Sum-frequency-generation spectroscopy of DNA films in air and aqueous environments

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Understanding the organization and orientation of surface-immobilized single stranded DNA (ssDNA) in aqueous environments is essential for optimizing and further developing the technology based on oligonucleotide binding. Here the authors demonstrate how sum-frequency-generation (SFG) spectroscopy can be used to compare the structure and orientation of model monolayers of ssDNA on gold in air, D_2O, and phosphate buffered saline (PBS) solution. Films of adenine and thymine homo-oligonucleotides showed significant conformational changes in air versus aqueous environments in the CH stretching region. The thymine films showed changes between D_2O and PBS solution, whereas the SFG spectra of adenine films under these conditions were largely similar, suggesting that the thymine films undergo greater conformational changes than the adenine films. Examination of thymine films in the amide I vibrational region revealed that molecules in films of nonthiolated DNA were lying down on the gold surface whereas molecules in films of thiol-linked DNA were arranged in a brushlike structure. Comparison of SFG spectra in the amide I region for thiol-linked DNA films in air and D_2O also revealed substantial conformational changes. © *2008 American Vacuum Society.* [DOI: 10.1116/1.3064107]

I. INTRODUCTION

DNA microarrays are detection tools which take advantage of the specific binding properties of DNA and RNA. They are currently used in applications such as measuring expression levels, assessing genetic variability, and disease profiling.[1–4] DNA microarrays are created by attaching thousands of single strands of DNA of a known sequence to a surface. When single stranded DNA (ssDNA) of an unknown sequence is passed over the microarray, complementary strands will bind and activate a fluorophore which can be visually detected and quantified, allowing for the processing of thousands of genetic tests simultaneously.

Methods currently used to examine model monolayers of DNA *in situ* include ellipsometry and surface plasmon resonance,[5–7] as well as fluorescence and radioactivity based techniques to examine the thickness, mass, optical properties, and hybridization of these films.[8,9] Spectroscopic techniques used for molecular level based characterization include ultrahigh vacuum (UHV) based x-ray photoelectron spectroscopy (XPS)[10] and near edge x-ray absorption fine structure spectroscopy (NEXAFS),[9,11] as well as Fourier transform infrared (FTIR) spectroscopy in air.[12] These methods offer a substantial understanding of the chemical composition, surface density, orientation, and ordering of DNA films under nonaqueous conditions. However, the ability to characterize ssDNA films in aqueous environment is important because it allows for the examination of these films *in situ*—the conditions under which they are actually used. Furthermore, the ability to compare results obtained *in situ* and *ex situ* can provide a way to determine whether the results obtained in air or under UHV conditions are applicable in aqueous environments.

The nonlinear optical technique of sum-frequency-generation (SFG) spectroscopy has emerged in recent years as a suitable method for characterizing biological molecules at interfaces.[13–23] In SFG spectroscopy, a visible and an infrared beam coincide in time and space at an interface to produce a signal at the sum-frequency of the two incident beams. The selection rules of this process dictate that this SFG signal can only be generated in a non-centrosymmetric environment, effectively resulting in the suppression of isotropic bulk signal. This feature makes SFG spectroscopy inherently surface specific[24] with submonolayer resultion, allowing for the tracking of subtle modifications even in ultra thin films. Being an all optical technique, SFG is also noninvasive and able to be applied under aqueous conditions, making this technique useful for the characterization of DNA films *in situ*.

Measurements of immobilized ssDNA films by SFG spectroscopy in air have recently been reported.[25–27] Other SFG experiments involving DNA have also been performed by Wurpel and co-workers, who used λ-phage DNA bound to a cationic lipid monolayer at the air-water interface to study screening effects of counterions by detecting water signals in the OD stretching region at different concentrations.[28] Here we present the first report of the characterization of model monolayers of immobilized ssDNA on gold in air, D_2O, and phosphate buffer solution in both the CH stretch and amide I regions.

II. EXPERIMENTAL SETUP

HPLC-purified custom thymine and adenine homo-oligonucleotides were purchased from Operon Biotechnologies (Cologne, Germany) and VBC Genomics (Vienna,

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Austria). All oligonucleotides were used without further purification or removal of the S-$(CH)_6OH$ protective group in accordance with previously published work.[10] Unmodified $(dT)_5$, 5′-thiol-modified $(dT)_5$-SH, and 5′-thiol-modified $(dA)_5$-SH (henceforth referred to as T_5, T_5-SH, and A_5-SH, respectively) were used for these experiments.

Substrates of 100 nm thick polycrystalline gold films on silicon wafers were cleaned in warm piranha solution (70% H_2SO_4/30% H_2O_2) for 10 min and rinsed thoroughly with HPLC-grade water immediately prior to DNA deposition. Gold wafers were placed in 2 mL of 1 M phosphate buffered saline (PBS) solution containing a 5 μM concentration of T_5, T_5-SH, or A_5-SH and incubated for 24 h at room temperature.

After the incubation, all samples were rinsed with flowing ultrapure water to remove excess buffer salt and dried under flowing nitrogen. SFG measurements in air were performed within 24 h. The same films were then immersed in D_2O (pH=6.8) or 1 M PBS-H_2O solution (pH=7.2) in order to obtain the solution spectra. The T_5, T_5-SH, and A_5-SH systems were selected in order to allow comparison to similar films that have been thoroughly characterized using other methods.[29,11] The quality of all DNA films was verified with FTIR and XPS.

SFG spectra were recorded using a broadband femtosecond SFG spectrometer overlapping a broadband infrared and a narrow band visible beam. All spectra were recorded in ssp polarization in the order of increasing wavelengths (SFG, visible, and infrared) and each spectrum was obtained after 5 min of data accumulation. The dependence of the intensity of the SFG signal (I_{SF}) on the infrared frequency (ω_{IR}) is given by[30]

$$I_{SF} \propto |\chi^{(2)}| I_{IR} I_{VIS} \tag{1}$$

with

$$\chi^{(2)} = \chi^{(2)}_{NR} + \chi^{(2)}_{R} = \chi^{(2)}_{NR} + \sum_k \left| \frac{A_k}{(\omega_{IR} - \omega_k) + i\Gamma_k} \right| e^{i\phi_k}, \tag{2}$$

where $\chi^{(2)}_{NR}$, $\chi^{(2)}_{R}$ are the nonresonant and resonant contributions, respectively, to the surface nonlinear susceptibility, ϕ_k is the phase the resonant contribution, A_k is the amplitude, and Γ_k is the damping constant width of a given vibration mode with frequency ω_k. Because the SF signal is proportional to $\chi^{(2)}$ in Eq. (1), the spectral line shape of the SFG signal is affected by interference between the terms given in Eq. (2). SFG spectra shown in this work were fitted to Eq. (1) and normalized using the nonresonant background contribution obtained from the fit (resembling the spectral profile of the IR beam). The SFG intensity was then plotted as the absolute value of the resonant SFG contributions.

Measurements in D_2O and PBS were performed in a liquid cell in which the sample was probed through a prism (half-cylindrical CaF_2 crystal) located above the sample (Fig. 1). Characterizing systems in biologically relevant environments requires the use of specific sample cells. One possible way to achieve this is to probe the interface directly at the base of a prism via an evanescent field in a manner similar to an ATR experiment. However, in this arrangement, the film of interest must be on the base plate of the prism, which can be tedious and requires that the costly prisms be cleaned after use. In order to overcome these limitations we designed the sample cell shown in Fig. 1. In this arrangement, the distance between the sample and the prism can be varied and the pathway for the IR light through the liquid film in between the sample and the prism can be minimized.

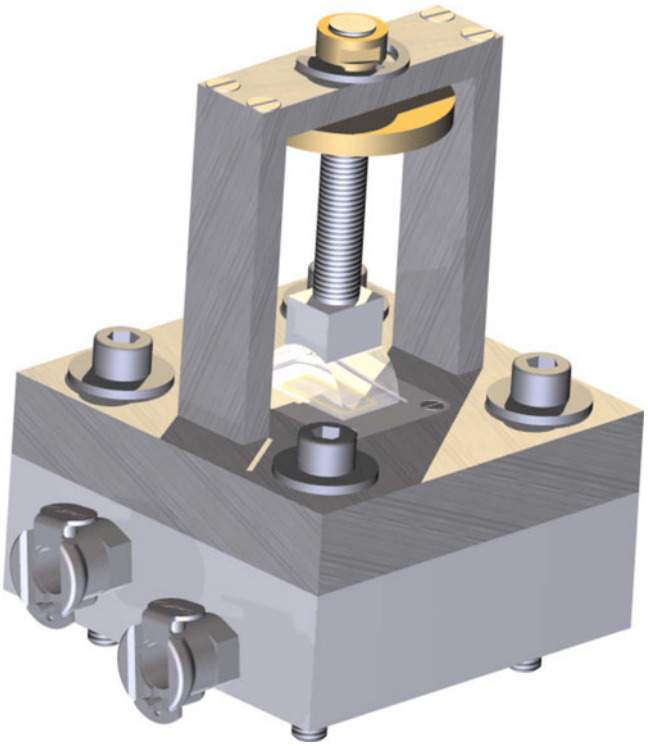

FIG. 1. (Color online) Measurement cell for *in situ* characterization of liquid/solid interfaces. The surface is probed through a half-cylindrical CaF_2 crystal located on top of the sample. The liquid is entrapped between the crystal base plate and the sample surface.

III. SFG SPECTRA OF FILMS IN THE CH STRETCHING REGION

Figure 2 shows the SFG spectra of T_5-SH in air, PBS, and D_2O in the CH stretching region. These spectra reveal contributions from CH vibrations emanating from the thiol linker groups, the sugar backbones, the thymine rings, and the methyl groups in the thymine nucleobases. Given this large number of CH oscillators contributing to the overall signal in various vibrational modes, we refrain here from making specific global peak assignments. However, our spectra of T_5-SH reveals features similar to previously published results examining films of ssDNA in air.[25,26]

The methyl groups in the thymine bases give rise to in-plane and out-of-plane antisymmetric CH_3 stretching vibrations located at 2968 cm^{-1} (r^-_{ip}) and 2954 cm^{-1} (r^-_{op}), respectively.[31] These peaks are distinguishable in all T_5-SH spectra and the ratio between them reveals information about the orientation of the methyl groups: the in-plane vibration has an infrared transition dipole moment along the bond be-

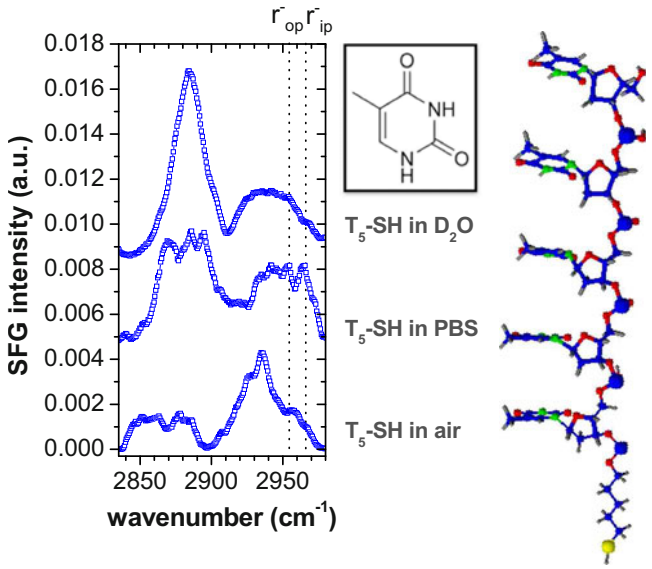

FIG. 2. (Color online) SFG spectra of T_5-SH in the CH stretching region in air (lower curve), PBS (middle curve), and D_2O (upper curve). All spectra were recorded in ssp polarization in the order of increasing wavelengths (SFG, visible, and infrared). The sketch on the right shows a single strand of T_5-SH.

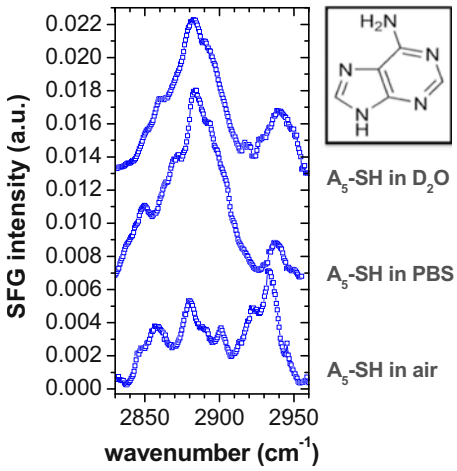

FIG. 3. (Color online) SFG spectra of A_5-SH in the CH stretching region in air (lower curve), PBS (middle curve), and D_2O (upper curve).

tween the methyl group and the thymine ring, whereas the out-of-plane infrared transition dipole moment is perpendicular to it. In the ssp polarization combination used, infrared transition dipole moments perpendicular to the surface have the strongest contribution to the SFG spectra while those parallel to the surface do not contribute. Therefore, the presence of prominent out-of-plane contributions, together with weak in-plane vibrations in the SFG spectra of T_5-SH in air, indicates that the orientations of the methyl groups are more parallel to the surface in this film. This is in agreement with previous NEXAS data on similar films,[11] despite the fact that the NEXAFS measurements were performed under UHV. Upon placing the samples in PBS the orientation of the methyl groups are changed in a way such that the ratio between the in-plane and out-of-plane peak is very similar. This suggests that there is no significant overall orientation of the methyl groups in the film, which may be related to the higher mobility of the DNA strands in the buffer solution and henceforth a higher rotational freedom through the main axis of the thymine base.

It is interesting to note that the ratios of in-plane and out-of-plane antisymmetric methyl vibrations in D_2O and in air are comparable (upper and lower curves in Fig. 2), indicating that in both cases the thymine bases are oriented parallel to the surface. However, the significant change in the overall spectra between these two conditions indicates that the conformation of the sugar backbone itself differs substantially between air and solution. This change is most clearly visible in the strong increase in intensity of the main peak at around 2880 cm^{-1} from air to D_2O. This peak results from an overlap of methylene contributions from the sugar backbone and methyl symmetric vibrations of the thymine base. However, in the polarization combination used, the symmetric methyl stretch would only contribute significantly if the methyl groups possessed an overall orientation perpendicular to the surface in D_2O. Given the fact that the antisymmetric methyl in-plane and out-of-plane contributions revealed a more parallel orientation of the bases in both air and D_2O (as previously mentioned), this cannot be the case. Therefore, we assume that the increase of the peak at 2880 cm^{-1} is primarily due to a rearrangement of the CH groups within the sugar backbone.

When the T_5-SH film is placed in PBS, the peak at 2880 cm^{-1} decreases markedly in comparison to D_2O. This decrease in intensity may indicate that when the film is placed in PBS, the ions present in the solution electrostatically shield the charged groups in the sugar backbone, which would allow the molecules to adopt a more flexible configuration and lead to a more random orientation of the ssDNA strands. Another consideration in the interpretation of these data is the *p*H of the D_2O solution (6.8), which is slightly more acidic than the PBS buffer (7.2). Earlier work has shown that protons can also serve to shield the electrostatic charges in a film of ssDNA.[32] Therefore, we would expect that if the slightly more acidic condition of the straight D_2O had any effect, it would be to shield the electrostatic charges of the DNA strands in a way similar to the effect of the buffer solution. The fact that we see such a drastic change in D_2O versus the PBS solution, despite the increased presence of these protons, suggests that the slight acidity is only playing a minor, if any, role in this phenomenon.

Figure 3 shows an A_5-SH film in air, D_2O, and PBS. Recent work on films of A_5-SH have shown that these molecules do not form densely packed brushes as T_5-SH films do, as the adenine bases are strongly attracted to the gold substrate and tend to bind to it.[9,12] The more subtle changes in this film between air and aqueous environments (PBS or D_2O) may therefore reflect that more of the DNA molecules in this film are lying down with their adenine bases bound to the substrate. Exposure to liquid would still allow some mo-

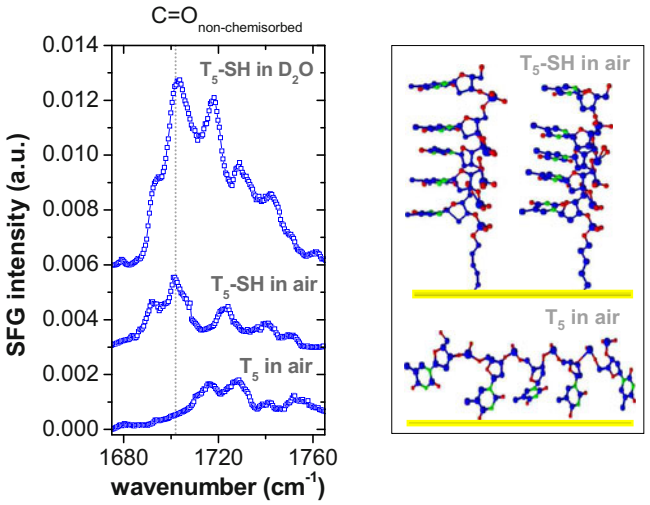

FIG. 4. (Color online) SFG spectra of thymine homo-oligonucleotide films in the Amide I region: T_5 in air (lower curve), T_5-SH in air (middle curve), and T_5-SH in D_2O (upper curve). The sketch on the right illustrates the different conformations observed for T_5 and T_5-SH in air.

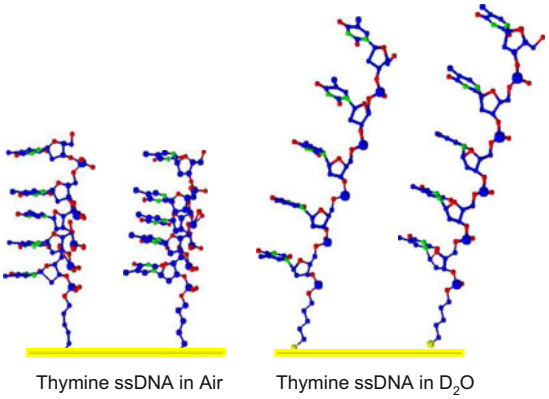

FIG. 5. (Color online) Graphic representation of the proposed arrangement of thymine single stranded DNA molecules in air and in D_2O.

bility in the strands, but the changes between air and D_2O, or even D_2O and PBS, would not be as dramatic as the changes observed in the T_5-SH film.

IV. SFG SPECTRA OF FILMS IN THE AMIDE I REGION

SFG spectral signatures in the amide I region are due to carbonyl and amide stretches, and therefore give complementary information to data obtained in the CH stretching region. The advantage of the amide region is that vibrational signatures here stem only from the bases themselves. This results in SFG spectra with fewer overlapping contributions. Figure 4 shows a T_5-SH film in air and D_2O, as well as a non-thiol-modified T_5 film, in the amide I region. Clear differences are evident between the T_5 and the T_5-SH films in air. The peak at around 1700 cm^{-1} is assigned to non-chemisorbed thymine as previously demonstrated by FTIR studies.[10] The fact that this peak is present in the T_5-SH film indicates that the DNA strands are arranged in a brushlike structure with free carbonyl groups. The much lower intensity of this peak in the T_5 film, however, shows that the majority of the carbonyl groups are attached to the surface. This is in agreement with previous work characterizing similar films using FTIR and XPS, which has shown clearly distinguishable signatures from the chemisorbed and non-chemisorbed carbonyls in T_5 and T_5-SH films, respectively.[11]

The SFG spectra of the T_5-SH film in D_2O (upper curve) show significant increases in peak intensity versus the T_5-SH film in air. Analyses of the spectra in the CH stretching region show that the methyl groups are parallel to the surface (Fig. 2). Therefore, since the opposing carbonyl group will also be more parallel to the surface, one possible explanation for this peak increase is that due to the higher ordering of the backbone in D_2O the DNA strands are considerably tilted, leading to an orientation of the carbonyl groups in the molecular axis of the thymine base more perpendicular to the surface (Fig. 5).

V. CONCLUSIONS

These data demonstrate the application of SFG spectroscopy to films of ssDNA immobilized on gold in both air and aqueous environments. The SFG data support previous findings using XPS, NEXAFS, and FTIR. In addition, the SFG spectra show the possibility of characterizing changes in orientation and conformation of ssDNA films in both D_2O and PBS in the CH stretching and amid I regions.

The spectra show distinct differences in the conformation of films in air and solution, especially for films of thymine homo-oligonucleotides. These data suggest that in both air and D_2O the bases of thymine ssDNA are oriented parallel to the surface in a largely stacked arrangement. In D_2O, however, the backbones of the DNA molecules appear to stretch away from the surface, which may decrease the flexibility of the sugar backbone, resulting in a more ordered conformation. This would explain the observed increased SFG response from the backbone methylene stretches. In the case of PBS, the contributions from the backbone methylene vibrations are significantly lower. Here, the electrostatic shielding may allow a more flexible configuration of the sugar backbone. Furthermore, the methyl groups in the DNA molecules in PBS showed less overall order in comparison to those in air and D_2O.

Films of adenine ssDNA also showed conformational changes between air and aqueous environments; however, the change between D_2O and PBS is not that severe in comparison to the thymine measurements. This is likely due to the tendency of these molecules to bind to the gold surface instead of arranging in a brushlike structure.[9,12] Therefore, the addition of ions has little effect on the overall SFG spectra.

Spectra in the amide I region show that films of thymine ssDNA without a thiol linker are attached to the surface via the carbonyl groups, whereas in the thiol-linked film they are arranged in a brushlike structure with free carbonyl groups.

In D_2O the conformation changes, suggesting that the backbone gets more ordered. This may lead to a tilted alignment of the ssDNA strands and a subsequent increased peak intensity of the carbonyl groups.

This work adds SFG to the techniques currently available for the characterization of ssDNA films in solution, further building on the SFG results obtained by Stokes *et al.*, who examined hybridization phenomena,[25] and Asanuma *et al.*, who studied the deformation of ssDNA films upon exposure to cations.[27] These results allow for further exploration of their findings, as well as results generated by XPS, NEXAFS, and FTIR, in aqueous environments, and may also serve to begin bridging the gap between *ex situ* and *in situ* characterization of ssDNA films.

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