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# Molecular Responses of Proteins at Different Interfacial Environments Detected by Sum Frequency Generation Vibrational Spectroscopy

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***Abstract:*** Sum frequency generation (SFG) vibrational spectroscopy has been applied to investigate molecular responses of bovine serum albumin (BSA) molecules adsorbed at different interfacial environments. Molecular level and in situ SFG studies demonstrate that albumin molecules have different adsorption behaviors when contact with fused silica, polystyrene, and poly(methyl methacrylate). Adsorbed albumin molecules exhibit different structural changes when exposed to different chemical environments, including air, water, and hydrophobic solvents. This paper provides direct molecular insight into protein responses to different interfacial environments.

## Introduction

Protein adsorption is the first process that happens when a biomaterial interacts with a biological system; the structure of adsorbed proteins determines finally whether an implanted material will be accepted or rejected by the body (biocompatible or not). Because of its critical importance to biomedical devices, protein adsorption on biomaterial surfaces has been studied by a myriad of surface-sensitive methods for decades.[1−3] However, it is still difficult to determine chemical and structural information of adsorbed protein molecules, and details about how protein molecules respond to different interfacial environments are still not clear. AFM and FTIR-ATR have been shown to be important tools for studying protein adsorption.[4−11] Unfortunately, they provide little chemical information or lack sufficient intrinsic surface sensitivity, respectively. As of yet, no thorough molecular-level interrogations of interactions between proteins and biomaterials in situ have been achieved.

Recently, SFG has been developed into a powerful tool to study various interfacial structures including molecules adsorbed at interfaces at the molecular level in situ.[12−16] However, the study of protein adsorption by SFG has been reported[17,18] but the SFG technique has not yet been widely applied in this area. This is perhaps because of the complicated structure of protein molecules. Unlike small adsorbed molecules, a layer of adsorbed proteins may not be treated as a simple interface. In general, under the dipole approximation, SFG signals come from materials without inversion symmetry within the optical field.[19−21] Typically, bulk materials have inversion symmetry, thus SFG signals can be canceled out and no net bulk signals are detected. Because an adsorbed protein layer cannot always be treated as a simple interface, a more general model, in particular thin-film theory, should be applied.[22] Under this theory, the local optical field across the protein thin film can be considered to vary according to the refractive indices across the film. To simplify the analysis here, we comparatively study bovine serum albumin (BSA) adsorption on three different substrates with similar refractive indices to avoid the spectral changes induced by the local field effect. We show that protein molecules behave differently at different interfaces.

## Experiment

Details of the SFG technique, our SFG setup, and the experimental geometry have been published and will be briefly reported here.[23−26]

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SFG is a process in which two input beams at frequencies $\omega_1$ (usually in the visible range) and $\omega_2$ (usually a tunable infrared beam) mix in a medium and generate an output beam at the sum frequency $\omega = \omega_1 + \omega_2$. If $\omega_2$ is scanned over the vibrational resonances of molecules, then the SFG signal is resonantly enhanced, thus producing a vibrational spectrum characteristic of the material. As a second-order nonlinear optical process, SFG spectral intensity will be zero in a medium with inversion symmetry under the electric-dipole approximation. SFG spectra can be detected from the material where the inversion symmetry is broken. Most bulk materials have inversion symmetry, thus they do not generate SFG signals. We believe that SFG can be applied to study orientation or conformation changes of interfacial protein molecules, where inversion symmetry is broken.

In our lab, sum frequency spectra were collected by overlapping a visible and a tunable IR beam on a surface or interface, at incident angles of 60° and 54°, respectively. The visible beam had a wavelength of 532 nm, and was generated by frequency-doubling the fundamental output pulses of 20 ps pulse width from an EKSPLA Nd:YAG laser. The IR beam, tunable from 1000 to 4300 $cm^{-1}$, was generated from an EKSPLA optical parametric generation/amplification and difference frequency system based on LBO and $AgGaS_2$ crystals. The diameters of both beams on the sample were about 0.5 mm. The sum frequency signal was collected by a photomultiplier tube. In this work, SFG spectra with the ssp polarization combination (s-polarized SFG output, s-polarized visible input, and p-polarized infrared input) were collected. The experimental geometry used to collect signals from polymer/protein solution and fused silica/protein solution interfaces was similar to that for collecting spectra from the polymer/water interface, which was described previously.[23,26]

BSA was purchased from Sigma (>99%, fatty-acid free). The BSA solution concentration was 1 mg/mL in deionized water with no buffer added. Each protein solution had a pH level near 7. Deuterated polystyrene (d-PS) and deuterated poly(methyl methacrylate) (d-PMMA) were purchased from Polymer Sources, Inc. and used as received. Polymer films were prepared by spin coating. A spin coater from Specialty Coating Systems was used to spin coat 2 wt % polymer/toluene solution at 3000 rpm on fused silica (1" diameter and 1/8" thickness, from ESCO Products) to make polymer films. The polymer films were dried at 80 °C for 12 h. Before contact with protein or polymer coatings, fused silica substrates were left in a potassium dichromate/sulfuric acid solution for 24 h, and subsequently heated in the same solution for 30 min. Then they were rinsed thoroughly using deionized water and dried in a dry $N_2$ stream. Fused silica has a very small contact angle, water can spread on it. The water contact angles for PS and PMMA are ~91° [27,28] and ~72°, [29,30] respectively. Thus, the three surfaces we will study here have very different surface hydrophobicity.

## Results and Discussion

We have collected SFG spectra of BSA molecules from the BSA solution/fused silica, BSA solution/d-PS, and BSA solution/d-PMMA interfaces. We also collected SFG spectra from adsorbed BSA molecules on fused silica, d-PMMA, and d-PS exposed to different environments, including air, water, and hydrophobic solvents. The BSA structure was widely studied.[31−35] Research shows that the degree of BSA adsorption and adsorption kinetics vary on different surfaces.[2,3,36] Here, using SFG, we demonstrate in situ that BSA molecules also have different structures at different interfaces.

We will only discuss the C−H stretching bands and ignore the broad OH peaks around 3200 and 3400 $cm^{-1}$, which mainly come from interfacial water molecules. C−H stretching results were also repeated and confirmed using BSA $D_2O$ solutions, where no spectral interferences between C−H signals and water O−H bands were involved. SFG C−H stretching signals have been used to determine conformations of small molecules at various interfaces.[13] Here, C−H signals come from the hydrophobic side chains of BSA molecules. Therefore, SFG spectra can be used to elucidate conformations of BSA molecules, e.g., they can be used to differentiate the "hydrophobic" or "hydrophilic" conformation of a protein molecule, and their relation to the hydrophobicity of the contacting media.

**BSA on Fused Silica.** SFG spectrum collected from the fused silica/BSA solution interface is shown in Figure 1a. No C−H stretching signals from albumin molecules can be detected.[17] This is due to either a lack of protein adsorption at this interface or no net alignment of functional groups such as methyl, phenyl, or methylene of adsorbed BSA. The fused silica was removed from the albumin solution and was washed by water to remove loosely deposited BSA. The spectrum was collected from the fused silica in air, and strong C−H signals were detected, indicating that albumin adsorbed to the fused silica interface (Figure 1b). The possibility that BSA molecules were transferred from the BSA solution/air interface to fused silica when the fused silica was removed from the solution can be excluded because the sample was washed several times by water before SFG spectra were collected in air. Because of the similar refractive indices of fused silica (~1.45), BSA solution (~1.34), and the adsorbed protein layer (~1.4), the local field differences across the film can be ignored. Thus, the conclusion can be made that the absence of C−H signals is due to the lack of net alignment of C−H groups at the fused silica/BSA solution interface. BSA at the fused silica/solution interface is in contact with hydrophilic environments at both sides. The resulting adsorbed protein layer adopts a hydrophilic configuration. The hydrophobic groups would tend to stay inside the BSA film with no net alignment, thus no SFG signal could be detected. When the adsorbed BSA molecules were exposed to air, which is hydrophobic, C−H signals were detected. This is possibly due to the fact that hydrophobic parts of BSA tend to face toward the air.

An SFG spectrum was also collected from the fused silica/water interface after we contacted the adsorbed BSA on fused silica with water. Again, C−H signals disappeared (Figure 1c), due to the recovery of the hydrophilic configuration of the adsorbed BSA layer. The C−H signals were detected again after the sample was exposed to air (Figure 1d), indicating that configuration changes are reversible. We can detect these reversible cycles repeatedly. The C−H signals changed only slightly each time when the sample was exposed to air, probably

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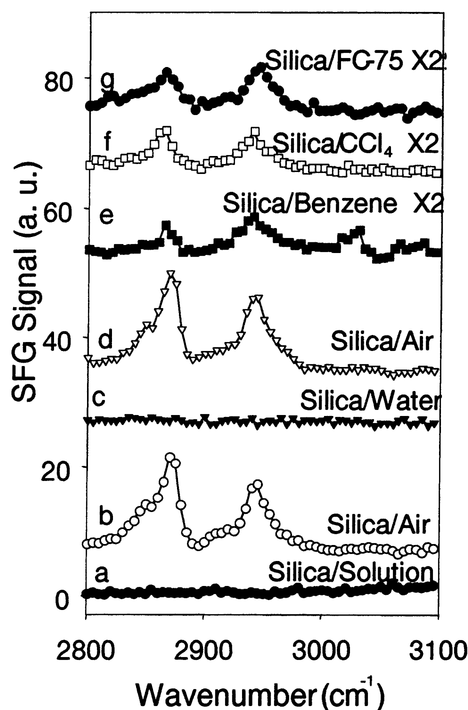


*Figure 1.* SFG Spectra collected from (a) silica/BSA solution interface; (b) silica/air interface after silica was removed from solution and washed by water; (c) silica/water interface after the sample contacted water again; (d) silica/air interface after the sample was again removed from water; (e,f,g) silica/benzene or silica/ $CCl_4$ or silica/FC-75 interface after procedures (a) and (b) and contacting the sample with benzene, $CCl_4$, or FC-75. Spectral intensities in 1e,f,g are multiplied by 2.

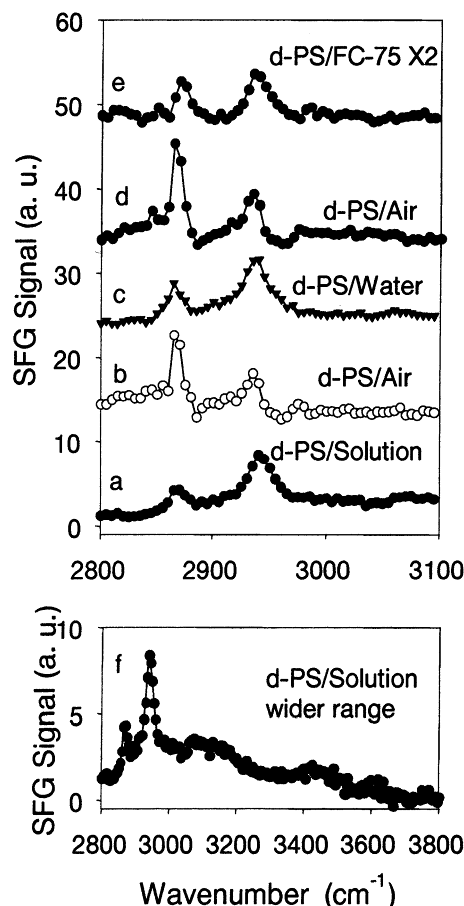


*Figure 2.* SFG spectra collected from (a) d-PS/BSA solution interface; (b) d-PS/air interface after the sample was removed from solution and washed by water; (c) d-PS/water interface after the sample contacted water; (d) d-PS/air interface again; (e) d-PS/FC-75 interface after procedures a and b and contacting the sample with FC-75; (f) d-PS/BSA solution interface in the wide spectral range. The spectral intensity in 2e has been multiplied by 2.

due to some slight desorption of adsorbed albumin molecules on the fused silica in water. When we contacted this fused silica sample with a hydrophobic liquid, such as benzene, carbon tetrachloride, or FC-75 (a hydrophobic fluorinated solvent from 3M), C−H signals were still visible and quite similar to one another (Figure 1e,f,g). This is different from the fused silica/water situation. FC-75 has a refractive index (~1.3) similar to water, and different from benzene (~1.5) and $CCl_4$ (~1.46). Therefore, we believe that differences in SFG spectra at hydrophobic solvent and water interfaces can be attributed to different configurations of interfacial BSA molecules, rather than different local field effects at these interfaces. When we removed the samples from these hydrophobic solvents and exposed them to air again, the spectra collected were similar to the ones collected before contact, indicating that the adsorbed BSA molecules on fused silica were not desorbed by contact with hydrophobic solvents. The SFG spectral intensity in air (Figure 1b,d) is much stronger than those in solvents (Figure 1e,f,g), mostly due to the different Fresnel coefficients for the different interfaces.

**BSA on d-PS.** An SFG spectrum has been collected from the deuterated polystyrene (d-PS)/BSA solution interface (Figure 2a,f). Strong C−H signals were detected, demonstrating that the adsorbed BSA film could not have a hydrophilic configuration at this interface. The d-PS surface is relatively hydrophobic, thus the hydrophobic components of BSA would adopt a preferential alignment. When the sample was removed from the BSA solution, washed by water, and exposed to air, SFG signals were still visible (Figure 2b). When we contacted the sample with water again, strong C−H signals were detected (Figure 2c), with different features compared to the spectrum collected from the d-PS/air interface. C−H signals repeatedly changed as we further alternately contacted the sample with water and air (e.g., Figure 2d). This phenomenon of reversible changes of C−H signals is similar to the fused silica/air or water interfaces. However, the details of these changes are different.

According to our calculations, for our sample geometry, the Fresnel coefficients of the BSA layer at the d-PS/air interface should be ~2 times greater than those at the d-PS/water interface. The intensity of SFG signal for the d-PS/air interface is therefore weighted about four times greater than that of the d-PS/water interface due to the difference in Fresnel coefficients. However, the magnitude of the observed BSA signal from the d-PS/air interface is comparable to that of the d-PS/water interface, which means that it is much weaker after normalization. The different SFG intensities of BSA molecules at the d-PS/air interface and the d-PS/water interface (after normalization) observed here indicate that they have different configurations at these two interfaces. We believe that at the d-PS/water interface, the hydrophobic methyl groups prefer to orient toward the d-PS. At the d-PS/air interface, the methyl groups can orient toward both air and d-PS sides and cause partial cancellation of SFG signals.

When we contacted the sample with FC-75 instead of water, C−H signals were still detected (Figure 2e), but with weaker

intensity (the intensity shown in Figure 2e was multiplied by a factor of 2). Because the refractive indices of water and FC-75 are very similar, different SFG intensities of BSA molecules at the d-PS/water interface and the d-PS/FC-75 interface indicate that they have different configurations at these two interfaces. As the d-PS/air interface, at the d-PS/FC-75 interface, methyl groups also can orient toward both d-PS and FC-75 sides to cause partial cancellation of the SFG signal.

Because FC-75 and air are hydrophobic contacting media, whereas water is hydrophilic, spectral features should be more similar for BSA molecules at the d-PS/air and d-PS/FC-75 interfaces. It is interesting to see that spectral features of BSA molecules at the d-PS/water interface are similar to those at the d-PS/FC-75 interface, but quite different from those at the d-PS/air interface. The distorted methyl symmetric stretching peak of BSA molecules at the d-PS/air interface and the undistorted methyl symmetric stretching peak at the d-PS/water and the d-PS/FC-75 interfaces indicate that interferences between the methyl symmetric stretching peak and the nonresonant background at various interfaces are different. One possible explanation for the undistorted methyl symmetric stretching peak of BSA molecules at the d-PS/water and d-PS/FC-75 interfaces is that the average absolute orientations of methyl groups perhaps align toward the d-PS in both cases. As mentioned, at the d-PS/water interface, methyl groups should orient toward the d-PS side due to the hydrophobicity of the d-PS. For the d-PS/FC-75 interface, the methyl groups of BSA molecules can orient toward both the d-PS and FC-75 sides, perhaps with less order at the FC-75 side than at the d-PS side. Thus, the overall orientation is toward the d-PS side, as the d-PS/water interface. Therefore, vibrational peaks of BSA molecules at the d-PS/water and d-PS/FC-75 interfaces can have similar interferences with the nonresonant background and generate similar SFG spectra.

For the d-PS/air interface, the methyl groups can orient toward both the air and d-PS sides. Probably due to the more ordered structure of methyl groups at the air side, the overall orientation of methyl groups at the d-PS/air interface orients toward the air. This shows that the preferential absolute orientations of methyl groups are in opposite directions at the d-PS/air and d-PS/water interfaces. Because of the different interferences between the methyl groups and the nonresonant background, the methyl symmetric stretching peak of BSA at the d-PS/air interface is distorted. This explains the different spectral features of BSA molecules at the d-PS/air and d-PS/water interfaces. This conclusion was also confirmed by our BSA adsorption studies on PS. The aromatic C−H signals of PS also have different interferences with the methyl symmetric stretches of BSA molecules at the PS/air and PS/water interfaces.

The behavior of BSA on d-PS is totally different from the case of BSA on fused silica. The spectral features of BSA molecules at the fused silica/air interface and the fused silica/FC-75 interface are similar. At both interfaces, methyl groups orient away from fused silica and toward the hydrophobic medium, air or FC-75.

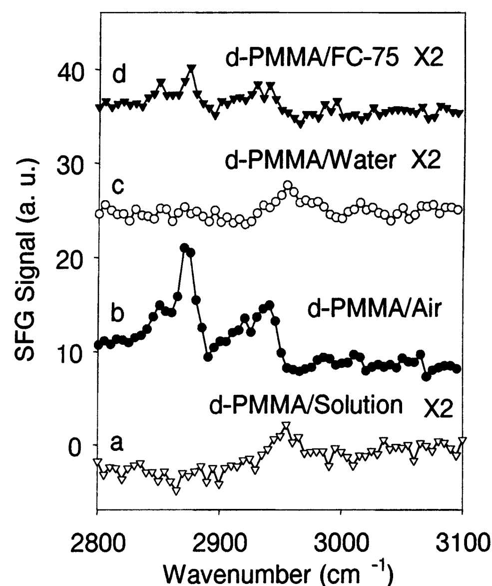


***Figure 3.*** SFG spectra collected from (a) d-PMMA/BSA solution interface; (b) d-PMMA/air interface after the sample was removed from solution and washed by water; (c) d-PMMA/water interface after the sample contacted water; (d) d-PMMA/FC-75 interface after procedures a and b and contacting the sample with FC-75; The intensities of the spectra in Figure 3a,c,d have been multiplied by a factor of 2.

**BSA on d-PMMA.** We have also studied BSA structures at the deuterated poly(methyl methacrylate) (d-PMMA)/BSA solution interface, and at the d-PMMA/air, d-PMMA/water, and d-PMMA/FC-75 interfaces (Figure 3a−d). The results are intermediate to those from interfaces involving fused silica and d-PS. This is reasonable since the surface of d-PMMA is dominated by ester methyl groups[24] and thus is more hydrophilic than d-PS, but not as hydrophilic as fused silica.

## Conclusion

Our SFG results show clearly that the configuration of adsorbed BSA films at different interfaces can be varied. The adsorbed BSA film can form a “hydrophilic” configuration between two hydrophilic media (fused silica/BSA solution), and a “hydrophobic” configuration between two hydrophobic media (polymer/air or polymer/hydrophobic solvent). When the film is in contact with two media of different hydrophobicity, the configuration exhibits a preferential alignment of hydrophobic groups. Configurational changes may include orientation and/or conformational changes of adsorbed BSA molecules, and they are closely related to the hydrophobicity of the contact media. Our results confirm that hydrophobic effects are a significant factor directing the adsorption behavior of globular proteins.

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