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Sum-frequency Generation Spectroscopy in Biosensors Technology

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1. Introduction

As there is need for detection of chemical and biochemical substances in many important areas (e.g. medicine, biotechnology, security, drug and food monitoring), the development of devices allowing such detection grows up rapidly. *Such devices are known under the generic name of "biosensor", revealing a combination of two components: a biological element and a sensor device. Practically, in a biosensor, a bioelement recognises an analyte and the sensor transduces the variations in the biomolecule into a measurable signal.* The selected bioelement is specific to the analyte, whose presence and concentration is expected to be determined.

Since the first preliminary devices, research groups from various areas worked together to develop more sophisticated, reliable and mature biosensors. *However, despite intensive attempts, these have not been so successful, partially due to a lack of technology for assembling biomolecules in an oriented, ordered, or site directed manner on solid surfaces. Indeed, many problems have to be fixed.* First of all, the active biomolecule (protein, antibody or nucleic acid) must be immobilised onto the selected surface at the appropriate surface density while maintaining its activity. The way they are immobilised (physical adsorption (Dreesen et al., 2004a; Ekblad & Liedberg, 2010; Kidoaki & Matsuda, 2002; Nakanishi et al., 2001) versus covalent binding (Brady & Jordaan, 2009; Frasconi et al., 2010; Gandhiraman et al., 2009; Stutz, 2009)) and the surface substrate properties (wettability, roughness... (Gandhiraman et al., 2010a)) are, amongst others, parameters affecting biomolecule density and activity. Also, once proteins are attached to surfaces, different factors are affecting their biomolecular recognition (specific binding), i.e. environment (Gubala et al., 2010), immobilisation strategy (Gandhiraman et al., 2010b), protein orientation (Xu et al., 2007; Araci et al., 2008)... Further, non-specific binding on biosensor surface is decreasing/affecting its limit-of-detection (Gandhiraman et al., 2009, 2010b). So, protein immobilisation/adsorption and orientation therefore appear to play a crucial role, highly affecting biosensor performances.

In this context, several analytical techniques were used to characterise adsorbed/immobilised proteins, to determine their identity, concentration, conformation and/or spatial density. However, relatively few data are available concerning protein orientation particularly. Indeed, this issue remains relatively marginal, mainly due to a lack of appropriate characterisation techniques availability. Despite its primary interest, protein orientation is generally only discussed *a posteriori* as an hypothesis to explain variable protein activity efficiency.

Since the last ten years, specifically dedicated investigations to protein orientation were performed. In such cases, adapted characterisation techniques were used. For example, the orientation of heme complex in *Cytochrome C* proteins were determined by Raman spectroscopy, therefore deducing the overall protein orientation (Mc Donald et al., 1996; Edminston et al., 1997; Keating et al., 1998; Yu et al., 2007; Sonois et al., 2009; Grosserueschkamp et al., 2009). Unfortunately, such investigations were limited to heme groups containing proteins. Characterisation techniques more easily applicable to a larger range of proteins were developed by Castner's (Xia et al., 2002; Wagner et al., 2003, 2004; Belu et al., 2003; H. Wang et al., 2004; Cheng et al., 2006; Baugh et al., 2010; Liu et al., 2010) and Aoyagi's (Aoyagi et al., 2008a, 2008b, 2009; Okada et al., 2008) groups. They successfully adapted time-of-flight secondary ion mass spectroscopy (ToF-SIMS) in order to discriminate protein orientation by precisely probing ionised fragments peaks associated to particular amino acids (by using principal component analysis). Unfortunately, this technique can only be performed under ultra-high vacuum conditions, which is not adequate for biological species.

Recently, an emerging technique, sum-frequency generation vibrational (SFG) spectroscopy was applied to the analysis of proteins adsorbed at various interfaces. The technique relies on a second-order nonlinear optical process forbidden in centrosymmetric media. Therefore, SFG is intrinsically specific to the interfacial media where the centrosymmetry of the bulk substrate is broken. Practically, in a SFG experiment, two pulsed laser beams (one in the infrared and the other in the visible range) are focused on a surface or an interface. The spatial overlap and temporal synchronisation of both incident laser pulses –the duration of which lays in the picosecond or femtosecond spectral ranges- induces the generation of SFG photons (Humbert et al., 2008; Y.R. Shen, 1989; Vidal & Tadjeddine, 2005; Williams & Beattie, 2002), which frequency is the sum of the frequencies of both initial ones (Howell et al., 2008; Ye et al., 2009). The SFG signal is enhanced while the IR beam excites a vibrational mode of the molecules adsorbed on the sample surface. Therefore, SFG allows identification of molecular species and chemical groups, but also provides information on the interfacial structures, such as molecular orientation. This all-optical technique presents the advantages of being non-invasive, order-sensitive, and of requiring small sample volumes. SFG can be performed in situ and in real-time, for a large variety of substrates (metals, insulators, semiconductors) and of buried interfaces (solid-liquid, liquid-liquid, liquid-gaseous, solid-high pressure) (Buck et al., 2001; Z. Chen et al., 2002; Lambert et al., 2005; H.F. Wang et al., 2005; Z. Chen et al., 2007; Kubota et al., 2007).

A few number of research group have demonstrated the possibility to record SFG spectra from proteins, leading to the differentiation of protein conformations, (sub)structures and functional groups orientation distributions (J. Wang et al., 2002a, 2002b, 2003a, 2003b, 2004a, 2004b, 2005, 2007; Koffas et al., 2003; J. Kim et al., 2004; Dreesen et al., 2004b; Paszti et al., 2004; X. Chen et al., 2005a; Clarke et al., 2005; Mermut et al., 2006; Phillips et al., 2007; York et al., 2007; Weidner et al., 2009, 2010; Baugh et al., 2010; Boughton et al., 2010; Ye et al., 2010; Nguyen et al., 2010). We believe it is important to summarise these protein related SFG studies and make it accessible to researchers in the fields of biosensors, biomaterials and biomedical diagnostic devices. Although we tried to be as exhaustive as possible regarding the literature, authors apologise for any possible omission.

In this review, we propose to describe this technique as well as its application and utility in biological species analysis and biosensor technology. After a brief introduction to the SFG

we illustrate the performance of SFG using selected studies on protein thin films. Thereafter, we present precursor studies using SFG spectroscopy as a detection system in biosensor devices. In each section, we shall detail what kind of information can be extracted from SFG data, based on recent experimental results. Finally, SFG prospects in biosensor, biomedical diagnostic devices or biomaterials will conclude this review.

2. Sum-frequency generation - theoretical background

Detailed theoretical and technical considerations on SFG spectroscopy can be found in numerous reviews and books (Shen, 1984; Tadjeddine & Peremans, 1998; Lambert et al., 2005; Zheng et al., 2008). We shall briefly describe the necessary theoretical background for the understanding of the following sections. We'll then focus on the SFG phenomenon and its application to the investigations of adsorbate properties. Sum-frequency generation vibrational spectroscopy is a second-order nonlinear optical process. Just like a low-intensity external electric field induces a linear polarisation in materials such as

$$\vec{P} = \epsilon_0 \chi \vec{E} \quad (1)$$

(where $\epsilon_0, \chi, \vec{E}$ respectively represent the dielectric permittivity of free space, the electric susceptibility of the material and the external electric field), when this electric field is intense enough, higher-order terms in the polarisation can no longer be neglected and become sufficiently large to be observed. The polarisation can therefore be expressed as :

$$\vec{P} = \epsilon_0 \chi^{(1)} \vec{E} + \epsilon_0 \chi^{(2)} \vec{E} \vec{E} + \epsilon_0 \chi^{(3)} \vec{E} \vec{E} \vec{E} + \dots \quad (2)$$

where $\chi^{(n)}$ represents the n^{th} -order susceptibility, which is a tensor with 3^{n+1} components. Let's consider two incident electromagnetic waves polarised in the Z direction and propagating in the X direction such as:

$$E_1 = E_{1z} \cos \omega_1 t \quad \text{and} \quad E_2 = E_{2z} \cos \omega_2 t \quad (3)$$

(the spatial phase kx in the cosinus is ignored for simplicity). We shall try to evaluate the components of the polarisation, based on the above-mentioned equation (2) limited to the 1st and 2nd-order susceptibilities. For example, the component along the X axis of the polarisation can be rewritten as

$$\begin{aligned} P_x &= \epsilon_0 \chi_{xz} (E_{1z} \cos \omega_1 t + E_{2z} \cos \omega_2 t) + \epsilon_0 \chi_{xzz}^{(2)} (E_{1z} \cos \omega_1 t + E_{2z} \cos \omega_2 t)^2 \\ &= P_x^L + P_x^{NL} \end{aligned} \quad (4)$$

with P_x^L and P_x^{NL} are respectively the linear and nonlinear terms of the X component of the polarisation. The nonlinear terms proportional to $\chi^{(2)}$ in equation (4) can be expressed as:

$$P_x^{NL} = \epsilon_0 \chi_{xzz}^{(2)} \frac{1}{2} (E_{1z}^2 + E_{2z}^2) \quad (5)(a)$$

$$+ \epsilon_0 \chi_{xzz}^{(2)} \frac{1}{2} (E_{1z}^2 \cos 2\omega_1 t + E_{2z}^2 \cos 2\omega_2 t) \quad (b)$$

$$+ \epsilon_0 \chi_{xzz}^{(2)} E_{1z} E_{2z} \cos(\omega_1 + \omega_2) t \quad (c)$$

$$+ \epsilon_0 \chi_{xzz}^{(2)} E_{1z} E_{2z} \cos(\omega_1 - \omega_2) t \quad (d)$$

The nonlinear term is now composed of:

- Two components $E_{1z}^2/2 + E_{2z}^2/2$ at nul frequency, i.e. the term (a) in equ. (5). This process is called *optical rectification* and is responsible of the apparition of a static bias voltage at the edges of the material.
- One component at the frequency $2\omega_1$ and one component at the frequency $2\omega_2$, i.e. the term (b) in equation (5). This phenomenon is called *second-harmonic generation (SHG)* and is doubling the frequency of the incident beams.
- One component at a frequency $(\omega_1 + \omega_2)$, i.e. the term (c) in equation (5). This is the *sum-frequency generation (SFG)* phenomenon.
- One component at a frequency $(\omega_1 - \omega_2)$, i.e. the term (d) in equation (5). This phenomenon is called *difference-frequency generation (DFG)*.

This equation explains the origin of the SFG signal. Indeed, as the nonlinear polarisation oscillates at the SFG frequency in the probed material, it will emit an electromagnetic radiation at that frequency. So, practically, in a typical SFG spectroscopy experiment, two input pulsed laser beams (at frequencies ω_1 and ω_2) are focused and overlapped spatially and synchronised temporally on a sample surface (J. Wang et al., 2002a). The laser beams are intense enough to reveal 2nd-order components of the sample susceptibility. These will mix the frequencies of both input beams and give rise to nonlinear effects like optical rectification, SHG, SFG and DFG. We only focus here on the generated SFG light. Generally, ω_1 and ω_2 are in the visible and infrared range, respectively. Interestingly, the SFG signal is enhanced while the IR beam excites a resonance mode of molecules on the sample surface (the condition is that the vibration must be both infrared and Raman active). Therefore, if ω_2 is scanned over the SFG-active vibrational resonances of adsorbates on the sample surface, the SFG signal is resonantly enhanced, thus producing a vibrational spectrum characteristic of the adsorbates (J. Wang et al., 2002a). It therefore allows identification of molecular species and chemical groups, but also provides information about interfacial structures, such as order and orientation distribution of functional groups.

2.1 SFG signal properties

Several important properties of the SFG spectroscopic signal makes it very advantageous for surface analysis:

1. It can be demonstrated that this technique is intrinsically surface-sensitive. This is a consequence of the fact that 2nd-order nonlinear processes, including SFG spectroscopy, are forbidden in media presenting an inversion symmetry. As most usual materials present any inversion symmetry, they do not generate an SFG nonlinear signal from their volume. So only their surface or the interface between two media, which can be viewed as a symmetry break, is an important source of surface nonlinear polarisation, and therefore of SFG signal. Moreover, due to its high interface sensitivity, a reference spectrum is not required, unlike for surface IR spectroscopy for example.
2. As a photon based technique, it is not limited to vacuum but can also be performed through gases and transparent liquids and solids.
3. As a vibrational spectroscopy, it yields very specific physico-chemical information on the interface structure and composition via the molecular vibrations of the adsorbates.
4. From a general point of view, the SFG signal is proportional to the absolute square of the nonlinear susceptibility, $|\chi|^2$. Moreover, if the IR beam frequency is coinciding with

a vibrational resonance of adsorbates, the vibrationally enhanced SFG susceptibility can be estimated to be roughly proportional to the product of the IR and Raman transition moments. Therefore, the SFG signal is resonantly enhanced only if the vibrational mode is both Raman and IR active (Shen et al., 1994; Yenageh et al., 1995; Tadjeddine et al., 1995; Himmelhaus et al., 2000; Mani et al., 2004a).

5. The SFG response is sensitive to orientation and ordering of surface species (Himmelhaus et al., 2000; Rao et al., 2004). More precisely, the SFG spectrum of an isotropic layer adsorbed on a surface can be modelled, assuming a lorentzian response of molecular vibrations as

$$I_{ppp/sp}^{SFG} \propto |\chi_{ppp/sp}^{(2)NR,eff} + \chi_{ppp/sp}^{(2)R,eff}|^2 \quad (6)$$

where $\chi_{ppp/sp}^{(2)NR,eff}$ and $\chi_{ppp/sp}^{(2)R,eff}$ represent the effective non-resonant (NR) susceptibility of the interface and the effective resonant (R) contribution from the adsorbed molecular vibrations. The effective macroscopic resonant susceptibility $\chi_{ppp/sp}^{(2)R,eff}$ can be expressed as:

$$\chi_{ppp/sp}^{(2)R,eff} \propto \frac{\omega_{SFG}}{c} \frac{N_s}{\cos \theta_{SFG}} \sum_{i,j,k} F_{p/s}^{SFG,i} F_{p/s}^{Vis,j} F_{p/p}^{IR,k} \times \left\{ \sum_v \frac{1}{\omega_v} \frac{1}{(\omega_v - \omega_{IR} - i\Gamma_v)} \left\langle \sum_{l,m,n} T_{ijk}^{lmn} \frac{\partial \alpha_{lm}(\omega_{vis})}{\partial Q_v} \frac{\partial \mu_n}{\partial Q_v} \right\rangle \right\} \quad (7)$$

where $\partial \alpha_{lm}(\omega_{vis})/\partial Q_v$ and $\partial \mu_n/\partial Q_v$ are the Raman susceptibility and the infrared dipole associated to each vibration. T_{ijk}^{lmn} rotates the molecule in the surface coordinate system and the bracket $\sum_{lmn} \langle T_{ijk}^{lmn} \rangle$ represents a sum-average over the molecule orientation distribution. N_s is the density of molecules on the surface. Γ_v is the half width at half-maximum of the Lorentzian band shape. F are called the Fresnel factors and connect the electric field components of the three coherent beams to the fields inside the interfacial layer. Assuming an azimuthally isotropic ad-layer, only four components of the susceptibility tensor are independent. In principle, these components can be selectively probed with four sets of polarisations (*ppp*, *ssp*, *sps* and *pss*). However, on metallic surfaces, the *pss* and *sps* polarisation sets yield negligible SFG signals due to efficient screening of the IR beam component parallel to the surface. The *ssp* polarisation combination on metallic surface gives an SFG signal typically more than one order of magnitude weaker than that of the *ppp* polarisation combination. However comparing SFG spectra with these two different polarisation combination enables to refine the determination of the ad-molecule orientation as discussed in the following paragraph. By comparison, the IR absorption spectrum is obtained using the following equation:

$$Abs(\omega_{IR}) \propto N_s \sum_v \text{Im} \frac{1}{(\omega_v - \omega_{IR} - i\Gamma_v)} \sum_k |F_{IR,k}|^2 \left\langle \left(\sum_n T_k^n \frac{\partial \mu_n}{\partial Q_v} \right)^2 \right\rangle I^{IR} \quad (8)$$

So, interpreting SFG data requires the Raman tensor $\partial \alpha_{lm}(\omega_{vis})/\partial Q_v$ and IR dipole $\partial \mu_n/\partial Q_v$ to be determined for each vibrational mode. These molecular properties can be inferred from *ab initio* calculations and calculated SFG spectra can be compared to

experimental data, acquired for different polarisation combinations, to determine admolecule orientation (Cecchet et al., 2010a, 2010b).

3. SFG signal of proteins

In the last two decades, SFG vibrational spectroscopy has been applied to structure and orientation analysis of adsorbed molecules (Cecchet et al., 2010; Kudelski et al., 2005; Lin et al., 1995; Mani et al., 2004b; Watanabe et al., 1994). For example, alkanethiol molecules adsorbed on gold, silver or platinum surfaces were extensively studied (Yeganeh et al., 1995; Kudelski et al., 2005; Humbert et al., 2006; Dreesen et al., 2006a; Sartenaer et al., 2007). SFG allowed the determination of molecular adsorption and orientation phenomenon to be better understood. Although the understanding of protein adsorption on the molecular level is crucial for design of future applications in coating technology or biosensor device for example, SFG spectroscopy was applied only recently to the investigation of biological samples such as phospholipid bilayers (Pohle et al., 1999; X. Chen et al., 2007; X. Chen et al., 2010; Kett et al., 2010), model peptides (York et al., 2007; Phillips et al., 2007) or amino acids (Mermut et al., 2005). Several proteins like bovine serum albumin (BSA) (J. Wang et al., 2003b), immunoglobulin G (IgG) (X. Chen et al., 2005b), collagen (Rocha et al., 2007) or fibrinogen (Jung et al., 2003) were investigated by SFG at various surfaces/interfaces.

Initially, such studies mainly focused on -CH and -NH vibrational features (J. Wang et al., 2002a, 2002b, 2003a, 2004a; Paszti et al., 2004; Dreesen et al., 2004b; Clarke et al., 2006), providing, amongst others, structural informations about hydrophobic side chains. Nowadays, a few groups have demonstrated the possibility to detect SFG signals from protein amide I groups, leading to the differentiation of protein secondary structures and protein orientation. These three growing steps (C-H, N-H and amide I bands analysis) in the understanding and interpretation of protein SFG signal are detailed in the next three subsections.

3.1 CH bands

The C-H stretching vibration region (between 2800-3000 cm^{-1} for aliphatic and 3000-3100 cm^{-1} for aromatic C-H) is the most regarded region while studying adsorbed molecules or biological species. Proteins are composed of a succession of amino acids, where the most important amount of C-H groups are located in their side chains. The average orientation of such side chains, and even some kinetic changes can therefore be deduced.

Most SFG investigations on proteins are performed in their native environment, i.e. liquid (mainly water or PBS solutions). For example, the first analysis of protein layers by SFG spectroscopy was published in the 2000's by J. Wang et al. (J. Wang et al., 2002a, 2002b). Bovine serum albumin (BSA) layers (J. Wang et al., 2002a, 2002b) adsorbed on three different substrates (fused silica, deuterated-polystyrene (d-PS) and deuterated-polymethylmetacrylate (d-PMMA)). As depicted in Figure 1 a and c, no SFG vibration in the CH stretching region is observed at the fused silica/BSA solution or at fused silica/water interfaces, which was interpreted as a lack of protein adsorption or no net alignment of functional groups of adsorbed BSA proteins. After washing and drying, SFG spectra highlighted a strong C-H vibration when collected in air (Fig. 1 b and d), revealing for the first time adsorption of BSA molecules using SFG spectroscopy (J. Wang et al., 2002b). From these observations, orientation information was inferred. Indeed, in solution, adsorbed BSA molecules are in contact with two hydrophilic environments, silica and water (or BSA

solution). Therefore, BSA molecules adopt an hydrophilic conformation, i.e., the hydrophobic groups (mainly C-H) tend to stay inside the film with no net alignment: thus they induce no SFG signal. Oppositely, when exposed to air, hydrophobic parts of the molecules are assumed to face towards the air, which is also hydrophobic (J. Wang et al., 2002b). Such experiments were also performed on various substrates (PS, PMMA...) and various environments (hydrophobic solvents like benzene, carbon tetrachloride or FC-15...) (J. Wang et al., 2002b), revealing various protein conformations (including conformation and/or orientation changes) according to environmental conditions (substrate and surrounding medium). Moreover, the effect of pH solution for example on protein side chain conformation was also revealed (J. Wang et al., 2002a; G. Kim et al., 2002). This highlights the primary importance of hydrophobic effects towards protein adsorption behaviours. Since acidity also influences the orientation distribution of functional groups in proteins, this parameter is also important for properties like biocompatibility or molecular recognition efficiency.

On another hand, air/solid interfaces were also investigated and provided some interesting results. For example, ambient contaminations while working in air are of primary concern. In this context, SFG is totally adapted for studying effects of contaminations in air on protein adsorption. Such experiments were performed on titanium surfaces, where SFG was also able to determine the role of natural hydrocarbon contamination on protein adsorption behaviour. Actually, it highlighted its relatively poor influence in the protein adsorption phenomenon on Ti-oxide surfaces, only based on SFG C-H vibrations (Paszti et al., 2004).

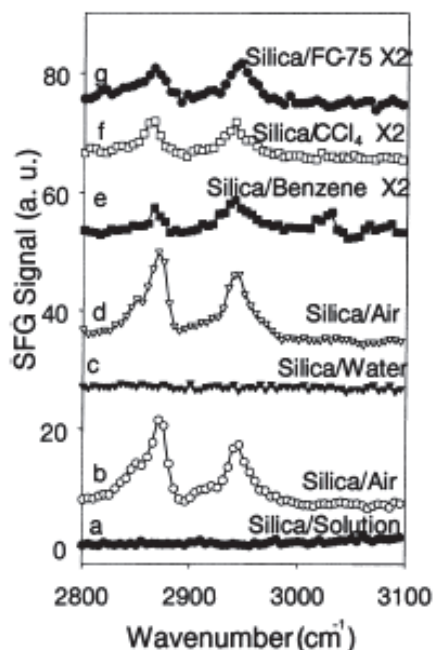


Fig. 1. SFG Spectra collected from (a) silica/BSA solution interface; (b) silica/air interface after silica was removed from solution and washed by water; (c) silica/water interface after the sample contacted water again; (d) silica/air interface after the sample was again removed from water; (e,f,g) silica/benzene or silica/ CCl_4 or silica/FC-75 interface after procedures (a) and (b) and contacting the sample with benzene, CCl_4 , or FC-75. Spectral intensities in spectra e,f,g are multiplied by 2. (a). Reprinted with permission from Ref (J. Wang et al., 2002b). Copyright 2002 American Chemical Society.

These examples highlight the importance for the careful analysis of SFG C-H vibration modes of adsorbed proteins. The experiments should be performed with caution and precision. The model used in the interpretation of data is important and needs to be selected considering each experimental parameter. Using this, C-H vibrational range in SFG spectra can provide very useful information on functional groups conformation of adsorbed proteins, protein and/or substrate surface changes.

3.2 NH bands

Another spectral region of interest in protein SFG spectra is the N-H vibrational range. Indeed, until 2009 and the investigations of Weidner et al. (Weidner et al., 2009, 2010), the attribution of this mode was unclear. The dominant peak near 3300 cm^{-1} observed in SFG spectra of adsorbed proteins or peptides was either attributed to amide A mode (related to protein backbone structure) or to amine resonances (related to side chains) (Jung et al., 2003; Clarke et al., 2005; Mermut et al., 2006; Phillips et al., 2007; York et al., 2007). In order to solve this controversy, Weidner et al. used isotope-labelled Lk α 14 peptides adsorbed on SiO_2 (negatively charged) and CaF_2 (positively charged) surfaces (Weidner et al., 2009). These peptides are composed of leucine (L) side chains (hydrophobic) and lysine (K) side chains (hydrophilic) assuming an α -helix structure. The amine groups of the lysine side chains were labelled with ^{15}N isotopes, which is supposed to result in an estimated 8 cm^{-1} red-shift of the NH_3 resonance frequency. Authors observed a red-shift of the $\sim 3300\text{ cm}^{-1}$ vibrational mode of about 9 cm^{-1} (on SiO_2) and of $\sim 13\text{ cm}^{-1}$ (on CaF_2), while comparing unlabelled and labelled peptides SFG spectra. This clearly demonstrates that this peak can be unambiguously associated to the NH_3 amine stretching mode. However it is still under controversy if its origin is associated to backbone, side chains or both NH groups orientation (G. Kim et al., 2002; Clarke et al., 2005).

3.3 Amide I band

The major drawback of the previous experiments is their difficulty to deduce overall structure, conformation or orientation of adsorbed proteins only from C-H and/or N-H SFG stretching vibrations. Previously, protein structures were successfully studied using infrared spectroscopy and Raman scattering. These investigations revealed that amide vibrational bands are very sensitive to protein secondary structures (Singh, 2000). Unfortunately, in situ detection of such bands is made difficult due to limited sensitivity of such techniques and possible interferences from the environment (i.e. water signals). In 2003, J. Wang et al. (J. Wang et al., 2003b) first demonstrated the possibility of using SFG to observe amide I signals from proteins or peptides adsorbed at solid/liquid interfaces. The advantages of using SFG rather than other spectroscopic techniques are its ability to provide the amide I signals without interference from the environmental signals (i.e. from water for example). Spectroscopic data on the amide also provide supplementary structural information since the related second-order nonlinear optical susceptibility tensor contains more elements than the linear susceptibility tensor. However, in order to improve the recording of the weak amide I SFG signal, Wang et al. (J. Wang et al., 2003b) adopted a “near total reflection” experimental configuration for their SFG set up using a CaF_2 prism, leading to an enhancement of the SFG intensity. SFG signature of amide I was obtained in situ from various proteins (Fig. 2 Left). All proteins presented very specific amide I SFG signature, which can be due to variations in protein surface coverage, orientation and secondary structure. The attribution of each SFG feature needed more investigations as detailed below.

3.3.1 β -turns structures

Further, at this stage, a correlation between SFG amide I spectral characteristics and protein interfacial structures, with particular attention to the β -sheets conformation, was still needed. This was performed by X. Chen et al. early after while demonstrating the feasibility of using SFG to identify protein/peptide secondary structures (β -sheets) (X. Chen et al., 2005a). Particularly, tachyplesin I, a peptide having an antiparallel β -sheet structure, was investigated. SFG spectra of tachyplesin I adsorbed on polystyrene surface are shown in Fig. 2 Right (a). Several characteristic peaks can be observed at 1664 cm^{-1} and 1688 cm^{-1} . After addition of dithiothreitol (DTT) in the solution, the SFG amide I region appears as presented in Fig. 2 Right (b). DTT is often used to elucidate disulfide bonds function in proteins, as it is known to reduce it. So, contacting proteins/peptides with DTT will induce a reduction of disulfide bonds and destroy the β -sheet structures. The disappearance of the 1688 cm^{-1} peak after contact with DTT demonstrated its relationship with β -sheet structures. This analysis first validated SFG as a powerful technique for determining the detailed β -sheet structures of proteins.

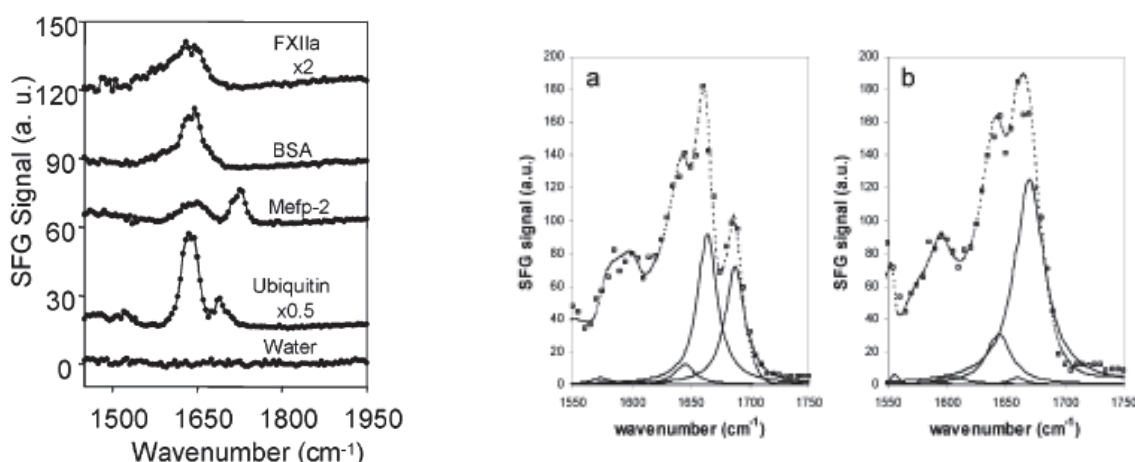


Fig. 2. (Left) SFG spectra collected from interfaces between polystyrene and various protein solutions. Reprinted with permission from Ref (J. Wang et al., 2003b). Copyright 2003 American Chemical Society. (Right) SFG spectra (squares) and fitting results (dotted lines) for tachyplesin I adsorbed at solution/polystyrene interface. The solid lines represent the component peaks used to fit the spectra. (a) SFG spectra obtained before contacting with dithiothreitol and (b) after contacting with dithiothreitol. Reprinted with permission from Ref (X. Chen et al., 2005a). Copyright 2005 American Chemical Society.

3.3.2 α -helix structures

Similarly to the β -sheets, X. Chen et al. (X. Chen et al., 2005a) investigated the SFG fingerprints of α -helical structures in adsorbed proteins. They focused on a peptide, MSI594, consisting of 24 amino acids residues and adopting a α -helical structure. In this case, the main peak in the SFG signal of the amide I region is centred at 1650 cm^{-1} , and was attributed to α -helical structure, as confirmed by ATR-FTIR experiments. Those examples clearly demonstrated that SFG amide I signals can differentiate various protein secondary structures, such as α -helices and β -sheets. Based on such considerations, α -helix and β -turn secondary structures of several proteins and peptides have been analysed by SFG spectroscopy (Weidner et al., 2010; Baugh et al., 2010; Boughton et al., 2010).

4. Protein orientation

Considering the above-mentioned investigations, we can now expect to infer the protein orientation from SFG data, as demonstrated by Clarke et al. on blood proteins like fibrinogen (Clarke et al., 2005; J. Wang et al., 2006). Such results are of primary interest in biomaterials science for example as it can help in-depth understanding of biocompatibility properties of the studied materials. Fibrinogen is a protein composed of a central hydrophobic domain (E domain, Fig. 3 Left) connected to two hydrophobic domains (D domains, Fig. 3 Left) by coiled coils of α -helices (Fig. 3 Left) (Clarke et al., 2005; J. Wang et al., 2006). The dominant contribution to SFG amide I signal (Fig. 3 Right) is associated to the presence of α -helices (at 1650 cm^{-1}). Minor contributions from β -sheets and turn structures are also observed and are in agreement with protein structure. The orientation of the protein parts can be deduced from those signals. Indeed, the majority of the α -helices can be found in the coiled coils. The strong SFG signal is assumed to appear when a large orientation of α -helices is occurring, i.e. an ordering of α -helices in coiled coils. This can be explained from the fact that : (1) the net dipole of an α -helix arises from the N- to the C-terminus; (2) the ssp (SFG signal is s-polarised, the visible light is s-polarised and the IR beam is p-polarised) polarisation combination is sensitive to functional groups presenting an orientation perpendicular to sample surface. In such a case, the authors conclude that the SFG signal originates from fibrinogen proteins adsorbed in a bent conformation (Clarke et al., 2005; J. Wang et al., 2006). Such orientation determination of protein substructures were also performed on tachyplesin I, where SFG combined with ATR-FTIR data allowed the quantitative determination of the tilt angle (θ) and the twist angle (ψ) of the β -sheet structure at various interfaces.

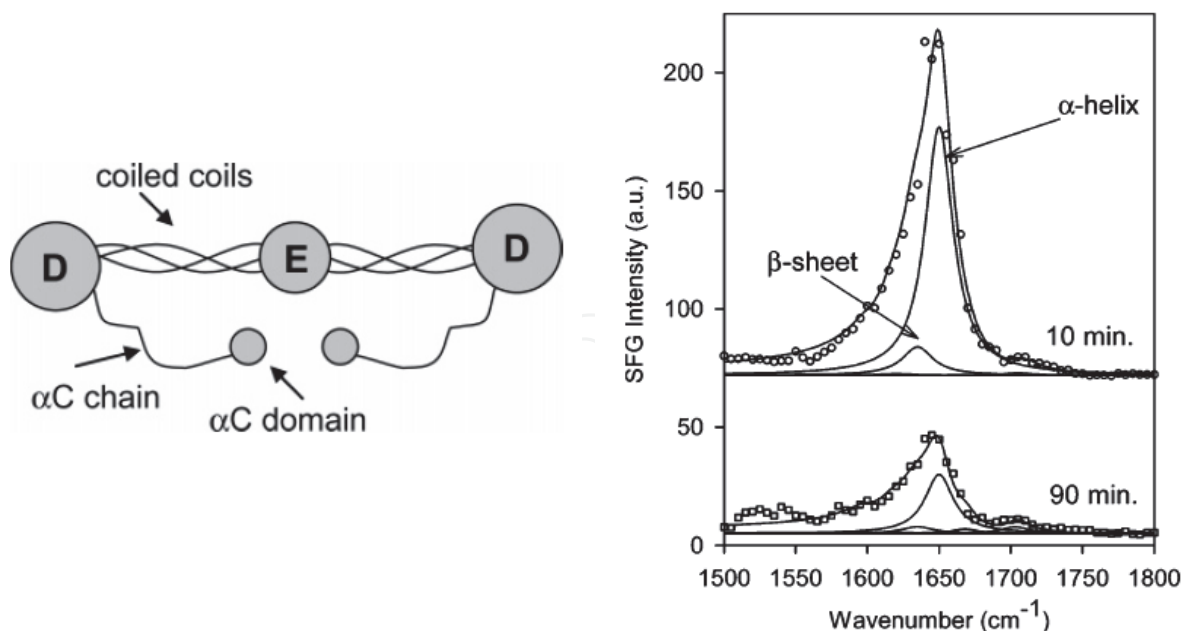


Fig. 3. (Left) Fibrinogen structure (with labelled domains). (Right) Amide I spectral range in SFG spectra of fibrinogen adsorbed at PBS/poly(ether urethane) interface collected after 10 and 90 min. Points are experimental data while lines are fitting results (or represent components of the fit). Reprinted with permission from Ref (Clarke et al., 2005). Copyright 2005 American Chemical Society.

Moreover, conformational changes with time were also observed. These changes encountered after adsorption on surfaces are of primary interest in biomedical materials and biomaterials science. For example, protein adsorption is the first phenomenon occurring when implants are integrated into living tissues. Moreover, these changes can also affect their recognition efficiency with regards to other biomolecules. Similar investigations were also performed on diverse proteins like bovine serum albumin, ubiquitin, factor XII and some other deuterated proteins (J. Wang et al., 2005).

4.1 Polarisation mapping

Since proteins are large molecules, their vibrational spectra can be composed of many vibrational modes, resulting in complex spectra, which may be difficult to interpret. In order to circumvent this difficulty, a new method, referred as *polarisation mapping method*, was developed by J. Wang et al. in 2005 (J. Wang et al., 2005). In this method, the input and output signals polarisations were modified compared to usual polarisations used (ssp or ppp). Indeed, it was shown that fitting parameters only derived from ssp and ppp fitted spectra may not always be reliable (J. Wang et al., 2004b). So, in the polarisation mapping method, SFG spectra are in this case collected using a p-polarised IR beam and a 45° polarised visible beam (i.e. between s- and p-polarisation). A continuous change of the SFG output signal polarisation is performed. Such configuration, combined with model calculations (J. Wang et al., 2005), allowed an improvement of reliability and resolution of SFG measurements, from which more structural information (orientation and orientation distribution of functional groups) can be deduced. This is particularly true for complex molecules like proteins or polymers.

Orientation of proteins/peptides on surface is relevant for applications where biological recognition events are involved. Indeed, protein/peptide orientations are crucial since recognition sites can be blocked due to inappropriate protein orientation on surface. This can drastically decrease the signal-to-noise ratio (and consequently degrade the limit-of-detection) of the derived biosensor or biomedical diagnostic device, for example. A first step in this direction was put forward by Ye et al. only recently (Ye et al., 2010). Using amide I SFG signal, they observed peptide orientation variations according to protein deposition method. Indeed, differences were noticed between physically adsorbed and chemically immobilised peptides regarding its orientation.

5. SFG as a biosensor technique

The analysis and information obtained using SFG on proteins/peptides using C-H, N-H and amide I vibrational modes have been described and its importance for biosensor revealed. Further, as only molecular groups in proteins/peptides at interfaces that have a net organisation will contribute to the measured SFG signal, it is expected to be an excellent probe to identify the side chains involved in biomolecular recognition events. Indeed, surface interactions can introduce significant ordering in the binding regions of proteins/peptides. However, in order to be applied to biosensors and/or biomedical diagnostic devices, biomolecular recognition should also be detected by SFG spectroscopy.

This was first highlighted by Dreesen et al. in 2004 (Dreesen et al., 2004a, 2004b). Indeed, the authors were interested in the vibrational information of an adsorbed derivated vitamin (biocytin) on diverse substrates (Au, Pt, Ag and CaF₂) and its subsequent recognition by a protein (avidin). The SFG signal (obtained in the total internal reflection configuration) in

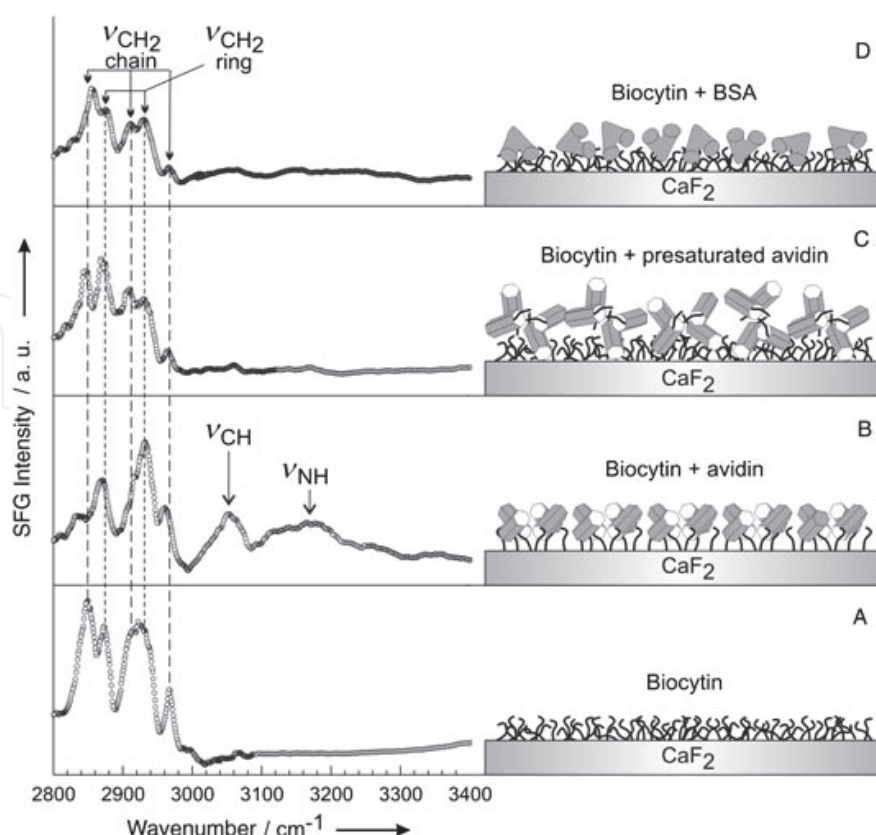


Fig. 4. SFG spectra of biocytin layers adsorbed on CaF_2 substrates (a) before and (b) after contacting with avidin solution, (c) presaturated avidin solution and (d) BSA solutions. Reprinted from Ref (Dreesen et al., 2004b), Copyright 2004, with permission from Elsevier.

the C-H vibrational range of the biocytin layer revealed, despite some structural gauche defects, that the adsorbed layer presents an organisation relatively similar to usual self-assembled monolayers of alkanethiols on gold surfaces (Dreesen et al., 2006a; Sartenaer et al., 2007). The biocytin layer only exhibits the SFG fingerprints of CH_2 groups included into the molecular backbone (CH_2 chain) and to the ureido ring (CH_2 ring). When comparing the SFG spectra of the biocytin layer alone to the (biocytin + avidin) layer, important changes were observed: (1) only CH_2 ring peaks are visible (CH_2 chain peaks are drastically reduced); (2) two signals in the $3000 - 3300 \text{ cm}^{-1}$ region appear. The disappearance of CH_2 chain peaks was explained by a reorganisation of biocytin conformation after avidin binding.

The most interesting feature demonstrating the biomolecular recognition between biocytin and avidin are the appearance of two peaks in the $3000 - 3300 \text{ cm}^{-1}$ region. Although an unambiguous attribution of those peaks is difficult, the authors' results demonstrated that the SFG signal observed is due to the interaction between both biological species. Indeed, complementary measurements were performed in order to address the specificity of the molecular recognition highlighted by the SFG signals observed: (1) immersing a biocytin layer in avidin solution presaturated with biotin (preventing recognition events between avidin in solution and the biocytin layer on surface); (2) immersing a biocytin layer in a BSA solution (for which no molecular recognition can occur with biocytin). The resulting SFG spectra recorded are similar to biocytin layers without spectral features associated to recognition events (i.e. in the $3000 - 3300 \text{ cm}^{-1}$ range) (Dreesen et al. 2004b).

Further, a new experimental setup, developed by Tourillon et al. (Tourillon et al., 2007, 2009), allowed to significantly enhance the SFG signal recorded, compared to usual external reflection configuration. Their concept was first demonstrated on self-assembled monolayers (SAMs) of alkanethiol (Tourillon et al., 2007). Indeed, authors first compared the SFG intensity on dodecanethiol SAMs adsorbed on a dense gold nanoparticle array in an external reflection and in a total internal reflection (TIR) configuration. Both exhibited clear SFG spectra but the TIR-SFG configuration presented intensities by one order of magnitude higher than external reflection configuration. This enhanced intensity SFG configuration was further applied to the recognition of biocytin molecules by avidin proteins (Tourillon et al., 2009). Again, they observed an excellent signal-to-noise as well as a high signal-to-background ratio. TIR-SFG spectrum of biocytinylated thiols adsorbed on the nanoparticles array only exhibit mainly CH bonds attached to the tetrahydrothiophene ring, CH₂ and a Fermi resonance-enhanced overtone of the 1550 cm⁻¹ band coming from amide II entities. These observations highlight a well ordered SAMs on gold nanoparticle surfaces. After immersing the sample in an avidin solution, drastic changes in TIR-SFG spectra were observed. The 2882 cm⁻¹, 2942 cm⁻¹ and 2975 cm⁻¹ peaks intensities greatly decreased and were associated to a reorganisation of the biocytinylated thiol layer in order to match the bonding pocket of avidin proteins. Oppositely, the 3079 cm⁻¹ band intensity increased while the 2859 cm⁻¹ peak was mainly unchanged. This indicates the molecular chains of the biocytinylated thiols remain unmodified and that only the apex biotin ring has to change its orientation for the recognition with avidin binding pocket. Finally, as previously tested, supplementary experiments were performed in order to address the specificity of the molecular recognition highlighted by the SFG. These recent results can lead to the emergence of a new label-free detection system for biosensor applications.

6. Conclusion

In this review, the recent experimental and theoretical developments in sum-frequency generation spectroscopy analysis of proteins and peptides adsorbed on surfaces were detailed. Our goal was to demonstrate the applicability and usefulness of such nonlinear optical spectroscopic technique to biological science and biotechnology.

Indeed, during the last 6 years, SFG spectroscopy was shown to be able to record the vibrational signature of biomolecule thin films through signals from protein –CH vibrations, allowing the determination of the “hydrophobic” or “hydrophilic” conformation of adsorbed proteins/peptides. The modification of surface structure and/or protein conformation was revealed as well. The N-H vibration mode (~ 3300 cm⁻¹) was also identified and appropriate peak attribution performed. Moreover, the amide I band of proteins was observed. This spectroscopic range is very interesting as it allows to identify (using adequate modelling) the presence, conformation and orientation distribution of some functional groups, but also of protein secondary structures (i.e. α -helix, β -sheets and turns). It allows to infer the overall protein orientation/conformation as well.

Based on such considerations, it can be reasonably assumed that recognition events between complementary biomolecules could also be detected, introducing SFG spectroscopy into the biosensor world. This exciting perspective was recently developed (Dreesen et al., 2004b; Tourillon et al., 2009) in unambiguously identifying the SFG fingerprint of molecular recognition events between biocytin molecules and avidin proteins.

This constitutes the basis for new developments of SFG spectroscopy in biotechnology. Indeed, in biosensor devices, the relationship between protein orientation and molecular recognition can for example now be determined on a wide range of substrates in a wide range of environments. The effects of the surface properties, environmental conditions, protein immobilisation procedures... could easily be related in situ to protein orientation and protein activity (recognition) only by using SFG spectroscopy. Further in biomedical devices, deeper understanding of the properties of materials biocompatibility can be inferred by analysing protein changes, conformation, orientation and activity once adsorbed on surfaces.

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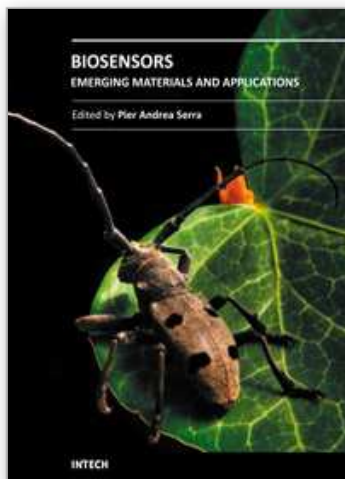
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