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Directions in peptide interfacial science

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I. INTRODUCTION

The evolution of biological surface science can be credited to the development of traditional surface-chemistry tools and techniques to investigate molecular and atomic-scale bonding, structure, conformation, physical properties (e.g., chemical, electronic, mechanical), and dynamics of adsorbates at various interfaces:[1] Both classical measurements of surface behavior and features (i.e., adsorption isotherms, surface areas, roughness, thickness, and topography) and modern spectroscopic-based techniques that provide information on elemental composition, oxidation state, depth profiling, and distribution of chemical species have shown applicability to the study of biomolecular interactions.[1] However, experiments that probe with electrons, atoms or ions require ultrahigh vacuum (UHV) or reduced pressures at the interface, and are thus intrinsically limited with regards to interfacial explorations in an aqueous environment, i.e., the study of at biomolecules the solid/water interface.[1]

A feature common to all biological molecules is the separation of their numerous hydrophobic and hydrophilic domains in aqueous media.[2] The hydrophobic effect is observed in an aqueous medium of amphiphilic molecules (or ions) containing polar/charged groups at one end, attached to a relatively large hydrocarbon moiety (segment).[3] The thermodynamic description of water-mediated clustering of hydrophobic units in biomolecules (i.e., to induce correct folding of protein molecules) varies significantly from interfacial reorganization of water molecules around small solutes.[4] Thus, factoring in hydration effects at the liquid/solid interface is essential from an experimental perspective in biological surface-science.

The recent surge of interest in surface-sensitive techniques that can be adapted to examine biointerfaces has primarily been motivated by the desire to understand how proteins adsorb on surfaces and the consequences of this surface interaction. Protein adsorption is key to the initiation of cellular activities, such as cell attachment, surface migration, differentiation, and proliferation (growth). Studying processes involved in biomolecule adsorption can give insight into achieving molecular control of surface properties (e.g., structural and chemical). Surface-directed control of bioadsorption has many applications, such as the design biomedical implant devices (nonfouling surfaces), biosensors and development of materials which encourage/prevent biomolecular and cell adhesion (i.e., surfaces designed to reduce inflammatory responses).[5] The extension of surface science studies on metals and oxide surfaces, which has opened the doors to understanding fundamental surface phenomena of adsorption and catalysis, to the biological arena has largely been facilitated by explorations of polymeric interfaces. Nonlinear optical techniques [e.g., sum-frequency generation (SFG)] nm-scale microscopic tools [e.g., atomic force microscope (AFM)], and quantitative adsorption sensors [e.g., quartz crystal microbalance (QCM)] have been particularly useful in this regard. This is because the methods used for studying adsorbed polymers at interfaces are compatible with examining the physical properties of biomolecules on surfaces *in situ*. Polymers can serve as "soft surfaces" to which surface-modifying endgroups can be attached in order to engineer the surface of biomedical devices that require compatibility.[6,7] For example, Surface-Modifying Endgroups™[6,7] may be attached to polymer-based materials for medical devices.[8] Such materials include polymethyl methacrylate, polyethylene terephthalate, polyamides (Nylon), polyterafluoro ethylene, polyurethanes, polypropylene, and polyvinyl chloride.[8,9] Furthermore, the findings of molecular-scale studies of polymers at interfaces provide a natural progression for understanding adsorption of biological random co-polymers, i.e., proteins. Proteins and peptides are similar to polymeric materials; their properties and behavior are significantly affected by their molecular structure or amino acid sequence.[10] The importance of surface chemistry in mediating protein adsorption is perhaps best exemplified in the demonstration of the affect of surface hydrophobicity in aqueous media. Specifically, well-defined functionalized self-assembled monolayers (SAMs) can serve as model substrates with controlled composition to achieve structural (and molecular-level) control of the adsorption process at the biological water/solid interface.[11]

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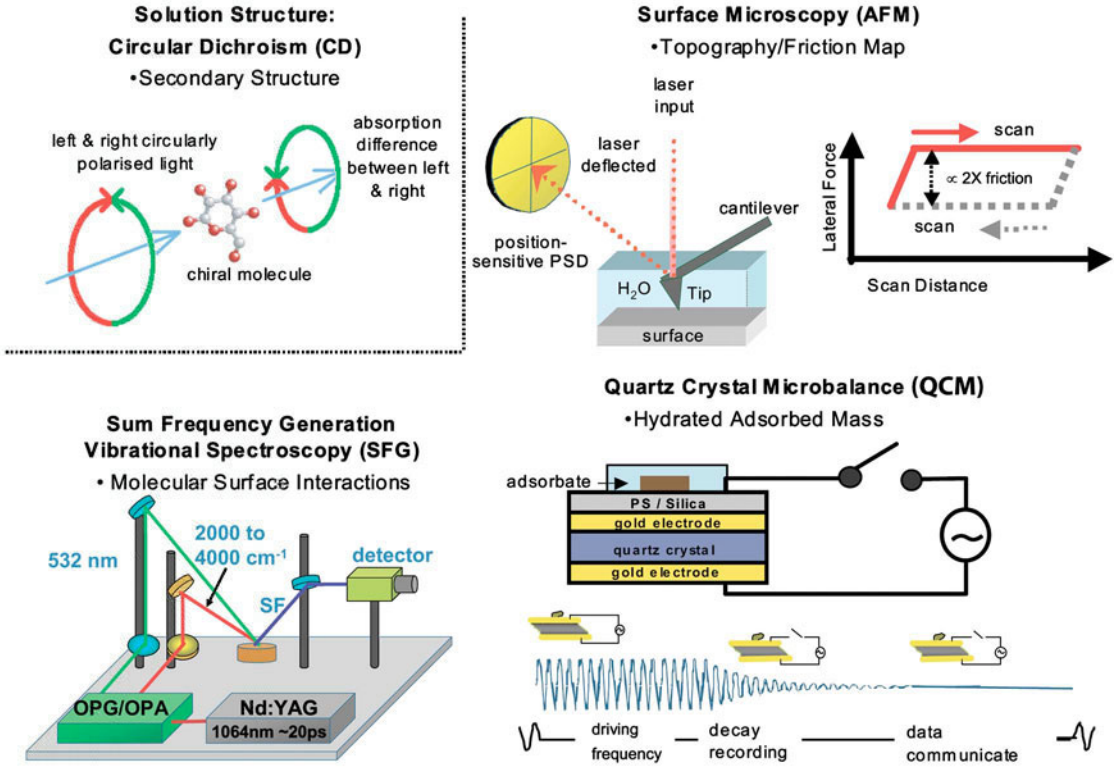

FIG. 1. (a) CD is a solution technique used for characterization of protein/peptide secondary structure. Techniques used to interrogate the solution/solid interface include: (b) AFM, a topographic and friction measurement tool; (c) SFG, a vibrational spectroscopic tool for probing molecular interactions at the surface; and (d) QCM, to measure the amount of adsorbed material.

II. CHARACTERIZATION TOOLS

The key in understanding adsorption processes at the molecular level is to examine the conformational transitions of the biomolecule incurred upon adsorption.[12–17] Analysis of solution phase (bulk) structure,[18–20] and how it changes upon adsorption, necessitates both the use of ultrahigh-sensitivity techniques and molecular spectroscopic instruments, as discussed below.

A. Structural analysis of biomolecules in solution

The most common nondestructive technique for three-dimensional secondary structure determination of biomolecules in solution is circular dichroism (CD) spectroscopy. It is a linear optical adsorption spectroscopy that has been widely utilized in the biological/biochemical sciences as an effective tool to identify the equilibrium secondary structure of proteins/peptides (i.e., α-helicies, β-sheets, random coils, etc.).[21,22] This technique is also recognized in quality control processes for characterization of biopharmaceutical materials. Furthermore, kinetic information regarding folding/denaturation processes of biomolecules with defined secondary structure can be obtained.[21,22] CD monitors the molecular absorption differences in right- and left-handed circularly polarized light. The CD spectra are typically scanned between 190 nm to 250 nm since the amide backbone transitions of the peptide dominates absorption in this spectral region.[21,22] The major advantages of CD is the rapid determination and discrimination of secondary structures, and the need for very dilute solutions (typically $\sim$1 μM and 10 μM for proteins and peptides, respectively).[21,22] Thus, CD remains the simplest method for identification of secondary structures in solution.

B. *In situ* biosurface science techniques

The key to analytical biosurface science is the extraction of various adsorption properties (on the nano- to mesoscale) under biologically-relevant conditions. To accomplish this task, complementary interfacial techniques (all of which are compatible with aqueous media) must be chosen. Critical to surface analysis of biomolecules at the biological interface is the acquisition of molecular-level information in order to derive physical properties and behavior of the adsorbate. Such properties include bonding interaction (energy and chemical nature), structure, conformation, and mechanics. Traditional UHV molecular surface characterization tools are less suitable for studying the resultant properties of adsorbed biomolecules in the presence of interfacial water. Furthermore, vacuum-based high-resolution surface spectroscopic tools invoke high energies (on the order of 1–10 keV) for sample excitation,[1] which can potentially disrupt the physical behavior or even damage the biomolecule adsorbate.

Alternatively, SFG is a molecular-scale nondestructive second-order nonlinear optical method that can provide an average orientation of surface-specific vibrational modes at

the relevant biomolecule/solid interface in water (Fig. 1). It involves the spatial and temporal combination of two laser frequencies (i.e., visible and tunable infrared beams) to induce a polarization at the sum frequency ($\omega_{SF}=\omega_{VIS}+\omega_{IR}$). The surface specificity of SFG signal arises from the fact that even-ordered nonlinear processes vanish in centrosymmetric media (e.g., bulk isotropic nonchiral liquids) but are necessarily broken at interfaces under the electric dipole approximation. SFG is a highly suitable surface technique because it combines high interfacial sensitivity, molecular specificity, low input energy, versatility in substrate material utilized, and flexibility of the interface interrogated (specifically, the biologically relevant water/solid interface).

A complete picture of interfacial adsorption phenomena, however, cannot be provided alone by SFG, which can yield an average orientation of biomolecules at an interface. SFG lacks the capability to explore local characteristics of the adsorbate, such as the morphology (roughness and topographic heights) and lateral properties (i.e., aggregation and domain formation).[8] Consequently, we can employ an *in situ* scanning probe technique, AFM, which provides spatial resolution over the surface (both long-range ordering and shorter-range local features of biomolecules on a surface).[23] As shown in Fig. 1, AFM works by raster scanning a nanoprobe across a surface and optically detecting the deflection (vertical) of the tip caused by topographical surface features. Furthermore, the tribological behavior and mechanical response (e.g., work of adhesion, elastic modulus, viscoelastic characteristic) of the adsorbed material can be characterized,[8,24] and the friction coefficient of the adsorbate determined by mapping the lateral deflection induced on a scanning tip.[9]

Biointerfacial science also requires the implementation of a complimentary technique that can sense adsorbed quantities of material, as a function of the surface chemistry. QCM is an ultrasensitive (nanogram-range) mass sensor that monitors the real-time change in adsorbed amount of material.[25,26] This is achieved by exciting a piezoelectric crystal to oscillation by applying an ac voltage and monitoring the resonant frequency of the crystal over time (Fig. 1). The resonant frequency is dependent on the total oscillating mass, and decreases as a function of the amount of adsorbed material to the crystal (including coupled water mass). By presenting different surfaces to a biomolecule, QCM can also differentiate between varying affinities of adsorption in response to specific surface chemistry [i.e., hydrophobic polystyrene (PS), versus hydrophilic silica (SiO_2)]. In the last ten years, QCM techniques have also advanced to incorporate measurements of energy dissipation changes during adsorption processes, which has been coined QCM-D, where D signifies the simultaneous measurement of "dissipation", that is energy lost over energy stored.[25,26] QCM-D measurements not only yield adsorbed quantity (and affinity) of hydrated material at the liquid/solid interface but directly provides the kinetics of the process and the system energy losses during the adsorption.[25,27,28] Thus, using our multitechnique approach, we can successfully answer three fundamental questions regarding *in situ* biomolecular adsorption: (a) What is the molecular bonding/structure of the adsorbate on the surface (SFG)? (b) How much biomaterial adsorbs over a given time and what is the relative adsorption affinity (QCM)? and (c) How does the adsorbed biomaterial appear in the lateral and vertical dimensions (AFM)?

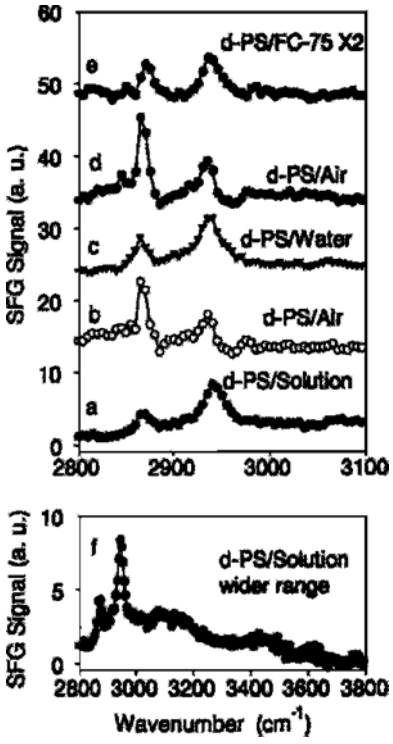

FIG. 2. SFG spectra from BSA protein adsorption showing different surface molecular interactions at various interfaces. Examined interfaces are: (a) BSA solution/PS-d_8; (b) air/PS-d_8 (after removing sample and washing with water; (c) water/PS-d_8 (after sample is rewet); (d) air/PS-d_8 (redried); (e) FC-75/PS-d_8 (where FC-75 is a fluorinated hydrophobic solvent from 3M). Structured water is also observed at the BSA solution/PS- d_8 interface showing broad resonances at 3200 cm^{-1} ("ice-like" water) and at 3400 cm^{-1} (H-bonded, "liquid-like" water) (Reprinted with permission—Ref. 30).

III. INTERFACIAL STUDIES OF BIOMOLECULES

A. Proteins at the biointerface

Initial approaches at exploring biointerfaces, particularly with regard to using SFG for molecular bonding information, has focused on biorelevent large macromolecule species, such as proteins. For example, Fig. 2 shows the protein bovine serum albumin (BSA) adsorbed on deuterated polystyrene (PS-d_8). This BSA experiment demonstrates that the resonant modes observed from the protein are interface dependent. However, molecular assignment of interfacial modes from SFG vibrational spectra of proteins is difficult because of the large number of contributing resonances.[29,30]

B. Amino acids at the biointerface

All levels of a proteins' structure (from primary, secondary, tertiary, to the quaternary level) influence how it binds to a surface.[31] Ultimately, the three-dimensional conformation of a protein, and its activity, is determined by complex inter-

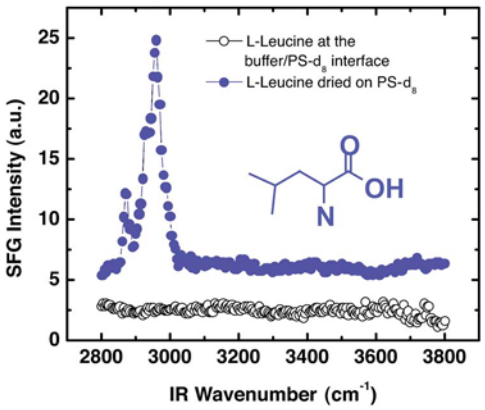

FIG. 3. SFG_{ssp} spectrum of *dried* L-Leucine amino acid (1 mg/mL) adsorbed on hydrophobic PS-d_8 (filled circle symbols) Symmetric and asymmetric CH_3 stretches are, respectively, observed at 2872 cm^{-1} and 2958 cm^{-1} from the Leucine side chain. Note that no detectable SFG signal was observed in the CH spectral region at the *wet* buffer/PS-d_8 interface (open circle symbols).

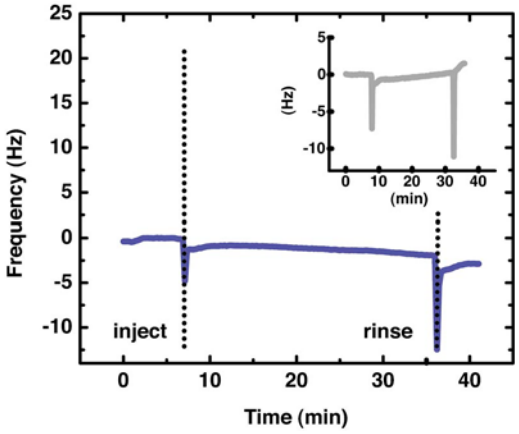

FIG. 4. *In situ* QCM measurement of 1 mg/mL L-Leucine amino acid adsorbed onto hydrophobic PS indicates negligible adsorption from buffer. Similarly, inset shows negligible adsorption of L-Leucine on the hydrophilic silica (SiO_2) from solution.

actions among side chains of amino acids, which dictates the chemical properties. SFG can also be used to probe individual amino acids, the building blocks of proteins, at various interfaces. This has been demonstrated before by SFG investigations of amino acids at the CCl_4/water, water/air, and water/electrode interfaces.[32–35] The obvious advantage of this approach to understanding biological interfaces is the size of the amino acid; it is possible to gather quantitative data of how the side chains of an amino acid orient at a liquid/solid interface. It would be ideal, therefore, to build up a molecular understanding of how the side chains of various amino acids influence its orientation at the biological interface. With detailed knowledge of amino acid behavior, one could then synthesize small peptides (for example, a dipeptide, i.e., a peptide that is composed of two amino acids) and compare, for example, the affect of the length or secondary structure on adsorption. The SFG spectra of a solution of 1 mg/mL Leucine at the buffer/PS-d_8 and air/PS-d_8 interface in presented in Fig. 3. The spectra demonstrate that in *pH* =7.4 standard phosphate buffered saline (PBS) solution, Leucine gives little SFG signal at the buried buffer/PS-d_8 interface. The absence of CH resonances of Leucine on PS-d_8 likely comes from one of two sources: A small number of adsorbed Leucine molecules or a lack of ordering of the adsorbate.

To determine the origin of the weak SFG signal, we performed *in situ* QCM adsorption experiments of Leucine (demonstrated in Fig. 4) and Lysine (data not shown) on both hydrophobic PS and hydrophilic SiO_2. The negligible decrease in frequency ($\leqslant$1 Hz) observed upon adsorption of Leucine and Lysine amino acid from PBS buffer solution on both the PS and SiO_2 surface indicates very little adsorption of weakly bound amino acid. Previous studies of amino acid adsorption on hydrophilic surfaces suggest that there is little free energy gain from the adsorption of an amino acid to a surface.[36] The small adsorbed quantity, independent of the surface hydrophobicity, explains the poor sum-frequency signal observed for Leucine at the buffer/PS-d_8 interface. Upon drying, strong CH signals are observed. The difference in spectral features between the buffer/PS-d_8 and air/PS-d_8 interface demonstrates the strong influence of water on the absorption of the amino acid. The lack of SFG signal at the buried solution/solid interface makes the above strategy difficult to implement, and raises the question: What is the difference in interfacial behavior between individual amino acids and proteins, which are entirely composed of amino acids? To answer this, we turn to small model polypeptides which contain one or two types of amino acids, as described below.

C. Model peptides at the biointerface

In the last ten years, "bottom-up" approaches in the nanosciences have popularized the idea of using molecular building blocks to obtain and control macroscopic properties of a material. Similarly, we have decided to employ a bottom-up strategy to understand real biological adsorbates by performing molecular-scale investigations of simple, model peptides on surfaces of controlled chemistry. Ideally, this approach would begin with the most simple precursors of biological molecules, amino acids. However, the poor adsorption affinity of amino acids at physiological *p*H, as demonstrated by Leucine and Lysine, on model hydrophobic surfaces and model hydrophilic surfaces prevents this simplistic approach. Short-chain model peptides are suitable intermediates for examining the influence of the amino acid molecular composition on the physical adsorption properties. Although the level of complexity of peptides is greater than that of amino acids, short-chain peptides can be designed with the appropriate primary sequence and chain length (via solid-state peptide synthesis)[37] to form secondary structural subunits found in more complex proteins by maximizing intramolecular and H bonding, and electrostatic interactions (i.e., α-helix, β-sheet). Directing selective folding/coiling of a peptide to a desired secondary structure can be achieved by the driving stable formation intramolecular hydrogen bonds in H_2O[38,39] and

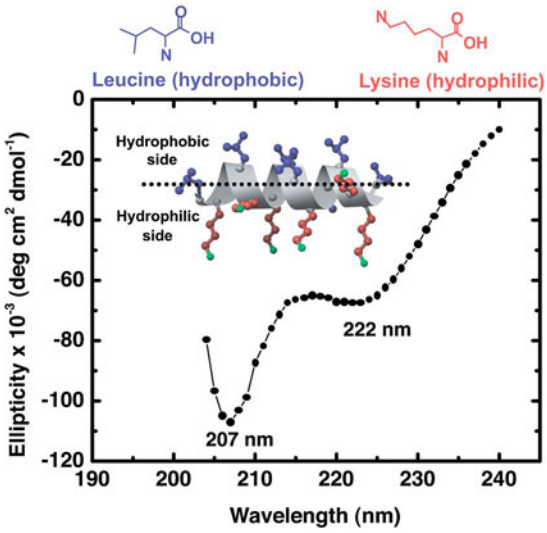

FIG. 5. CD spectrum of 140 μg/mL LK_{14} peptide in PBS buffer showing a typical α-helical secondary structure. Leucine side chains (L, where $R=(CH_3)_2$–CH–CH_2–) segregate to create a hydrophobic side. The hydrophilic side chains of Lysine (K, where $R=H_2N$–$(CH_2)_4$–) are isolated from the Leucine side chains to form an hydrophilic domain.

maximization of electrostatic interactions between amino acid side-chains.[40] Furthermore, abstracting conformational data of model peptides at an interface using SFG simplifies identifying the origin of the observed vibrational modes, since there are fewer, but similar resonances to those present in the parent protein.

Our goal is to determine how molecular bonds in model peptides (composed of a hydrophobic and a hydrophilic amino acid and systematically combined in different ways) respond to model surfaces of varying hydrophobicity *in situ* during adsorption process. This two-amino-acid scheme allows us to investigate the role of electrostatic, hydrophobic, H bonding, and van der Waals/dipole interactions (both intramolecular side-chain interactions of residues, and the affect on surface/side-chain interactions), as well as contributions such as aromatic-aromatic interactions (i.e., using phenyl-side chain amino acids, and phenyl-containing polymer surfaces). As a proof of principal, we synthesized a model amphiphilic 14-amino acid peptide composed of hydrophobic Leucine (L, where $R=(CH_3)_2$–CH–CH_2–) and hydrophilic Lysine (K, where $R=H_2N$–$(CH_2)_4$–) residues of sequence LKKLLKLLKKLLKL (LK_{14}), as illustrated in Fig. 5. The secondary structure of the peptide determined by the set of repeating dihedral angles (ψ,φ), defining the intramolecular H bonding and the spatial orientation of the peptide. The hydrophobic periodicity of 3.6 residues/turn (1.5 Å) and chain length of 14 is chosen to support the formation of an α-helical structure $(\phi=60°,\psi=45°)$[21] at a nonpolar (air)/polar (water) interface.[38] The secondary structure of this peptide in PBS solution can be confirmed by CD measurements (Fig. 5). The two residual ellipticity negative bands observed at 207 nm (large) and 222 nm (smaller) are representative of typical α-helical peptides, which correspond to an electronic transition that is independent of the length of the helix, and relating to a strongly H-bonded environment, respectively.[21] To examine the real-time affect of a hydrophobic (PS) versus a hydrophilic (SiO_2) surface on the adsorbed mass of LK_{14}, we have applied the QCM-D technique. It has been shown that the amount of hydrated LK_{14} peptide adsorbed and the adsorption timescale greatly differ as a function of the surface hydrophobicity as detected by QCM-D.[28] Specifically, on hydrophobic PS, a monotonic/single-step adsorption profile is exhibited on PS with little dissipative energy loss in the thin film that is produced in <2 min. Conversely, on SiO_2, a multistep (and multilayer) adsorption pattern is observed over >35 min, simultaneously incurring large dissipative energy loss through each adsorption step. AFM measurements of topographic morphology, roughness, and friction coefficient of adsorbed LK_{14} in the presence of buffer, confirms a single-step and multistep adsorption behavior (on PS and SiO_2, respectively), and the timescale of surface-induced lateral aggregation (and hence increase in surface roughness) that occurs only in the case of hydrophilic SiO_2. Furthermore, a significant difference in the mechanical properties of the adsorbed LK_{14} peptide has been observed. Specifically, AFM lateral force measurements demonstrate that the peptide friction (at the buffer/solid interface) on the SiO_2 surface is double that on PS.[28]

Using SFG, we can compare molecular interactions and ordering of LK_{14} at the solid/water interface. As shown in Fig. 6(a), ordered methyl groups of Leucine side chains are observed at the buffer/PS-d_8 interface (CH_3 v_s at 2869 cm^{-1}, CH or the Fermi resonance of a CH_2 mode at 2895 cm^{-1}, and the Fermi resonance of a CH_3 mode at 2935 cm^{-1}).[34] Conversely, Fig. 6(b) indicates solely ordered NH resonances detected at the buffer/SiO_2 interface. The differences in SFG spectra indicates that the amphiphilic LK_{14} peptide adapts completely different average orientations in response to a hydrophobic (buffer/PS-d_8) interface versus a hydrophilic (buffer/SiO_2) one. A significant difference is also observed in the structure of water on PS-d_8, a broad OH mode centered at 3092 cm^{-1} in Fig. 6(a), as compared to a much weaker OH mode at 3190 cm^{-1} on SiO_2, Fig. 6(b), in the presence of adsorbed LK_{14} peptide. It is also interesting to note that both AFM and QCM results suggest increasing amount of adsorbed peptide on a timescale of minutes on the hydrophilic SiO_2 surface while immediate adsorption is observed on hydrophobic PS-d_8. Preliminary SFG investigations probing the CH and NH spectral region (2800–3600 cm^{-1}) show a different trend. SFG spectra show a time-dependent increase (on the order of min) of surface-interacting modes exhibited at the buffer/PS-d_8 interface, Fig. 6(a), but not at the buffer/SiO_2 interface, Fig. 6(b). This is indicated by the growth of SFG peaks at 2869 cm^{-1} (CH_3 symmetric resonance) and 2935 cm^{-1} (methyl Fermi resonance) over 0.5 h period on PS-d_8, inset Fig. 6(a), but the absence of an observable time dependence in the relative SFG intensity of the NH mode on SiO_2 on the same timescale, Fig. 6(b). These results suggest that the surface-sensitive SFG signal changes come from various factors (including average molecular orientation and number of

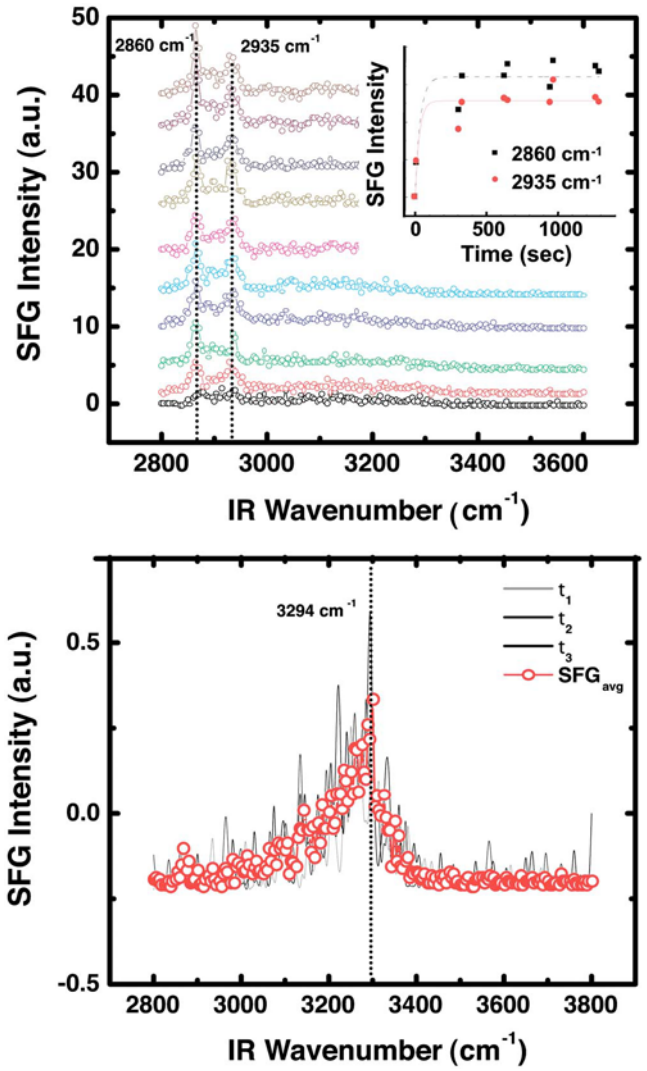

FIG. 6. (a) SFG_{ssp} spectra of LK_{14} peptide at the buffer/PS-d_8 interface showing increasing CH resonances at the surface (CH_3 υ_s at 2860 cm^{-1}, methyl Fermi resonance at 2935 cm^{-1}) as a function of time (ranging from 1 to 20 min). The lines plotted in the inset are meant to guide the eye. (b) A completely different SFG spectrum is observed for LK_{14} adsorbed at the buffer/SiO_2 interface, showing NH stretching at 3294 cm^{-1} (where t_1, t_2, t_3 are approximately 10, 15, and 20 min, respectively) and an OH mode from "ice-like" water centered at 3190 cm^{-1}.

oscillators). In other words, time dependence of the SFG signal on PS-d_8 is related to diffusion or kinetic effects, which influence the surface concentration and/or average molecular orientation. Current studies by the Samuel *et al.*[41] of LK_{14} adsorbed on a fluorocarbon surface do not show a time dependence in the CH region. The different dynamic aspects observed in the molecular bonding behavior (as examined by SFG), as compared to the measured adsorbed mass and morphology (obtained by QCM and AFM), demonstrate that each technique is also capable of providing unique kinetic information and thus warrants further investigations.

IV. CONCLUSIONS AND PERSPECTIVES

The understanding of biomolecular adsorption at biologically relevant interfaces is a highly complicated and interdisciplinary problem. There are many techniques available to surface scientists to measure physical adsorption properties, but few are suitable for studying biomolecules at the solid/liquid interface. We chose SFG for molecular sensitivity to examine interactions at the interface, AFM to understand morphological and mechanical interfacial properties, and QCM to quantify the adsorption. Our purpose in choosing this combination of *in situ* techniques is to combine complementary data to obtain a more complete understanding that cannot be obtained solely from employing one technique.

Our ultimate goal is to understand the interfacial behavior of proteins and other real biological species. Since large biomolecules are complicated in their molecular-scale bonding description, smaller model studies must be performed first. However, scaling studies to derive interfacial data from the specific molecular chemistry of amino acid side chains, (the fundamental constituents of proteins) is also challenging since amino acids do not have a strong adsorption affinity on model hydrophobic (PS) and hydrophilic (SiO_2) surfaces in *p*H 7.4 buffer.

We have found that model amphiphilic peptides (composed of one charged and one hydrophobic residue) have the ideal level of complexity for probing the amino acid side-chain interaction with surfaces of varying hydrophobicity. Moreover, de novo design of analagous model peptides having simple sequences used to control the desired secondary structure in solution, enable one to study the affect of various secondary structures on adsorption. Our current work suggests that this strategy is also amenable to "real peptides", which have known biological function, such as collagen and RGD cell-attachment peptides (composed of two and three different amino acids, respectively). Our future studies will aim to develop a molecular- and meso-scale database of adsorption behavior from model peptides and model surfaces. Furthermore, we are expanding our interfacial studies to biologically relevant surfaces, such as contact lenses and bioimplant polymers with Surface-Modified Endgroups.[42]

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