ARTICLE

Single particle characterisation in biologics: from mid-infrared micro-spectroscopy and mapping to spectral imaging

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Supplementary Information[1]

Background

Vibrational spectroscopic methods, like infrared spectroscopy, are powerful techniques for the chemical characterisation of materials. For example, mid-infrared spectroscopy is commonly used for the identification of chemical compounds, due to very specific features (absorptions) observed in the so-called fingerprint mid-infrared region ($< 1500\ cm^{-1}$). Depending on the technical equipment and set-up, for most experiments, bulk properties, i.e. average properties of the sample, are obtained. In order to register localised information from the sample, infrared spectrometers have been combined and linked to microscope systems (see Figure 1 in main article[1]). The first efforts towards this had already been undertaken in the 1940s and one of the first commercially available set-ups was proposed by Perkin-Elmer.[2] However, the performance of the first generation infrared micro-spectrometers was poor due to technical reasons (for more details see Griffiths and de Haseth[3]). It was not until the 1990s that the first mid-infrared micro-spectroscopy instruments became commercially available that had the reasonable performance necessary for micro-spectroscopic investigations.[2]

Micro-spectroscopic techniques

For the investigation of localised regions of a sample by infrared micro-spectroscopy, the following three approaches can be used: (i) single-spot, (ii) mapping or (iii) imaging technique.

(i) During single-spot experiments, the investigated sample region is delimited by the aperture of the microscope and a single infrared spectrum is obtained from the investigated sample area. Narrow-band mercury cadmium telluride (MCT) detectors, which are commonly used for mid-infrared micro-spectroscopy, often have a detector size of 250 µm × 250 µm, although smaller detectors are available with, for example, a 100 µm × 100 µm or a 50 µm × 50 µm-sized element.[3]

(ii) The mapping technique is a logical continuation from single-spot measurements, namely by recording sequential measurements of single-spots of adjacent regions of a sample. This is realised by moving each region of the sample into the beam focus of a microscope after the spectrum of the previous region has been measured.[4] This measurement step is repeated until the entire region of the sample has been investigated. The two approaches, single-spot and mapping experiments, can be used with the same infrared micro-spectrometric set up.

(iii) For imaging experiments, a specific array detector, a so-called focal plane array (FPA) detector which consists of a large number of pixels (for example, 64 × 64 up to 256 × 256), is used. Imaging requires the sample image to be focused onto an array detector, where the intensity of the infrared radiation from each region of the sample is measured at each pixel. Using a Fourier transform infrared (FT-IR) set-up, a full infrared spectrum is obtained from each pixel element.

As pointed out by Griffiths,[3,5] in a mapping experiment in which the sample is only moved in one specific direction (x or y), the measurement is called line mapping. Moving the sample in both directions (x and y), the measurement cannot be properly denoted as imaging, because the spectra were not acquired by an array detector.[3] Nevertheless, data evaluation and data treatment from a mapping study can be handled in a similar fashion as the data obtained from an imaging experiment.

The term hyperspectral imaging refers to a technique whereby images are recorded simultaneously with an array detector and for which, at each pixel, the spectrum over a large number of wavelengths is obtained. This is achieved using two-dimensional array detectors. By using the FT-IR spectroscopic technique, interferograms corresponding to different spatial regions of the sample are recorded by each detector element and subsequent Fourier transformation of these yields the desired hyperspectral data set.[3]

Measurement options

Mid-infrared micro-spectroscopy measurements can be made using

various sampling techniques, which are transmission, reflection or attenuated total reflection (ATR) mode.[2–4,6] In transmission experiments, for samples that can be sandwiched between two IR transparent windows (for example, CaF_2), the sample thickness should typically be in the range of *ca* 10 µm. The thickness of the sample needs to be as uniform as possible, especially for mapping and imaging experiments and particularly if information on component concentration composition is required. Homogeneous samples may be obtained by, for example, microtome sectioning. If it is not possible to obtain a sample of constant thickness, the ratio of the peak absorbance or integrated absorbance area of bands that can be assigned to particular chemical components has to be determined.[3,7] For single-spot measurements of very small particles, sample thickness homogeneity is of less importance.

Three types of external reflection experiments can be performed. (i) In transflection spectroscopy a sample (*ca* 5–10 µm thickness) is deposited onto an infrared reflective substrate and the transflection spectrum measured.[2] As shown by Merklin and Griffiths,[8] the disadvantage of this measurement option is based on additional reflected radiation effects, because radiation reflected from the front surface of the sample also reaches the detector and gives rise to a distortion of the pure transflection spectrum.[8]

The other types of reflection experiments, (ii) specular reflection, also known as front-surface reflection and (iii) diffuse reflection spectroscopy, are less appropriate for the characterisation of biological small samples (for more details on these reflection techniques, see Chalmers and Griffiths[2]).

ATR is a sampling approach that allows for the direct, non-destructive investigation of the surface layer of solid and liquid samples, provided sufficient contact can be achieved between the internal reflection element (IRE) and the sample. Materials used as IRE must have a higher refractive index (n) than the sample; two commonly used materials are: zinc selenide ($n = 2.4$) and Germanium ($n = 4$). The infrared beam is passed through an IRE so that it reflects at the internal surface in contact with the sample (lower n). The internal reflection of the IR beam forms an evanescent wave which penetrates into the sample. IREs are available with a single- or multi-reflection capabilities. The penetration depth of the beam into the sample depends on the wavelength of the radiation, the angle of incidence (which must be greater than the critical angle) and the relative refractive indices of the sample and IRE.[3,9] In the mid-infrared region, with ZnSe or Ge as the IRE, the penetration depth into biological samples lies in the approximate range 0.5–2 µm. An advantage of ATR is the limited path length into the sample, thereby avoiding strong attenuation of the infrared beam in highly absorbing media.[2] Using diamond as the IRE ($n = 2.4$), extremely hard samples can be investigated without damaging the ATR unit.

Detectors

The detectors used for mid-infrared micro-spectroscopy determine the infrared application experiments that can be undertaken as well as the signal-to-noise ratio (SNR) of the spectrum. Most standard FT-IR spectrometers are equipped with a deuterated triglycine sulfate (DTGS) detector. The main advantages of this type of detector are its low purchase costs and that it can be operated at ambient room temperature. Its main disadvantages are a low sensitivity for relatively weak signals as well as a slow response time. As an alternative, MCT detectors are used due to their higher sensitivity and higher response times. Narrow-band MCT detectors are, typically, 100-fold more sensitive than DTGS detectors, but most do not respond to radiation below *ca* 600 cm^{-1}. This cut-off can be extended to a lower wavenumber (450 cm^{-1}), but at the expense of sensitivity (cut-off: mid-band MCT *ca* 600 cm^{-1}, wide-band MCT *ca* 450 cm^{-1}).[3,5] For applications with biological samples, often mid-band MCT detectors are used.[5]

MCT detectors have to be operated at very low temperatures to attain their highest sensitivity, i.e. the detectors have to be cooled with liquid nitrogen or by cryo-coolers.[4]

For imaging experiments, focal plane array (FPA) detectors are used. These, as mentioned above, are composed of a certain number of pixels (for example, 64 × 64 and 256 × 256 pixels). Using an FT-IR spectroscopy set-up from each such experiment with an FPA detector, 4096 or 65536 spectra, respectively, are generated, with one full infrared spectrum associated with each pixel.

Infrared radiation sources

The infrared radiation is normally provided by an incandescent silicon carbide source such as a Globar which operates at very high temperatures of *ca* 1130°C. The spectral energy density of the infrared source has a strong impact on the signal-to-noise ratio of the recorded spectrum. This is of particular relevance when small particles are investigated with small apertures. In general, there are two alternative sources for infrared radiation that are much better for mid-infrared micro-spectroscopic investigations of very small samples, namely synchrotron radiation or free electron laser (FEL) (for more details see Chalmers and Griffiths[2] and Falta and Möller[10]). However, these facilities are typically available only within national research laboratories and only realisable in the case of samples that can be moved and transferred to such a facility.

Spatial lateral resolution

Based on the laws of optics, the spatial lateral resolution of a microscope is determined by the diffraction limit of the used radiation. The diffraction limited spatial resolution (d) depends on: (i) wavelength (λ) of the radiation, (ii) the refractive index (n) of the medium in which the optics are immersed, (iii) the angle (Θ) defining the most extreme ray exciting the optical system with respect to the central axis "the diffraction limited spatial resolution" can therefore be calculated as $d = (0.61 \cdot \lambda) / (n \cdot \sin\Theta)$.[1] The term $n \cdot \sin\Theta$ is called the numerical aperture. Thus, two objects are completely resolved if they are separated by $2d$, and barely resolved if they are separated by d. For most IR microscope objectives, the numerical aperture ranges between 0.5 and 0.7. For an ATR Cassegrainian[2]

optic, the largest numerical aperture is *ca* 0.6 and, thus, the diffraction limited spatial lateral resolution is approximately equal to the wavelength of the light, $d \approx \lambda$. Independent of whether a single spot, mapping or imaging experiment is performed, the lateral spatial resolution is limited by diffraction. For the mid-infrared region from 4000 cm^{-1} to 400 cm^{-1}, which corresponds to 2.5 µm to 25 µm, the lateral spatial resolution is approximately 5–10 µm. Objects smaller than 5–10 µm are very difficult to investigate properly by infrared microscopy.[2,4,5]

An advantage of using an ATR set-up is that the ATR crystal acts as a solid immersion lens so that the spatial resolution is increased by a factor equal to the refractive index of the ATR crystal (Ge: $n = 4$), compared with measurements performed in the transmission or trans-reflection mode.[5,7]

For imaging experiments, one has to remember that the FPA detectors are made of single pixel elements of finite sizes. As pointed out by Griffiths,[5] when the image of the element pixel at the sample plane becomes the limiting aperture, it can often become the limiting factor determining the ultimate spatial lateral resolution before wavelength limited diffraction effects become apparent. In the case for which the pixel element size at the sample plane is larger than the wavelength that is used in the measurement, the spatial lateral resolution of the system becomes pixel size-limited. However, in the case where the wavelength of the radiation being used to measure the sample is smaller than the pixel element size at the sample plane, the system is diffraction-limited.[3]

The microscope, as shown in Figure 1 in the main article,[1] may also be equipped with a focal plane array detector (FPA). This microscope design is such that two separate optic units are used to guarantee highly accurate, distortion-free images of the sample on the FPA detector when performing chemical imaging. However, this design also allows for a maximum of light throughput when the single element detector is used for single particle/spot characterisation. With the routinely available FPA, a sample area of 340 µm × 340 µm can be investigated generating 16384 full infrared spectra simultaneously (using a 128 × 128 FPA pixel element), at a pixel resolution of 2.7 µm (15× objective). For transmission and reflection measurements, the pixel resolution can be improved to 1.1 µm, by using a 36× objective.[4] This pixel resolution is required when a lateral resolution of 2.5 µm (at 4000 cm^{-1}) is needed for transmission or reflection experiments.

The need for better chemical characterisation of samples and particles has increased significantly over the last few years. However, until recently, infrared micro-spectroscopic set-ups were not commonly used due to their complexity and cost and, consequently, more user-friendly, lower cost FT-IR/microscope systems were needed, which could be operated fully automated. Therefore, compared to the more commonly available IR microscope set-ups, in which the microscope is coupled to the infrared spectrometer, stand-alone instruments with a fully "integrated" spectrometer using a permanently aligned interferometer have become available (for example, the one shown in Figure S1). As mentioned above, a number of samples are not transparent or reflective, but can, alternatively, be investigated in the ATR mode, which can be directly integrated into the microscope. Using ATR objectives, with a motorised ATR crystal, data acquisition is performed by positioning the ATR crystal into the IR focus by an encoded piezo drive. An integrated pressure control ensures the constancy of the pressure applied from the crystal to the sample which is essential for mapping and imaging experiments. In addition to an objective with a specific magnification (for example, 8×), a digital zoom has been implemented that allows one to increase the magnification (for example, up to 32×). With most infrared microspectrometers, the investigated samples can then be "documented" by capturing images using a highly resolving CCD camera.

Figure S1. Example of a fully automated FT-IR microscope (Bruker, LUMOS system), which includes a motorized ATR crystal within the objective.

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