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Amide or Amine: Determining the Origin of the 3300 cm^{-1} NH Mode in Protein SFG Spectra Using ^{15}N Isotope Labels

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Sum frequency generation (SFG) vibrational spectroscopy has been employed in biomaterials research and protein adsorption studies with growing success in recent years. A number of studies focusing on understanding SFG spectra of proteins and peptides at different interfaces have laid the foundation for future, more complex studies. In many cases, a strong NH mode near 3300 cm^{-1} is observed in the SFG spectra, but the relationship of this mode to the peptide structure is uncertain, since it has been assigned to either a backbone amide mode or a side chain related amine resonance. A thorough understanding of the SFG spectra of these first model systems is an important first step for future experiments. To clarify the origin of the NH SFG mode, we studied ^{15}N isotopically labeled 14-amino acid amphiphilic model peptides composed of lysine (K) and leucine (L) in an α-helical secondary structure (LKα14) that were adsorbed onto charged surfaces in situ at the solid−liquid interface. ^{15}N substitution at the terminal amine group of the lysine side chains resulted in a red-shift of the NH mode of 9 cm^{-1} on SiO_2 and 13 cm^{-1} on CaF_2. This clearly shows the 3300 cm^{-1} NH feature is associated with side chain NH stretches and not with backbone amide modes.

1. Introduction

The understanding of protein adsorption on the molecular level is crucial for the design of future bioactive surface coatings and interfaces.[1−3] Sum frequency generation (SFG) spectroscopy has proven to be an increasingly powerful technique to probe those films *in situ* at the solid−liquid interface. In a number of studies, SFG was used to measure the orientation and secondary structure of a variety of model peptides and proteins.[4−10] According to the "selection rules" of SFG, only molecular groups in proteins or peptides at an interface that have a net order will contribute to the measured SFG signal.[11] Since surface interactions can introduce significant ordering in the binding regions of proteins and peptides, SFG is an excellent probe to identify the side chains involved in these binding events[4,5,8,10] and, in some cases, also the orientation of adsorbed peptides.[12,13] Backbone related amide modes, which are sensitive to conformation,[14] have also been used to determine the secondary structure of adsorbed peptides.[5,9,15] Investigations of peptides and proteins on model surfaces aimed at identifying the basic principles of peptide orientation and binding chemistry lay the foundation for future experiments with more complex biological systems.

A number of SFG studies of adsorbed proteins and peptides report a dominant peak in the SFG spectra near 3300 cm^{-1}, which can be assigned to either an amide A mode related to the backbone or to side chain related amine resonances. Whether this mode is assigned to the backbone or to specific side chains makes a significant difference in the interpretation of the SFG data. Thus, resolving this uncertainty is essential to achieving a detailed understanding of SFG spectra needed for interpretation of more complex experiments. Cremer et al. have observed the NH feature in an SFG study of adsorbed fibrinogen and assigned it to amine modes of lysine or arginine side chains.[10] Chen et al. observed a similar time dependence of the 3300 cm^{-1} mode and backbone amide I modes in a kinetic study of fibrinogen adsorption and suggested the 3300 cm^{-1} mode is related to the peptide backbone.[15] They have also reported a SFG resonance near 3300 cm^{-1} for amine groups in urea.[16] In recent studies of LK model peptides on hydrophilic surfaces, Somorjai et al. have tentatively assigned the feature to a backbone mode[6,7] but have also suggested it is related to NH side chain modes in earlier studies.[4]

To put this controversy to rest and to unequivocally assign this spectral feature, we performed an SFG isotope-labeling study of the LKα14 peptides adsorbed onto negatively charged SiO_2 and positively charged CaF_2 surfaces. These model peptides are comprised of hydrophobic leucine (L) and hydrophilic lysine (K) side chains designed to assume an α-helical secondary structure with the hydrophobic leucines and the positively charged lysines on opposite sides of the helix. This results in an amphiphilic and rigid rod-like peptide.[17] The 3300 cm^{-1} feature has been observed in SFG studies of LKα14 adsorbed onto different charged surfaces previously.[4−7] In the current study, the amine groups of the lysine side chains were isotopically labeled with ^{15}N. Substituting ^{14}N with ^{15}N should

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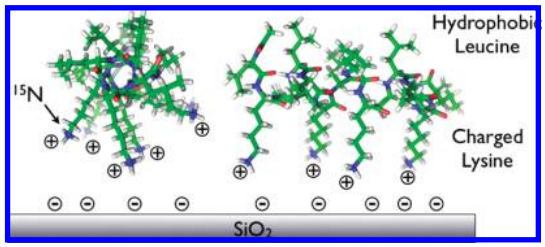
SCHEME 1: Idealized Drawing of the Synthetic LKα14 Peptide Used in This Study Indicating Its α-Helical Secondary Structure and Suggested Side Chain Interactions with a Silica Surface[a]



[a] This amphiphilic α-helical peptide has charged lysine and hydrophobic leucine residues on opposite sides. ^{15}N was used to label the lysine side chains.

result in an estimated red-shift of ca. 8 cm^{-1} for the resonance frequency of the NH_3 group.[18]

2. Experimental Details

Details of our SFG setup are published elsewhere and will only be briefly discussed here.[19] The visible beam from an EKSPLA Nd:YAG laser with a wavelength of 532 nm and the tunable IR beam from an EKSPLA optical parametric generation/amplification unit were focused at the sample with energies of 150 and 200 μJ/pulse for the visible and IR beams, respectively. The spectra were collected with 400 shots per data point in 4 cm^{-1} increments. The bandwidth of the IR laser light was near 1 cm^{-1} in the CH region and less than 6 cm^{-1} at higher frequencies, which is significantly narrower than the spectral features observed. The SFG spectra were normalized by the product of the IR and visible pump beam intensities. The input angles of the visible and IR pump beams inside the prism were 47 and 58° versus the surface normal, respectively. These angles are below the critical angles for total internal reflection (TIR) for the SiO_2− or CaF_2−water interface (66 and 68°, respectively). This is of particular importance for the present study, since optical artifacts have been observed at the SiO_2−water interface in TIR geometry.[20] All spectra were collected using s-polarized visible, s-polarized SFG, and p-polarized IR beams.

One side of the prism was brought into contact with the PBS buffer solution, and the interface was probed through the back side of the film. Subsequently, the peptide solution was injected into the buffer without exposing the SiO_2 surface to the air−water interface. The concentration of the peptide solutions used in this work was 0.05 mg/mL. Sets of labeled and unlabeled samples were measured on the same day to ensure comparable conditions. Further details of the peptide adsorption procedures have been described previously.[5,12,21]

For a more quantitative spectra analysis, we used the following expression to fit the SFG intensity:

$$I_{SFG} \propto |\chi^{(2)}|^2 = \left| \chi_{NR}^{(2)} e^{i\phi} + \sum_{\nu} \int_{-\infty}^{\infty} \frac{A_\nu e^{i\phi_\nu} e^{-[\omega_L - \omega_\nu/\Gamma_\nu]^2}}{\omega_{IR} - \omega_L + i\Gamma_L} d\omega_L \right|^2 \quad (1)$$

$\chi^{(2)}$ is the second order nonlinear susceptibility of the nonresonant background, A_ν is the strength of the νth vibrational mode, ϕ denotes the phase of the respective mode, and ω_{IR} refers to the frequency of the incident IR field. The integral is over Lorentian lines, centered at ω_L with a width Γ_L, having a Gaussian distribution. Since the expected resonance position shifts upon ^{15}N are comparatively small (<10 cm^{-1}), identical peak widths were used for all peaks and the CH and OH resonances were fit using identical peak positions and widths for all spectra.

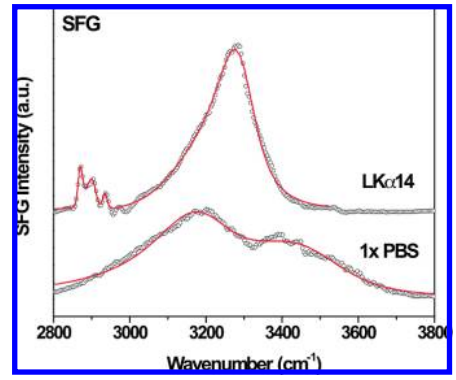


Figure 1. SFG spectra of the PBS−SiO_2 interface before and after injection of 0.05 mg/mL LKα14 along with the corresponding fits.

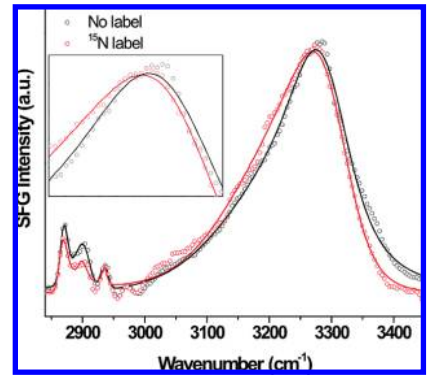


Figure 2. SFG spectra of LKα14 at the SiO_2−1×PBS buffer interface with and without ^{15}N isotopically labeled lysine side chains. For the labeled sample, the NH mode is red-shifted by ca. 9 cm^{-1}. The inset shows a detailed view of the NH stretching region.

3. Results and Discussion

Figure 1 displays SFG spectra of the SiO_2−PBS buffer interface before and after injection of LKα14. The spectrum of clean SiO_2 in PBS exhibits two broad resonances centered near 3200 and 3400 cm^{-1} related to water molecules with tetrahedral coordination and water molecules with a less ordered structure, respectively.[22−24] After peptide injection, the strong NH mode near 3300 cm^{-1} becomes visible along with weaker resonances in the CH stretching region near 2868, 2901, and 2930 cm^{-1} assigned to the symmetric CH_3, CH, and CH_3 Fermi resonances, respectively. The latter modes are most likely related to the leucine side chains.[25,26] Upon peptide adsorption, the water mode near 3400 cm^{-1} disappears, while the water mode near 3200 cm^{-1} decreases noticeably in intensity. These results are in accord with those reported by Somorjai et al., the only difference being the clear detection of CH modes in our spectra,[4,6,7] which is explained by the improved signal/noise ratio due to the different experimental geometry and greater number of laser shots per data point of the data presented here.

Figure 2 shows SFG spectra of LKα14 with unlabeled (black trace) and ^{15}N-labeled (red trace) lysine side chains adsorbed at the SiO_2−buffer interface along with fits of eq 1 to the data. The 3300 cm^{-1} feature is noticeably shifted in the spectrum of the ^{15}N-labeled peptides. At the same time, the peak positions

TABLE 1: Fitting Results for SFG Spectra from LKα14 Peptides Adsorbed onto SiO_2 and CaF_2 Surfaces

mode	frequency/cm^{-1}
SiO_2	
1×PBS: $^{14}NH_3$	3299
1×PBS: $^{15}NH_3$	3290
10×PBS: $^{14}NH_3$	3296
10×PBS: $^{14}NH_3$	3289
OH	3187
CH_3 - sym	2868
CH_3 - FR	2930
CH_3 - asym	2961
CH	2901
CaF_2	
$^{14}NH_3$	3316
$^{15}NH_3$	3303

of the CH modes remain unaltered, which confirms that the small shift for the labeled peptide is not related to minor shifts of the energy scale (small intensity changes of the CH modes observed in the two spectra are likely related to slight differences of the side chain order). Pertinent fitting results are summarized in Table 1. The fits reveal the NH peak position of the ^{15}N labeled peptides is red-shifted by 9 cm^{-1}, decreasing from 3299 to 3290 cm^{-1}. The experiment was repeated three times, and the variation of the peak shift was ±2 cm^{-1}. This shift of the resonance position is in excellent agreement with the estimated resonance position shift of 8 cm^{-1} for 15N substitution in NH_3. Most importantly, it proves that the 3300 cm^{-1} feature is an NH_3 amine stretching mode related to the lysine side chains. The positively charged lysine most likely becomes ordered upon interaction with the negatively charged SiO_2 surface. This result is in good agreement with recent studies of LK peptides on self-assembled monolayers which conclude that the lysine side chains are oriented toward negatively charged surfaces.[12,13]

Since the NH peak strongly interferes with the broad water resonance near 3190 cm^{-1}, the experiments described above were repeated in a 10× PBS buffer solution. It has been shown that higher ionic strength buffers greatly reduce the intensity of water peaks in LK peptide spectra.[6] SFG spectra of ^{15}N-labeled and unlabeled LKα14 on SiO_2 in a 10× PBS buffer are shown in Figure 3. The water shoulder is reduced in intensity compared to the 1× PBS spectra, and the NH mode is more pronounced. The red-shift of the ^{15}N-labeled peptides is clearly

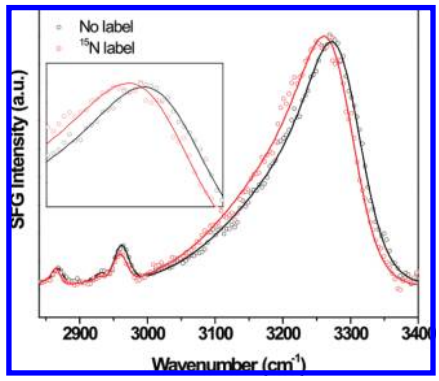


Figure 3. SFG spectra of LKα14 at the SiO_2−10×PBS buffer interface with and without ^{15}N isotopically labeled lysine side chains. The intensity of the water mode near 3200 cm^{-1} is reduced at this ionic strength which provides a more well-defined NH mode compared to the spectra taken in 1×PBS. For the labeled sample, the NH mode is red-shifted by ca. 7 cm^{-1}. The inset shows a detailed view of the NH stretching region.

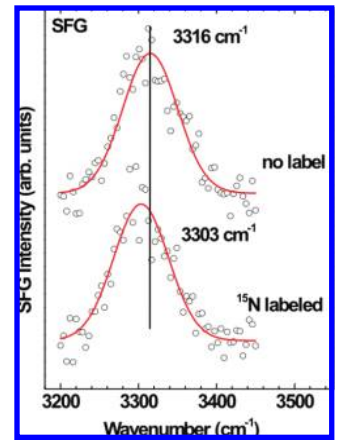


Figure 4. SFG spectra of LKα14 at the CaF_2−1×PBS buffer interface with and without ^{15}N isotopically labeled lysine side chains. For the labeled sample, the NH mode is red-shifted by ca. 13 cm^{-1}.

visible. Fits of the spectra show a red-shift of 7 cm^{-1}, in good agreement with the results for 1× PBS buffer.

The NH mode is also visible for LKα14 adsorbed onto positively charged surfaces. Figure 4 shows NH region SFG spectra of labeled and unlabeled LKα14 at the CaF_2−1×PBS buffer interface (CaF_2 is positively charged at neutral pH). The NH stretching modes are located near 3316 and 3303 cm^{-1} for the labeled and unlabeled peptides, respectively (for amide I and survey spectra before and after peptide adsorption, see the Supporting Information). Similar to the SiO_2 surface, the ^{15}N label shifts the NH mode by 13 cm^{-1}. The slightly higher peak position of the NH modes on CaF_2 compared to the SiO_2 surface and the different shift upon isotopic substitution is not surprising in view of the different surface chemistry and polarity. It is unclear how the positively charged lysine side chains interact with the positively charged CaF_2 surface, but from the presence of the NH mode in the spectrum, it can be concluded that they assume a certain degree of orientational order once the peptides bind to the surface. It is possible they point away from the interface because of charge−charge interactions. Alternatively, they could be oriented toward the surface and are bound to the surface via salt bridges. While the NH mode is comparatively low in intensity, we observed a very strong signal near 1658 cm^{-1} in the amide I region. This shows that the peptides preserve their α-helical conformation on the CaF_2 surface.[9,27,28] The strength of the signal also indicates a high overall film order (see the Supporting Information). Future SFG experiments, using positively charged amine terminated self-assembled monolayers on gold as a substrate, could help to elucidate the absolute orientation of the lysine side chains by using the gold nonresonant background as a phase reference.[12]

4. Summary

^{15}N isotopically labeled lysine was used to clarify the origin of the SFG mode near 3300 cm^{-1} observed in a variety of protein and peptide samples. SFG spectra of LKα14 adsorbed at the SiO_2−PBS interface with ^{15}N-labeled and unlabeled lysine side chains showed an ca. 9 cm^{-1} red-shift of the NH resonance for the labeled peptides. Analogous measurements at the CaF_2−PBS interface resulted in a red-shift of 13 cm^{-1}. This directly proves that the 3300 cm^{-1} NH peak is associated with the terminal amine groups of the lysine side chains and not with backbone amide modes. The ability to differentiate between side chain and backbone modes in model peptides of reduced complexity will be important for future SFG experiments with

more complex proteins and other biomolecules. This experiment emphasizes the powerful combination of isotope labeling with SFG spectroscopy to elucidate the structure of complex biointerfaces.

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Supporting Information Available: Details of the peptide synthesis and SFG data for LKα14 adsorbed onto CaF_2. This material is available free of charge via the Internet at http://pubs.acs.org.

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