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ARTICLE *in* JOURNAL OF PHYSICAL CHEMISTRY LETTERS · AUGUST 2010

Impact Factor: 7.46 · DOI: 10.1021/Jz100742j

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In Vitro Characterization of Surface Properties Through Living Cells

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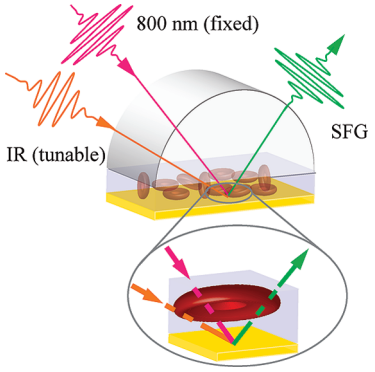
ABSTRACT The ability to probe an interface beneath a layer of living cells in situ without the need for labeling and fixation has the potential to unlock some of the key questions in cell biology and biointerfacial phenomena. Here, we show that vibrational sum frequency generation (SFG) spectroscopy can be used to detect alkanethiol self-assembled monolayers (SAMs) buried underneath a layer of living erythrocytes (ECs). SFG spectra with and without ECs showed the spectral signatures typical of these SAMs, indicating that the signal was being generated solely by the SAM and was not influenced by the presence of cells. Direct comparison of infrared spectroscopy to SFG measurements of cells adhered on a fibronectin layer showed that the SFG signal emanated solely from this layer. These results have important implications for the characterization of surfaces in biomedical, environmental, and industrial applications.

SECTION Surfaces, Interfaces, Catalysis



The determination of surface properties at solid/liquid interfaces such as chemical composition, molecular ordering, dynamics, and conformation is central to the understanding of the development of biofilms and the interaction of cells with a given substrate.[1−3] In particular, the in situ monitoring of the interphase between a substrate and the cellular layer is of great interest as it allows determination of changes in surface properties upon cell adhesion. Typically, this is accomplished by labeling and fixing the cell samples, which may result in their disruption and in the loss of valuable information. In this work, we show that the surface specificity of vibrational sum frequency generation (SFG) spectroscopy permits the in situ characterization of an interfacial layer through living cells, without the need for fixation or labeling. These results will be useful for those seeking to apply this system to investigate layers under living cells with high surface specificity.

SFG spectroscopy has proven well-suited to probing various interfaces.[4,5] In recent years, this technique has been applied to the in situ investigation of biomolecules,[6] including peptides,[7,8] proteins,[9] and DNA.[10,11] Previous work in our lab has demonstrated the ability of SFG spectroscopy to detect a substrate through a layer of fixed cells.[12] In order to test the ability of this technique to probe a substrate through living cells, a well-characterized self-assembled monolayer (SAM) was probed underneath of a layer erythrocytes (ECs). Highly ordered SAMs of deuterated dodecanethiol ($CD_3(CD_2)_{11}$−SH, *d*-DDT) were chosen as they have a distinctive spectral signature in the CD stretching vibrational region (2000−2300 cm^{-1}). These deuterated layers give little to no signal in the CH stretching region (2800−3000 cm^{-1}), allowing us to detect any contribution from the cells or cellular debris. In addition, we investigated undeuterated dodecanethiol ($CH_3(CH_2)_{11}$−SH, DDT) in the CH region to confirm that the typical spectral signatures of an alkanethiol SAM remained unchanged even in the presence of cells. Living ECs were selected as the biological barrier since they contain the majority of molecule types encountered in most biological systems. These cells are known to have no direct interaction with the surface offered to them and will lay flat on it if given enough time to settle (Figure 1).[13] ECs do not deposit an extracellular matrix (ECM) of their own or modify surface chemistry, allowing them to act as biological barriers without altering surface properties.[14] Additionally, these cells contain a large amount of hemoglobin, which makes them optically dense in comparison to most other thin biological layers. If these cells pose no obstacle to the generated SFG signal, many biological barriers, either pro- or eukaryotic, become eligible for use with this technique.

A scheme of the SFG setup and the measuring cell has been previously described.[15] A crudely purified solution of ECs in phosphate-buffered saline (PBS) was placed in between the SAM substrate and an optically transparent prism. All samples were measured using a femtosecond broad-band SFG spectrometer in ppp polarization. A ppp configuration (both input beams as well as the SFG beam are p-polarized) was chosen as it gives a higher SFG response and it probes more $\chi^{(2)}$ tensor elements simultaneously than other polarization configurations. It is thus more likely to probe additional contributions from the

Received Date: June 2, 2010
Accepted Date: July 7, 2010
Published on Web Date: July 12, 2010

DOI: 10.1021/jz100742j | *J. Phys. Chem. Lett.* **2010**, *1*, 2339–2342

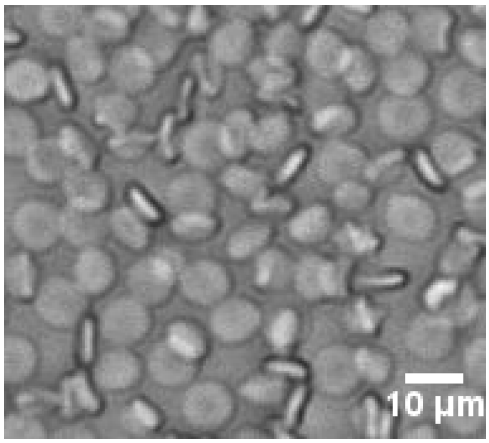


Figure 1. Phase contrast micrograph of erythrocytes after 10 min of settling (20× magnification).

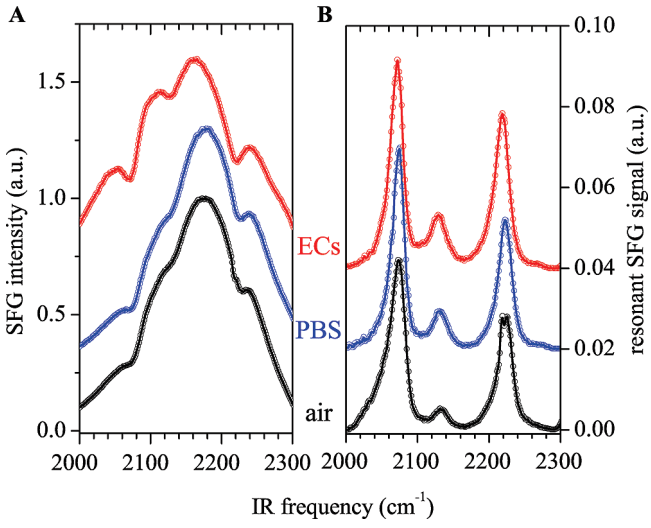


Figure 2. SFG spectra of a *d*-DDT SAM recorded in air (black), sterile PBS (blue), and PBS containing ECs (red). (A) Spectra containing nonresonant contributions in the CD stretching region. (B) Processed spectra showing only resonant contributions in the same spectral region. The CD peaks remain unchanged upon addition of ECs.

sample. Spectra were acquired in 2.5 s, background-subtracted, and normalized to the nonresonant background. First, the presence of the *d*-DDT was confirmed by measurement in the CD stretching region (Figure 2). CD stretches of *d*-DDT SAMs were probed in air, sterile PBS, and PBS with ECs. In Figure 2A, the Gaussian-like envelope reflects the spectral profile of the femtosecond IR pulse, whereas the resonant peaks appear as dips in the spectra with a phase difference of π. These peaks at 2072, 2133, and 2222 cm^{-1} are established to be CD stretching vibrations of the terminal deuterated methyl group (CD_3) in the *d*-DDT SAM.[16] The lack of deuterated methylene (CD_2) vibrations in the spectrum is related to the presence of an inversion center in between these groups within the alkane chain and indicates the absence of kinks or gauche defects. Figure 2B shows the resonant contributions after data processing of the spectra shown in Figure 2A (see Supporting Information for

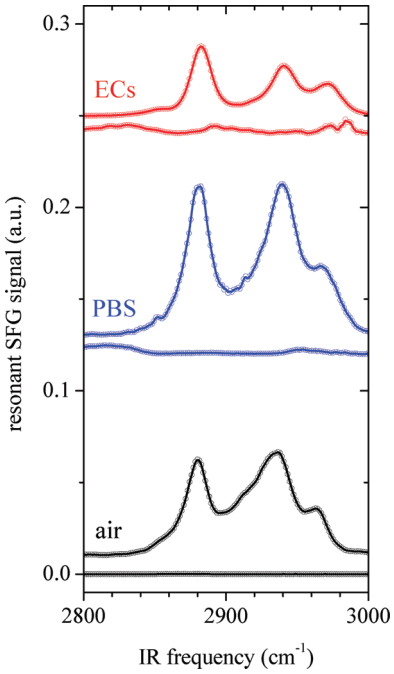


Figure 3. Resonant SFG spectra of *d*-DDT (lower curve) and DDT SAMs (upper curve) recorded in air (black), sterile PBS (blue), and PBS containing ECs (red). The CH peaks of the DDT SAMs show the typical spectral features upon addition of ECs, and only weak CH signatures show up in the presence of PBS and ECs.

details and references). The lack of appearance of new CD_2 peaks due to gauche defects in the film indicates that the film structure is not significantly affected by the presence of ions, cells, or cellular debris. A more thorough analysis of the ratio between the asymmetric and symmetric CD_3 stretching vibrations yielded values of 0.55, 0.63, and 0.77 for air, PBS, and PBS with ECs, respectively. This translates to a calculated tilting angle variation[17] of about 2°, indicating a negligible reorientation of the terminal CD_3 head group.

Measurements in the CD spectral region show that the *d*-DDT SAM can be detected through a cellular layer; however, this region does not have contributions from cellular components. To verify whether we can detect signatures of the alkanethiol SAM even in the CH region, we further compared resonant SFG spectra of deuterated and undeuterated SAMs in air (black), PBS (blue), and PBS with ECs (red) (Figure 3). The lower and upper curves correspond to the measurement of *d*-DDT and DDT SAMs, respectively. The spectrum of the *d*-DDT SAM taken in air is a flat line, as one would expect, whereas the DDT spectrum shows the features typical of an alkanethiol SAM in the CH region. The same holds true for the PBS spectra, with the exception of very weak features in the *d*-DDT SFG spectra, which are attributable to a slight hydrocarbon contamination of the PBS solution. Most interestingly, the spectrum with the ECs on top of the *d*-DDT SAM also does not show any significant spectral contributions other than those which are comparable in intensity with the PBS spectra. The two peaks at higher wavenumbers are related to fitting artifacts of the initial broad-band spectra as the IR frequency was at the edge of the Gaussian-like envelope (see Supporting

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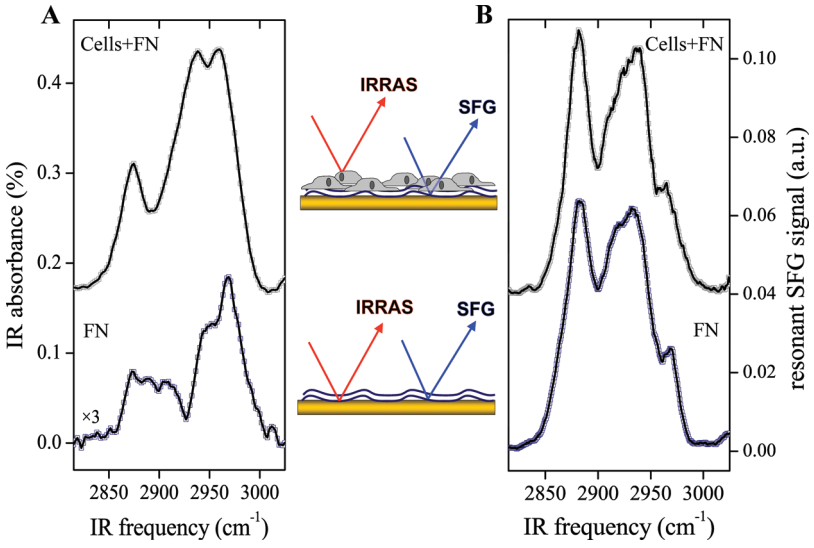

Figure 4. (A) IRRAS spectra of fibronectin (FN) samples with (upper curve) and without (lower curve) adherent cells in the CH stretching region. Spectra differ from one another due to the presence of the cells. (B) SFG spectra of the same samples in the same spectral region. In contrast to the IRRAS spectra, these spectra are similar, indicating that SFG is probing exclusively the layer between the cells and the substrate.

Information). This is also supported by the fact that these signatures are not observed in the DDT spectra, which show only the typical features of the SAM. If the cellular structures were contributing to the spectra, one would expect to see additional peaks appearing in the CH region, which are not observed. The lack of other spectral contributions is surprising considering the fact that the crudely purified EC solution used in these experiments contains not only whole cells but also many small CH-containing debris, including sugars, peptides, and membrane fragments. It could be argued that this debris would give rise to an SFG signal due to the build up of a locally ordered layer at the interface driven by the amphiphilic character of these molecules. This might be the source of the very weak signals detected in the CH region; however, the peak intensities are 1−2 orders of magnitude lower than the signal generated by the SAM. These results show that SFG spectroscopy is able to probe buried interfaces even through a cellular layer.

Beyond their use as a biological barrier, ECs have limited prospects in contributing to the field of biointerfaces. Adherent eukaryotic cells or bacterial biofilms have a much more active influence on the surface to which they adhere. The former were chosen as prominent representatives of these two medically and industrially relevant groups. As eukaryotic cells do not adhere to alkanethiol SAMs,[18] fibronectin (FN) was chosen as it is a well-established system for cell adhesion and because of its abundance in the ECM. FN-coated gold wafers with and without a confluent layer of adherent fibroblast cells were examined using both SFG and infrared reflection−absorption spectroscopy (IRRAS) in the CH stretching region. IRRAS was employed to estimate the influence of the absorption of the IR pulse by the layer of adherent cells. In order to compare the same samples using both SFG and IRRAS, all samples were fixed using ice-cold acetone/methanol. A comparison between IRRAS and SFG spectroscopy results are shown in Figure 4. For the IRRAS measurements, there is a clear difference between the spectra of samples containing only FN and samples with cells adhered on top of the FN, as was expected (Figure 4A). The spectrum of FN alone (lower curve in Figure 4A) shows a number of distinct peaks, while the spectrum of FN with adherent cells (upper curve in Figure 4A) shows fewer peaks at different positions. SFG spectra, however, show nearly no differences between samples of FN and samples of cells adhered on FN (Figure 4B). Both spectra show four peaks with similar relative intensities for both sample types. From these data, it can be concluded that the SFG signal originates solely from the FN layer and that none of the cellular structures above it are contributing significantly to the spectrum. Furthermore, the absorbance measured by IRRAS is less than 0.3% (Figure 4). Since the IR beam in an IRRAS experiment passes through the cellular layer twice (before and after the reflection) and the fact that SFG signals are produced at the interface when the IR light has passed through the cellular layer only once, we assume an upper limit of the absorption of IR light prior to the sample to be less than 0.15%. Furthermore, the IRRAS experiments were performed at a more oblique incident angle of 80°, in comparison to 60° for the SFG measurements. Therefore, the influence of IR absorption prior to the surface in SFG can be regarded as negligible.

In summary, this work demonstrates how SFG spectroscopy can be used to investigate interfaces under a layer of living cells without the need for labeling or fixation. Furthermore, these results demonstrate that this technique is able to penetrate through biological barriers as long as they are optically transparent. The groundwork presented here opens the possibility for use of this technique in diverse systems including the growth and development of biofilms in situ, the corrosive activity of bacteria and fungi on metal surfaces, or the interaction of cells with nanopatterned surfaces.

DOI: 10.1021/jz100742j | J. Phys. Chem. Lett. 2010, 1, 2339–2342

SUPPORTING INFORMATION AVAILABLE Details about erythrocyte isolation, cell culture, sample preparation, and experimental setup. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT The authors thank Michael Grunze for continuous support and fruitful discussions, Sabine Müller from the animal facility for providing the ECs, and Suat Oezbek for useful discussions. M.-O.D. thanks Hartmut Hoffmann-Berling International Graduate School (HBIGS) of Molecular and Cellular Biology, C.H. acknowledges financial support from a U.S. NSF Graduate Research Fellowship, and P.K. acknowledges funding from the Deutsche Forschungsgemeinschaft (KO 3618-1 and 2).

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