**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 Ca2+ 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 ago1,2. 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, etc3-5. 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 structures9-26.

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 spectra27-29. 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 results3,9-15,30.

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 substrate9, formation of silk fibroin (SF) secondary structures on the polystyrene (PS) surface and water structures surrounding model short-chain oligonucleotide duplex16,21, 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. 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.
   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.
   3. Set the incident angles of two input beams to be 53° (IR) and 64° (visible), respectively, versus the surface normal.
   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.
   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.
   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**
   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 simultaneously31,32. This provides the opportunity for us to probe the SFG vibrational signal from only one interface. Here, the PHEMA film on the CaF2 prism was taken as an example9.
   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

(1)

where denotes the effective second-order nonlinear susceptibility tensor.

**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.

(2)

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

* 1. Apply a thin-film model to calculate the Fresnel coefficients in this case. Here only brief calculation procedures are presented.
     1. For the prism/polymer interface, use

(3)

(4)

(5)

The meaning of each parameter shown is presented below.

* + - 1. *ωi* denotes the beam frequency.
      2. *tp* and *ts* denote the overall transmission coefficients and can be expressed as

(6)

(7)

* + - 1. *tp12* and *ts12* denote the linear transmission coefficients of the light beam at the prism/polymer interface.
      2. *rp23* and *rs23* denote the linear reflection coefficients of the light beam at the polymer/medium interface.
      3. *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

(8)

* + - 1. *λ* represents the wavelength of the light beam and *d* is the polymer film thickness.
      2. *φ1* and *φ2* represent the incident angles at the prism/polymer interface and the polymer/medium interface respectively.
      3. *n1* and *n2* represent the refractive indices of the prism and polymer film respectively.
      4. *n12* *represents* the refractive indices of the polymer interfacial layers for the prism/polymer.
    1. For the polymer/medium interface, use

(9)

(10)

(11)

* + - 1. *Δ* represents the phase difference of the light electrical fields at two interfaces.
      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.
      3. The expression of such phase difference for the output SFG, the input visible and the input infrared beams can be separately written as

(12)

(13)

(14)

* 1. 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.
     1. For the prism/polymer interface, the expressions of the total Fresnel coefficients for both *ssp* and *ppp* polarization combinations are presented as follows.
        1. For *ssp*, the equation is

(15).

* + - 1. And for *ppp*, the equation is

(16)

(17)

(18)

(19).

* + - 1. *t10* and *t01* denote the linear transmission coefficients at the air/prism and prism/air interfaces respectively.
    1. For the polymer/medium interface, the expressions of the total Fresnel coefficients for both *ssp* and *ppp* polarization combinations are described as follows.
       1. For *ssp*, the equation is

(20).

* + - 1. For *ppp*, the equations are

(21)

(22)

(23)

(24).

* 1. 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 CaF2 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 CaF2 prism/PHEMA interface.

1. **Chiral SFG polarization combination**
   1. For the normal achiral interface, commonly, use C∞v symmetry in terms of ensemble average33,34. With operation of inversion symmetry, the nonzero second-order nonlinear susceptibility tensor components can be deduced, which are χxxz, χxzx, χzxx, χyyz, χyzy, χzyy and χzzz (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, χzyx, χzxy and χyzx under the consideration of non-electronic resonance. Therefore, by using *psp*, *pps* and *spp* polarization combinations, chiral SFG spectra can be collected33,34.
2. **Sample preparation**
   1. Preparation of PHEMA film
      1. Dissolve PHEMA powder (see **Table of Materials**) in anhydrous ethanol to prepare the solution with 2 wt% and 4 wt% respectively.
      2. Before deposition of the PHEMA films, soak the CaF2 right-angle prisms in the toluene solvent firstly and then wash them with ethanol and ultrapure water (18.2 MΩ·cm).
      3. Afterwards, expose the substrates (CaF2 right-angle prisms) to oxygen plasma to remove possible organic contaminants by plasma cleaner (see **Table of Materials**).
         1. First turn on the plasma cleaner and put the substrates in it.
         2. Then turn on the vacuum pump to vacuumize the cleaner. Input the oxygen in it.
         3. Finally set 4 minutes for cleaning. After that, preserve the clean substrates for the sequential PHEMA film preparation.
      4. Then prepare the PHEMA films on the CaF2 prisms by a spin-coater (see **Table of Materials**). Adjust the film thicknesses by the solution concentration and spin speed.
         1. Immobilize the CaF2 prism on the sucking disc of spin-coater.
         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).
      5. Anneal all the prepared PHEMA films in a vacuum oven at 80 °C overnight.
   2. Preparationof silk fibroin (SF)

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

* + 1. Place7.5 g of silk cocoons of *B. mori* in the boiling sodium carbonate (Na2CO3, 0.02 M) aqueous solution (3 L) for 30 min. Remove the fibrous SF to a clean container.
    2. Washthe 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.
    3. Drythe fibrous SF sample in a vacuum oven at 60 °C overnight.
    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.
    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.
  1. Preparationof short-chain oligonucleotide duplex
     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).
     2. Mix2 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.
     3. Preparation of DPPC & d-DPPC monolayer by a Langmuir−Blodgett (LB) trough
        1. Attach the right-angle CaF2 prism to a homemade sample holder with one prism face perpendicularly dipped into the aqueous environment of the LB trough.
        2. Afterwards, inject the mixed lipid solution prepared before onto the water surface until the surface pressure reached a certain value below 34 mN·m−1.
        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·m−1 was reached.
        4. Lift the prism with a lipid monolayer out of the water at a rate of 1 mm/min vertically.
     4. Preparation of the other lipid monolayer
        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).
        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·m−1 was reached.
     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.
  2. Lorentzequation
     1. Use the Lorentz equation to fit the SFG spectra to extract the vibrational information for a **specific** vibrational mode.

(25)

where represents the intensity of the *qth* vibrational mode, represents the resonant frequency, denotes the half width at half maximum (HWHM) and 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 CaF2 substrate is smooth and cannot be penetrated by PHEMA molecules, leading to a sharp CaF2/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 CaF2/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 CaF2 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 Ca2+ 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 Ca2+ 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 CaF2 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 Ca2+ 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 Ca2+ concentration. However, the achiral water vibrational signals were strongly affected when the Ca2+ 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 Ca2+ ions, in the normal biological condition.

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**DISCLOSURES:**

We have nothing to disclose.

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