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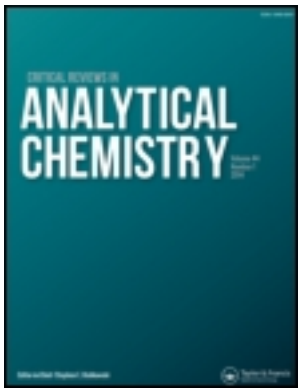

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Biomedical Investigations Using Fourier Transform-Infrared Microspectroscopy

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Biomedical Investigations Using Fourier Transform-Infrared Microspectroscopy

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One of the most exciting recent developments in infrared spectroscopy has been the coupling of the spectrometer to an infrared microscope. The combination of the new infrared spectrometer and a microscope was a natural thought of scientists in these fields. This development has been so rewarding and so useful in solving today's chemical problems that infrared microspectroscopy has quickly become a significant subclassification of infrared spectroscopy. Infrared microspectroscopy has a much longer history than the recent enthusiasm would imply, however. The great interest in the use of infrared spectroscopy to solve biomedical problems that occurred in recent years shortly spread into the medical and biological fields. The aim of this review is to discuss the new developments in applications of FT-IR microspectroscopy in biomedical analysis, covering the period between 2008 and 2013.

Keywords Biomedical applications, infrared mapping and imaging, infrared miscroscopy

INTRODUCTION

Infrared (IR) spectroscopy is one of the oldest and most versatile analytical chemical techniques. IR was discovered in the early 1800s, however, only at the end of the century were the first IR spectra published (Abney and Festing, 1881; Nichols, 1893). In the late 1800s, Michelson developed his interferometer for studying the speed of light (Michelson and Morley, 1887). Some 70 years earlier in France, Jean Baptiste Joseph Fourier had developed the mathematical technique that served to convert the output of the Michelson interferometer into a spectrum. Later, at the end of 1940s, the great advantage of combining an IR spectrometer with a microscope had been pointed out in order to extract accurate molecular information from small areas of a sample (Blout et al., 1949; Gore, 1949). With the introduction of Fourier transform-infrared (FT-IR) spectroscopy, the use of IR expanded continuously for qualitative and quantitative analyses in many disciplines (Bunaciu et al., 2010, in press; Chalmers and Griffiths, 2002; Marcelli et al., 2012; Movasaghi et al., 2008).

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It was pointed out by P. B. Fellgett (1958) that in principle interferometry also provides a multiplex advantage using broadband sources in that an interferogram records the superimposed signals from all the different wavelengths presented to the detector. The separate signals at each wavelength can again be recovered from the combined interferogram by Fourier transform mathematical analysis.

In 1954 the first commercial IR microscope was made available by Coates et al. (1953). The original IR spectrometers were significantly improved in the following years with the use of high-quality detectors and the introduction of new electronic components, such as microprocessors. Nowadays, the high sensitivity of the most recent generation of microscopes opens new experimental opportunities. Moreover, improved performance, such as enhanced frequency accuracy, ultimate signal-to-noise ratios, and high data acquisition speed, combined with modern computational methods, allows the ultimate investigation of many biochemical systems. In addition, the development of alternative vibrational spectroscopic techniques, e.g., reflection measurements and attenuated total reflection (ATR) probes, extended the analysis to thick, opaque, small and/or otherwise "difficult" samples to be investigated in transmission geometry.

There are two optical constants to be determined, so two measurements are needed at each wavenumber. Alternatively, one

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of these measurements may be replaced by the Kramers-Kronig transform and a value of n at a single wavenumber. These requirements can be met in a variety of ways and many different methods have been used. The methods used before 1970 and the results obtained were presented by Young and Jones (1971). The methods currently used involve the use of external reflection, which is the best method for very strong absorption by nonvolatile materials; ATR spectroscopy, which is far more sensitive than external reflection; ellipsometry, which should perhaps be more widely used; dispersive Fourier-transform spectrometry, which has been widely used in the far-IR spectrum; and transmission spectroscopy, which is the most suitable for weak absorption (Chalmers and Griffiths, 2002).

Fourier transform-infrared (FT-IR) spectroscopy is commonly used to obtain the molecular fingerprint of a sample. The method has been used for more than 50 years to study a host of chemical and biomedical problems, and now in many laboratories is often both the first and the last method employed in an analytical procedure.

IR microscopes require reflecting optics in order to avoid wide-region achromatism. Such microscopes were first built by C. R. Burch in 1947 and manufactured by Perkin-Elmer at an early stage. IR microscopes have become very widely used since the development of FT-IR. They are applied to obtain spectra of very small samples or of small parts of larger samples.

The biological and biomedical applications of FT-IR spectroscopy started with the analysis of simple biomolecules. Later, the availability of Fourier transform methods running on desktop computers and more sensitive detectors and microscopy setups made possible the investigation of more complex biosystems. The increase of possible applications deeply transformed the community of biospectroscopists, stimulated by modern chemical and statistical approaches (Fernandez et al., 2005; Petibois and Deleris, 2006). The latter have been introduced to facilitate the analysis of spectral data as well as the classification of IR spectra representing different components in a biosample, for instance, different cells within a tissue biopsy. Due to intrinsic complexity and dishomogeneity, many absorption modes from different molecules overlap, making the classical approach useless to perform stringent molecular analysis in biomedical samples, except for a few notable cases.

In recent years, applications of microspectroscopic techniques in biological and biomedical investigations have increased a great deal, and particularly clinical investigations related to malignancy and cancer detection by microspectroscopic means have attracted attention from many researchers from clinical and nonclinical fields. Research has been carried out on a number of natural tissues using spectroscopic techniques, including FT-IR microspectroscopy. All these techniques are relatively simple, reproducible, and nondestructive and only small amounts of material are required. In addition, these techniques require minimum sample preparation and provide molecular-level information, allowing investigation of functional groups, bonding types, and moleculat conformations.

An important aspect in infrared spectroscopy is the identification of spectral patterns, e.g., spectral bands that are characteristic for certain sample constituents (Levin and Bhargava, 2005; Salzer at al., 2000). Based on these specific marker bands the distribution of a chemical entity can be investigated. However, chemometrical analysis of the data is also used to extract information from the huge amount of infrared data.

The objective of this article is to review new developments in applications of FT-IR microspectroscopy in biomedical investigations, covering the period between 2008 and 2013. Prior to a review on this subject, it is useful to give a short introduction to the concept of the FT-IR microscopy and imaging followed by discussion of the quantitative and qualitative biomedical investigations of the technique.

INFRARED AND IMAGING THEORY

As it is known, FT-IR spectroscopy is based on the absorption of infrared light by vibrational transitions in covalent bonds. The IR spectrum of complex biological samples contains all the contributions of different vibrating molecules such as proteins, lipids, and nucleic acids, depending on the natural composition of probed samples (Petibois and Deleris, 2006).

The mid-IR (MIR) range contains bands due to vibrations of the major classes of biological molecules, such as carbohydrates, organophosphates, phospholipids, and proteins, and provides considerable information about the structures and relative concentrations of these molecules within a biological sample (Miller and Dumas, 2006). The mapping of an individual formalin-fixed and air-dried cell was carried out by *synchrotron radiation* (SR) microspectroscopy FT-IR. The selected area was raster-scanned by collecting a series of spectra from a 18 $\times$ 15 point grid (10 μm $\times$ 10 μm area, 2 μm step, 256 scans within the interval 4000–600 cm^{-1}, scanner velocity 20 kHz, spectral sensitivity 8 cm^{-1}, one background area every five sample spectra) on a single-channel 100 μm $\times$ 100 μm mercury cadmium tellurium (MCT) detector (Miller and Dumas, 2006). The spectra were assembled and integrals were calculated within the interval of wavenumbers between 3000 and 2800 cm^{-1} (symmetric and antisymmetric stretching of CH_3 and CH_2 groups in lipid molecules) and between 1690 and 1480 cm^{-1} (amide I and amide II modes in proteins), allowing obtaining the two-dimensional distributions of lipid and proteins, respectively. The intensity scale of false colors extends from the lowest absorbance (blue) to the highest absorbance (red-violet) values in the two-dimensional contour maps superimposed on the underlying object. False color images and/or maps of the content of biological molecules are generated by integrating the intensity or area of one or more of these bands, thereby illustrating the distribution of these biochemicals (Figure 1).

In mapping experiments, point-by-point spectra are measuring sequentially from a defined region in the sample as it is scanned, whereas in imaging experiments, multiple spectra are recorded simultaneously at different points using a focal plane

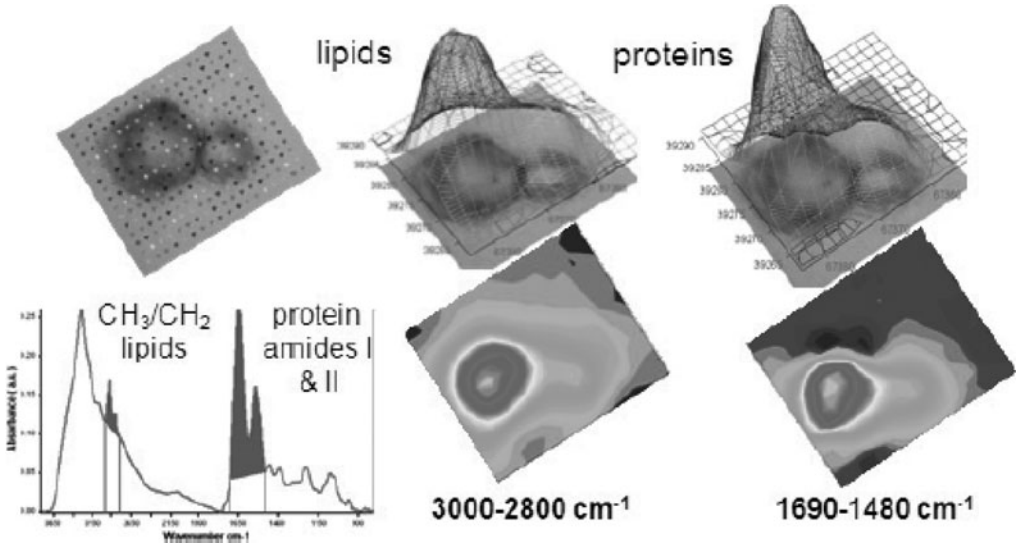

FIG. 1. Fourier transform-infrared microspectroscopy on individual cells.

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array (FPA) detector in FT-IR spectroscopy (as can be seen in Figure 2). At each grid position of the focal plane array detector, a spectrum (e.g., that in the middle of the figure) is recorded that corresponds to a particular area of IR irradiation on the sample.

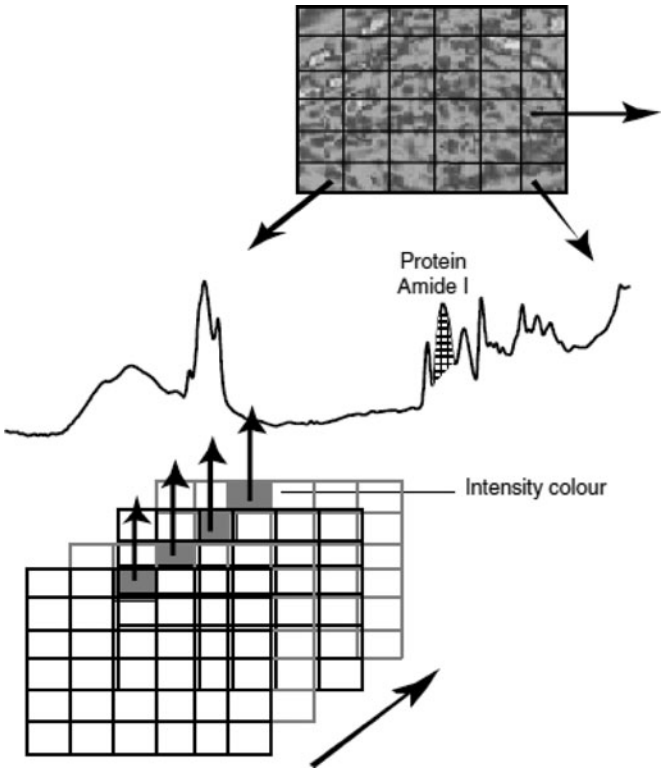

FIG. 2. Focal plane array FT-IR imaging technique showing multiple grids.

A series of false color grids, shown at the bottom of the figure, can be constructed based on the intensity, area, or relative diagnostic band ratios, resulting in a functional group map showing the distribution of specific band(s) of interest on the sample. An example of such a false color image is given at the top of Figure 2; it corresponds to the grid below where the protein amide I band has been integrated, as shown in the spectrum. Modern FT-IR instruments have the capabilities of imaging relatively large areas (macro-imaging, 2×2 mm^2), which can then be used to find areas of interest for high-spatial-resolution (5–10 μm) imaging of areas of around 170×170 μm^2. Even larger areas can be imaged by producing a mosaic that stitches together a number of areas (Carter et al., 2009).

Imaging methods developed during the past decade may represent the most significant advances in the application of vibrational spectroscopies. By the word *imaging* we refer to the large set of computer-based techniques to visually represent an object for different purposes, from the simple collection of data to the characterization of a surface or a material to the the study of complex dynamic phenomena to medical diagnostics. When imaging is performed by a spectroscopic method, the small differences in the composition, typically associated with small differences among spectra collected in different spatial locations, may significantly enhance experimental capabilities and easily highlight chemical or molecular components, and the morphology and/or the texture of a sample (Marcelli et al., 2012).

FT-IR spectral imaging techniques maintain all the advantages of FT-IR spectroscopy: energy calibration, high spectral resolution, and high throughput. FPA (focal plane arrays) add a multichannel advantage and the highest time resolution, which could be overcome only by dispersive systems coupled to array detectors (Snively et al., 2004).

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FT-IR spectroscopy is a powerful tool for the simultaneous analysis of a diverse range of biomolecules within intact cells, in vitro. This optically based, bioanalytical technique measures the transitions in vibrational modes (mainly stretching and bending) of the functional groups of biomolecules as a result of absorption and subsequent excitation by low-energy IR photons (0.05–0.5 eV). The functional group vibrations are representative of cellular constituents such as lipids, proteins, carbohydrates, and a variety of phosphorylated molecules, which include nucleic acids. The resulting IR spectrum of the cell comprises a series of peaks as a function of the wavenumber that depict the state of chemical bonding, which includes intra- and intermolecular hydrogen bonding, van der Waals interactions, steric factors, and inductive or resonance effects.

Infrared spectroscopic imaging can be performed in transmission, reflection, transflection, and attenuated total reflection (ATR) mode. It should also be mentioned that the introduction of indium antimonide focal plane array (InSb FPA) detectors allowed spectroscopic imaging to be performed in the near-infrared region, which is sometimes also called "infrared imaging." In contrast to other imaging modalities, such as visible microscopy, fluorescence microscopy, and magnetic resonance tomography imaging, which result in only one data point or a few data points per image, a FT-IR spectroscopic image generally has hundreds of data points per image pixel. This large data set provides a world of information, which has to be selectively reduced. FT-IR spectroscopic imaging allows one to record chemical images at a spatial resolution close to the diffraction limit. The majority of the applications are in biochemical and medical areas. The way from the raw spectroscopic data set to a colorful image requires data processing and multivariate data analysis techniques. Data preprocessing is necessary to compensate for variations in the sample thickness or so-called dispersion artifacts due to variations in the sample refractive index (Steiner and Koch, 2009). FT-IR spectroscopy is a relatively new method that has received great attention as a new field of analytical chemistry. The greatest benefit of this technique lies in the high molecular sensitivity combined with a spatial resolution down to a few micrometers. Another advantage is the ability to probe samples under native conditions, which allows new insights into samples without the need for fixation, stains, or an additional marker.

A number of recent reviews have high lighted various aspects of vibrational spectroscopic mapping and the imaging of cells and tissues, as well as the use of complementary microscopic imaging/mapping (Carter et al., 2009; Eichart et al., 2007; Srinivasan and Bhargava, 2007).

APPLICATIONS

A wide range of biomedical investigations have been covered by FT-IR microspectroscopy analysis. These investigations include: articular cartilage, all types of colon tissue, cancer detection, living cells, glucose measurement, histopathology, oxygen tissue saturation, prostatic calculi screening, skin diffusion, urinary crystals, and other.

Colon tissue constitutes a valid model for the comparative analysis of soft tissue by Raman and FT-IR imaging because it contains four major tissue types: muscle tissue, connective tissue, epithelium, and nerve cells (Krafft et al., 2008). Raman microscopic images were recorded in the mapping mode using 785 nm laser excitation and a step size of 10 μm, while FT-IR microscopic images that were composed of 4, 8, and 9 individual images of 4096 spectra each were recorded from the same regions using a FT-IR spectrometer coupled to a microscope with a focal plane array detector.

Cancer is a multistep process resulting from the accumulation of irreversible and transmittable genetic aberrations together with the concurrent presence of epigenetic alterations in susceptible cells (Bellisola and Sono, 2012; Straton, 2011). Diagnosis of cancer is one of the most important avenues of medical research and has become the main aim of biomedical investigations.

A nondestructive method employing FT-IR microspectroscopy coupled with ATR objective for the analysis of histopathological specimens was described by Rehman et al. (2010). Malignant breast tissue specimens were analyzed to demonstrate the hypothesis that chemical changes taking place in biological tissue can be reliably and reproducibly identified. FT-IR analysis of tissue sections provided distinct spectra that can be used to distinguish between the nuclear grades of DCIS (ductal carcinoma in situ) and IDC (invasive ductal carcinoma) of the breast. It was concluded that FT-IR could objectively and reproducibly discriminate between DCIS and IDC grades without sample destruction, and, in the future, applications of FT-IR approaches should become feasible in the nondestructive express classification of grades and diagnosis of breast carcinoma.

Another simple and rapid method for the detection of breast cancer with IR-spectroscopy was developed (Backhaus et al., 2010). The method needs only 1 μL of a serum sample. The serum sample is dried on a suitable sample carrier such as a Si-plate. After drying the IR spectrum is measured. Every disease leaves a typical fingerprint in the IR spectrum of serum. This typical fingerprint can be used to identify different patient groups. The identification system can be trained by classification methods, using two independent classification methods, cluster analysis and artificial neural networks.

FT-IR microspectroscopy is widely perceived as a future technology for cancer detection and grading. Malignant melanoma, an aggresive skin cancer, is accessible to noninvasive IR radiation–based surface probes for its identification and grading. The article discusses the differences in the IR spectra of melanoma tissues and the surrounding epidermis in skin biopsies with the main objective of identifying diagnostic parameters and suitable methods of computational/statistical analysis (Hammody et al., 2008).

Congenital cystic adenomatoid malformations (CCAMs) are benign masses of nonfunctional lung tissue developing from an overgrowth of the terminal bronchioles with subsequent

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suppression of alveolar growth. For the first time CCAMs have been studied by Raman mapping and FT-IR imaging (Krafft et al., 2008). The authors discuss how the combined application of Raman mapping and FT-IR imaging might improve the differential diagnosis of lung malformations and how both modalities might be applied to other bioanalytical and biomedical problems in the future.

FT-IR spectroscopy is widely accepted nowadays as a technique with high potential for diagnosis of cancerous tissues. Another article presents an example of the investigation of peritoneal metastases by FT-IR microimaging (Untereiner et al., 2009). Peritoneal malignancies are generally secondary localizations of primary visceral cancers such as ovarian, stomach, or colon cancers. Microimaging also permits revelation of the heterogeneity of the tissue; it was possible to localize precisely the cancerous areas and to distinguish, on the basis of their spectral signatures, the peritumoral neighboring connective tissue close to the carcinomatous areas from the connective tissue distant from the cancerous area. These spectral differences could be useful as complementary information to study molecular changes associated with the malignancy.

Mid-IR FT-IR microscopy is a suitable and objective analytical method to investigate the chemical composition of urinary crystals, which can be distinguished from insoluble drug metabolites also excreted within urine. Kidney stones can be a source of considerable morbidity for pregnant women. There are many articles confirming that different stone compositions predominate for different age and sex cohorts (Ross et al., 2008). A comparative study of two FT-IR techniques was applied to urinary stones analysis: single reflection diamond attenuated total reflection (ATR) and KBr pellet transmission (KPT) (Benramdane et al., 2008). The two methods allow the identification of all stone components. The ATR technique is more useful and rapid in identifying the species without sample pretreatment. Nevertheless, KPT is more appropriate for component determination in urinary stones. The stone constituents were first identified by ATR, and in a second step, the proportion of each species present in the stone was determined by KPT in a whole stone mixture.

Distribution of chemical components in mixed oxalatic, phosphatic, and uratic urinary stones was analyzed by means of infrared microspectroscopy (Pucetaite et al., 2013). The specular reflection from the surface of the cross-sectioned stones was used for infrared spectroscopic imaging. It was found that the reflection spectra consist of specular and diffuse reflection components and the former exhibits *Reststrahlen* bands, which resemble first derivative of the spectral bands in the absorption spectrum. In order to use the measured spectra for chemical imaging they were corrected for asymmetry by subtracting the diffuse reflection component and applying the *Kramers-Kronig* transform to the specular reflection component.

An important study was conducted to investigate the molecular changes associated with glioma tissue using FT-IR microspectroscopic imaging (Beljebbar et al., 2008). Structural changes were mainly related to quantitative and qualitative changes in lipid content, protein, and nucleic acids, which can be used as spectroscopic markers for pathology, and a model was elaborated to quantify these chemical changes. This approach could, in the future, contribute to enhancing diagnostic accuracy, improving the grading and prognosis, and playing a vital role in therapeutic strategy and monitoring.

Infrared spectroscopy was applied also to study changes in solid teeth tissues of persons exposed to low (0.12–0.2 Gy) and high (0.5–1.7 Gy) doses of ionizing radiation during their work in the Cernobyl zone after the accident (Darchuk et al., 2008). Changes in the organic and inorganic matrix of teeth were noted for both high and low level radiation doses. The results demonstrated that the pathological effect of radiation touches upon all dental tissue and obviously all bone systems of irradiated people.

FT-IR imaging was used to investigate amide anisotropies at different surfaces of a three-dimensional cartilage or tendon block (Ramakrishnan et al., 2008). The anisotropies of amide I and amide II components were studied in order to probe the orientation of the collagen fibrils at different tissue surfaces.

Articular cartilage (AC) exhibits specific zonal structure that follows the organization of the collagen network and concentration of tissue constituents (Kobrina et al., 2012). The aim of the study was to investigate the potential of unsupervised clustering analysis applied to FT-IR microspectroscopy to detect depth-dependent structural and compositional differences in intact AC. Seven rabbit and eight bovine intact patellae AC samples were imaged using FT-IR microspectroscopy, and normalized raw spectra were clustered using the fuzzy C-means algorithm. Differences in mean spectra of clusters were investigated by quantitative estimation of collagen and proteoglycan (PG) contents, as well as by careful visual investigation of locations of spectral changes. Clustering revealed the typical layered structure of AC in both species. FT-IR microspectroscopy in combination with cluster analysis allowed detailed examination of spatial changes in AC.

Brain tissue is characterized by high lipid content (Dreissig et al., 2009). Its content decreases and the lipid composition changes during use of the existing diagnostic tools to determine tumor type and grade. A quantification model for the main lipids of porcine brain tissue on partial least squares regression was developed.

FT-IR spectra of four major lipoprotein classes, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and two classes of high density lipoprotein (HDL_2 and HDL_3), were analyzed to obtain their detailed spectral characterization (Krilov et al., 2009). Information about the protein domain of a particle was obtained from analysis of the amine I band. Characterization of spectral details in the FT-IR spectrum of natural lipoprotein is necessary to study the influence of external compounds on its structure.

There is a growing interest for screening antitumor drugs for their mechanism of action on cancer cells. Screening for

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"modes of action" presents a technical challenge that is beyond the practical capability of conventional methods used in cellular or molecular biology. The infrared spectrum of cells exposed to anticancer drugs could offer an opportunity to obtain a fingerprint of all molecules present in the cells and with a high sensitivity observe the metabolic changes induced by the drugs. The spectra could thus be used as signatures to provide evidence of global effects of a compound on all of the cell constituents.

In the 1980s, it was demonstrated that the FT-IR spectrum of bacteria provides a unique fingerprint that allows the identification of bacteria species (Naumann et al., 1988, 1991). In the 1990s, it was shown that tumor cells can be distinguished from their normal counterparts by observation of their FT-IR spectra (Cohenford and Rigas, 1998; Wood et al., 1998). Later, with advances in instrumentation and in multivariate statistics, tumor cell lines with different biological behavior could be separated. For instance, the aggressiveness of different glioma cell lines was successfully predicted from their FT-IR spectra when no molecular biology means were available for this purpose (Gaigneaux et al., 2004). Similarly, the distinction of premalignant from malignant cells also became available using specific parameters obtained from FT-IR spectra, making it a rapid and reagent-free method (Sahu and Mordechai, 2005). Gasper et al.(2009) suggested that the FT-IR spectrum of cells exposed to anticancer drugs could offer a unique opportunity to obtain a fingerprint of all molecules present in the cells and to observe, with high sensitivity, the metabolic changes induced by potential anticancer drugs. Zwielly and coworkers (2009) investigated the ability of FT-IR microscopy to define spectral changes between drug-sensitive and drug-resistant human melanoma cells. These results strongly support the potential of developing FT-IR microspectroscopy as a simple, reagent-free method for the identification of drug-resistant cells. Rapid detection of tumors resistant to a particular drug should contribute to the ability of the physician to choose an effective treatment protocol.

FT-IR imaging and microspectroscopy have been extensively applied in the identification and investigation of both healthy and diseased tissues. FT-IR can be used to determine the biodistribution of several molecules of interest (carbohydrates, lipids, proteins) for tissue analysis, without the need for prior staining of these tissues (Yang et al., 2011). Molecular structure data, such as protein secondary structure and collagen triple helix exhibits, can also be obtained from the same analysis. Thus, FT-IR imaging has been proposed as a new tool integrating both molecular and histopathological assessment to investigate the degree of pathological changes in tissues.

CONCLUSIONS

Throughout this article we have attempted to demonstrate the broad applicability and versatility of IR microspectroscopy in many disciplines of biomedical investigations and over enormous variations in physical scale. In spite of the reduced interest for the application of vibrational spectroscopy in some research areas, in principle there is growing interest and general enthusiasm about the possibility of applying vibrational microspectroscopy to biomedical research. Relevant developments have certainly occurred in this field during the past decade. However, no preclinical or clinical trials of FT-IR spectromicroscopy applications have been performed to date. This poor consideration of IR techniques is most probably due to the limited background of clinicians and biomedical researchers with the concepts and methods of IR spectromicrosopy as well as to the delay in translating the results of research with IR sources into validated applications easy to use in conventional biomedical laboratories and for clinical diagnoses/prognoses. These results suggest that IR spectroscopy in combination with intelligent mathematical evaluation tools can be a good tool for the diagnosis of all types of cancer. These approaches could, in the future, contribute to enhancing diagnostic accuracy, improvig grading and prognosis, and playing a vital role in therapeutic strategy and monitoring.

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