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Perspective

Probing Orientations and Conformations of Peptides and Proteins at Buried Interfaces

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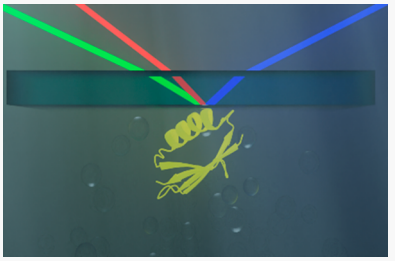
ABSTRACT: Molecular structures of peptides/proteins at interfaces determine their interfacial properties, which play important roles in many applications. It is difficult to probe interfacial peptide/protein structures because of the lack of appropriate tools. Sum frequency generation (SFG) vibrational spectroscopy has been developed into a powerful technique to elucidate molecular structures of peptides/proteins at buried solid/liquid and liquid/liquid interfaces. SFG has been successfully applied to study molecular interactions between model cell membranes and antimicrobial peptides/membrane proteins, surface-immobilized peptides/enzymes, and physically adsorbed peptides/proteins on polymers and 2D materials. A variety of other analytical techniques and computational simulations provide supporting information to SFG studies, leading to more complete understanding of structure−function relationships of interfacial peptides/proteins. With the advance of SFG techniques and data analysis methods, along with newly developed supplemental tools and simulation methodology, SFG research on interfacial peptides/proteins will further impact research in fields like chemistry, biology, biophysics, engineering, and beyond.



The behavior of proteins and peptides at interfaces plays important roles in many applications and research fields. For example, it has been widely known that protein adsorption is related to the biocompatibility of a biomaterial.[1] Marine biofouling starts to occur when adhesive proteins of marine organisms interact with a ship's hull surface.[2] The surface-immobilized enzyme structure influences the performance of a biosensor using enzymes as the biorecognition component.[3] Advanced microelectronics can be constructed using 2D materials; their interactions with proteins at interfaces are widely encountered from diagnostics to optogenetics.[4] Membrane protein structures determine their functions and their interactions with drugs—more than 50% of drugs target membrane proteins.[5] Antibody drugs have been developed to cure many different diseases; their interactions with various surfaces during storage and administration may lead to the loss of their functions.[6]

Molecular behavior is determined by molecular structure. Excellent results have been obtained from research on protein structures in bulk environments (e.g., in solution or in crystal form) using X-ray diffraction, nuclear magnetic resonance (NMR) spectroscopy, cryo-electron microscopy, etc.[7−9] However, it is difficult to examine molecular structures of proteins at interfaces in situ because of the lack of appropriate techniques. In the past 30 years, sum frequency generation (SFG) vibrational spectroscopy has been developed into a powerful tool to probe surfaces and interfaces,[10−20] including buried interfaces such as solid/liquid and liquid/liquid interfaces. SFG vibrational spectroscopy (SFG in short) is a second-order nonlinear optical spectroscopic method, which can selectively probe surfaces and interfaces with a submonolayer specificity by providing their vibrational spectra. The superb surface/interface selectivity of SFG is due to the selection rule of a second-order nonlinear optical process: only a medium without inversion symmetry can generate an SFG signal under the electric dipole approximation.[10−20] Most bulk materials have inversion symmetry, and therefore, they cannot produce an SFG signal. Inversion symmetry is broken at a surface/interface, leading to the detection of SFG signals from the surface/interface. SFG has been successfully applied to investigate molecular interactions of peptides and proteins at solid/liquid interfaces in the last 20 years.[21−31] In a typical SFG experiment, two input pulsed laser beams—a frequency tunable infrared beam and a frequency fixed visible beam—spatially and temporally overlap at an interface, generating a signal which has the sum frequency of the two input beams.[10−20] The SFG signal intensity is plotted against the infrared input wavenumber to produce a vibrational spectrum of the interface. Figure 1 shows the sample geometry used to collect SFG spectra from interfacial protein molecules and the energy level diagram of the SFG process.

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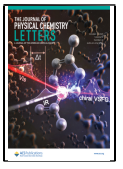

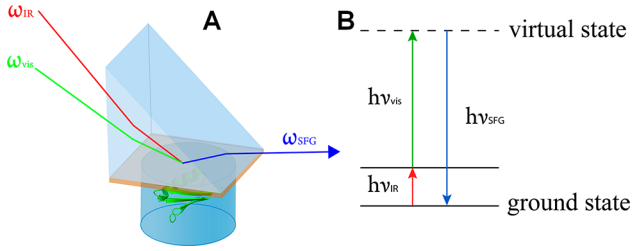

Figure 1. (A) Near-total-reflection geometry used in SFG experiment; (B) SFG energy level diagram

In the past 30 years, SFG vibrational spectroscopy has been developed into a powerful tool to probe surfaces and interfaces, including buried interfaces such as solid/liquid and liquid/liquid interfaces.

SFG studies on interfacial proteins initially started from examining physical interactions between protein side chains and polymer surfaces.[32−34] SFG was then applied to study amide I signals,[35−43] which can provide backbone structural information on interfacial peptides/proteins, and systematic ways to analyze SFG amide I signals have since been developed.[35−43] We have used SFG to deduce orientations of membrane peptides and proteins, probe physical interactions between peptides/proteins and various surfaces including polymers, silicone oils, and 2D materials, and investigate surface-immobilized antimicrobial peptides and enzymes on self-assembled monolayers (SAMs) and polymer materials.

It was demonstrated in our early research that SFG could directly probe protein molecules at solid/liquid interfaces in situ in real time by collecting SFG C−H stretching signals of protein side chains.[33,34] SFG studies indicated that protein side-chain ordering at interfaces depends on the hydrophobicity of the contact medium.[33,34] A "thin-film model" was adopted to interpret SFG data and ensure that SFG probes the structure of the entire protein layer at the interface, instead of only the protein/contact substrate and/or protein/solution interfaces.[44] We then focused on the SFG examinations of protein amide I modes,[35−43] showing that SFG could differentiate different protein secondary structures at interfaces,[36] detect chiral signals from interfacial peptides and proteins,[45] and deduce orientations of different secondary structures such as straight α helix,[38] bent α helix,[42] 3−10 helix,[38] and β-sheet[39] with polarized signals. A methodology was developed to determine interfacial protein orientation by using the SFG signals contributed from α-helical domains of a protein.[40] For this method, a computer software package was developed to find all the helical structures in a protein, and SFG amide I signal strength ratio of the spectra collected with different polarization combinations such as ssp (s-polarized SFG signal, s-polarized input visible, and p-polarized input IR), ppp, and sps could be calculated from the helical structures in the protein as a function of protein orientation. The calculated SFG signal strength ratio can be compared to the experimentally collected data to generate a heat map to determine the protein orientation.[40] Here a heat map is a 2D plot which shows the matching score between the measured and calculated data as a function of protein orientation. Each location inside the heat map corresponds to a protein orientation. A higher matching score indicates a better match between the calculated and the measured data. The location with the highest matching score is the most likely protein orientation. In such an approach, the protein was assumed to have a similar structure at the interface to the crystal structure, so the calculation was based on the protein crystal structure. Such an approach could be validated by minimal protein structural changes at the interface, which can be tested using coarse grain molecular dynamics (MD) simulation.[46,47] If an

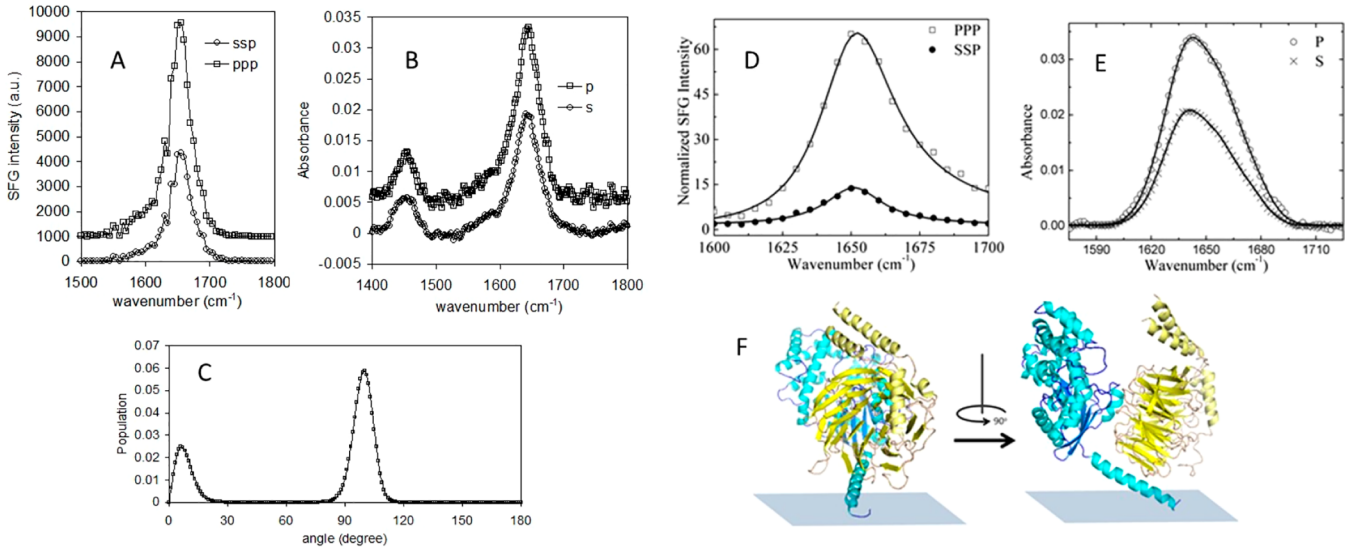

Figure 2. SFG spectra (A) and ATR-FTIR spectra (B) collected from melittin associated with a lipid bilayer. Orientation distribution of interfacial melittin could be deduced from combined SFG and ATR-FTIR study (C). SFG spectra (D) and ATR-FTIR spectra (E) collected from G$\alpha\beta\gamma$ associated with a lipid bilayer. Schematic showing the membrane orientation of G$\alpha\beta\gamma$ deduced from combined SFG and ATR-FTIR study (F). Reproduced with permission from refs 37 and ref 41. Copyright American Chemical Society 2007 and 2013.

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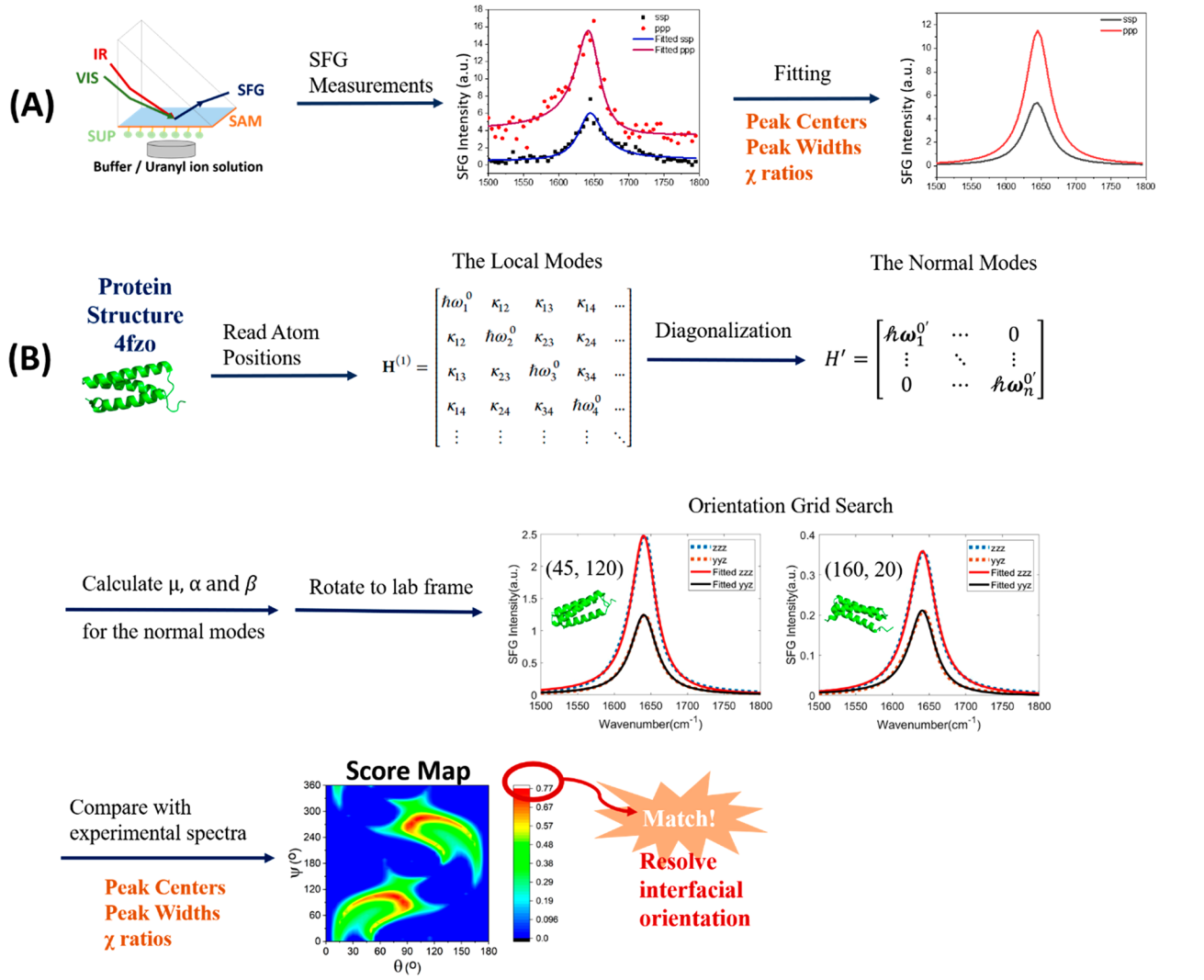

Figure 3. Flowchart of (A) SFG measurement process and (B) Hamiltonian matching process, using a uranyl ion binding protein as an example.[43] The input protein structures of panel B can be obtained by protein crystal structures and MD simulated structures. Here μ, α, and β are IR transition dipole, Raman polarizability, and SFG hyperpolarizability, respectively. Reproduced with permission from ref 43. Copyright 2021, American Chemical Society.

MD simulation shows that a protein undergoes substantial conformational changes at the interface,[43] the crystal structure cannot be used to calculate SFG signal strength. The protein structure obtained from MD simulations should be used to calculate SFG signal strength ratio to determine protein orientation (as well as the conformation) at interfaces.[43]

In addition to SFG, we also applied attenuated total reflectance (ATR)-FTIR spectroscopy as a supplemental tool to study interfacial peptides/proteins.[37,41] ATR-FTIR measures different orientation parameters compared to SFG, thus providing additional independent measurements to SFG. For example, for an α helix, the tilt angle θ (the angle between the principal axis of the helix and the surface normal) defines its orientation. SFG measures $\langle \cos\theta \rangle$ and $\langle \cos^3\theta \rangle$ ("$\langle\ \rangle$" means "average"), while ATR-FTIR measures $\langle \cos^2\theta \rangle$. The combined ATR-FTIR and SFG studies could deduce a complex orientation distribution of a peptide[37] or more accurate orientation of a protein at an interface (Figure 2).[41]

Recently, we adopted a Hamiltonian approach to calculate SFG spectra to compare to the experimentally measured data to determine interfacial protein orientation and conformation.[43] Differing from our earlier SFG data analysis method for protein orientation determination, which used the SFG signals contributed from the protein α-helical components,[40,41] here the Hamiltonian approach calculates SFG spectra using all the amino acids in various secondary structures in the protein.[43] First, a Hamiltonian matrix was constructed with the uncoupled amide I stretching peak center (e.g., 1640 cm^{-1}) as the diagonal elements. The off-diagonal elements were calculated by the coupling between each pair of the amino acids in the protein. The matrix can then be diagonalized to find the normal modes of the amide I signals and the related peak centers. The polarized SFG signal strength of each normal mode can be calculated with uncoupled IR dipole transitional moment and Raman polarizability. With a certain peak width for the SFG signal generated from each normal mode, the entire SFG amide I spectrum for an interfacial protein can be calculated. Such a Hamiltonian approach can be used to calculate SFG spectra of an interfacial protein based on its crystal structure or MD simulated structure as a function of protein interfacial orientation. The calculated spectra can be compared with the reconstructed SFG spectra from the fitted

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experimentally measured SFG resonant spectra (after deconvoluting the nonresonant contribution) to generate a heat map to match the calculated and experimental spectra. The highest matching score in the heat map determines the most likely orientation of the protein. Figure 3 shows the schematic of this process.[43] The crystal or simulated structure which generates the highest matching score in the heat map is the most likely protein conformation. The comparison between the calculated and reconstructed experimental spectra can be done by using spectral fitting parameters (e.g., peak center and peak width) and the signal intensity ratio of the spectra collected (calculated) for different polarization combinations such as ssp, ppp, or sps.[43] This method has been applied to study the conformation and orientation of a uranyl ion binding protein.[43]

Membrane-Associated Peptides and Proteins. Antimicrobial peptides (AMPs) have been extensively researched to replace traditional antibiotics to treat infectious diseases.[48,49] Bacteria can develop drug resistance against traditional antibiotics, but not AMPs, because AMPs target and disrupt bacterial cell membranes. In order to understand the interaction mechanisms of AMPs with bacterial cell membranes and the selectivity of AMPs in distinguishing between bacterial and mammalian cell membranes, SFG was applied to study a variety of AMPs interacting with solid-supported lipid bilayers serving as models for cell membranes.[50] Bacterial and mammalian cell membranes were modeled with negatively charged and zwitterionic lipid bilayers, respectively, on solid substrate supports. Using an α-helical magainin 2 AMP as a model, SFG results demonstrated that AMP can be associated with and insert into a model bacterial membrane, but not a model mammalian cell membrane at the same solution concentration.[51] By increasing the peptide concentration, AMP can be associated with the model mammalian cell membrane but tilt on the membrane surface instead of inserting into the membrane.[51] This research demonstrated that the excellent selectivity of AMPs in killing bacteria (but not mammalian cells) is due to their active disruptions of bacterial cell membranes. Using a magainin 2 analogue, MSI-78, as an AMP example, SFG studies showed that AMP adopts varied orientations in model bacterial membrane at different peptide solution concentrations, varying from lying down, to tilting, to having multiple orientations with the increase of AMP solution concentrations.[52] With the help of ATR-FTIR measurements, we successfully determined a complex multiple-orientation distribution, using melittin as an example.[37] In this study, a maximum entropy trial orientation distribution function was used, which could flexibly fit into different multiple orientation distributions when different measured parameters were obtained.[37] In addition to the "straight" helical peptides such as magainin 2 and MSI-78, the bent α-helical peptide LL-37, the only AMP which can be generated by human beings, was also investigated.[42] Methods for SFG orientation determination for two kinds of bent helices, with or without a separation segment between the two helical components, were developed and successfully applied to study the orientations of LL-37 in different lipid bilayers.[42] In addition to the α-helical peptides, the 3−10 helical structure, e.g., alamethicin (which has an α-helical segment and a 3−10 helical segment) was also examined.[53−56] It was found that alamethicin could insert into a liquid-phased lipid bilayer, but not a gel-phased lipid bilayer.[56] Under an electric potential, alamethicin could change its orientation in the lipid bilayer.[54] Alamethicin has been extensively used as a model for ion channel proteins.[57] This SFG research demonstrates the feasibility for future SFG studies on larger ion channel proteins in physiologically relevant environments in situ to elucidate the ion gating mechanisms.

In addition to the helical structure, SFG has also been applied to study antiparallel β-sheets, using tachyplesin I as a model. SFG chiral signals were successfully detected from tachyplesin I at the interface using the spp and psp polarization combinations.[39,45] Such a β-sheet structure has D_2 symmetry; the orientation analysis method was developed by combining ATR-FTIR and SFG measurements to determine the tilt and twist angles of a β-sheet structure.[39]

In the above studies, substrate-supported lipid bilayers (e.g., on solid CaF_2 or silica prisms) were used as models for cell membranes. The interactions between the bilayers and the solid substrates may change the lipid bilayer behavior and the interactions with biomolecules, leading to errors in the SFG experiments. To eliminate this possibility, we compared the SFG results obtained from lipid bilayers supported on a CaF_2 substrate with those from lipid bilayers on a polymer cushion (e.g., a spin-coated poly(L-tactic acid) or PLLA film) on CaF_2. We also compared the interactions between these two types of lipid bilayers and an AMP, cecropin P1. The comparisons indicate that the lipid bilayer−solid substrate (e.g., CaF_2) interaction is minimal and can be ignored (because of a water layer between the lipid bilayer and the solid substrate).[58] Therefore, solid-substrate-supported lipid bilayers are good models for cell membranes.

SFG has been applied to study membrane proteins, e.g., the orientation of $G_{\beta\gamma}$ associated with a model cell membrane.[59] Heterotrimeric guanine nucleotide-binding proteins (G proteins) are important membrane proteins that participate in signal transduction and relay signals sensed by G protein-coupled receptors (GPCRs) to downstream effectors.[60] The G protein has three subunits (Gα, Gβ, and Gγ, with Gβ and Gγ forming a tightly associated dimer). To simplify the SFG data analysis, it was hypothesized that the $G_{\beta\gamma}$ can only rotate around the y-axis (while the y-axis is on the surface, perpendicular to the plane of incident/outgoing beams for SFG experiments), so the tilt angle versus the surface normal could determine the protein orientation. The tilt angle was then deduced by using the ratio between the SFG ssp and sps spectra (dominated by contributions from the α helix part of the protein) based on the data analysis of the helical part of the protein.[59] We then studied the cytochrome b5 associated with model cell membranes.[61] Similar to those collected from $G_{\beta\gamma}$, here SFG spectra were also mainly contributed by the helical section of the protein. The SFG signals could be analyzed based on the helical structure, showing that the linker in cytochrome b5 is crucial to enable the protein to insert into the cell membrane.

With the developed computer software package, we successfully determined the membrane orientation of Gβ1γ2-GPCR kinase 2 (GRK2) complex, which is a very complicated protein, demonstrating the feasibility of using SFG to study large and complex proteins.[40] With the combined SFG and ATR-FTIR studies, the membrane orientation of $G\alpha_i\beta_1\gamma_2$ heterotrimer could be uniquely determined, showing that $G\beta_1\gamma_2$ binds to $G\alpha_i$ without a significant change in orientation.[41] Similar to SFG and ATR-FTIR, we also studied the effect of lipid composition in the cell membrane on Gβ1γ2-GRK2 complex orientation. It was found that a particular lipid

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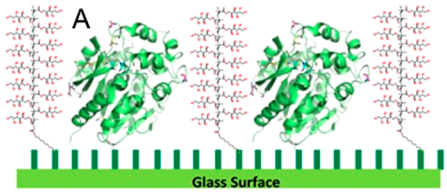

B

	Turnover number s^{-1}	Relative values %
Free HLD (aqueous phase)	185	100
Immobilized HLD (aqueous phase)	76.9	44
Lyophilized HLD (vapor phase)	0.44	0.24
Immobilized HLD (vapor phase)	3.12	1.7
Immobilized HLD+PSMA (vapor phase)	17.3	9.4

Figure 4. (A) Schematic showing co-immobilized hydromimetic polysorbitols and enzyme haloalkane dehalogenase (HLD). (B) Comparison of HLD activity for dehalogenation of 1-bromopropane at 37 °C under various conditions. The activity of immobilized HLD with polysorbitols is about 40 times higher compared to that of lyophilized HLD in air. Reproduced with permission from ref 78. Copyright 2017, American Chemical Society.

composition, phosphatidylinositol-4′,5′-bisphosphate (PIP_2), uniquely interacts with GRK2 pleckstrin homology (PH) domain to orient the G$\beta1\gamma2$-GRK2 complex on the membrane to better interact with activated GPCRs.[62] In addition to the GRK2 complex, the membrane orientation of GRK5 was also deduced with combined SFG and ATR-FTIR approach.[63] Previously, it was proposed that GRK5 can be associated with cell membranes using its N-terminus, its C-terminus, or both, but no experimental evidence was found to support those proposals. The membrane orientation of GRK5 was also a matter of speculation. We successfully deduced the GRK5 orientation using SFG and showed that GRK5 is associated with the membrane through its C-terminus.[63] It is worth mentioning that the determination of the orientations of membrane-bound proteins such as G-proteins and GRKs provides critical knowledge for understanding how the cell membrane facilitates interactions between signaling proteins, how these protein molecules are aligned for communications or other functions, and how higher-order signaling scaffolds are assembled.[40]

Surface-Immobilized Peptides and Proteins. Surface-immobilized peptides and proteins are widely used for antimicrobial coatings, biosensors, biochips, fuel cells, etc.[64−66] Chemical immobilization of peptides and proteins on surfaces can better control peptide/protein orientations and avoid the loss of biomolecules from the substrate surface compared to physical adsorption. SFG was first used to compare the chemical immobilization and physical adsorption of cecropin P1 on a polystyrene maleimide surface. As expected, the chemically immobilized peptide via the cecropin P1 terminal cysteine group, which reacted with the surface maleimide group, adhered to the surface more tightly and with controlled orientation.[67] For chemical immobilizations, SFG studies indicated that the same peptide immobilized with different termini could adopt varied orientations due to the different interactions among the different peptide ends, the solution, and the substrate surface. For example, with SFG and MD simulation studies, it was found that the C- and N-terminus cysteine-modified cecropin P1 molecules immobilized on maleimide terminated SAM adopt different orientations.[68] Similarly, MSI-78 molecules with C- or N-terminus modified azido group immobilized on an alkyne-terminated SAM also exhibit different orientations.[69] MD simulations clearly interpreted the SFG observations: both peptides have a hydrophilic terminus and a hydrophobic terminus. When the hydrophilic end was immobilized on a SAM surface, the hydrophobic terminus more favorably interacted with the hydrophobic SAM to enable the peptide to lie down on the surface. Oppositely, when the hydrophobic end was immobilized, the hydrophilic terminus liked to interact with water, making the peptide stand up.[70] It was also found that the peptides that were immobilized on a more hydrophilic substrate could have more flexible orientations.[71] The bacteria testing results showed that peptides standing up more in water could kill more bacteria or kill bacteria faster.[68,69] However, when the surface-immobilized peptides on SAM or polymer surface interacted with live bacteria, it was found by SFG that the peptides lay down more,[72,73] showing that the bacterial killing mechanisms for surface-immobilized peptides and free peptides in solution are different. The peptides in free solution can insert into bacterial cell membranes to form pores to disrupt cell membranes to kill bacteria. Differently, the surface-immobilized AMPs could not form pores in bacterial cell membranes because they are not long enough to penetrate the cell membranes. We believe that they need to stand up more on the surface in water or solution to capture/interact with the incoming bacteria. During the interaction process with bacteria, AMPs lie down on the surface to expose more positive charges to kill bacteria.[72,73]

SFG was also applied to compare the chemically immobilized enzymes with those physically adsorbed on a surface, using β-Gal as an example.[74] Physical adsorption may lead to the denaturation of the protein due to strong hydrophobic protein−surface interactions. Chemical immobilization via a selected cysteine on maleimide-terminated SAM could orient the protein to adopt a preferred orientation.[74] When enzymes were immobilized via different immobilization sites, they exhibited varied orientations, leading to different activities.[75] The surface-immobilized enzyme with the active domain far from the surface exhibits a higher activity, while the substrate surface's hydrophobicity also influences the immobilized enzyme orientation. If the enzyme's active domain is near the surface after the immobilization, the enzyme immobilized on a more hydrophilic surface has a more flexible orientation, leading to a higher activity.[76] It was also shown by SFG, MD simulations, and enzymatic activity measurements that an enzyme immobilized via a rigid structural unit, e.g., a helix instead of a coil, or immobilized via multiple neighboring immobilization sites has a high thermal stability.[46,77]

Surface-immobilized enzymes are widely used in air to detect toxic molecules. Typically, an enzyme in air has a much lower (e.g., 3 orders of magnitude lower) activity compared to that in aqueous solution. We found that by co-immobilizing enzymes with hydromimetic molecules (e.g., polysorbitols), the co-immobilized hydromimetic moieties can protect the immobilized protein's native structure in air (at 50% humidity level). Compared to the freeze-dried enzyme in air, the activity of the surface-immobilized enzyme with co-immobilized polysorbitols was enhanced by 40 times (Figure 4).[78] SFG clearly showed that without co-immobilized hydromimetic molecules, the enzyme structure collapsed, leading to the absence of SFG amide I signals. With the co-immobilized

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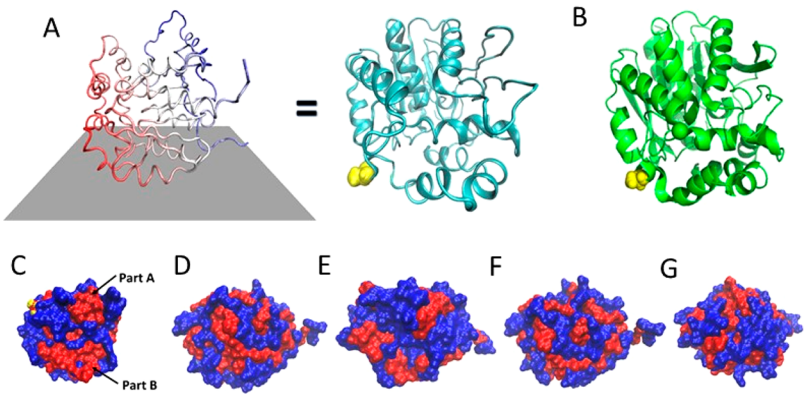

Figure 5. (A) The stabilized HLD (A141C) structure on MoS_2 surface plotted in the ribbon form obtained by MD simulation. (B) One of the most likely HLD (A141C) orientations on MoS_2 deduced from the SFG and ATR-FTIR results. The simulated and experimentally deduced orientations match with each other well. (C) Hydrophobicity surface mapping of the HLD surface that adsorbs onto the MoS_2/WS_2 surface (top-down view). (D−G) Hydrophobicity surface mapping of the HLD along the residue109−residue159 rotation axis at 0°, 90°, 180°, and 270°, respectively. Hydrophobic amino acids are labeled in red, while hydrophilic amino acids in blue. Parts A and B in panel C are two large hydrophobic patches on HLD which enable favorable interactions with MoS_2 for adsorption. Reproduced with permission from ref 85. Copyright 2018, American Chemical Society.

hydromimetic polysorbitols, strong SFG amide I signals from surface-immobilized enzymes in air could be detected.[78]

It is worth mentioning that MD simulations serve as a powerful supplemental tool for SFG studies. For example, MD simulation results on surface-immobilized peptides via different immobilization sites and different substrate hydrophobicity were well-correlated to the SFG conclusions,[70,71,79] providing further understanding of peptide−surface interactions. MD simulations on surface-immobilized proteins could confirm whether or not SFG data analysis should be performed based on the protein crystal structure.[43] MD simulation results could also confirm the protein orientations deduced from SFG experiments.[46]

Physical Adsorption of Peptides and Proteins. SFG has been applied to study proteins that are physically adsorbed onto various surfaces. This is important, for example, for understanding the biocompatibility which is determined by the interactions between physically adsorbed proteins and biomaterial surface and the sensitivity and selectivity of biosensors based on biological molecules adsorbed on 2D materials. SFG studies showed that fibrinogen, an important blood protein, adsorbed on a polystyrene surface adopts a broad orientation distribution,[80] and it exhibits a bent structure on biomedical materials.[80] The bent angle can change as a function of time because of the protein−surface interactions.[81] SFG studies also showed that another blood protein, FXII, adopts a more ordered orientation on negatively charged polymer surfaces, facilitating the FXII activation on negatively charged surfaces and leading to blood coagulation through the intrinsic pathway.[82]

We have systematically investigated interactions between peptides/proteins and 2D materials such as graphene and MoS_2. SFG results showed that the two α-helical AMPs discussed above, cecropin P1 and MSI-78, adopt different orientations at the graphene/peptide solution interface.[83] Cecropin P1 stands up on the graphene surface, with a tilt angle of ∼30° vs the surface normal, while MSI-78 lies down. MD simulations indicated that the lying-down orientation of MSI-78 on graphene is caused by the strong $\pi-\pi$ interactions between graphene and the aromatic amino acids on the same side of the helical MSI-78 molecule. After mutating two of the aromatic amino acids with nonaromatic amino acids, such $\pi-\pi$ interactions could be greatly reduced, leading to a standing-up pose for the MSI-78 mutant on graphene, which is confirmed by both SFG studies and MD simulations.[83] Because of the similar strong $\pi-\pi$ interactions, protein GB1 denatured on graphene, as evidenced by both MD simulations and SFG studies.[47] With the mutation of two aromatic amino acids to nonaromatic amino acids, the mutant protein GB1 could retain its native structure on graphene.[47]

Different 2D materials have different interactions with peptides/proteins. The combined studies on interactions between peptides/enzymes with MoS_2 using SFG and MD simulations demonstrated that the dominating interaction is not the $\pi-\pi$ interaction, but the hydrophobic interaction. To enable a peptide to stand up on the MoS_2 surface, one end of the peptide should contain multiple charged amino acids, which have favorable interactions with water.[84] To ensure that an enzyme can be adsorbed to the MoS_2 surface with a preferred orientation (with the active domain facing the aqueous solution), the protein surface should have a hydrophobic patch on the opposite side of the active domain to enable the strong hydrophobic interaction with the hydrophobic MoS_2 (Figure 5).[85] It was found that enzymes have similar interactions with the WS_2 surface as those with MoS_2.[85]

SFG has also been applied to study protein drugs interacting with silicone oil surfaces to understand protein aggregation on silicone oil. Various protein drugs including fusion protein, monoclonal antibody, and bispecific antibody were studied at the silicone oil/protein solution interfaces in situ.[86,87] It was found that proteins do adsorb onto silicone oil with order. Surfactant wash or mixing surfactant with protein in the solution could reduce or even minimize the protein adsorption onto silicone oil.[86,87] It is thus important to include surfactants in the protein drug formulation to minimize the protein−silicone oil interaction and prevent protein aggregation. Similarly, SFG studies also showed that surfactants can disrupt the protein ordering and/or adsorption amount at the corn oil/water interface, facilitating water−corn oil separation.[88]

The presented SFG studies demonstrate that SFG is a powerful and unique tool to elucidate molecular structures and

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The presented SFG studies demonstrate that SFG is a powerful and unique tool to elucidate molecular structures and the behavior of peptides and proteins at buried interfaces in situ. However, SFG studies on interfacial proteins are still relatively new, and extensive research in this field needs to be done in the future.

the behavior of peptides and proteins at buried interfaces in situ. However, SFG studies on interfacial proteins are still relatively new, and extensive research in this field needs to be done in the future. Our perspectives on future SFG studies on peptides/proteins are presented below.

Combined SFG Studies with Other Analytical Tools. As with every analytical tool, SFG has limitations, and it is important to combine SFG studies with other techniques, including experimental methods and computer simulations. For example, SFG can measure the peptide/protein structure at interfaces, but it cannot measure the amount of interfacial biomolecules. The combined use of SFG with techniques that can measure the amount of molecules at interfaces such as quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) could provide a more complete picture of interfacial peptides/proteins.

SFG measurements provide only limited numbers of measured structural parameters. As we discussed above, combined SFG and ATR-FTIR studies can determine more complicated orientation distributions or more accurately measure the protein orientation because SFG and ATR-FTIR measure different structural parameters.[37,41] By combining higher-order nonlinear optical spectroscopic techniques with SFG, additional structural information can be measured.[89] For example, four-wave mixing spectroscopy is able to detect polarized signals from a monolayer of molecules with the use of a commercial SFG spectrometer.[90] Thus, it is feasible to combine SFG with linear ATR-FTIR and higher-order nonlinear spectroscopic techniques (e.g., four-wave mixing) in the future to study interfacial peptides/proteins. Recently, it has been reported by Hore and colleagues that surface (SFG)−bulk (Raman or IR) vibrational correlation spectroscopy provides more information than SFG alone,[91] which can be applied to study interfacial protein behavior in the future. Circular dichroism (CD) spectroscopy can also provide supplementary secondary structural information on peptides and proteins on surfaces/at interfaces to SFG. For example, combined SFG and CD studies showed that MSI-78 lies down on graphene with an α-helical structure.[83]

There are many advantages to combining SFG studies with MD simulations, as we demonstrated multiple times above. Current SFG data analysis relies on protein structural models, e.g., the crystal structure. For "rigid" proteins, MD simulations can confirm that the protein at an interface has a similar structure to the crystal structure, validating the approach to use the protein crystal structure for SFG data analysis.[46] Computer simulation results on protein orientation can also be compared to the protein orientation deduced by SFG: if they are matching, the simulation results can further confirm the SFG conclusion.[46] For proteins with possible interfacial conformational changes, the protein orientation deduced from polarized SFG study based on the protein crystal structure is not reliable. MD simulations could possibly provide these changed protein conformations for SFG data analysis. According to the matching scores of the calculated and experimentally measured SFG spectra, both protein orientation and conformation can be determined.[43] In the future, MD simulations and SFG experiments should be combined to deduce the protein interfacial structure more reliably. Recently, it was reported that a developed computational algorithm can quite accurately predict a protein structure in solution using the protein sequence.[92] This will substantially impact the MD simulations of proteins in different chemical environments including interfaces, facilitating SFG studies on interfacial proteins.

SFG spectroscopy measures the average conformation and orientation of proteins at interfaces. Extensive research has been performed to investigate single-molecule behavior in situ at the solution interface using a variety of analytical tools such as atomic force microscopy (AFM) and fluorescence microscopy.[93,94] In the future, it is necessary to combine SFG and single-molecule analytical techniques to measure the interfacial protein behavior at both ensemble and single-molecule levels to obtain a more complete picture.

It is important to understand the interfacial protein structure−function relationships. The combined studies on interfacial protein structure with SFG and interfacial functions (e.g., measuring enzymatic activity by fluorescence spectroscopy, UV−vis absorption, electrochemical analysis, etc.) can provide important knowledge on such relationships.

Many new analytical techniques and new simulation methodologies have been developed quickly in recent years and will be developed in the future. It is always necessary to consider the combined use of SFG and these new techniques to study interfacial biomolecules.

More Accurate Data Analysis Methods. Biological molecules such as peptides and proteins are much larger than typical inorganic and organic molecules. Their structures can be very complicated, and the data analysis on their SFG spectra can be complex. Currently, we are using the Hamiltonian approach to calculate SFG spectra collected from interfacial proteins. But this approach is solely based on the amide bonds and does not consider side-chain, solvent, and surface effects.[43] In the future, more accurate coupling coefficients need to be well-tuned by considering the side chains and the solvent effects, because the side chains may induce amide I frequency shifts, and the solvents may change the inter- and intrahydrogen bonding strength of proteins and peptides.[95] Signals generated from the surface within the amide I frequency region need to be calculated in addition to protein/peptide signals in order to gain a more complete picture.[95,96] Efforts can also be put into developing more systematic computer packages to calculate 1D vibrational spectra (e.g., IR, Raman, and SFG spectra) and 2D vibrational spectra (e.g., 2D IR and 2D SFG spectra) simultaneously, as they follow similar principles.[96,97] This will benefit the general research of vibrational spectroscopies. In summary, more efforts need to be invested in the development of more accurate data analysis, which can be widely applicable to study many different kinds of peptides and proteins at interfaces.

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J. Phys. Chem. Lett. 2021, 12, 10144−10155

Study SFG Spectra in Various Frequency Regions beyond Amide I. The current SFG studies on proteins are mainly focused on the investigations of C−H/N−H/O−H stretching and amide I frequency regions. The linear vibrational spectroscopic techniques such as infrared spectroscopy and Raman spectroscopy have been applied to study proteins in many other frequency regions. For example, it has been shown that amide II and amide III spectra can provide unique structural information on proteins. SFG has been used to study amide III modes of biological molecules interacting with lipid bilayers, showing that SFG amide III signals can be used to study interfacial protein folding and better identify secondary structures along with SFG amide I signals.[98,99] It was found that SFG amide II signal can also provide important structural information on interfacial peptides/proteins.[100−102] For example, a strong correlation between the SFG amide II signals and the formation of β-sheet oligomers/fibrils was reported.[100] In the future, SFG studies should cover more spectral frequency regions to obtain structural information on peptides and proteins at interfaces.

Chiral SFG Studies. Protein molecules are chiral molecules; the detection of chiral SFG signals from interfacial peptides and proteins could provide unique structural information. One important advantage to using second-order nonlinear optical spectroscopy to study chiral molecules is that the chiral signal can have similar intensity compared to regular (or achiral) signal, which is very different from linear optical spectroscopy.[103,104] For the first time, we successfully detected chiral SFG amide I signals from peptides and protein at interfaces[45] and later combined chiral and achiral SFG signals to determine antiparallel β-sheet orientation at the interface.[39] Yan and colleagues have performed extensive research on chiral SFG studies on a variety of interfacial peptides and proteins and demonstrated that the SFG chiral amide I and amide A signals can be used to distinguish different secondary structures.[24,105−107] In the future, more chiral SFG studies can be conducted, providing additional independently measured SFG second-order nonlinear optical susceptibility components. With the help of MD simulations and the Hamiltonian SFG data analysis methodology, the combined chiral and achiral SFG studies on proteins will provide more measured parameters to deduce protein structures at interfaces.

SFG Studies on Isotope-Labeled Proteins. Isotope labeling has been widely used in studying protein structures with various analytical tools such as infrared spectroscopy and NMR.[108,109] Isotope labeling provides unique opportunities for SFG studies, because it can break local symmetry to enhance SFG signals for local isotope-labeled units. In addition, because of the coherent nature of the SFG signal, isotope labeling may substantially change the SFG signals collected from the nonlabeled protein segments. One protein can be labeled with different amino acids and different combinations of amino acids, greatly increasing the independently measured structural parameters. SFG has been applied to study isotope-labeled α-helical peptides at an interface.[110,111] With similar approaches, SFG should be combined with isotope-labeled peptides and proteins containing various secondary structures to determine detailed structures of peptides/proteins at interfaces.

Advanced SFG Techniques. Advanced SFG techniques such as phase-sensitive SFG and time-resolved SFG have been developed quickly in recent years.[96,112−118] Phase-sensitive SFG can be used to determine the absolute orientation (up or down) of a protein at an interface, providing additional directly observed experimental evidence to probe protein−surface interactions at buried interfaces. Time-resolved SFG could provide ultrafast vibrational dynamics and vibrational coupling information on different segments inside the protein molecules at interfaces. As reported by Ye and colleagues, time-resolved SFG was used to study the vibrational relaxation of interfacial proteins.[119] Zanni's research group has developed 2D-SFG to obtain additional coupling and dynamical parameters of interfacial proteins.[96] These advanced SFG techniques can provide structural information or probe interfacial interaction which regular SFG cannot, leading to further understanding of interfacial protein structure, behavior, and dynamics.

Interfacial proteins play significant roles in many applications ranging from biomedical materials and antifouling coatings, to biosensors and biofuel production. It has been challenging to investigate interfacial proteins because of the lack of appropriate analytical methods in the past. SFG has been demonstrated to be a powerful and unique technique to study interfacial proteins in situ at the buried solid/liquid or liquid/liquid interfaces at the molecular level. With the combined study of MD simulations and the Hamiltonian approach, SFG can provide important information regarding protein interfacial orientation and conformation, leading to in-depth understanding of interfacial protein molecular behavior and molecular interactions. SFG has been successfully applied to study cell membrane-associated peptides and proteins, surface-immobilized AMPs and enzymes, and physical interactions between peptides/proteins and a variety of solid surfaces including polymer and 2D material surfaces.

Combined with other experimental tools that can measure independent structural parameters and/or properties, SFG can provide more detailed structural information on interfacial peptides and proteins, and it can deduce the structure−function relationships of these interfacial biomolecules. With the isotope-labeled method, SFG can probe local structural information and more detailed global structure of peptides/proteins at interfaces. With chiral detection and probing SFG signals in different frequency regions, additional independently measured structural parameters could be obtained to determine the interfacial peptide/protein structure more accurately. With advanced SFG techniques such as phase-sensitive SFG and time-resolved SFG, absolute orientation of interfacial biological molecules can be determined, and ultrafast dynamics of interfacial proteins can be elucidated, providing more complete knowledge of molecular behavior and molecular interactions of interfacial proteins. With the continued success in SFG studies on interfacial peptides/proteins, we believe that SFG will be developed into a

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significant analytical tool to study biological molecules at interfaces, which is important for many research areas and applications covering chemistry, biology, biophysics, medical science, materials science, engineering, and beyond.

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