

Localized IR spectroscopy of hemoglobin

Fiona Yarrow · James H. Rice

Received: 31 May 2010 / Revised: 25 September 2010 / Accepted: 29 September 2010 / Published online: 15 October 2010
© European Biophysical Societies' Association 2010

Abstract IR absorption spectroscopy of hemoglobin was performed using an infrared (IR) optical parametric oscillator laser and a commercial atomic force microscope (AFM) in a novel experimental arrangement based on the use of a bottom-up excitation alignment. This experimental approach enables detection of protein samples with resolution much higher than that of standard IR spectroscopy. Presented here are AFM-based IR absorption spectra of micron-sized hemoglobin features.

Keywords Proteins · Infrared spectroscopy · AFM · Condensed phase

Absorption spectroscopy is a widely applied technique for chemical characterization. This method is able to detect both luminescent and nonluminescent materials and provide chemical specific information. An extensively used form of absorption spectroscopy is infrared (IR) absorption 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, enabling 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 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. Thus, spectroscopy is a useful method to investigate lipid structure and conformation.

While IR absorption studies of biosystems have been well established in the study of proteins, there remain 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 (Abbe 1873; Rice 2007). 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 IR absorption frequency of 2,000 cm^{-1}). This means that IR absorption spectroscopy technology cannot be applied to study features that are smaller than approximately 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 (Hammiche et al. 2004a, b; Hammiche et al. 1999). One particular method based on this approach, referred to as AFMIR, measures IR absorption directly by 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 (Dazzi et al. 2005; Houel et al. 2007; Mayet et al. 2008; Hill et al. 2009; Rice 2010). This enables IR absorption spectra of features as small as the AFM cantilever tip to be recorded. Using this approach, study of quantum dot nanomaterials with spatial resolution of 60 nm has been reported (Houel et al. 2007). To date the experimental

F. Yarrow · J. H. Rice (✉)
NanoPhotonics Research Group, School of Physics,
University College Dublin, Belfield, Dublin, Ireland
e-mail: james.rice@ucd.ie

methodology of AFMIR has utilized the attenuated total internal reflection arrangement in combination with IR cyclotron radiation or a top-down configuration using a customized IR laser source.

Here, we outline work performed on an experimental method for IR surface spectroscopy that samples directly via a novel bottom-up optical arrangement using a commercial laser system. The advantage of this setup is that it uses a commercial laser rather than synchrotron radiation and allows use of commonly used substrates such as glass or mica. In this letter, this novel AFMIR setup is applied for the first time to study hemoglobin. The work presented here demonstrates that AFMIR can be applied to study micron-sized aggregations of protein.

Methods

The AFMIR experimental setup is shown in Fig. 1a. The experimental configuration consists of an optical parametric oscillator (OPO) laser and an AFM. The excitation IR radiation is directed upward in a novel configuration 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 tunable over >3.0–3.6 μm . The

output power was ca. 2 mW. The laser was focused to a relatively large spot of ca. 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 $100 \times 100 \mu\text{m}^2$ 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 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

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 (Dazzi et al. 2005; Rice 2010). As an IR laser source is tuned into resonance with a vibration mode, 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. 1b). A small area of the sample surveyed in the AFM topography image (marked α in Fig. 1b) was selected for study. The hemoglobin feature in this area was ca. 1 μm higher than the surrounding layer and has a diameter of ca. 200 nm. The lateral size of the sample area probed is proportional to the size of the AFM tip (i.e., around 20 nm).

Figure 2a shows the oscillation of the AFM cantilever following absorption of the IR laser pulse by the sample. The intensity of the cantilever oscillation changes on resonance ($2,960 \text{ cm}^{-1}$) and off resonance ($2,810 \text{ cm}^{-1}$) with the C–H stretching mode of hemoglobin. The intensity of the oscillation of the cantilever as a function of wavelength was recorded. The resulting AFMIR spectrum of hemoglobin is shown in Fig. 2b.

The AFMIR spectrum is shown alongside the FTIR spectrum of hemoglobin. The two spectra show very similar features. The AFMIR spectra shown in Fig. 2b 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 (marked α , β , χ , δ , ϵ , ϕ) on a broad background.

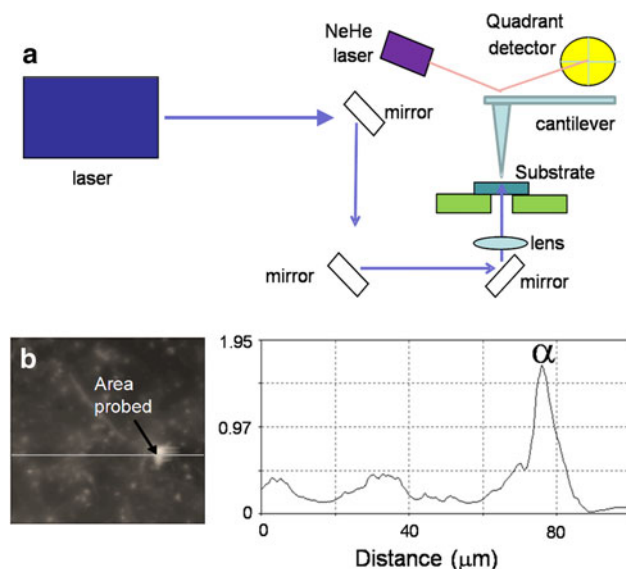


Fig. 1 **a** Schematic drawing of the experimental setup. **b** AFM image of hemoglobin sample on a glass surface ($100 \times 100 \mu\text{m}^2$ area). *Left* topography; *right*, height profile recorded at the position of the *white line*. The *arrow* shows the region probed by AFMIR, which corresponds to α

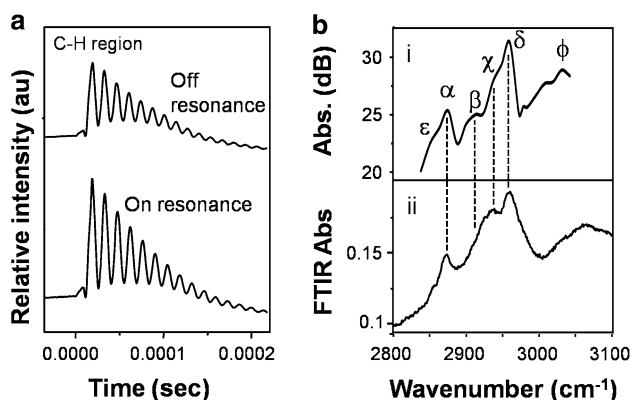


Fig. 2 **a** Plot of AFM tip oscillations on ($2,960\text{ cm}^{-1}$) and off ($2,810\text{ cm}^{-1}$) resonance, **b** IR absorption spectra for hemoglobin

The position of these peaks corresponds to crystalline hemoglobin. Kuenstner et al. (2000) 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. The bands seen in the spectra arise from N–H and C–H vibrations. The amide B band at $3,061\text{ cm}^{-1}$ is assigned to an intramolecular hydrogen-bonded N–H stretching or to an overtone band (i.e., $2 \times 1,541\text{ cm}^{-1}$) (Kuenstner et al. 2000). The band at $2,960\text{ cm}^{-1}$ is due to aliphatic C–H stretching. Changes in the relative intensities in some peaks are present when comparing the AFMIR and FTIR spectra. Normalizing to the peak at δ , the peak at χ is reduced in intensity, while the peak at β is reduced compared with α . These changes in relative intensity may be associated with the micron-sized particle measured inducing small changes in the conformation of the protein.

Studies of smaller, nanosized protein layer features were undertaken. Figure 3a shows an AFM image of hemoglobin sample on a glass surface. Two regions were probed which were ca. 150 nm different in height (denoted by α and β). Studies of these two regions showed that difference in the AFM tip oscillations on (region β) and off (region α) the heme ledge could be seen at a wavelength of $2,960\text{ cm}^{-1}$, which corresponds to on resonance (Fig. 3b). The thinner part of the sample showed a weaker AFM tip oscillation than the thicker part of the sample (i.e., regions denoted by α and β). This shows that small, nanosized features can be discerned using AFMIR. Changing the wavelength to a wavelength that corresponds to off resonance (i.e., $2,810\text{ cm}^{-1}$) led to the same effects in the oscillations as shown in Fig. 2a.

In conclusion, IR absorption spectroscopy of hemoglobin was performed using an IR optical parametric oscillator laser and a commercial atomic force microscope in a novel configuration. This experimental approach enables detection of micron-sized protein features. This resolution

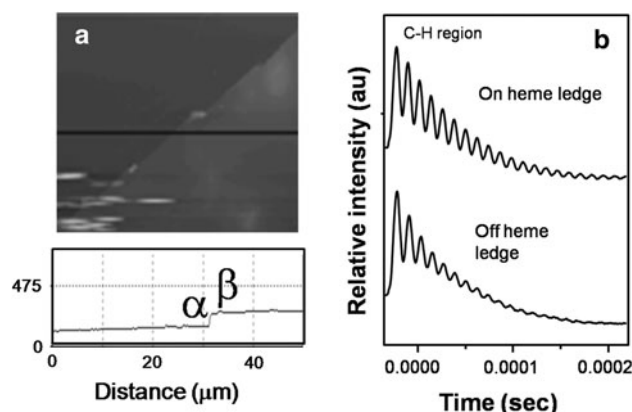


Fig. 3 **a** AFM image of hemoglobin sample on a glass surface ($500 \times 500\text{ nm}$ area). *Top*, topography; *bottom*, height profile recorded at the position of the *black line*. The areas probed are denoted by α and β . **b** Plot of AFM tip oscillations on resonance ($2,960\text{ cm}^{-1}$) on and off the heme ledge (β and α , respectively)

cannot be achieved by standard IR spectroscopy. This methodology for IR spectroscopy can potentially be applied to study other protein structures on the micro- and nanometer length scales.

References

- Abbe E (1873) Beitrage zur theorie des Mikroskops und der mikroskopischer Wahrnehmung. Arch Mikroskop Anat 9:413–487
- Dazzi A, Prazeres R, Glotin F, Ortega JM (2005) Analysis of nanochemical mapping performed by an AFM-based (“AFMIR”) acousto-optic technique. Ultramicroscopy 107:1194–1200
- Hammiche A, Pollock MH, Reading M, Claybourn M, Turner PM, Jewkes K (1999) Photothermal FT-IR spectroscopy: a step towards FT-IR microscopy at a resolution better than the diffraction limit. Appl Spectrosc 53:810–815
- Hammiche A, Bozec L, Pollock HM, German M, Reading M (2004a) Progress in near-field photothermal infra-red microspectroscopy. J Microsc 213:129–134
- Hammiche A, Bozec L, German MJ, Chalmers JM, Everall NJ, Poulter G, Reading M, Grandy DB, Martin FL, Pollock HM (2004b) Mid-infrared microspectroscopy of difficult samples using near-field photothermal microspectroscopy. Spectroscopy 19:20–40
- Hill G, Rice JH, Meech SR, Kuo P, Vodopyanov K, Reading M (2009) Nano-infrared surface imaging using an OPO and an AFM. Opt Lett 34:431–433
- Houel J, Sauvage S, Boucaud P, Dazzi A, Prazeres R, Glotin F, Ortega JM, Miard A, Lemaitre A (2007) Ultraweak-absorption microscopy of a single semiconductor quantum dot in the midinfrared range. Phys Rev Lett 99:217404
- Kuenstner JT, Norris K, Kalasinsky VF (2000) Spectrophotometry of human hemoglobin in the midinfrared region. Biospectroscopy 3:225–232
- Mayet C, Dazzi A, Prazeres R, Allot F, Glotin F, Ortega JM (2008) Sub-100 nm IR spectromicroscopy of living cells. Opt Lett 33:1611–1613
- Rice JH (2007) Beyond the diffraction limit: far-field fluorescence imaging with ultrahigh resolution. Mol Bio Syst 3:781–793
- Rice JH (2010) Nanoscale optical imaging by atomic force infrared microscopy. Nanoscale 2:660–667