Vibrational Sum Frequency Generation Spectroscopy: A Tool to Probe Complex Biological Interfaces

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Abstract:

In this mini-review article, we discuss the potential application of vibrational sum frequency generation (VSFG) spectroscopy to probe complex biological interfaces. We will provide the basic principle of VSFG spectroscopy and explain why it can exclusively probe different interfaces by giving some specific examples of air/water interfaces, and interactions of nanoparticles (NPs) with different lipid membranes. Further, we will explain how VSFG can be used to probe the aggregation and ice nucleation properties of specific proteins. Combining the heterodyne and time-resolved measurements, the VSFG experiments can provide in-depth information about the structure and dynamics at different interfaces. Such information can be useful for various applications from fundamental understanding to translational research.

1. Behaviour of biomolecules at the interface

Biomolecules such as proteins, peptides, amino acids, polysaccharides, lipids, and nucleic acids are important natural machinery as they drive key biological processes such as sensing, drug transport, immune response etc. Most of these processes action occurs at the interface of biomembranes, generally known as biological interface[1].The interfacial water present at these biological interfaces play an important role in the hydration of the membranes, proton transfer to biomolecules etc. which ultimately makes the biological interface comprising of three components i.e. water, membranes and biomolecules[2]. It is well known that the processes taking place at interfaces are distinguished from those in the bulk, and therefore it is expected that biomolecules may show a different behaviour at the interface due to the unique hydrophobic hydrophilic environment present at the interfaces. The interfacial behaviour of such biomolecules can be further tuned to different interfacial conditions such as pH, the concentration of biomolecules, and the chemical potential of a particular interface etc. Therefore, probing the structure and dynamics of the biomolecules at the interface may provide a deeper understanding of the interfacial processes to exploit the optimization and development of novel materials for biomedical applications[1].

Over the years, several techniques have emerged such as solid-state NMR, atomic force microscopy (AFM), surface-enhanced Raman scattering (SERS), and surface plasmon resonance (SPR) that give information about biomolecules adsorbed at interface. However, the molecular level information about the structure and dynamic of the biomolecules at the interface remains poorly understood because of the difficulties involved in probing extremely thin interfaces. Recently, the Vibrational Sum Frequency Generation (VSFG) spectroscopic

technique has evolved as an efficient surface-sensitive tool to monitor the structure and dynamics of interfacial biomolecules.

2. Sum Frequency Generation (SFG) Spectroscopy

SFG is a coherent, non-linear spectroscopic method. It is highly sensitive to interfacial molecules. It is active in a non-centrosymmetric medium and possesses the characteristics of Raman-IR spectroscopy. In SFG two incident photons of frequency ω_1 and ω_2 spatially and temporally combine at interfaces and produces a photon of summed frequency (ω_1+ω_2). The interaction of the photons and material induces a polarization within a material which is equal to

$$P = \chi^{(1)}E^{(1)} + \chi^{(2)}E^{(2)}$$

where the first term is responsible for linear absorption and the second term is for the sum frequency generation method. The intensity of the SFG signal depends on the susceptibility tensor and the electric field of the two incident beams $I_{SFG}(\omega 1 + \omega 2) = \left(\chi^{(2)}E_1E_2\right)^2$. Here

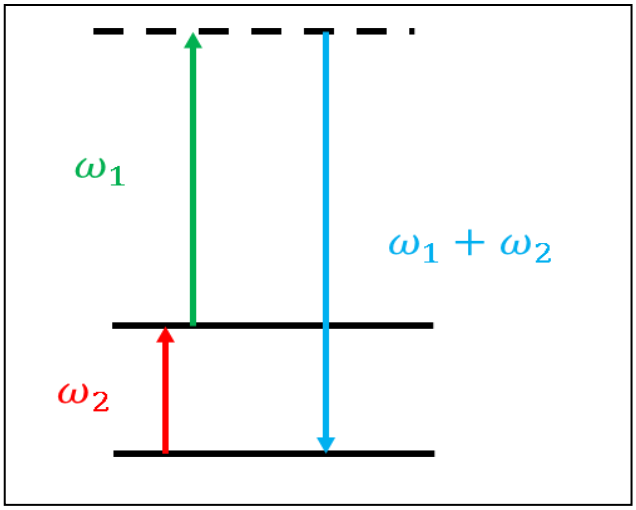

Figure 1: *Energy-level description of Sum Frequency Generation.*

$\chi^{(2)}$is a second-order susceptibility term. In a centrosymmetric environment, all directions are equivalent and the value of$\chi^{(2)}_{ijk}$ for two opposing directions must, therefore, be identical, viz., $\chi^{(2)}_{ijk} = \chi^{(2)}_{-i,-j,-k}$. However, as $\chi^{(2)}_{ijk}$ is a third-rank tensor, a change in the sign of the three subscripts is simply equivalent to reversing the axis system, and the physical phenomenon $\chi^{(2)}_{ijk}$ describes must, therefore, reverse sign, $\chi^{(2)}_{ijk} = -\chi^{(2)}_{-i,-j,-k}$.

In the VSFG process, two pulsed laser beams, one tunable infrared and the other a visible beam are used. Here one of the incident beam photons is in resonance with a vibrational transition of interest and gives characteristics of the molecule. The electronic analogue of VSFG spectroscopy known as electronic SFG has a potential to probe the electronic states present at the interfaces. The density of state information obtained from ESFG measurements can be used for optimizing the efficiency of various optoelectronic devices. We have also developed ESFG measurements in our laboratory at IIT Roorkee, but for this article we have restricted the discussion to VSFG applications. The intensity of the SFG signal at a certain frequency is dependent on the non-resonant signal and resonant signal. The measured VSFG intensity is proportional to the square of the second order nonlinear susceptibility $\chi^{(2)}$ of the sample and the intensities ofthe visible and infrared beams.

$$I_{SFG}\,\alpha\left|\chi^{(2)}\right|^2 I_{VIS}I_{IR} \qquad (1)$$

When the frequency of the incident infrared field is resonant with the vibrational mode n, the VSFG field can be resonantly enhanced. Thus, the susceptibility $\chi^{(2)}$ consists of non resonant (NR) and resonant(RES) terms[3,4].

$$I_{SFG}\ \alpha \left| A_{NR} e^{i\phi} + \sum_{j=1}^{N} \frac{A_j}{(\omega_{IR} - \omega_j) + \Gamma_j} \right|^2 \qquad (2)$$

where A_{NR} represents the amplitude of the non resonant susceptibility, φ_{NR} is its phase, A_n is the amplitude of the nth vibrational mode with resonant frequency ω_n, and Γ_n is the line width of the vibrational transition.

3. Water at interfaces

Water molecules in the bulk are stabilized by a tetrahedral bonding network with their neighbours. At interfaces, this molecular arrangement is not possible, due to increased surface energy. Each interfacial water molecule tries to have more number of hydrogen bonds to minimize the excess surface free energy. This can be achieved if surface water molecules assume a hexagonal lattice-type structure, with the surface terminated by free -OH bonds, which is the characteristic of the free water-air interface[3]. The vibrational spectra of interfacial water is shown in fig. 2(a), peak at 3700cm^{-1} represents free -OH while peaks at 3200cm^{-1} and 3400cm^{-1} correspond to strongly and weakly hydrogen-bonded water molecules at the interface. The unique structure of interfacial water turns out to be important for understanding the surface chemistry of mineral-water interface, atmospheric aerosols, membrane biochemistry etc. Moreover, anything which passes through biological membranes has to interact with interfacial water. The water then determines the fate of any biomolecule as to how it will interact with membranes. The interfacial water molecules facilitate energy transfer between membrane leaflets of DPPC, which is the most ubiquitous phospholipid membrane. This was attributed to the emergence of extended vibrational modes in the membrane due to the hydration of lipid head groups[5]. The presence of water near the surface of biomolecules is crucial for maintaining sufficient structural flexibility so that the necessary motion of biomolecules can take place.

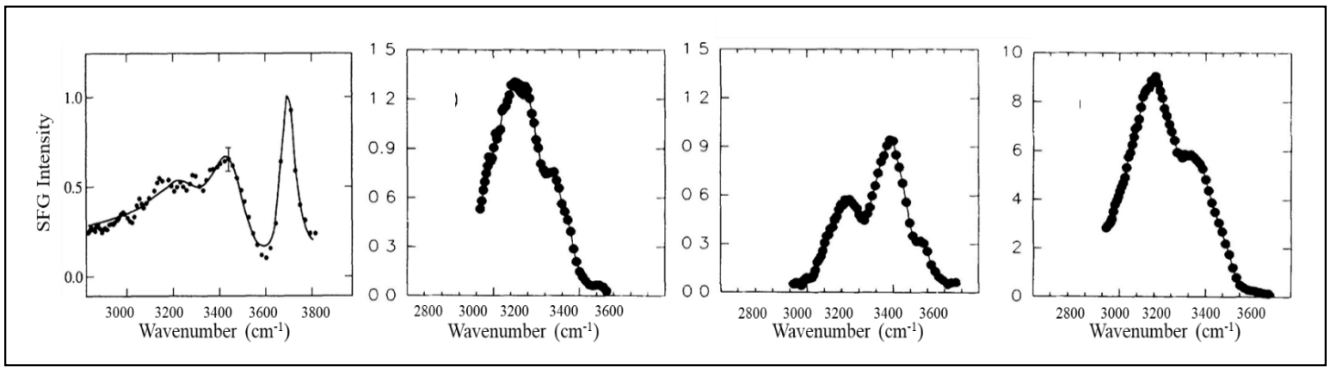

Figure 2: *Vibrational SFG spectra of water at (a) free water-air interface, showing a peak at 3700 cm^{-1} corresponding to free –OH (b) water in contact with quartz surface at pH 1.5, free -OH signal diminished due to ionization of interface (c) water in contact with quartz at pH 5.6 and* ***(d)*** *at pH 12.3.Reproduced with permission from ref* [3,4]*. Copyright 1993 by Physical Review Letters.*

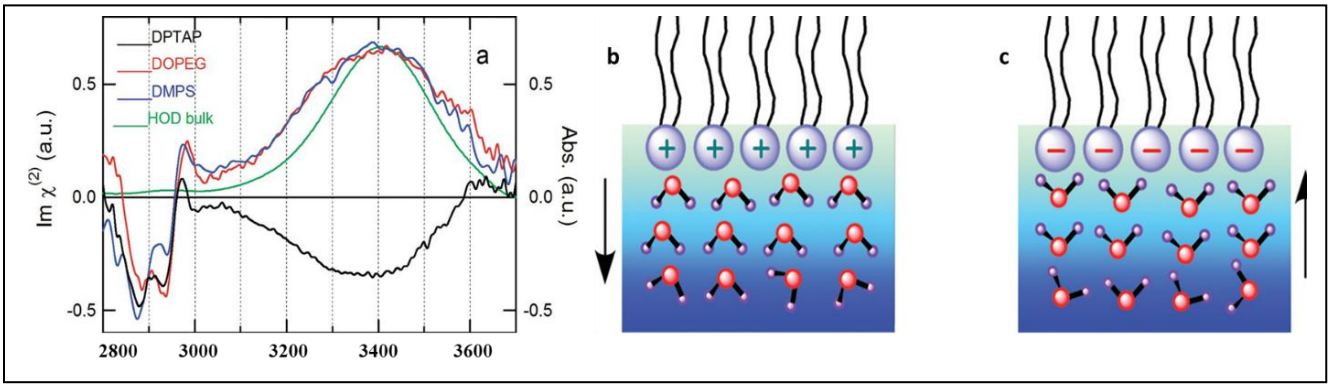

Figure. 3: *(a) HD-VSFG spectrum in the -OH stretch region of water molecules at differently charged lipid monolayers, (b) In the presence of positively charged lipid, water dipoles are oriented away from the interface while, (c) In the presence of negatively charged lipid, water dipoles are oriented towards the interface.Reproduced with permission from ref [6]. Copyright 2010 by American Chemical Society.*

Apart from the free water-air interface, buried water interfaces can apply to many surface sciences such as electrochemistry, micelle formation, membrane stability etc. For the first time, Shen *et al.* presented the SFG spectra of a fused quartz/water interface under various conditions[3,4]. The results indicate that interfacial water molecules can interact with quartz by two forces: hydrogen bonding and electrostatic attraction which result from the ionization and deionization of surface silanol (SiOH) groups. At a high pH value of 12.3, the surface silanol groups are all ionized, and the negative surface charge produced by quartz aligns some water molecules towards itself giving a strong SFG signal (fig.2d). However, if the quartz surface was neutral (pH 1.5, fig. 2b), the water molecules formed hydrogen bonds with quartz surface pointing their oxygen towards the surface. In this case, the signal intensity was low compared to high pH value therefore it was expected that only 1 or 2 monolayers of water was oriented. At intermediate pH value of 5.6, the surface was partially ionized due to which some of the water molecules are pointing towards quartz surface while some point away from it ultimately, disturbing the order of interfacial water molecules which can be corroborated to even more decrease in SFG intensity as compared to pH 1.5 and pH 12.3(fig. 2c).

The interfacial water structure can undergo an order-disorder-order pattern depending on the pH of the bulk solution. At specific pH, when the surface becomes completely ionized, more water molecules can be aligned thus giving rise to an intense SFG signal. In addition, surfactants/lipid membranes can also orient water molecules at the interface depending upon the charge of lipid membrane. Tahara *et al.* have demonstrated that water is oriented differently at different charged lipid membranes using heterodyne-detected vibrational sum frequency generation (HD-VSFG) spectroscopy[6]. The beauty of HD-VSFG measurements is that they can provide information about $\chi^{(2)}$ otherwise lost in simple VSFG measurements as it measures $\chi^{(2)2}$. The direct measurement of $\chi^{(2)}$ allows for retrieval of the phase information in the HD-VSEFG measurements. For the negatively charged lipid DOPEG and DMPS, the Im $\chi^{(2)}$

spectrum has positive sign (fig. 3) which represent that the water dipole is oriented towards the interface (H-up orientation) while for positively charged lipid DPTAP, the Im $\chi^{(2)}$ spectrum shows negative sign which represent that the water dipole is oriented away from the interface (H-down orientation). So, the net charge on lipid head groups governs the net orientation of water molecules at the interface.

4. Interfacial water mediates the interaction between Gold Nanoparticles and bio membranes

The surface charge of gold nanoparticles (GNPs) influences their interaction with the biological membranes. It is reported that anionic and cationic GNPs follow different cell internalization pathways, which predominantly rely on the surface charge of GNPs. The interactions of NPs with cell membrane are governed by the nature of the molecules, surface characteristics, and solution (Water) environment. Having considerable attention aimed at NPs membrane interaction it becomes important to know the role of interfacial water. Gahtori*et al.* used Vibrational Sum Frequency Generation (VSFG) spectroscopy to show the effect of NPs charge to perturb the interfacial potential and stern layer configuration at lipid-water interface[7]. Cationic and anionic GNPs were prepared and their interaction with model lipid membranes (1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG, negative charged), 1,2-dipalmitoyl-3-trimethyl ammonium-propane (DPTAP, positively charged), and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, zwitterionic) was monitored. It was found that cationic GNPs induced a significant change in the water spectra when interacted with negatively charged DPPG lipid (fig.4a), while anionic GNPs do not bring any noticeable change in the water spectra.

For DPTAP being a positively charged lipid monolayer, there were counter intuitive trends in the water spectra. Both cationic and anionic GNPs show depreciation in water intensity, moreover cationic GNPs show more depreciation compared to anionic GNPs (fig. 4b). The results were explained on the basis of stern layer configuration. The stern layer formed at DPTAP monolayer consists of charged-lipid-surface/water/Cl^- ions transform to charged-lipid-surface/water/Cl^- ions/water/cationic GNPs

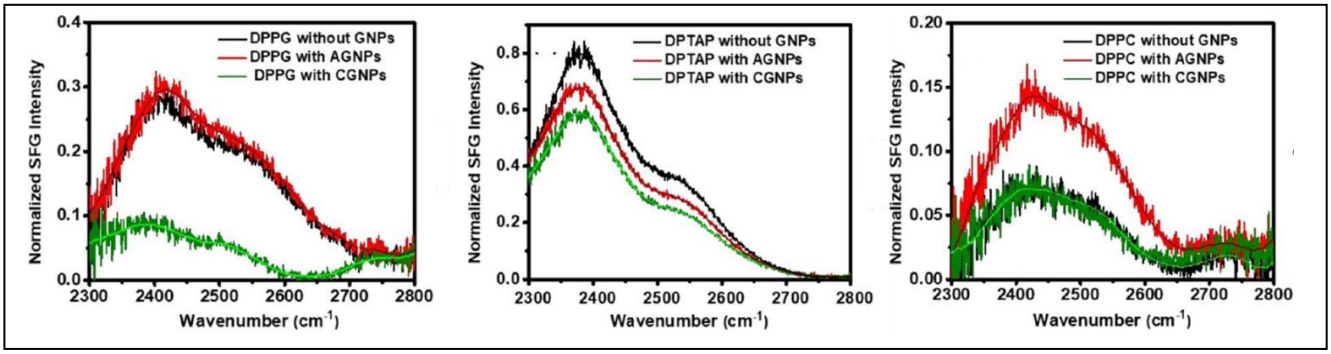

Figure 4: *Interfacial water SFG spectra when cationic and anionic GNPs are added in presence of (a) negatively charged DPPG monolayer (b) positively charged DPTAP monolayer and (c) zwitterionic DPPC monolayer. Reproduced with permission from ref[7]. Copyright 2021 by American Chemical Society.*

in presence of cationic GNPs which results in the cancellation of the net dipole of water molecules near DPTAP monolayer and SFG intensity of water stretch vibrations decreases. In the presence of anionic GNPs, Cl^- ions of lipid membranes be likely pushed toward the lipid head groups because of repulsion between Cl^- ions and anionic GNPs thus making the interface positively charged DPTAP monolayer/Cl^- ions/water/anionic GNPs. This configuration also results in the decrement of SFG intensity. In the case of zwitterionic lipid monolayer, DPPC, it was observed that anionic GNPs enhance the water SFG intensity (fig. 4c). DPPC composed of both positive and negative charged head groups with the positive group facing towards the water surface. When anionic GNPs were added to the monolayer, they attracted toward the positive head group thereby neutralizing the effect of positive charge, in this way only the negative charge of the lipid remains at the interface. The effect of neutralization of charge by anionic GNPs increases the interfacial water alignment which results in an increment of SFG intensity. Whereas in the presence of a cationic GNPs, there are only subtle changes in the dipolar orientation profile which results almost no change in the VSFG spectrum. This study reveals, how interfacial water mediates the interaction between GNPs with different lipid membranes.

5. Hydration and lipid-mediated lysozyme oligomerization at the model cell membrane

Protein aggregation is a cause of many neurodegenerative disorders such as Alzheimer, Parkinson diseases. In presence of cell membranes, the aggregation is extensively enhanced due to protein misfolding in the cellular environment. To understand the molecular-level insights of protein aggregation induced by membranes, it is essential to study the role played by lipid chains, interfacial water molecules and protein hydration near the lipid/water interface. Four major factors are relevant to understand protein aggregation near the lipid membrane. These are (a) conformational change in protein while in contact with lipids, (b) accumulation of protein at the interface, (c) change in the orientation of

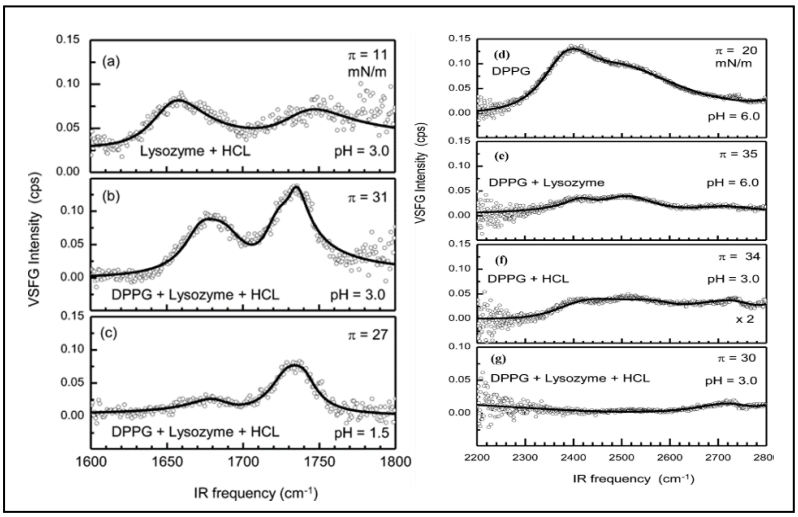

Figure 5: *VSFG spectra in the C=O region for (a) lysozyme monolayer at pH3 (b)DPPG in presence of lysozyme at pH 3, peak at 1685 cm-1 was attributed due to the formation of small aggregates of lysozyme(c) DPPG in presence of lysozyme at pH 1.5,the decrease in intensity of aggregates was due to the departure of lipid-lysozyme aggregates domains from the interface.(d-g) VSFG spectra in the O-D stretching region, as lysozyme was added to the DPPG monolayer interface, the water intensity decreased, which indicated significant dehydration of lipids. Reproduced with permission from ref[2]. Copyright 2014 by American Chemical Society.*

protein upon interaction with lipids, and (d) templating effects of the membrane. Rzeznicka *et al.* studied an aggregation of lysozyme oligomers at a negatively charged DPPG lipid monolayer while varying the pH[2]. At low pH conditions, basic amino acids of the protein backbone get protonated which may increase the side chain charge repulsions thus exposing the hydrophobic and aggregation-prone sites to the water. This facilitates the protein-protein interaction which ultimately leads to aggregation.

It was found that, at the air/water interface, lysozyme maintains its native structure even at pH 1.5 because in an acidic environment most of the amino acids are protonated which induces repulsion between side chains of lysozyme and consequently prevents aggregation. However, in presence of lipids at the air/water interface the aggregation of lysozyme starts as the solution pH is reduced (fig.5a-c). The process of aggregation was mainly derived from the dehydration of lipids and an increase in lipid order. The DPPG lipid, being negatively charged, attracts protonated, positively charged side chains of lysozyme towards itself, which can be observed by the interfacial water SFG spectra (fig. 5d-g) that decreases when lysozyme is added at the DPPG-lipid/water interface. The extra added lysozyme molecules adsorb at the peripheral sites of the DPPG monolayer which ultimately results in the dehydration of lipids and an increase in the lipid order. The increase in lipid order decreases the area occupied by each lipid molecule which in turn reduces the distance between lysozyme oligomers adsorbed at the peripheral sites of DPPG. In this way, lysozyme molecules act as coupling units which favor aggregation.

6. Ordered water structure facilitates ice nucleation

The ice nucleation process starts when small ice

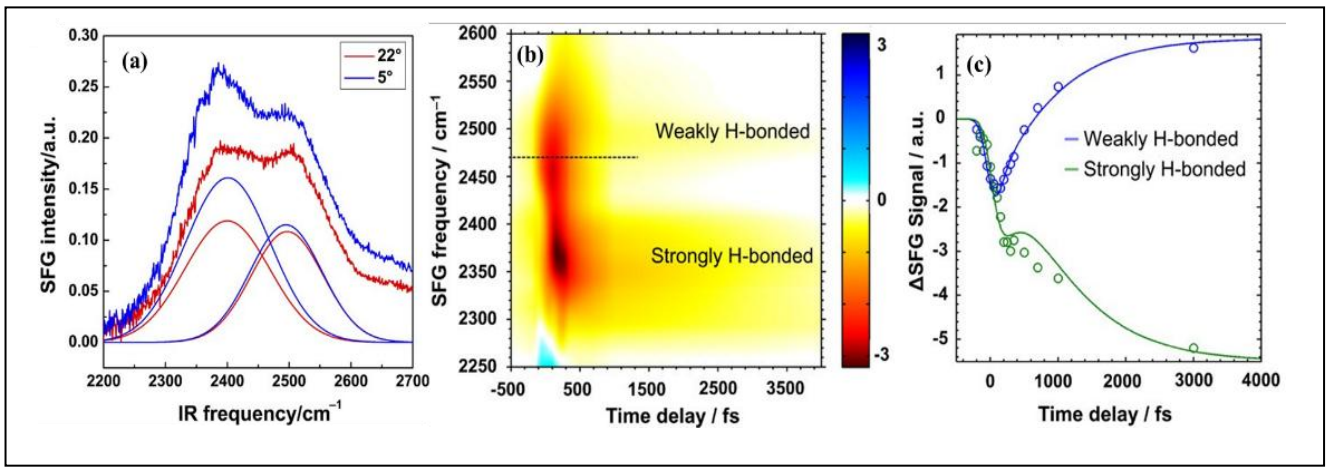

Figure 6: *(a)VSFG spectra of water near P. syringae bacteria surface, as the temperature was reduced to 5^{o}C the strongly hydrogen bonded population of water molecules increases(b) time-resolved SFG spectrum of the bacteria-water interface after excitation with a 2470cm^{-1} pulse, the signal bleach is very intense in the low-frequency region that corresponds to strongly hydrogen-bonded water molecules(c)time-resolved bleach for strongly and weakly hydrogen-bonded water molecules. Reproduced with permission from ref[5]. Copyright 2016 by American Association for the Advancement of Science.*

crystal embryos form on the protein surface. The ice crystals are formed which further facilitated by the ordered water arrangement at the protein surface. Protein "in a Z" is a membrane protein, present at the outer cell membrane of *P. syringae* bacteria which is known to form ice crystals close to the melting point of ice. The bacteria are used for artificial snow production in winter sports around the world. For farmers, ice nucleation can be devastating as it can damage the crops. To test the model, of forming ice crystals near the protein surface, experimentally, Pandey *et. al* studied the interfacial water arrangement around the ice-nucleating protein in a Z using SFG spectroscopy[5]. It was found that the two conditions favor ice nucleation: (a) arrangement of water in an ordered structure, (b) removal of latent heat due to phase transition. To test the first condition, SFG spectra were taken in the O-D range. Fig.6a shows the temperature dependence of interfacial water in the presence of in a Z protein. When the temperature was lowered from 22^oC to 5^oC, the strongly hydrogen-bonded population of water molecules at $2390cm^{-1}$ increased, whereas the weakly hydrogen-bonded population at $2500cm^{-1}$ remained unchanged. Also, using MD simulations it was found that, at lower temperatures the water is ordered which is mainly driven by hydrophilic end groups of the protein whereas inner (hydrophobic) regions of the protein did not show a pronounced effect. So, the commonly assumed fact that the presence of only hydrophilic sites promote ice nucleation was not sufficient to explain the ice nucleating properties of *P. syringae* bacteria, but a pattern of hydrophobic-hydrophilic regions can induce optimum water alignment and thus ice nucleation.

Near hydrophilic regions, the water molecules are strongly aligned and therefore their mutual dipole-dipole interaction is increased. The strong hydrogen bonding network promotes energetic coupling between the dipoles which results in effectively removing heat away from that region ultimately promoting ice nucleation. The long-range coupling of water dipoles has a direct impact on the removal of latent heat which is the second requirement of ice nucleation. Using time-resolved IR pump/SFG probe spectroscopy, the group has explored the possibility of ice nucleation by estimating vibrational energy transfer dynamics of interfacial O-D groups (fig. 6b-c). At 5^oC, the energy transfer from weakly hydrogen-bonded to strongly hydrogen-bonded water molecules were more efficient in the case of *P. syringae* sample compared to the ice-inactive lysozyme water interface which was used in control experiments. Therefore, the study reveals that the mechanism involved in the ice nucleation of *P. syringae* bacteria is optimized for temperatures close to the freezing point of ice.

7. Conclusion

This review has highlighted the potential of SFG spectroscopy to elucidate the orientation and conformation of biomolecules such as protein/peptides and lipid membranes at the interface. Depending upon the different surface environment like the presence of charge and pH at the interface, there is an average preferential orientation of water molecules which govern the organization of biomolecules. The ideas, concepts, and knowledge learned from these bio interfaces at such molecular level can be useful for understanding of complex biomolecular system.

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About the authors

Mr. Vineet Gunwant *joined the Department of Chemistry, IIT Roorkee as a PhD. scholar under the guidance of Prof. Ravindra Pandey in the year 2020. He did his Bachelor's and Master's degrees in Physical Chemistry from Kumaun University Nainital.Currently his PhD. work is focused on probing structure and dynamics of complex protein at air/water interface with the help of vibrational sum frequency generation spectroscopy.*

Ms. Preeti Gahtori *is currently working as a Ph.D. scholar in the Department of Chemistry, IIT Roorkee under the supervision of Prof. Ravindra Pandey. She did her Bachelor's and Master's degrees in Chemistry from Delhi University. Her work is primarily related to the investigation of the interaction between the nanoparticles with the cell membranes with the help of vibrational sum frequency generation spectroscopy.*

Prof. Ravindra Pandey *is currently working as an Assistant Professor in the Department of Chemistry at IIT Roorkee. He received his PhD in Physical Chemistry from Indian Institute of Science, Bangalore in 2012 under the guidance of Prof. Puspendu K. Das. His PhD work was focused on probing the equilibrium geometry of weakly interacting systems in solution by hyper-Rayleigh scattering. From July 2012 to September 2014, he has worked with Prof. Mischa Bonn at the Max Planck Institute for Polymer Research, Mainz, Germany as a postdoctoral fellow. He probed the role of ice nucleating proteins in controlling the order and dynamics of interfacial water molecules. From October 2014 to April 2017, he has worked with Prof. Sean T. Roberts at the University of Texas at Austin, USA, where he demonstrated that electronic*

	sum frequency generation can be used to noninvasively probe the interfacial electronic structure of organic semiconductor films. Ravindra has joined IACS Kolkata in April 2017 and then moved to IIT Roorkee in April 2018. The primary goal of his research is to use the tools of ultrafast nonlinear spectroscopy to provide a comprehensive picture of the interfacial structure and dynamics at the complex interfaces. He has been awarded the Ramanujan Fellowship in 2017 by the Department of Science and Technology and ASEM-DUO award in 2019.