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Interfacial Molecular-level Structures of Polymers and Biomacromolecules Revealed via Sum Frequency Generation Vibrational Spectroscopy

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TITLE:

Interfacial Molecular-level Structures of Polymers and Biomacromolecules Revealed via Sum Frequency Generation Vibrational Spectroscopy

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KEYWORDS:

SFG, Fresnel coefficients, interfacial molecular-level structures, polymers, biomacromolecules, chiral structure, spatial confinement.

SUMMARY:

Being comprehensively utilized, sum frequency generation (SFG) vibrational spectroscopy can help to reveal chain conformational order and secondary structural change happening at polymer and biomacromolecule interfaces.

ABSTRACT:

As a second-order nonlinear optical spectroscopy, sum frequency generation (SFG) vibrational spectroscopy has widely been used in investigating various surfaces and interfaces. This non-invasive optical technique can provide the local molecular-level information with monolayer or submonolayer sensitivity. We here are providing experimental methodology on how to selectively detect the buried interface for both macromolecules and biomacromolecules. With this in mind, interfacial secondary structures of silk fibroin and water structures around model short-chain oligonucleotide duplex are discussed. The former shows a chain-chain overlap or spatial confinement effect and the latter shows a protection function against the Ca^{2+} ions resulting from the chiral spine superstructure of water.

INTRODUCTION:

Development of sum frequency generation (SFG) vibrational spectroscopy can be dated back to the work done by Shen et al. thirty years ago[1,2]. The uniqueness of the interfacial selectivity and sub-monolayer sensitivity makes SFG vibrational spectroscopy appreciated by a large number of researchers in the fields of physics, chemistry, biology, and materials science, etc[3-5]. Currently, a broad range of scientific issues related to surfaces and interfaces are being investigated using SFG, especially for complex interfaces with respect to polymers and biomacromolecules, such as the chain structures and structural relaxation at the buried polymer interfaces, the protein secondary structures, and the interfacial water structures[9-26].

For polymer surfaces and interfaces, thin-film samples are generally prepared by spin-coating to obtain the desired surfaces or interfaces. The problem arises due to the signal interference from the two interfaces of the as-prepared films, which leads to inconvenience for analyzing the collected SFG spectra[27-29]. In most cases, the vibrational signal only from one single interface, either film/substrate or film/the other medium, is desirable. Actually, the solution to this problem is quite easy, namely, to experimentally maximize the light fields at the desirable interface and minimize the light fields at the other interface. Hence, the Fresnel coefficients or the local field coefficients need to be calculated via the thin film model and to be validated with respect to the experimental results[3,9-15,30].

With the above background in mind, some polymer and biological interfaces could be investigated in order to understand fundamental science from the molecular level. In the following, taking three interfacial issues as examples: probing poly(2-hydroxyethyl methacrylate) (PHEMA) surface and buried interface with substrate[9], formation of silk fibroin (SF) secondary structures on the polystyrene (PS) surface and water structures surrounding model short-chain oligonucleotide duplex[16,21], we will show how the SFG vibrational spectroscopy helps to reveal the interfacial molecular-level structures in connection to the underlying science.

PROTOCOL:

1. SFG experimental

1.1. Use a commercial picosecond SFG system (**Table of Materials**), which provides a fundamental 1064 nm beam with a pulse width of ~20 ps and a frequency of 50 Hz, based on an Nd:YAG laser.

1.2. Convert the fundamental 1064 nm beam into a 532 nm beam and a 355 nm beam by using second and third harmonic modules. Directly guide the 532 nm beam as an input light beam and generate the other input mid-infrared (IR) beam covering the frequency range from 1000 to 4000 cm^{-1} through the optical parametric generation (OPG)/optical parametric amplification (OPA)/difference frequency generation (DFG) process.

1.3. Set the incident angles of two input beams to be 53° (IR) and 64° (visible), respectively, versus the surface normal.

1.4. To detect the polymer interfacial structures (either film/substrate interface or film/the other medium interface), use ssp (s-polarized sum-frequency beam, s-polarized visible beam and p-polarized infrared beam) and ppp polarization combinations.

1.5. To detect the interfacial protein secondary structures and water structures surrounding DNA, besides ssp and ppp, use chiral spp and psp polarization combinations were used.

1.6. To ensure the samples were not damaged, control the infrared and visible pulse energies to be ~70 and ~30 μJ, respectively. A schematic of the SFG process with the energy level diagram was shown in **Figure 1**. **Figure 2** shows the SFG system in our clean room.

2. Fresnel coefficients

2.1. Use right-angle prisms as substrates for all the experiments discussed here. There exist two interfaces for a polymer film on the solid substrate, i.e., polymer surface in air and polymer/substrate interface. Both can generate SFG signals since inversion symmetry is broken at both interfaces. Therefore, a collected SFG spectrum is an interfered one. However, the local field coefficients or the Fresnel coefficients at the two interfaces can be adjustable by varying either the incident angles or the film thickness one at a time or simultaneously[31,32]. This provides the opportunity for us to probe the SFG vibrational signal from only one interface. Here, the PHEMA film on the CaF_2 prism was taken as an example[9].

2.2. As shown in **Figure 3**, employ the right-angle prism geometry to detect the SFG signals generated from the bottom PHEMA film. The SFG output intensity in the reflected mode is expressed as

$$I(\omega) \propto \left|\chi_{eff}^{(2)}\right|^2 \quad (1)$$

where $\chi_{eff}^{(2)}$ denotes the effective second-order nonlinear susceptibility tensor.

2 $\chi_{eff}^{(2)}$ consists of three parts, namely, the prism/polymer interface, the polymer/bottom medium interface (bottom medium includes gas, liquid or solid.) and the nonresonant background, as shown in the following equation.

$$\chi_{eff}^{(2)} = F_{prism/polymer}\chi_{prism/polymer} + F_{polymer/medium}\chi_{polymer/medium} + \chi_{NR} \quad (2)$$

Here the bottom medium could be air, water or something else. *F* represents the corresponding Fresnel coefficient responsible for the local field correction.

2.3. Apply a thin-film model to calculate the Fresnel coefficients in this case. Here only brief calculation procedures are presented.

2.3.1. For the prism/polymer interface, use

$$L_{xx}^{prism/polymer}(\omega_i) = t_p(1 - r_{p23}e^{2i\alpha})\frac{cos(\phi_2)}{cos(\phi_1)} \quad (3)$$

$L_{yy}^{prism/polymer}(\omega_i) = t_s(1 + r_{s23}e^{2i\alpha})$ (4)

$L_{zz}^{prism/polymer}(\omega_i) = t_p(1 + r_{p23}e^{2i\alpha})\frac{n_1 n_2}{n_{12}^2}$ (5)

The meaning of each parameter shown is presented below.

2.3.1.1. ω_i denotes the beam frequency.

2.3.1.2. t_p and t_s denote the overall transmission coefficients and can be expressed as

$t_p = \frac{t_{p12}}{1+r_{p12}r_{p23}e^{2i\alpha}}$ (6)

$t_s = \frac{t_{s12}}{1+r_{s12}r_{s23}e^{2i\alpha}}$ (7)

2.3.1.3. t_{p12} and t_{s12} denote the linear transmission coefficients of the light beam at the prism/polymer interface.

2.3.1.4. r_{p23} and r_{s23} denote the linear reflection coefficients of the light beam at the polymer/medium interface.

2.3.1.5. α represents the phase difference between a reflective beam and its secondary *reflective* beam after it propagates across the polymer thin film and then reflects back, which can be expressed as

$\alpha = \frac{2\pi}{\lambda}n_2 d\, cos(\phi_2)$ (8)

2.3.1.6. λ represents the wavelength of the light beam and d is the polymer film thickness.

2.3.1.7. ϕ_1 and ϕ_2 represent the incident angles at the prism/polymer interface and the polymer/medium interface respectively.

2.3.1.8. n_1 and n_2 represent the refractive indices of the prism and polymer film respectively.

2.3.1.9. n_{12} *represents* the refractive indices of the polymer interfacial layers for the prism/polymer.

2.3.2. For the polymer/medium interface, use

$L_{xx}^{polymer/medium}(\omega_i) = t_p e^{i\Delta}(1 - r_{p23})\frac{cos(\phi_2)}{cos(\phi_1)}$ (9)

$L_{yy}^{polymer/medium}(\omega_i) = t_s e^{i\Delta}(1 + r_{s23})$ (10)

$L_{zz}^{polymer/medium}(\omega_i) = t_p e^{i\Delta}(1 + r_{p23})\frac{n_1 n_2}{n_{23}^2}$ (11)

2.3.2.1. Δ represents the phase difference of the light electrical fields at two interfaces.

2.3.2.2. Because the pulse width for our input beams is ~20 ps, the error from the time delay associated with the dispersion effect can be neglected.

2.3.2.3. The expression of such phase difference for the output SFG, the input visible and the input infrared beams can be separately written as

$$\Delta_{SF} = \frac{2\pi n_{2,SF} d}{\lambda_{SF}\, cos(\phi_{2,SF})} \quad (12)$$

$$\Delta_{VI} = \frac{2\pi n_{2,VI} d}{\lambda_{VI}\, cos(\phi_{2,VI})} - \frac{2\pi n_{1,VI} d}{\lambda_{VI}} (tan(\phi_{2,VI}) + tan(\phi_{2,SF}))\, sin(\phi_{1,VI}) \quad (13)$$

$$\Delta_{IN} = \frac{2\pi n_{2,IN} d}{\lambda_{IN}\, cos(\phi_{2,IN})} - \frac{2\pi n_{1,VI} d}{\lambda_{IN}} (tan(\phi_{2,IN}) + tan(\phi_{2,SF}))\, sin(\phi_{1,IN}) \quad (14)$$

2.4. From the above discussion, for the prism-polymer film-medium (1-2-3) system, express the total Fresnel coefficients for the prism/polymer and polymer/medium interfaces as the following equations, for *ssp* and *ppp* polarization combinations. Of course, both interfaces are considered azimuthally isotropic.

2.4.1. For the prism/polymer interface, the expressions of the total Fresnel coefficients for both *ssp* and *ppp* polarization combinations are presented as follows.

2.4.1.1. For *ssp*, the equation is

$$F_{ssp,yyz}^{prism/polymer} = L_{yy,SF}^{prism/polymer} t_{s10,SF} L_{yy,VI}^{prism/polymer} t_{s01,VI} L_{zz,IN}^{prism/polymer} t_{p01,IN}\, sin(\phi_{1,IN}) \quad (15).$$

2.4.1.2. And for *ppp*, the equation is

$$F_{ppp,xxz}^{prism/polymer} = -L_{xx,SF}^{prism/polymer} t_{p10,SF} \cos(\phi_{1,SF}) L_{xx,VI}^{prism/polymer} t_{p01,VI} \cos(\phi_{1,VI}) L_{zz,IN}^{prism/polymer} t_{p01,IN} \sin(\phi_{1,IN}) \quad (16)$$

$$F_{ppp,xzx}^{prism/polymer} = -L_{xx,SF}^{prism/polymer} t_{p10,SF} \cos(\phi_{1,SF}) L_{zz,VI}^{prism/polymer} t_{p01,VI} \sin(\phi_{1,VI}) L_{xx,IN}^{prism/polymer} t_{p01,IN} \cos(\phi_{1,IN}) \quad (17)$$

$$F_{ppp,zxx}^{prism/polymer} = L_{zz,SF}^{prism/polymer} t_{p10,SF} \sin(\phi_{1,SF}) L_{xx,VI}^{prism/polymer} t_{p01,VI} \cos(\phi_{1,VI}) L_{xx,IN}^{prism/polymer} t_{p01,IN} \cos(\phi_{1,IN}) \quad (18)$$

$$F_{ppp,zzz}^{prism/polymer} = L_{zz,SF}^{prism/polymer} t_{p10,SF} \sin(\phi_{1,SF}) L_{zz,VI}^{prism/polymer} t_{p01,VI} \sin(\phi_{1,VI}) L_{zz,IN}^{prism/polymer} t_{p01,IN} \sin(\phi_{1,IN}) \quad (19).$$

2.4.1.3. t_{10} and t_{01} denote the linear transmission coefficients at the air/prism and prism/air interfaces respectively.

2.4.2. For the polymer/medium interface, the expressions of the total Fresnel coefficients for

both *ssp* and *ppp* polarization combinations are described as follows.

2.4.2.1. For *ssp*, the equation is

$$F_{ssp,yyz}^{polymer/medium} = L_{yy,SF}^{polymer/medium} t_{s10,SF} L_{yy,VI}^{polymer/medium} t_{s01,VI} L_{zz,IN}^{polymer/medium} t_{p01,IN} \sin(\phi_{1,IN}) \quad (20).$$

2.4.2.2. For *ppp*, the equations are

$$F_{ppp,xxz}^{polymer/medium} = -L_{xx,SF}^{polymer/medium} t_{p10,SF} \cos(\phi_{1,SF}) L_{xx,VI}^{polymer/medium} t_{p01,VI} \cos(\phi_{1,VI}) L_{zz,IN}^{polymer/medium} t_{p01,IN} \sin(\phi_{1,IN}) \quad (21)$$

$$F_{ppp,xzx}^{polymer/medium} = -L_{xx,SF}^{polymer/medium} t_{p10,SF} \cos(\phi_{1,SF}) L_{zz,VI}^{polymer/medium} t_{p01,VI} \sin(\phi_{1,VI}) L_{xx,IN}^{polymer/medium} t_{p01,IN} \cos(\phi_{1,IN}) \quad (22)$$

$$F_{ppp,zxx}^{polymer/medium} = L_{zz,SF}^{polymer/medium} t_{p10,SF} \sin(\phi_{1,SF}) L_{xx,VI}^{polymer/medium} t_{p01,VI} \cos(\phi_{1,VI}) L_{xx,IN}^{polymer/medium} t_{p01,IN} \cos(\phi_{1,IN}) \quad (23)$$

$$F_{ppp,zzz}^{polymer/medium} = L_{zz,SF}^{polymer/medium} t_{p10,SF} \sin(\phi_{1,SF}) L_{zz,VI}^{polymer/medium} t_{p01,VI} \sin(\phi_{1,VI}) L_{zz,IN}^{polymer/medium} t_{p01,IN} \sin(\phi_{1,IN}) \quad (24).$$

2.5. After calculating the Fresnel coefficients using the sandwiched model, plot them as a function of the film thickness, as shown in **Figure 4**.

NOTE: In this case, there exists a thickness range for collecting the SFG signal from the CaF_2 prism/PHEMA interface with neglectable contribution from the other interface, which is around 150 nm. Similarly, a suitable thickness can be chosen for detection of the PHEMA/bottom medium interface with neglectable contribution from the CaF_2 prism/PHEMA interface.

3. Chiral SFG polarization combination

3.1. For the normal achiral interface, commonly, use $C_{\infty v}$ symmetry in terms of ensemble average[33,34]. With operation of inversion symmetry, the nonzero second-order nonlinear susceptibility tensor components can be deduced, which are χ_{xxz}, χ_{xzx}, χ_{zxx}, χ_{yyz}, χ_{yzy}, χ_{zyy} and χ_{zzz} (the existing terms can be further reduced if an isotropic interface is assumed, which means x and y are the same). However, for the chiral interface, situation will be different. The chiral interface possesses the C_{∞} symmetry, only the rotation symmetry operation is allowed. In this case, besides the normal achiral terms, more second-order nonlinear susceptibilities will be nonzero, which can be termed as the chiral terms, namely, χ_{zyx}, χ_{zxy} and χ_{yzx} under the consideration of non-electronic resonance. Therefore, by using *psp*, *pps* and *spp* polarization combinations, chiral SFG spectra can be collected[33,34].

4. Sample preparation

4.1. Preparation of PHEMA film

4.1.1. Dissolve PHEMA powder (see **Table of Materials**) in anhydrous ethanol to prepare the solution with 2 wt% and 4 wt% respectively.

4.1.2. Before deposition of the PHEMA films, soak the CaF_2 right-angle prisms in the toluene solvent firstly and then wash them with ethanol and ultrapure water (18.2 MΩ·cm).

4.1.3. Afterwards, expose the substrates (CaF_2 right-angle prisms) to oxygen plasma to remove possible organic contaminants by plasma cleaner (see **Table of Materials**).

4.1.3.1. First turn on the plasma cleaner and put the substrates in it.

4.1.3.2. Then turn on the vacuum pump to vacuumize the cleaner. Input the oxygen in it.

4.1.3.3. Finally set 4 minutes for cleaning. After that, preserve the clean substrates for the sequential PHEMA film preparation.

4.1.4. Then prepare the PHEMA films on the CaF_2 prisms by a spin-coater (see **Table of Materials**). Adjust the film thicknesses by the solution concentration and spin speed.

4.1.4.1. Immobilize the CaF_2 prism on the sucking disc of spin-coater.

4.1.4.2. Drop one drop of the PHEMA solution prepared before onto the clean substrates at 1,500 rpm for 1 min (film thickness 2 wt% for 100 nm and 4 wt% for 200 nm).

4.1.5. Anneal all the prepared PHEMA films in a vacuum oven at 80 °C overnight.

4.2. Preparation of silk fibroin (SF)

NOTE: The protocol suggested by Kaplan et al.[35] was adopted.

4.2.1. Place 7.5 g of silk cocoons of *B. mori* in the boiling sodium carbonate (Na_2CO_3, 0.02 M) aqueous solution (3 L) for 30 min. Remove the fibrous SF to a clean container.

4.2.2. Wash the obtained fibrous SF with deionized water for three times under stirring in order to remove the sericin molecules and leave only the SF molecules in the fibrous sample.

4.2.3. Dry the fibrous SF sample in a vacuum oven at 60 °C overnight.

4.2.4. Afterwards, dissolve the degummed fibrous SF sample in a lithium bromide (LiBr, 9.3 M) aqueous solution (1 g of SF was solved in ~4 mL of LiBr solution.) and incubate it at 60 °C for 2 h

under stirring.

4.2.5. Dialyze the SF solution against deionized water (3,500 Da dialysis bags) for 3 days to remove the dissolved LiBr. Change new deionized water three times every day. Finally store the processed SF solution at 4 °C for later SFG experiments.

4.3. Preparation of short-chain oligonucleotide duplex

4.3.1. Order the single-stranded oligonucleotide sample with its 3'-end modified by cholesterol-triethylene glycol (Chol-TEG) (5'-GCTTCCGAAGGTCGA-3') from a commercial corporation (see **Table of Materials**) as well as the complementary one. For each single strand, dissolve 10 nmol of the sample powder in 0.5 ml ultrapure water. Then mix them together to form the duplex oligonucleotide solution (10 nmol/mL).

4.3.2. Mix 2 mg of 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 2 mg of deuterated DPPC (d-DPPC) and dissolve them in 1 mL of chloroform to prepare the lipid solution.

4.3.3. Preparation of DPPC & d-DPPC monolayer by a Langmuir–Blodgett (LB) trough

4.3.3.1. Attach the right-angle CaF_2 prism to a homemade sample holder with one prism face perpendicularly dipped into the aqueous environment of the LB trough.

4.3.3.2. Afterwards, inject the mixed lipid solution prepared before onto the water surface until the surface pressure reached a certain value below 34 $mN \cdot m^{-1}$.

4.3.3.3. After the surface pressure levels off, use two Teflon barriers to compress the lipid monolayer at a ratio of 5 mm/min until a surface pressure of 34 $mN \cdot m^{-1}$ was reached.

4.3.3.4. Lift the prism with a lipid monolayer out of the water at a rate of 1 mm/min vertically.

4.3.4. Preparation of the other lipid monolayer

4.3.4.1. To facilitate the assembly of the duplex oligonucleotide and the lipid molecules via the hydrophobic interaction (cholesterol and a lipid alkyl chain), mix the duplex oligonucleotide solution with the lipid solution in a molar ratio of 1:100 (oligonucleotide to lipid).

4.3.4.2. Inject the mixed lipid and duplex oligonucleotide solution onto the water surface in a homemade Teflon container until a surface pressure of 34 $mN \cdot m^{-1}$ was reached.

4.3.5. Finally, put the lipid monolayer at the bottom of the prism in contact with the lipid monolayer with inserted duplex oligonucleotides on the water surface to form the final sample

for the SFG measurement.

4.4. Lorentz equation

4.4.1. Use the Lorentz equation to fit the SFG spectra to extract the vibrational information for a **specific** vibrational mode.

$$\chi_{eff}^{(2)} = \chi_{NR}^{(2)} + \sum_q \frac{A_q}{\omega_{IR} - \omega_q + i\Gamma_q} \quad (25)$$

where A_q represents the intensity of the *qth* vibrational mode, ω_q represents the resonant frequency, Γ_q denotes the half width at half maximum (HWHM) and ω_{IR} represents the scanning frequency of the incident IR beam.

REPRESENTATIVE RESULTS:

In the Fresnel coefficient part of Protocol Section, we have shown that, theoretically, it is feasible to selectively detect only one single interface at one time. Here, experimentally, we confirmed that this methodology is basically correct, as shown in **Figure 5** and **Figure 6**.

Figure 5 shows the buried interfacial PHEMA structure after water intrusion with a ~150 nm PHEMA hydrogel film and **Figure 6** shows the surface structure in water with a ~430 nm PHEMA hydrogel film. Panels A and B correspond to the CH and CO ranges respectively for both figures. At the buried interface, all the observed vibrational peaks are sharp and clear. The reason is that the CaF_2 substrate is smooth and cannot be penetrated by PHEMA molecules, leading to a sharp CaF_2/PHEMA interface. However, at the surface, because water molecules can interact with PHEMA and diffuse into the bulk, the PHEMA/water interface would be not as sharp as the buried one. Therefore, different spectral profiles are observed for these two interfaces.

Figure 1. Schematic show of the SFG process (left panel) with the energy transition diagram (right panel).

Figure 2. The SFG system in the lab.

Figure 3. Schematic shows the light propagation path in prism for SFG experiment. The numbers 0, 1, 2 and 3 represent the air, prism, PHEMA and bottom medium (the bottom medium can be air, solid or liquid.), respectively. Reproduced from Li, X.; Li, B.; Zhang, X.; Li, C.; Guo, Z.; Zhou, D.; Lu, X. Macromolecules 2016, 49, 3116−3125 (ref 9). Copyright 2016 American Chemical Society. This figure has been modified from [9].

Figure 4. Calculated Fresnel coefficients as a function of the film thickness for the prism geometry in water for *ssp* and *ppp* polarization combinations. Panels **A1** to **C1** correspond to the CH range and Panels **A2** to **C2** correspond to the CO range. Reproduced from Li, X.; Li, B.; Zhang, X.; Li, C.; Guo, Z.; Zhou, D.; Lu, X. Macromolecules 2016, 49, 3116−3125 (ref 9). Copyright 2016 American Chemical Society. This figure has been modified from [9].

Figure 5. *ssp* and *ppp* spectra of the CaF_2/PHEMA interface after water exposure. A: CH and

OH range; B: CO range. The black curves are the fitted results by using Lorentz equation. The spectra have been offset for clarity. Reproduced from Li, X.; Li, B.; Zhang, X.; Li, C.; Guo, Z.; Zhou, D.; Lu, X. Macromolecules 2016, 49, 3116−3125 (ref 9). Copyright 2016 American Chemical Society. This figure has been modified from [9].

Figure 6. *ssp* and *ppp* spectra of the PHEMA surface on CaF_2 prism. A: CH and OH range; B: CO range. The sample was placed into contact with water. The black curves are the fitted results by using Lorentz equation. The spectra have been offset for clarity. Reproduced from Li, X.; Li, B.; Zhang, X.; Li, C.; Guo, Z.; Zhou, D.; Lu, X. Macromolecules 2016, 49, 3116−3125 (ref 9). Copyright 2016 American Chemical Society. This figure has been modified from [9].

Figure 7. Normalized chiral (*psp*) SFG spectra in the amide I (Panel A) and N-H (Panel B) ranges for the PS/SF solution (90 mg/mL) interface before and after adding methanol. The dots are experimental data and the solid lines are the fitted curves. Spectra have been offset for clarity. Reproduced from Li, X.; Deng, G.; Ma, L.; Lu, X.; Langmuir 2018, 34, 9453−9459 (ref 16). Copyright 2018 American Chemical Society. This figure has been modified from [16].

Figure 8. Normalized chiral (*psp*) SFG spectra in the amide I (Panel A) and N-H (Panel B) ranges for the PS/SF solution (1 mg/mL) interface before and after adding methanol. The dots are experimental data and the solid lines are the fitted curves (blue). Spectra have been offset for clarity. Reproduced from Li, X.; Deng, G.; Ma, L.; Lu, X.; Langmuir 2018, 34, 9453−9459 (ref 16). Copyright 2018 American Chemical Society. This figure has been modified from [16].

Figure 9. Achiral (*ssp*, A) and chiral (*spp*, B) SFG spectra for the duplex oligonucleotide-anchored lipid bilayer in contact with the Ca^{2+} solutions with different concentrations (from 0.6 mM to 6 mM). The data points were approximately fitted by using the Lorentz equation. The change of the integrated area for the water vibrational signals as a function of the Ca^{2+} concentration was presented (*ssp,* C; *spp*, **D**). All the spectra have been normalized and offset for clarity. Reproduced from Li, X.; Ma, L.; Lu, X.; Langmuir 2018, 34, 14774−14779 (ref 21). Copyright 2018 American Chemical Society. This figure has been modified from [21].

DISCUSSION:

To investigate the structural information from a molecular level, SFG has its inherent advantages (i.e., monolayer or sub-monolayer sensitivity and interfacial selectivity), which can be applied to study various interfaces, such as the solid/solid, solid/liquid, solid/gas, liquid/gas, liquid/liquid interfaces. Although the equipment maintenance and the optical alignment are still time-consuming, the payoff is significant in that the detailed molecular-level information at the surfaces and interfaces can be obtained.

Probing Poly(2-hydroxyethyl methacrylate) Surface and Buried interface in Solution: As we demonstrated above, the light field coefficients can be adjusted. We can confirm this experimentally. At the buried interface with the substrate, because the CaF_2 substrate surface is smooth and cannot be penetrated by PHEMA molecules, this interface is a sharp one. However, at the surface with water, water molecules can interact with PHEMA molecules and diffuse into

the bulk. Hence this interface is blurry, and not as sharp as the buried one. Therefore, different SFG spectral profiles would be observed for these two interfaces. Our experimental SFG data did prove this, indicating the capability to selectively probe the buried interface with the substrate or the surface in solution.

Interchain Interaction or Confinement effect on Formation of Silk Fibroin Secondary Structures: A key factor is the critical overlapping concentration (C^*). For SF, C^* is ~1.8 mg/mL. Experimentally, for the SF solution of ~90 mg/mL (above C^*), no chiral (*psp*) SFG vibrational signals were detected at the SF solution/PS interface unless an inducing agent-methanol was added, as shown in **Figure 7**. But, for the SF solution of ~1 mg/mL (below C^*), chiral (*psp*) SFG vibrational signals can be directly detected without adding methanol, as shown in **Figure 8**, which indicates that the ordered secondary structures have already been formed at the SF solution/PS interface. Since C^* is a threshold concentration for the chain-chain overlap, the chain-chain interaction or the spatial confinement has to be taken as a regulating factor here for the formation of SF secondary structures at the interface.

Water Molecular Structures Surrounding Short-chain Oligonucleotide Duplex: For a short-chain oligonucleotide duplex in the water solution, chiral water SFG vibrational signals correspond to the hydration layer of the chiral spine in the minor groove. Achiral water SFG vibrational signals mostly correspond to the water layer surrounding the oligonucleotide duplex chain and the bilayer (the chiral spine of the water layer also contributes)[33]. In a Ca^{2+} concentration range from 0.6 to 6 mM, as shown in **Figure 9**, we found, there was no obvious change for the chiral water vibrational signals in terms of the Ca^{2+} concentration. However, the achiral water vibrational signals were strongly affected when the Ca^{2+} concentration was changed. This indicates that the chiral spine of the water layer closely binding to the oligonucleotide duplex may protect the oligonucleotide from the Ca^{2+} ions, in the normal biological condition.

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DISCLOSURES:

We have nothing to disclose.

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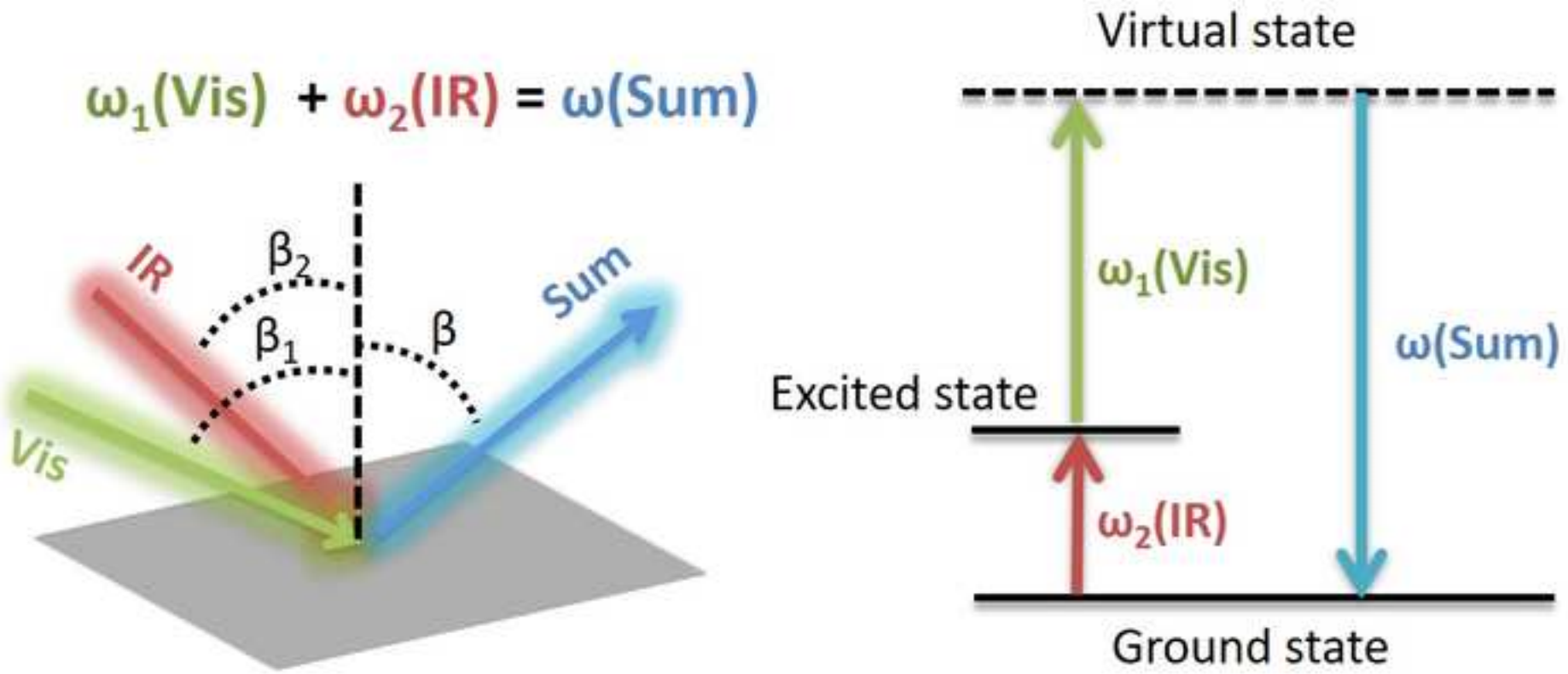

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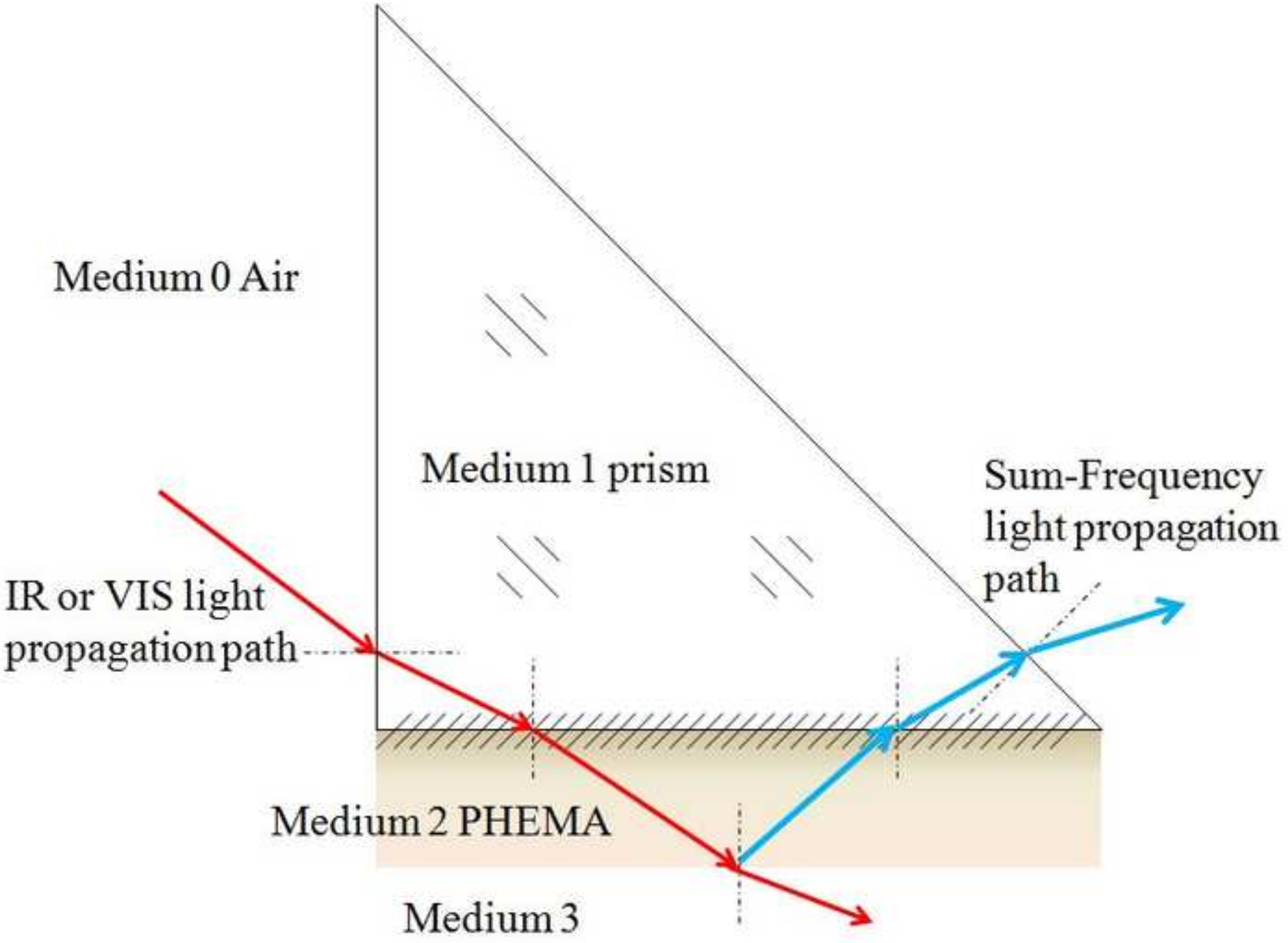

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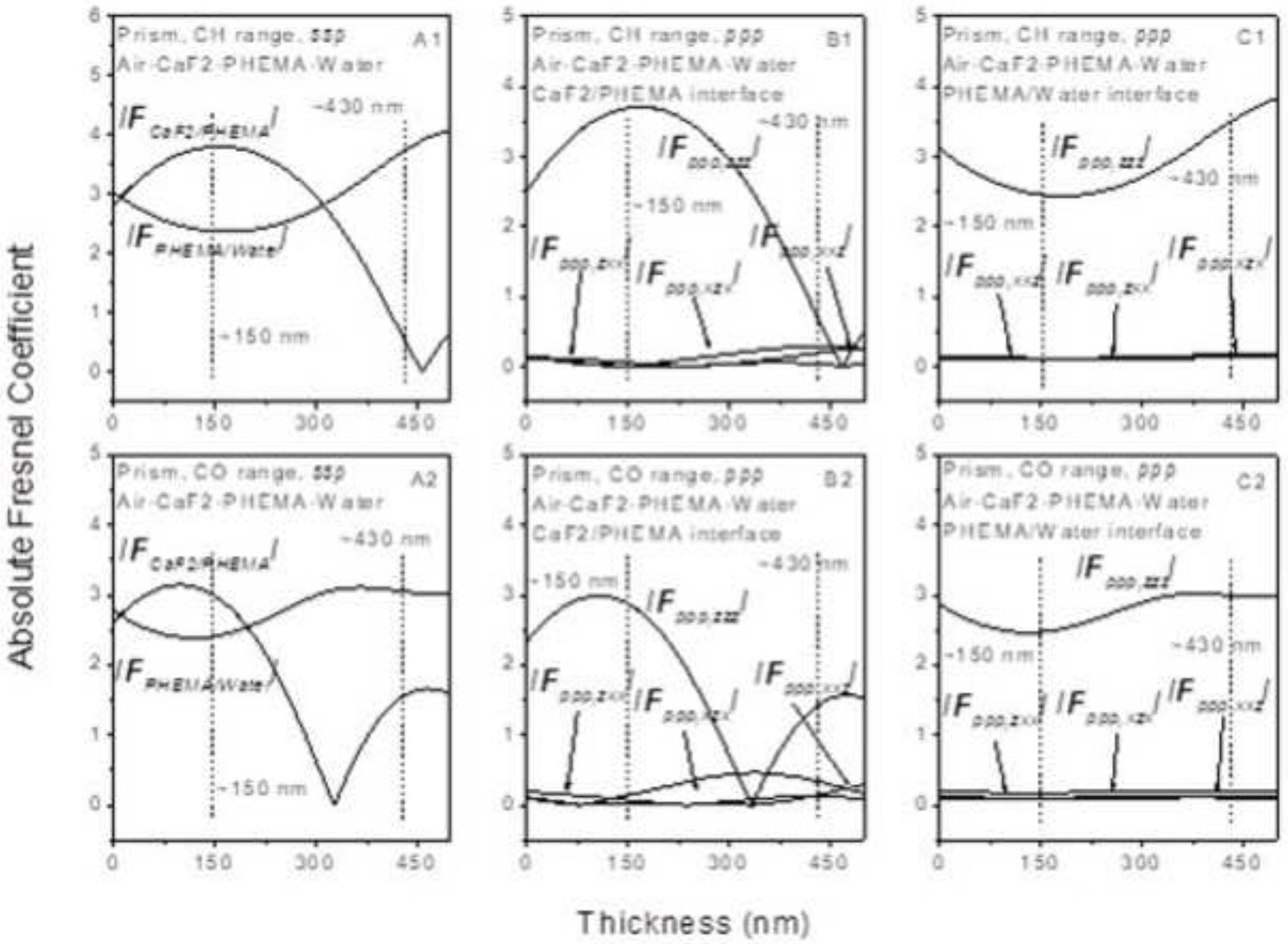
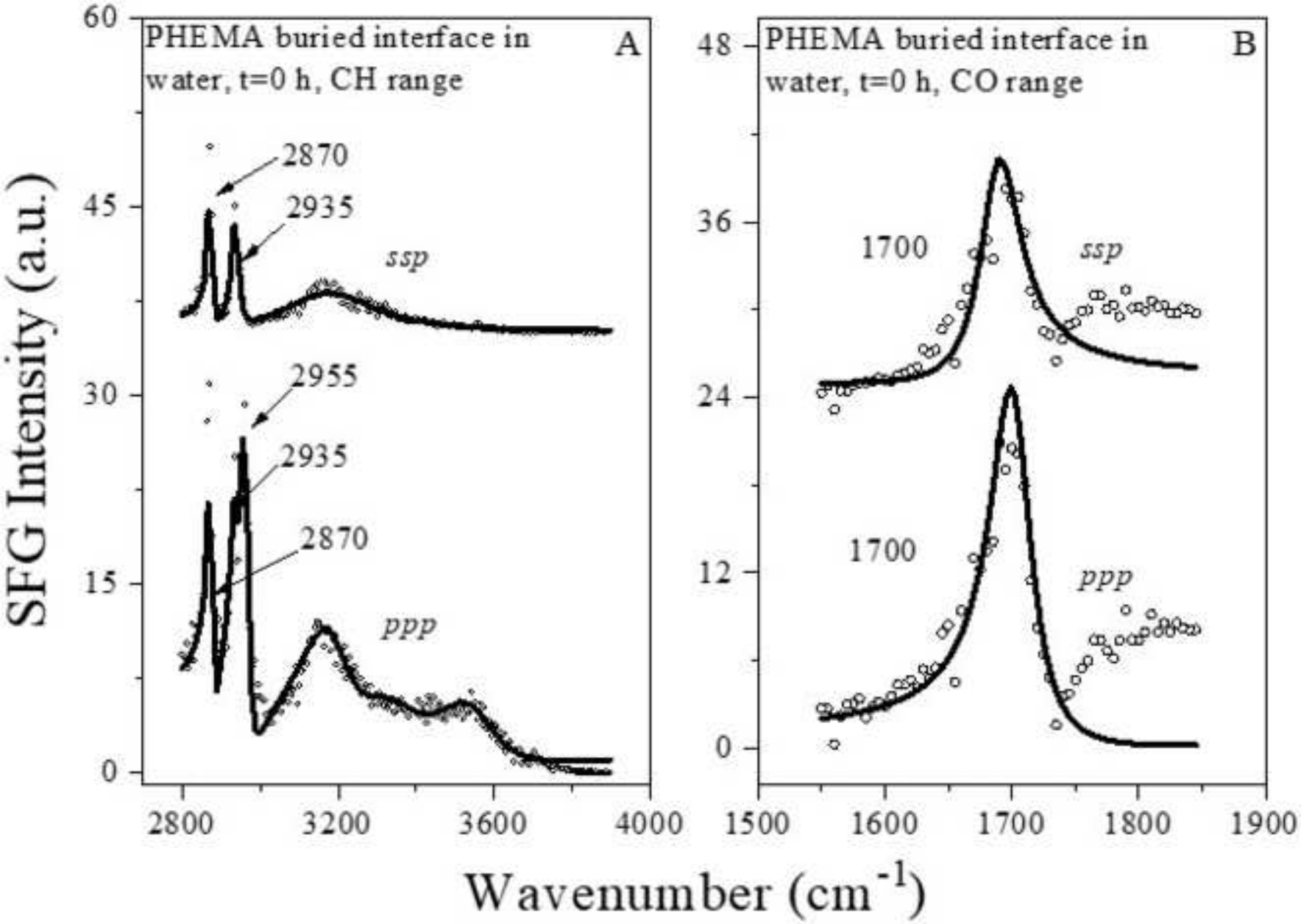


Figure5



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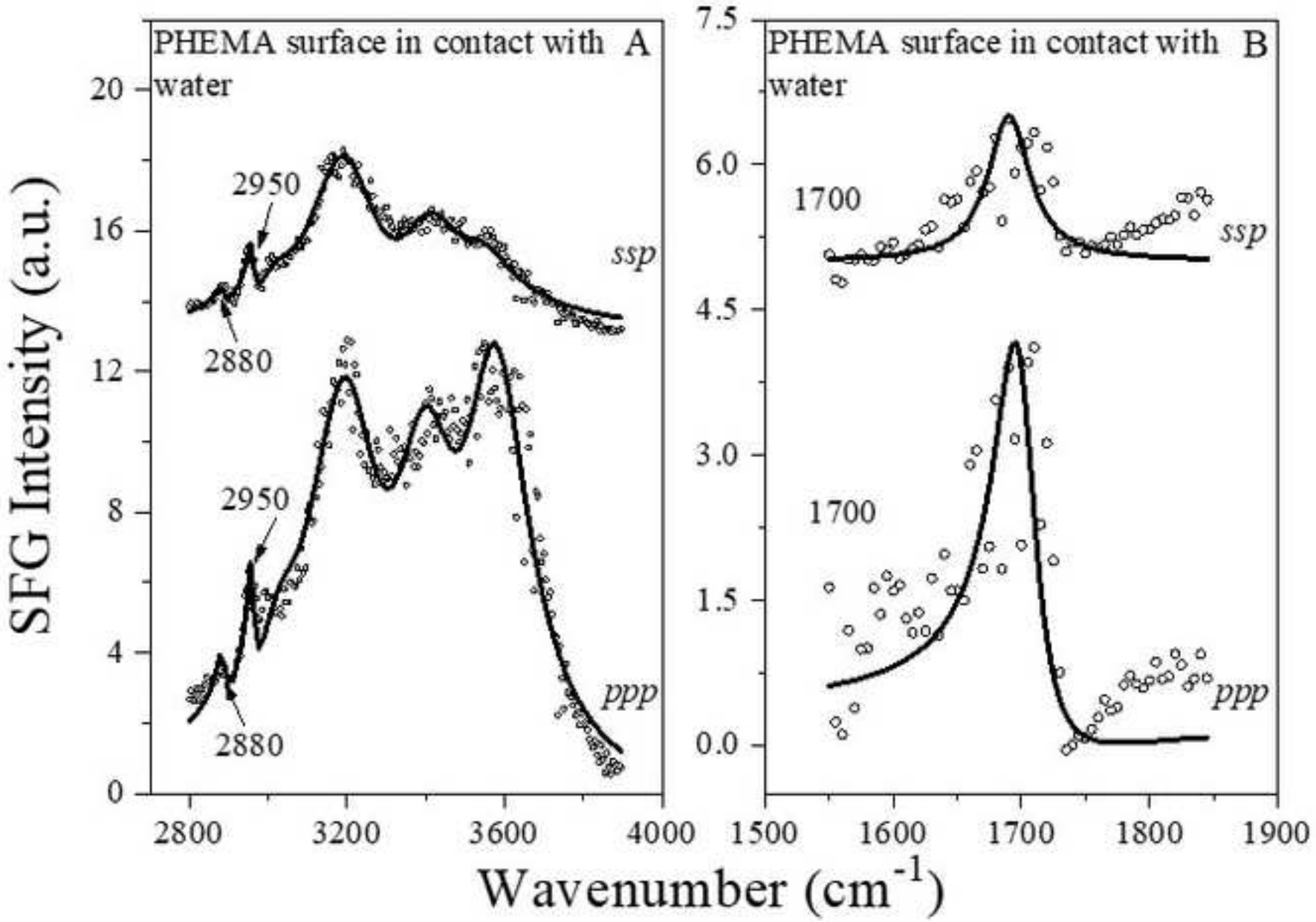

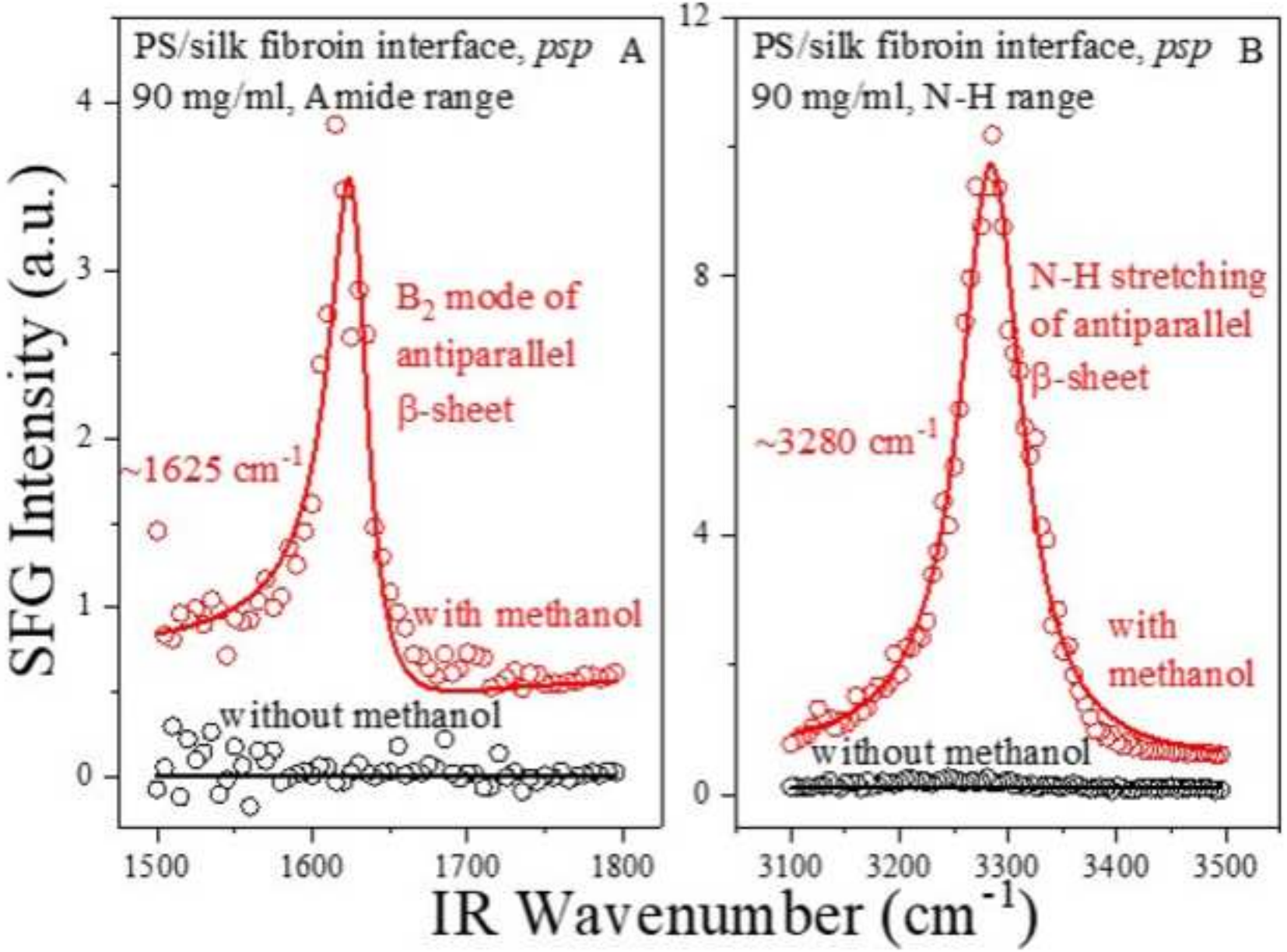

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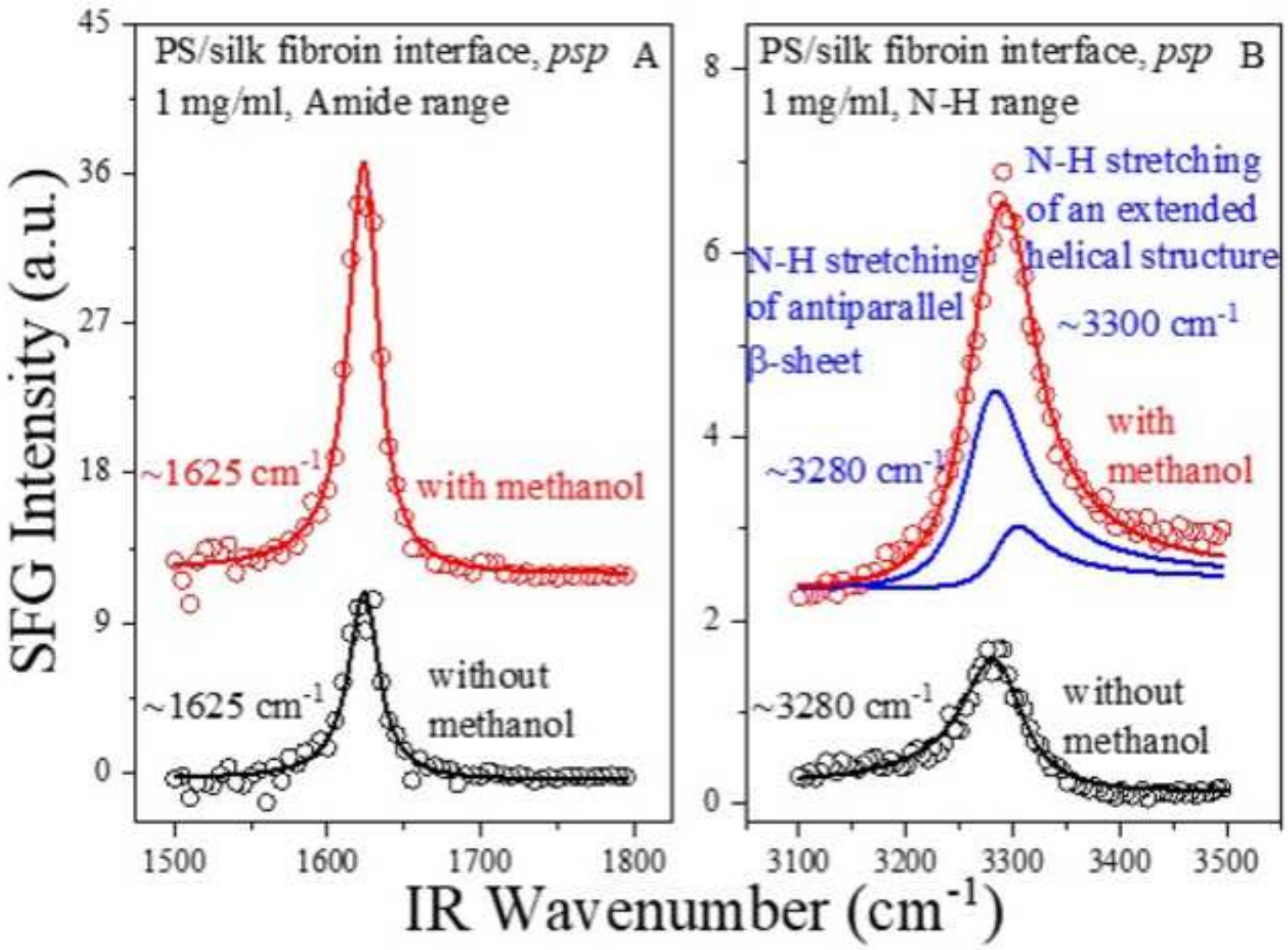

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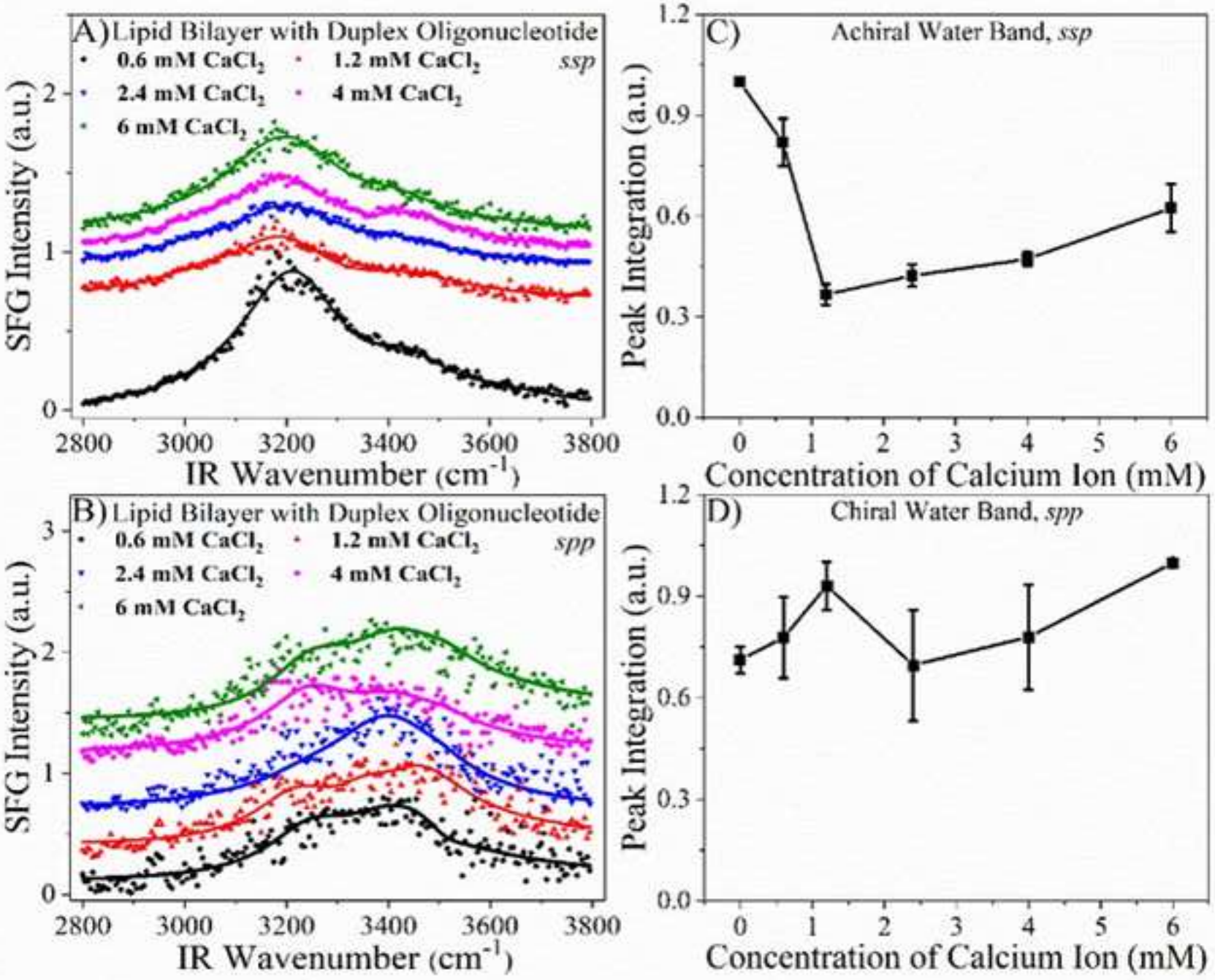

Name of Material/ Equipment
1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)
Anhydrous ethanol
CaF2 prism
Calcium chloride anhydrous
deuterated DPPC (d-DPPC)
Electromagnetic oven
Langmuir-Blodgett (LB) trough
Lithium bromide anhydrous
Milli-Q synthesis system
Plasma cleaner
Poly(2-hydroxyethyl methacrylate) (PHEMA)
Polystyrene
Silk cocoons
Single complementary strand of oligonucleotide
Single strand of oligonucleotide
Sodium carbonate anhydrous
Spin-coater
Step profiler
Sum frequency generation (SFG) vibrational spectroscopy system

Company	Catalog Number	Comments/Description
Avanti Polar Lipids, Inc.	850355P-1g	
Sinopharm Chemical Reagent Co., Ltd	100092680	≥99.7%
Chengdu YaSi Optoelectronics Co., Ltd.		
Sinopharm Chemical Reagent Co., Ltd	10005817	≥96.0%
Avanti Polar Lipids, Inc.	860345P-100mg	
Zhejiang Supor Co., Ltd	C21-SDHCB37	
KSV NIMA Co., Ltd.	KN 2003	
Sinopharm Chemical Reagent Co., Ltd	20056926	
Millipore		Ultrapure water
Chengdu Mingheng Science&Technology Co., Ltd	PDC-MG	Oxygen plasma cleaning
Sigma-Aldrich Co., LLC.	192066 MSDS	Mw = 300 000
Sigma-Aldrich Co., LLC.	330345 MSDS	Mw = 48 kDa and Mn = 47 kDa
		From *Bombyx mori*
Nanjing Genscript Biotechnology Co., Ltd.	H03596	5'-CGAAGGCTTCCAGCT-3'
Nanjing Genscript Biotechnology Co., Ltd.	H04936	3′-end modified by cholesterol-triethylene
Sinopharm Chemical Reagent Co., Ltd	10019260	≥99.8%
Institute of Microelectronics of the Chinese Academy of Scien	KW-4A	For the prepartion of ploymer films
Veeco	DEKTAK 150	For the measurement of film thickness
EKSPLA		A commercial picosecond SFG system

glycol(Chol-TEG) (5′-GCTTCCGAAGGTCGA-3′)

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Author(s): Xu Li, Liang Ma, Xiaolin Lu*

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"we", "you", "our" etc.).

Author reply: We have revised the text.

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15. Lines 73-215: Please note that calculations are not appropriate for filming; therefore I suggest unhighlighting these steps.

Author reply: We agree with comment and unhighlighted these steps.

16. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to publishbed material specifying how to perform the protocol action. See examples below.

17. Line 220: Please list an approximate volume and concentration of the solution to prepare.

Author reply: We have added the concentration.

18. Line 226: Please describe how this is done. What are the substrates?

Author reply: We added the necessary information.

19. Lines 229-231: Please describe how to prepare the PHEMA films using a spin-coater and specify the film thickness as well as the solution concentration and spin speed used in this step.

Author reply: We did this.

20. Line 233: How many films are produced?

Author reply: One film was prepared on one substrate. And we repeated the experiment for at least 4 or 5 times.

21. Line 238: What volume of Na2CO3 solution is used?

Author reply: The solution volume used was 3 L.

22. Line 241: Is the SF product removed from the solution in step 2.1 and placed in a container with deionized water? Please clarify. What volume of deionized water is used?

Author reply: Yes, it is. We have clarified it.

23. Line 246: What volume of LiBr solution is used?

Author reply: 1 g SF was solved in ~4ml LiBr solution.

24. Line 249: How often is the deionized water changed during dialysis?

Author reply: The deionized water was changed 3 times every day.

25. Line 288: Please describe how to perform the SFG measurement. For instance, please revise the paragraph in lines 55-72 to a stepwise protocol showing how the measurement is done.

Author reply: In the SFG experiment, we only need to adjust the optical path. It is difficult to present how to optimize the optical path by adjusting the rotary knobs. We sincerely hope the editor can understand this.

26. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Author reply: We added the necessary information in the discussion part.

"To investigate the structural information from a molecular level, SFG has its inherent advantages, saying, monolayer or sub-monolayer sensitivity and interfacial selectivity, which can be applied to study various interfaces, such as the solid/solid, solid/liquid, solid/gas, liquid/gas, liquid/liquid interfaces. Although the equipment maintenance and the optical alignment are still time-consuming, the payback is significant in that the detailed molecular-level information at the surfaces and interfaces can be obtained."

27. References: Please do not abbreviate journal titles.

Author reply: We have corrected them.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

General Comment: In this manuscript, the authors demonstrate an experimental methodology to selectively detect the buried interface, which could be potentially useful to avoid the interference by the 2 interfaces of a thin film. Instead of the conventional method of using with different incident angles to selectively detect the interface in SFG field, in this journal, the authors fixed the incidental angle, and calculated Fresnel coefficient of different polarizations as a function of thickness, then choose the suitable thickness to selectively detect the substrate/film interface or film/air interface.

I think it is a good paper to be published in JoVE after answer the follow questions and minor corrections in the manuscript.

Author Reply: We thank the reviewer for such comments. The interference effect can

lead to difficulty to analyze the SFG spectra, which is the reason why we introduced a methodology to solve it.

Major Concerns:

Comment 1: Selectively detecting the 2 interfaces (film/substrate and film/air) by using 2 different incidental angles is quite well-known in the SFG field - for example, in the paper of Langmuir, 2015, 31 (45), pp 12401-12407; the authors used incident angles (with respect to the surface normal of the sapphire prism face) of 42°, 16°, and 2° to probe the Polymer/air, Polymer/H2O (and Polymer/D2O), and Polymer/sapphire interfaces, respectively. Since the refractive index of mediums are different, thus the calculated incident angles are also different and could be used to distinguish the interfaces/surfaces. How did the author compare their methodology of using 2 thicknesses to differentiate the 2 interfaces vs. the conventional method of using 2 different incidental angles to differentiate the 2 interfaces? What is the advantage of the authors method?

Author Reply: We thank the reviewer for raising such a good question. We carefully read this paper, Langmuir, 2015, 31 (45), pp 12401-12407. The methodology by adjusting incident angles is an alternative way in this case. We cannot say which one (theirs and ours) is better since both are feasible and useful. For ours, to detect two single interfaces respectively, two samples have to be prepared with the appropriate thicknesses. For theirs, one sample with the chosen thickness should be enough. However, changing the incident angles will definitely change the optical path. The authors (Langmuir, 2015, 31 (45), pp 12401-12407) have to play with it. We sincerely hope the reviewer can be satisfied with our reply.

Comment 2: In the 2nd paragraph of discussion portion, the authors discussed the results of 2 different conformation of below and above the critical concentration. Isn't it better to support authors results by providing SFG spectra evidence of below this threshold concentration (ordered secondary structure) and above the threshold concentration (overlapped secondary structure)?

Author Reply: We thank the reviewer for raising such a positive comment. We added the corresponding SFG spectra.

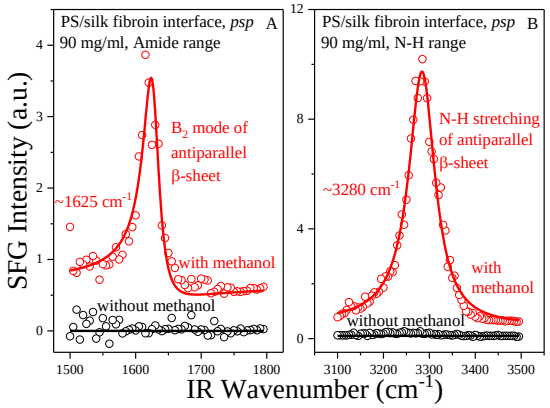

Figure 7. Normalized chiral (*psp*) SFG spectra in the amide I (Panel A) and N-H (Panel B) ranges for the PS/SF solution (90 mg/mL) interface before and after adding methanol. The dots are experimental data and the solid lines are the fitted curves.

Spectra have been offset for clarity.

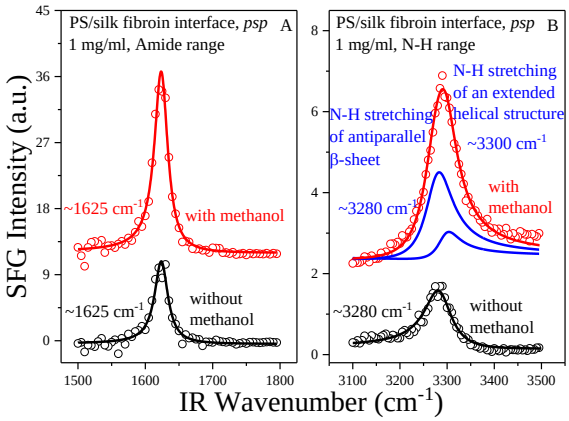

Figure 8. Normalized chiral (*psp*) SFG spectra in the amide I (Panel A) and N-H (Panel B) ranges for the PS/SF solution (1 mg/mL) interface before and after adding methanol. The dots are experimental data and the solid lines are the fitted curves (blue). Spectra have been offset for clarity.

Comment 3: In the 3rd paragraph of discussion portion, instead of just explanation, shall the authors prove the experiment data / spectra evidence to prove the addition of of Ca2+ concentration effect on the water SFG vibrational signals?

Author Reply: Yes, according to this comment, we added the corresponding SFG spectra.

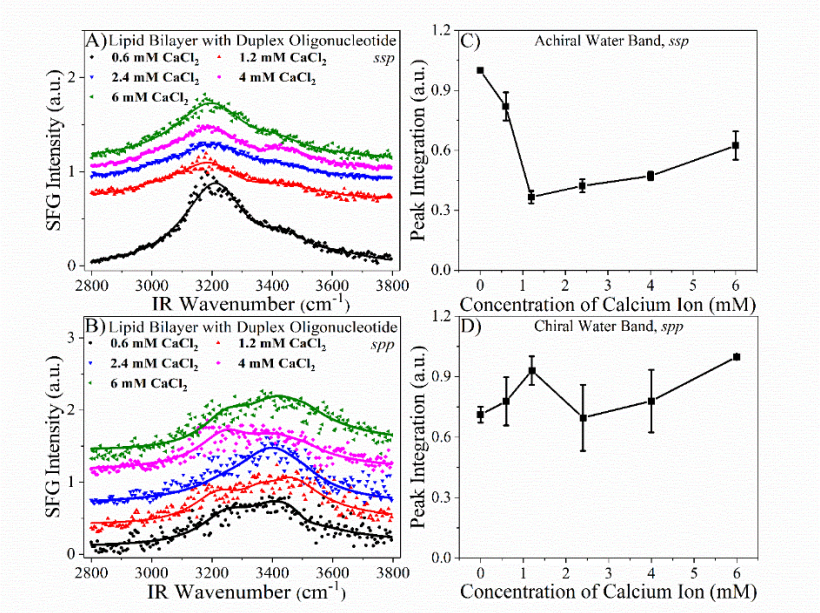

Figure 9. Achiral (*ssp*, A) and chiral (*spp*, B) SFG spectra for the duplex oligonucleotide-anchored lipid bilayer in contact with the Ca^{2+} solutions with different concentrations (from 0.6 mM to 6 mM). The data points were approximately fitted by using the Lorentz equation. The change of the integrated area for the water vibrational signals as a function of the Ca^{2+} concentration was presented (*ssp,* C; *spp*, D). All the spectra have been normalized and offset for clarity.

Minor Concerns:

Comment 1: How did the authors get the peak position labeled in Figure 5 and 6? It would be better for the authors fit the spectra with Lorentzian equation and then get the accurate peak positions.

Author Reply: We thank the reviewer for raising such a good comment. We fitted the spectra with Lorentz equation and the results are shown in Figure 5 and 6.

Reviewer #2:

Manuscript Summary:

General Comment: This manuscript describe the overall protocol of the analysis of the sum-frequency spectra using regional formulation, preparation of the samples on the specific prism, and the analysis of the results. I therefore would recommend acceptance in the JOVE if the authors can fully address my suggestions described below, which would improve the quality of the manuscript.

Author Reply: We thank the reviewer for the positive comment. SFG is a powerful tool to study the molecular-level structures at the surfaces/interfaces. Here, we provided the protocol by summarizing our recent experimental results.

Minor Concerns:

Comment 1: It would be better to show the spectral fitting procedures.

Author Reply: Yes, according to this comment, the spectral fitting procedures were added in the protocol section.

Lorentz Equation

The Lorentz equation was used to fit the SFG spectra to extract the vibrational information such as the intensity, half width at half maximum (HWHM) for a specific vibrational mode.

$$\chi_{eff}^{(2)} = \chi_{NR}^{(2)} + \sum_q \frac{A_q}{\omega_{IR} - \omega_q + i\Gamma_q} \quad (25)$$

where A_q represents the intensity of the *qth* vibrational mode, ω_q represents the resonant frequency, Γ_q denotes the half width at half maximum (HWHM) and ω_{IR} represents the scanning frequency of the incident IR beam.

Reviewer #3:

Manuscript Summary:

General Comment: Clear and concise summary.

Author Reply: We thank the reviewer for this positive comment.

Major Concerns:

Comment 1: Context of the work is only briefly introduced. A more comprehensive background, with particular emphasis to the SFG technique, its uniqueness and usefulness in the investigation of material interface properties should be given. More specific information can be added as to what "fundamental science from the molecular level" can be understood. Also, it is not clear how the actual samples were selected and what is the specific aim of the study.

Author Reply: We added contents in light of the reviewer's comment, "Currently, a

broad range of scientific issues related to surfaces and interfaces are being investigated using SFG, especially for complex interfaces with respect to polymers and biomacromolecules, such as the chain structures and structural relaxation at the buried polymer interfaces, the protein secondary structures and the interfacial water structures[9-26].”

Minor Concerns:

Comment 1: More consistency e.g. sometimes authors refer to "prism" other times to "substrate" (e.g. point 3.1.6). More clarity as to what is the "bottom medium" in these measurements (e.g. point 2 and Fig.3).

Author Reply: We thank the reviewer for this constructive comment. The prism and the substrate are the same thing. We believe that the audiences can understand this. To clarify the “bottom medium”, we added one note in a bracket, “(the bottom medium can be air, liquid or solid.)” at point 2 and the legend of Fig.3.

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Title: Detecting Surface Hydration of Poly(2-hydroxyethyl methacrylate) in Solution in situ

Author: Xu Li, Bolin Li, Xiaodong Zhang, et al

Publication: Macromolecules

Publisher: American Chemical Society

Date: Apr 1, 2016

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Title: Interchain Overlap Affects Formation of Silk Fibroin Secondary Structures on Hydrophobic Polystyrene Surface Detected via Achiral/Chiral Sum Frequency Generation

Author: Xu Li, Guozhe Deng, Liang Ma, et al

Publication: Langmuir

Publisher: American Chemical Society

Date: Aug 1, 2018

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BACK | CLOSE WINDOW

Title: Calcium Ions Affect Water Molecular Structures Surrounding an Oligonucleotide Duplex as Revealed by Sum Frequency Generation Vibrational Spectroscopy

Author: Xu Li, Liang Ma, Xiaolin Lu

Publication: Langmuir

Publisher: American Chemical Society

Date: Dec 1, 2018

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