation between the $\chi_{\text{ppp}}/\chi_{\text{ssp}}$ value and α -helix orientation, assuming a δ - or Gaussian distribution.⁷⁷ We did this by summing over the Raman polarizability and IR transition

dipole moments of the amino acids comprising the helix according to Tsuboi et al.'s amide I coordinates⁷⁸ and Pauling et al.'s α helix structure.⁷⁹ We calculated the hyperpolarizability tensor element ratios β_{aac}/β_{ccc} and β_{aca}/β_{ccc} for α -helices having 31 amino acids. Using these parameters, the ratio of χ_{ppp}/χ_{ssp} as a function of α -helix orientation angle (θ) was calculated for CP1c (Figure 3). Similar calculations have been performed on other α -helices with a range of different lengths, and the results indicate that small variations in the length of the α -helix will not significantly alter the relations used for orientation analysis.⁷⁷

The deduced orientation angle range for chemically immobilized CP1c is very close to the magic angle value in SFG (39.2°).⁸⁰ Thus, additional measurements were obtained using polarized ATR-FTIR spectroscopy to better characterize molecular orientation. The ATR-FTIR spectra after several rinses using DI water are shown in Figure 4. The intensity ratio (R) of the polarized ATR-FTIR spectra of immobilized CP1c on PS-MA surface was measured to be 1.7–1.8. From this R value, the orientation angle is determined to be about 35°–39° (assuming an orientation distribution $\sigma \leq 10^\circ$); this value is well correlated to the SFG result. Further, because this value is far away from the magic angle in ATR-FTIR (54.7°),⁸¹ we conclude that chemically immobilized CP1c is tilted on the PS-MA surface with a narrow orientation distribution. Here both SFG and ATR-FTIR orientation analysis methods for α -helical structure are used to determine CP1c orientation. These techniques measure different and complementary orientational parameters: SFG measures $\langle \cos\theta \rangle$ and $\langle \cos^3\theta \rangle$ (" $\langle \rangle$ " means average), while ATR-FTIR measures average $\langle \cos^2\theta \rangle$. Thus, the good agreement between the results obtained using these two methods supports our conclusions regarding the α -helical secondary structure and ordered orientation of CP1c.

SFG spectra were also collected from a PS/CP1c solution interface using ssp and ppp polarization combinations (Fig. 5a). Without the chemical coupling between the thiol groups and maleimide groups of PS-MA, CP1c can only be loosely adsorbed onto the PS surface, allowing for a comparison to the chemically immobilized peptide layers described above. The spectra of the physically adsorbed peptides are dominated by the 1650 cm^{-1} peak, indicating that CP1c adopts an α -helical structure at the PS/liquid interface. The intensity ratio of the ppp and ssp spectra is similar to that of CP1c at the PS-MA/peptide solution interface prior to washing, suggesting a complex or "multiple" orientation of interfacial CP1c. After the PS surface was removed from the peptide solution and exposed to air, ssp and ppp SFG spectra were collected in air (Fig. 5b). No discernible SFG signal could be observed, showing that CP1c adsorbed on the PS surface in air has no orientation order. This surface is even more random than the PS-MA/CP1c surface in air, where weak yet observable SFG signal could be seen. After washing the PS surface twice, the PS surface was immersed in buffer, and SFG spectra were again collected from the PS/buffer interface. Only very weak SFG signal can be observed after washing twice (Fig. 5c). Further washing of the sample with DI water or buffer made the SFG signal almost completely disappear, demonstrating that the physically adsorbed CP1c molecules are only loosely deposited and can be washed away. By contrast, further washing did not change the SFG amide I signals obtained from chemically immobilized CP1c on PS-MA (Fig. 2c).

Cysteine groups have been widely used for the chemical immobilization of proteins onto a solid support, e.g., onto a gold surface or a surface containing maleimide functionalities, enabling the construction of many biosensors and biochips.^{26,82–84} Cysteine residues can be genetically introduced into a specific site of the target protein, allowing for control of protein orientation through the coupling of the thiol group to the gold surface or maleimide surface in either thiolate or disulfide form.^{26,85} In this study, the addition of DTT to the CP1c solution prevents the formation of the disulfide bonds between CP1c molecules, preserving the reactivity of the thiol group. The thiol moiety in cysteine residue on the C-terminus of CP1c has a strong affinity with the maleimide moieties on the PS-MA surfaces,

promoting the covalent coupling between CP1c and the PS-MA surface, as shown in Fig. 1B. We believe that on the PS-MA surface, CP1c molecules are chemically immobilized, allowing observable SFG signals after the washing process. Furthermore, our spectral fitting results indicate that the orientation of immobilized peptides is different from the orientation of the adsorbed peptides before washing, supporting both the presence of chemically immobilized peptides and the hypothesized effect of chemical immobilization methods on peptide orientation.

Conclusion

In this research, we successfully detected differences in the orientation of physically adsorbed (e.g., at the PS or PS-MA/peptide solution interface) and chemically immobilized CP1c (e.g., on PS-MA after washing by buffer or water for several times to rinse away the loosely deposited peptide). The chemical environment (e.g. air vs. water) was also found to affect peptide orientation. We further demonstrated that the combination of SFG and ATR-FTIR experiments lead to more reliable measurements of the orientation of chemically immobilized biological molecules. Reliable, surface-sensitive orientation measurements will support a more detailed understanding of the relationship between adsorption/immobilization methods, biomolecular structure/orientation, and biosensor performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A). (4-Maleimidobutylamino)methyl polystyrene; B). Schematic showing S-H groups linking to Maleimide groups.

Figure 2. SFG amide I signals collected from: a). PS-MA/CP1c solution interface; b). PS-MA surface (with CP1c) in air; c). PS-MA/buffer interface after the PS-MA surface in b) was washed four times using DI water and then contacted with buffer.

Figure 3. Relations between the SFG susceptibility tensor component ratio and the α -helix orientation angle with different orientation distributions. The measured orientation angle is about 35° assuming a narrow angle distribution.

Figure 4. ATR-FTIR spectra of CP1c immobilized on PS-MA surface in contact with water after washing the surface by DI water for several times.

Figure 5. SFG amide I signals collected from: a). PS/CP1c solution interface; b). PS (with CP1c) surface in air; c). PS/buffer interface after the PS surface in b) was washed twice using DI water and then contacted buffer.