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IR absorption nanoscopy and nanoimaging of hemoglobin

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Abstract

IR absorption spectroscopy of hemoglobin was performed using an IR optical parametric oscillator laser and a commercial atomic force microscope. This experimental approach enables detection of protein samples with a resolution that is much higher than that of standard IR spectroscopy. Presented here are AFM based IR absorption spectra and images of micron sized hemoglobin features.

Introduction

Absorption spectroscopy is a widely applied technique for chemical characterisation. This method is able to detect both luminescent and non-luminescent materials and provide chemical specific information. An extensively used form of absorption spectroscopy is infrared absorption (IR) spectroscopy. This measures specific frequencies in the infrared region of the electromagnetic spectrum at which constituent parts of molecules corresponding to specific types of molecular bonds vibrate. This makes possible structural elucidation and compound identification of materials. As a consequence, IR absorption is extensively used as an analytical tool.

IR spectroscopy has been widely applied for the molecular structure characterization of lipids and proteins. As outlined IR spectroscopy detects molecular vibrations accompanied by changing molecular dipole moments. As a consequence the vibration frequencies that are detected are sensitive to molecular conformation.

While IR absorption studies of biosystems have been well established in the study of proteins there remains a number of limitations in the information that IR absorption spectroscopy can provide. For example, when studying micron or nanosized features IR absorption spectroscopy is limited by the diffraction limit [1,2]. The maximum image resolution in optical microscopy is found to be ca. $\lambda/2$. As a result of diffraction, the image resolution will be 2.5 μm when imaging in the infrared using electromagnetic radiation at 5 μm (corresponding to an IR absorption frequency of 2000 cm^{-1}). This means that IR absorption spectroscopy technology cannot be applied to study features that are approximately smaller than a few microns.

A newly emerging method for IR absorption spectroscopy using a photothermal based methodology enables IR spectroscopy of smaller amounts of materials than is currently possible using established IR absorption methods. This new method uses an Atomic Force Microscope (AFM) cantilever tip as the detection mechanism [3-10]. One particular method based on this approach, referred to as AFMIR, measures IR absorption directly via measuring local transient deformation in the sample via the AFM cantilever which is induced by an IR pulsed laser tuned at a vibration absorbing

wavelength [5-11]. This enables IR absorption spectra to be recorded of features as small as the AFM cantilever tip.

Here, we outline work performed on an experimental method for IR surface spectroscopy that samples directly via a bottom-up optical arrangement. In this letter AFMIR set-up is applied to IR image and obtain IR spectra haemoglobin with nanoscale resolution. The work presented here demonstrates that AFMIR can be applied to study micron sized aggregations of protein.

Methods

The experimental configuration consisting of an optical parametric oscillator (OPO) laser and an AFM. The excitation IR radiation is directed upward using gold coated mirrors to direct the laser light. The sample was mounted onto a glass slide to facilitate this optical arrangement. IR radiation was generated using an OPO laser (Cohesion) based on a periodically poled LiNbO₃ crystal emitting IR laser radiation that is tuneable over > 3.0 to 3.6 μm . The output power was c.a. 2 mW. The laser was focused to a relatively large spot of c.a. 500 μm on the sample in order to cover the entire area probed. The energy was low enough to avoid damaging the sample. An AFM (Veeco Explorer system) was used with a scanner with lateral and vertical dimensions of 100 x 100 μm and 8 μm respectively. The AFM is operated in contact mode enabling simultaneous IR and topography measurements. Silicon nitride tips mounted on a V-shaped cantilever with a nominal spring constant of 0.05 N/m (Veeco) were used. A force setpoint of 1 - 3 nN was used. Samples were prepared on standard microscope glass slides. A Fourier Transform IR (FTIR) spectrometer (Varian model 3100) was used to record a reference IR spectrum.

Results

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The AFM tip was positioned over the sample with the tip in contact with the sample surface. Following absorption of the incident radiation, the energy absorbed is dissipated through thermal and acoustic mechanisms. Propagating acoustic waves create a deformation in the surface topography which can be detected by displacement of the AFM tip [5]. As the wavelength of the IR laser source is tuned into resonance with a vibration mode of the sample, absorption of IR radiation increases. The response of the cantilever tip was monitored following the application of the IR radiation. AFMIR studies of a deposited layer of hemoglobin on a glass slide were undertaken. An AFM topography image of the surface was recorded (as shown in Fig 1a). A small area of the sample surveyed in the AFM topography image (marked α in Fig 1a) was selected for study. The hemoglobin feature in this area was c.a. 1 μm higher than the surrounding layer and has a diameter of c.a. 200 nm. The lateral size of the sample area probed is proportional to the size of the AFM tip (i.e. around 20 nm). The AFMIR spectrum is shown alongside the FTIR spectrum of hemoglobin. The two spectra show very similar features. The AFMIR spectra shown in Fig 1b possess a wavelength resolution of 15 cm^{-1} , while the FTIR based spectrum has a wavelength resolution of 2 cm^{-1} . Comparing the AFMIR and FTIR spectra shows that they possess similar spectral features. Both spectra show the presence of peaks on a broad background. The position of these peaks corresponds to crystalline hemoglobin. Kuenstner et al reported the position of peaks for crystalline hemoglobin to be 2871.5, 2960.2 and 3060.5 cm^{-1} which match the position of the peaks seen in both the AFMIR and FTIR spectra [12]. The bands seen in the spectra arise from N-H and C-H

vibrations. The amide B band at 3061 cm^{-1} is assigned to an intramolecular hydrogen-bonded N-H stretching or to an overtone band (i.e. $2 \times 1541\text{ cm}^{-1}$) [12]. The band at 2960 cm^{-1} is due to aliphatic C-H stretching. Changes in the relative intensities in some peaks are present when comparing the AFMIR and FTIR spectra.

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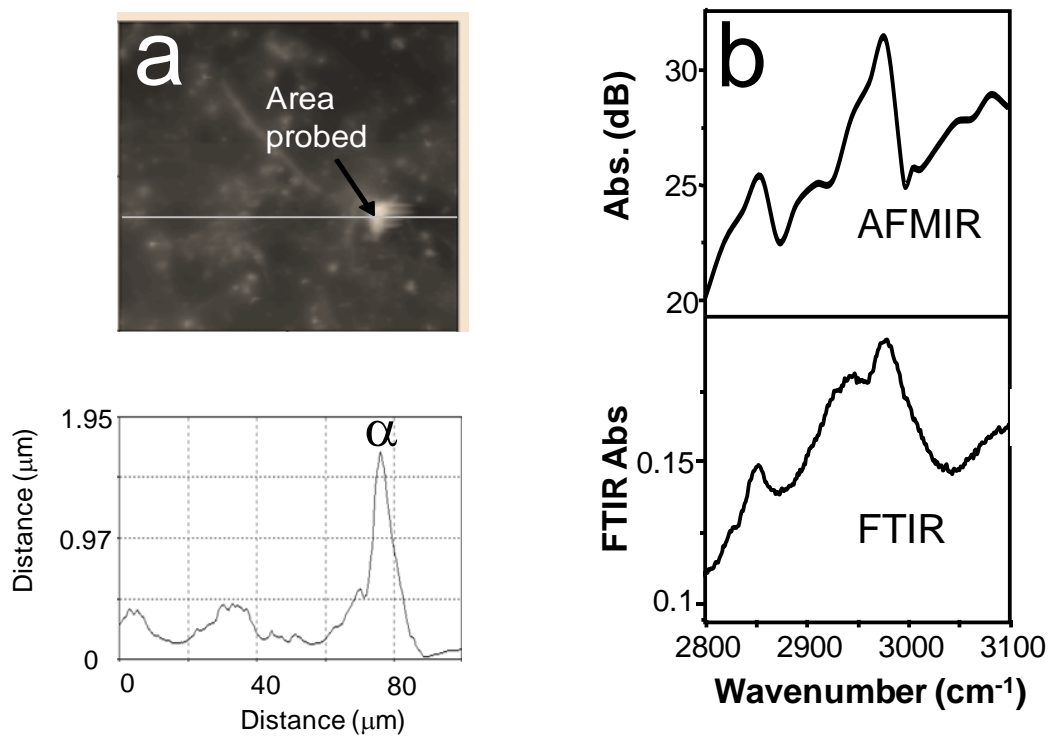


Fig 1. a) AFM topography image, b) IR absorption spectra for haemoglobin, i) is the AFMIR spectrum and ii) is the FTIR spectrum taken using a commercial FTIR instrument recording the IR spectrum from the bulk of the sample.

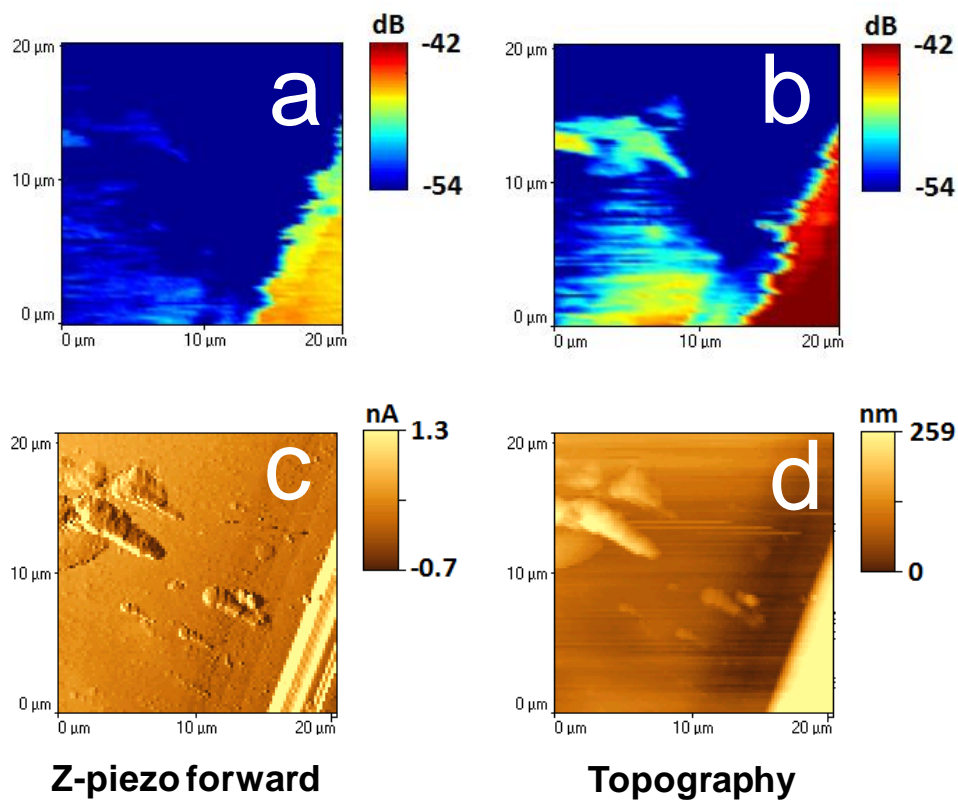


Fig 2. a) AFM topography image, b) IR absorption spectra for haemoglobin, i) is the AFMIR spectrum and ii) is the FTIR spectrum taken using a commercial FTIR instrument recording the IR spectrum from the bulk of the sample.