Surface Analysis

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Tip-Enhanced Raman Spectroscopy of Single RNA Strands: Towards a Novel Direct-Sequencing Method

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The sequencing of DNA or proteins is procedurally complex and requires sophisticated analytical techniques.[1,2] DNA sequencing while a very powerful method requires separation and visualization methods to recognize specific DNA fragments.[3] Furthermore, all the established methods require substantial amounts of DNA and fail to directly read the base composition of the strand. A method that utilizes the inherent information of the distinct bases present in DNA or RNA without the need of further labeling is therefore desirable. Recent approaches in this direction include pulling single DNA strands through nanopores, detecting certain electric properties, and then deducing the sequence.[4–6] Attempts were also made to directly sequence DNA by using a scanning tunneling microscope.[7,8] The main challenge with STM methods is always the low contrast and usually a statistic approach is necessary to evaluate the data. Our studies demonstrate that by using near-field optical techniques in combination with vibrational spectroscopy gives high contrast and the direct identification of bases on a single isolated RNA strand becomes feasible. Standard Raman spectroscopy makes the identification of the base components straight forward, however, the lateral resolution and the sensitivity of the method are far from the single-strand or even single-base detection levels required for a sequencing method. Herein we show that tip-enhanced Raman scattering (TERS) provides several advantages over conventional Raman spectroscopy: in just a few seconds acquisition time it gives high sensitivity at lateral resolution down to a few tens of nucleobases.[9,10] These properties allow TERS mapping along a poly(cytosine) RNA strand. The results demonstrate the potential of the method to identify and sequence the composition of polymeric biomacromolecules (DNA, RNA, peptides).

TERS spectra of a single-stranded RNA homopolymer of cytosine (poly(C)) have been measured with a spatial resolution down to a few nucleobases. The basic experiment is shown in Figure 1. A standard transmission TERS setup is used to focus a laser onto a silver-coated atomic-force microscope (AFM) tip, while the sample is moved independently, the sample surface is thus always in focus. Similar arrangements have been used to enhance infrared or Raman spectra of nanoscale materials, such as polymer samples,[11]

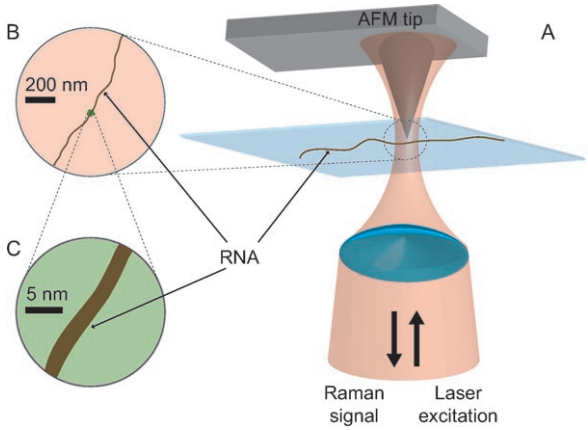

Figure 1. A) The tip-enhanced Raman scattering (TERS) experiment along a single strand of RNA. B) Higher magnification of the area approximately corresponding to the size of the laser spot. C) Magnification corresponding to the interaction area of the TERS probe tip.

molecular monolayers,[12] or single carbon nanotubes.[13] In Figure 2 the topography image of a single-stranded RNA cytosine homopolymer is shown. To avoid Raman scattering from compounds other than RNA, the use of buffer solutions and other chemicals was kept to a minimum. However, as a

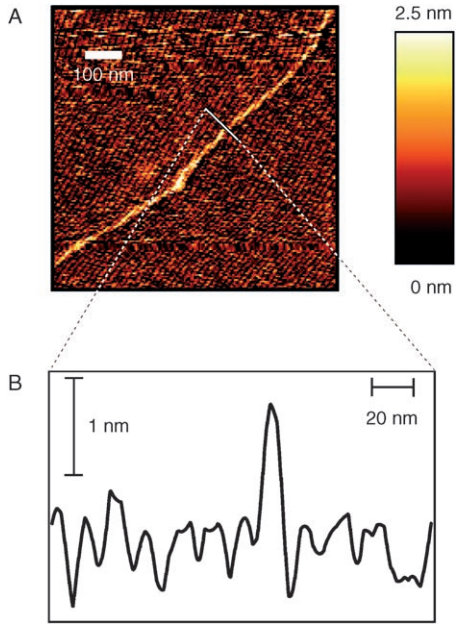

Figure 2. Topography of a cytosine single-stranded RNA homopolymer. A) AFM height image ("intermittent" contact) of a single poly-(cytosine) RNA strand on freshly cleaved mica. The image is baseline corrected. B) Height profile through the RNA along the line indicated in (A).

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result, entangling of the strands was a major issue and an extensive search for the linear single RNA strands needed for the measurements is required. The height of the strand shown in the profile measurement (Figure 2) corresponds to the known diameter of RNA, which strongly supports the identification of single strands. The effective area probed by an AFM tip is a convolution of tip and sample features. Hence, the measured strand width of approximately 10 nm can be largely assigned to the silver-coated AFM tips and corresponds well with the tip diameter of < 20 nm determined by SEM. The length of the homopolymer chain appears longer than expected and the small nodule-like feature in the center of the strand indicates that most likely two strands are attached to each other. However, these features had no impact on our further experiments as the length of the strand was not relevant. It is important to note that apart from the RNA no further topographic features are present. Thus other compounds (e.g. buffer crystals, etc.) can be ruled out for this particular sample area, making the evaluation of the Raman spectra amenable and without ambiguity.

Previous TERS measurements performed on single, nanometer-sized crystals or on monolayers of nucleobases demonstrate that the individual fingerprint of each nucleobase and the characteristic vibrations required for the distinction of each compound can be found easily.[14,15] In contrast to these previous investigations, the technical tolerances for a TERS experiment on a single DNA or RNA strand are much more stringent. The field-enhancing tip must be placed and held over the homopolymer during the whole laser exposure for the Raman experiment. It is crucial that the sample drift is nullified so that all subsequent measurement points are also on the RNA strand. In our experiment we measured TERS spectra at seven different positions along the single-strand RNA chain (Figure 3). The results show all the main spectral features of cytosine. Observed fluctuations of band intensities and positions exceeding statistical variations will be discussed below. Most importantly the results demonstrate the stability of the setup because the RNA could always be readily located. To show that the probe tip was not contaminated, reference measurements at sample sites without RNA were also measured. In these measurements no indication of RNA Raman bands or any other Raman bands, apart from silicon (arising from background scattering of the AFM cantilever) and mica (substrate material), could be found. This comparison also allows an estimation of the actual Raman enhancement. For this estimation we follow essentially the argument outlined in Refs. [16,17]

To estimate the enhancement, the number of molecules responsible for the signal both in the TERS experiment and in the reference case must be considered. The diameter of laser focus was 1 μm. In the reference case we assume that one RNA strand is stretched along the diameter of the laser focus (as indicated in Figure 1B). The signal of the unenhanced 1-μm-long RNA chain obtained when the tip was positioned next to this strand (Figure 3B spectrum position 8) is then used as the reference spectrum. In our experiments, no signal was detectable at this position under conditions identical to those for the other measurements.

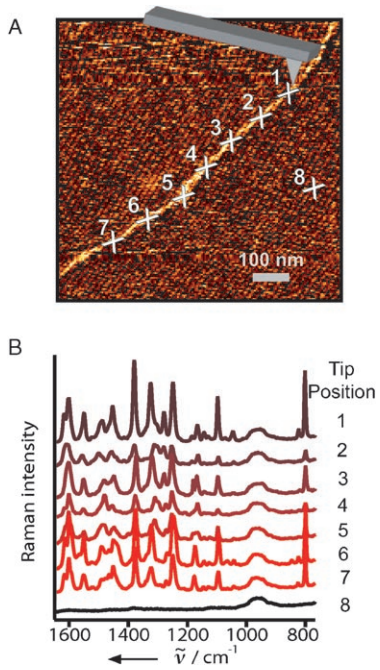

Figure 3. TERS experiment along an RNA strand. A) Topography image (same as in Figure 2A) showing seven adjacent spots corresponding to the positions of the TERS experiments and one additional spot for the reference measurement (position 8). B) The Raman spectra of the positions in (A).

In the TERS spectrum only a small RNA fragment of 20 nm length is responsible for all the signal intensity (see inset in Figure 1C). Hence, the relationship between the number of bases responsible for the Raman signal in the reference (ca. 3000 bases) and the TERS case (ca. 30–60 bases) is approximately 50-fold. In conjunction with the relationship between the signal-to-noise ratio (SNR) of the reference and the TERS spectrum, which is about 200:1, we can estimate an overall enhancement of the TERS signal of at least 10^4. This is a very cautious estimation as it does not take into account the duty cycle of the oscillating tip and all the parameters considered (tip size, SNR) are estimated very conservatively. However, in terms of sensitivity, the present TERS experiments are already very satisfactory. The SNR of approximately 200 stems from 30–60 bases underneath the TERS tip. Assuming a homogeneous signal enhancement, every single nucleobase then contributes with a SNR of 3–7 to the spectrum, making every base distinguishable. This situation means that single-base sensitivity has been achieved, which is one of the prerequisites required for sequencing using TERS.

A closer look at the spectra of the RNA strand (Figure 3B) shows slight changes in band intensities and positions. Band intensity changes resulting from different concentrations of RNA can be easily ruled out; the AFM images simply do not indicate the presence of multiple strands or other visible traces of molecules that interfere with the Raman spectra. A better explanation for intensity changes is the dependency of the enhancement on minuscule distance changes between probe and sample.[18–20] As the sample in the experiments is very small, probe positioning variations

even below a nanometer can be enough to cause intensity changes. In our case, the single RNA strand has a defined direction and even specific bends in the orientation of neighboring bases can induce changes in intensities and intensity ratios of distinct bands. As long as the interaction between the adjacent bases does not vary strongly, band shifts should be negligible and the band positions should be the same for all cytosine groups.

Figure 3 shows that the positions of some Raman bands also shift slightly. Again the positioning of the TERS probe with respect to the molecules provides a plausible qualitative explanation. It has been shown by Watanabe et al. that a strong dependence exists between the local position of a silver atom and the orientation of the probed molecule.[21,22] In most chemically relevant conditions the silver atom and analyte molecule will arrange themselves in the energetically most favorable position.[21,22] However, because of restricted flexibility, the RNA is fixed to the substrate and the silver coated TERS probe is held in a certain position, the system is forced into a specific arrangement. This arrangement will cause the bands to shift slightly from positions known from either solution or single-molecule experiments. One last difference between a standard Raman microscope experiment and a near-field Raman experiment such as TERS is the change in polarization direction and electromagnetic-field distribution. Longitudinal fields play an important role in the near-field and therefore new selection rules can arise.[23] The same effects can occur with strong field gradients at metal surfaces, as shown by Ayars et al.[24] All these effects can explain the variations occurring in the spectra. More important from a practical viewpoint is that the spectra could always be attributed to Raman bands assigned to cytosine in the literature.[25,26] Hence a distinction from other compounds should be straight forward and even sequencing of natural RNA strands will become feasible.

It is of note that it is not necessary to laterally resolve single bases. While the evaluation of the data is easier when only a few bases contribute to the overall signal, it is already sufficient in principal if single-base sensitivity can be achieved in one spectrum. If the measured data consists of the spectra of several adjacent nucleobases, then the sequence of the strand can be deduced by moving the TERS probe laterally in intervals of at least one base-to-base distance (Figure 4). Spectral changes from one position to the next then can be attributed to variations in the sequence. In conclusion we believe that TERS provides a powerful tool to directly sequence chainlike biomolecules, it provides high sensitivity, contrast, and lateral resolution. Further experiments on other biomolecules such as peptides will follow.

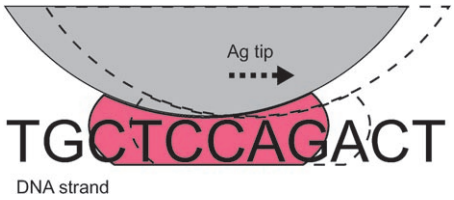

Figure 4. Direct base-sequencing procedure using TERS. Sequence information can be obtained by laterally shifting the probe in intervals of one base-to-base distance. The pink area refers to the enhancing site of the first position.

Experimental Section

A single-stranded RNA homopolymer of cytosine with a length range between 290–434 bases (GE Healthcare Europe GmbH, Germany) was used in this experiment without further purification. The RNA was dissolved in an organic chemical buffering agent to maintain a physiological pH value, HEPES (20 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and magnesium chloride (20 mM, $MgCl_2$; both Sigma-Aldrich) to fix the single-strand phosphate site onto mica (BAL-TEC). The concentration of the poly(cytosine) was 10^{-5} M. The RNA homopolymer solution (1 μL) was dropped onto a mica sheet and left to dry in an Argon atmosphere. Prior to the TERS experiments residual HEPES–Mg solution was removed from the sample by rinsing with doubly distilled water and the sample was dried again.[27]

The general setup of the TERS instrument has been described elsewhere.[9,14] For the TERS experiment a noncontact-mode silicon cantilever AFM tip (NSG10, NT-MDT) was coated with 20 nm of silver (99.99% pure, Balzers Materials) by thermal evaporation (BAL-TEC MDS 020 BAL-TEC GmbH) at an evaporation rate of 0.06 nm s^{-1}. The tips were then stored under argon and were used within two days.

For all TERS measurements the laser intensity at the sample was set to 1 mW (530.9 nm Krypton Ion) and the acquisition time was 15 s. All the spectra shown are raw data, no baseline-correction or smoothing procedures were applied. The signal-to-noise ratio of the TERS spectra was obtained by dividing the intensity of the most intense Raman band by two-times the standard deviation of the noise level measured in a signal-free section of the spectrum.

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