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Vibrational spectroscopies to investigate concretions and ectopic calcifications for medical diagnosis

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ABSTRACT

As asserted by numerous authors, Fourier Transform (FT)-Raman and FT-Infrared spectroscopies constitute two useful tools for bio-medical diagnostics. We can distinguish two different kinds of application, namely, tissue disorders analyzed using statistical methods, and detection of ectopic calcifications through precise analysis of the shape and the position of absorption bands. In this contribution, we present some new results regarding these research themes, as well as recent experimental developments, such as the ability to perform nanometer scale near-field infrared microscopy, which have already led to major scientific breakthroughs.

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1. Introduction

Infrared (IR) spectroscopy is an advantageous, non-destructive and label-free technique for chemical analysis [1–3]. IR absorption spectroscopy directly probes vibrational energy levels and phonons of materials, providing chemical as well as structural information by comparison with data bank reference spectra [4–9]. In regards to the medical field, IR microscopy, when combined with morphological description, has already demonstrated that it is possible to obtain significant clinical information regarding microcrystalline pathologies [10–16] through the analysis of concretions [17–28] as well as of ectopic calcifications in different human tissues [29–38]. Different scientific breakthroughs have been obtained in other research fields in which IR is now routinely used at the hospitals. Among these, we can cite the characterisation of lipids in graft liver at the Paul-Brousse hospital [39–41], of different biological tissues at the Reims hospital [42–48], and of concretions and ectopic calcifications in kidney biopsies for 30 years at the Necker hospital and subsequently at the Tenon hospital [9,12,16,21,24,29].

The introduction of Fourier Transform-IR (FT-IR) spectroscopy into the hospital environment is intimately related to experimental practicalities. Initially, the experimental procedure to collect IR spectra was quite laborious, requiring several minutes to collect data with sufficient signal-to-noise to extract significant clinical information. Thanks to various developments in laboratory apparatus, it is now possible to collect data rapidly (seconds to milliseconds) on subcellular sized crystals. Such low acquisition times allow the medical community to analyse large numbers of biological samples. For example, at the Necker and Tenon hospitals, more than 75,000 kidney stones have been characterized. Such opportunities also facilitate

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epidemiologic studies and help to relate urolithiasis to other major public health problems such as type II diabetes. Indeed, we have started an investigation on more than 200 patients after kidney transplantation in order to establish a possible correlation between the presence of ectopic calcifications and kidney function. The acquisition of a latest generation IR spectrometer at the Tenon hospital has also offered the opportunity to characterize ectopic calcifications in more than 800 biopsies of different organs (not only kidney but also breast and thyroid) originating from different hospitals in France, Belgium, Italy, Switzerland, and Canada.

In this contribution, we wish to present information that clinicians and more especially anatomopathologists can obtain through the conventional experimental setups, which already exist in some hospitals. Also, we will present some very recent developments in the area of near-field imaging, which have made it possible to collect IR spectra and images at about 20 nm resolution.

2. Some basic equations related to IR spectroscopy

We will not present here the theoretical formalism underlying IR spectroscopy but only some basic relevant equations [49,50]. In transmission, neglecting the reflection losses, the transmitted light (T) is a function of the absorption coefficient (α_{abs}) and of the path length of light in the sample:

$$T = I/I_0 = e^{-\alpha_{abs}} \cdot l,$$

where I_0 and I are the light intensity of the incident and transmitted beams, respectively and l is the sample thickness. If the sample is homogeneous and weakly absorbing, scattering is negligible, and the absorbance is defined as:

$$A = \gamma \cdot c \cdot l = -\log T,$$

where $\alpha_{abs} = \gamma \cdot c$, γ is the molar absorptivity and c is the molar concentration of the absorbing species. Another key point regarding IR spectroscopy is related to lateral resolution of IR microscopes, which is limited by light diffraction. This limit can be estimated by the Rayleigh criterion:

$$\xi = 2 \cdot \lambda/\mathrm{NA},$$

where ξ is the lateral resolution and NA is the numerical aperture of the microscope objective (the more commonly found equation with $\xi = 0.61\lambda/\mathrm{NA}$ does not hold true for reflective objectives such as Cassegrain and Schwartzschild due to the central obscuring of the primary mirror). For mid-IR spectroscopy, the values of λ are between 25 μm (400 cm^{-1}) and 2.5 μm (4000 cm^{-1}). Due to signal-to-noise constraints, diffraction limited resolution is not achieved with conventional instruments and it is necessary to use synchrotron radiation as the source of photons [51–56] to attain this limit. The most recent instrumental developments try to go beyond this limit by using other physical principles. We will see that it is possible now to collect IR spectra at the nanometer scale.

3. The FTIR spectrum of a biological tissue

It is convenient to conceptualize an IR spectrum as two regions: 4000–1000 cm^{-1} known as the functional group region, and <1000 cm^{-1} known as the fingerprint region, even if for many chemists the dividing line is in fact 1450 cm^{-1}. The functional group region contains relatively few peaks, which are typically associated with the stretching vibrations of functional groups (Fig. 1), denoted by the Greek symbol ν. In the fingerprint region, the spectra usually consist of bending vibrations within the molecule, denoted by the Greek letter β. This region is the key to the data analysis because it is here that each distinct compound produces its own unique pattern of peaks, effectively a fingerprint. In Fig. 1B, the different, well understood, absorption bands of the calcium phosphate apatite are assigned [8,9,57,58]. The ν_1 and ν_3 P–O stretching vibration modes occur at 960–962 cm^{-1} and 1035–1045 cm^{-1}, respectively.

From an experimental point of view, a FTIR spectrum can be collected using a wide range of sampling techniques. Those which we use at the hospital are depicted in Fig. 2. FTIR spectra are usually collected in the transmission mode (Fig. 2a), in which case kidney stones are ground with an agate mortar and pestle. Then almost 3 mg of the resultant powder is pelleted with spectroscopy grade dry KBr (~300 mg).

In the case of kidney biopsies, tissues were simply deposited on low-e slides (a glass slide on, which a metal layer has been deposited making them IR reflective). Such metal coated slides allowed us to measure IR spectra of biopsies deposited on the glass slide in the so-called *trans*-reflexion (or transflexion) mode (Fig. 2b). The dimension of the spot can be as low as 10 μm using a synchrotron source and thus, several thousands of IR spectra can be collected allowing high-resolution chemical mapping of the sample to be performed. Note that such mapping can be performed in transmission mode (and thus without such support). Then, through the selection of a specific IR band (red arrow on Fig. 2c), it is possible to determine the spatial distribution (Fig. 2d) of a single chemical phase (here whewellite) and overlay it with an optical image (Fig 2e).

Attenuated Total Reflectance (ATR) is another geometry developed independently in the 1960s by Harrick and Fahrenfort [59,60] and used at the hospital. FTIR-ATR spectroscopy historically has been used for samples which are too thick for transmission measurements (Fig. 2f,g). In this sampling technique, information is derived from an evanescent wave that extends the surface of an infrared transparent crystal and penetrates only a short distance into the sample, i.e. around 1–3 μm depending on wavelength and crystal refractive index. We may distinguish two kinds of experimental setup, namely, macro and micro FTIR-ATR. In macro-ATR spectroscopy, the whole sample is measured at once at depths of 1–3 μm, giving no spatial resolution but excellent spectral quality. In the micro-ATR configuration, the coupling of the IR microscope and the ATR probe allows spatial resolution as fine as 2–4 μm [61,62] to be achieved, thanks to the high refractive index crystal, which reduces the lateral beam spot size.

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