IBOM MEDICAL JOURNAL
Vol.15 No.1 January, 2022. Pages 54 - 61
www.ibommedicaljournal.org

Vibrational spectroscopy: A novel technique changing the face of clinical diagnosis

Effiong BO

Department of Medical Biochemistry, Faculty of Basic Medical Sciences, University of Uyo, Akwa Ibom State, Nigeria.

Abstract

The application of spectroscopy to provide insight to biological questions bothering on pathology in plant and animal tissues is a rapidly growing field known as biospectroscopy. Biospectroscopy has significantly changed the face of biological and toxicological research, from clinical diagnosis to environmental toxicology. Techniques involved have become potential tools for non-invasive optical tissue diagnosis and have been applied to study a wide variety of pathologic states. Cellular biomolecules absorb the mid-IR (λ = 2 -20 μm) via vibrational transitions that are derived from individual chemical bonds. Within the mid-IR range, 1800 – 900 cm^{-1} is regarded as the biochemical-cell fingerprint region, because it contains the fundamental vibrational modes of the structures present in biological specimens. The vibrational modes of key chemical bonds may thus be exploited to understand intracellular mechanisms as the biochemical-cell fingerprint of the material under study, is produced with direct association between peaks and chemical bonds. What makes it important for toxicological research in developing countries? **1)** The relative simplicity of the technique with regards to the equipment **2)** Ease of sample preparation/handling **3)** Low cost and potentially reagent free. This technique will prove particularly useful in situations where a broad spectrum, exploratory Tox-Screen is required to observe the effect of a compound on a variety of biomolecules.

Keywords: Clinical spectroscopy, Biospectroscopy, infrared spectroscopy, biochemical cell fingerprint, Tox-Screen

Introduction

Vibrational Spectroscopy has gained significant attention in biological research in the last twenty years. Vibrational spectroscopic techniques have become potential tools for non-invasive optical tissue diagnosis and have been applied to study a wide variety of pathologic states.[1,2] The application of different forms of spectroscopy to understand biological phenomena or provide answers to biological questions, is defined as biospectroscopy. The field is relatively novel and has expanded over the last two decades. Biospectroscopy has been applied within a diverse range of research investigations. The biggest advances have been made especially in clinical diagnosis and in more recent times, environmental monitoring/toxicology. It has also been applied to a wide variety of sample types including human, animal and plant tissue.

The principle of biospectroscopy is based on the knowledge that biomolecules that contain chemical bonds with an electric dipole moment are infrared (IR) active.[3] Cellular biomolecules absorb the mid-IR (λ = 2 -20 μm) via vibrational transitions that are derived from individual chemical bonds.[4] Within the mid-IR range, 1800 – 900cm^{-1} is regarded as the biochemical-cell fingerprint region, because it contains the fundamental vibrational modes of the structures present in biological specimens.[5] The vibrational modes of key chemical bonds may thus be exploited to understand intracellular mechanisms as the biochemical-cell fingerprint of the material

Corresponding Author: Blessing O. Effiong, PhD

Department of Medical Biochemistry,
University of Uyo,
Akwa Ibom State, Nigeria
Email: blessingobinaju@uniuyo.edu.ng, Phone: +234(0)38622159

under study, is produced with direct association between peaks and chemical bonds.[6]

Within the West African region and with specific reference to Nigeria, the technique could greatly advance the scope of research currently being carried out in various fields. However, this review focuses on the applications of vibrational spectroscopy within biological and clinical sciences. It aims to introduce the basic techniques of biospectroscopy e.g., Fourier-transform IR (FTIR), attenuated total reflection FTIR (ATR-FTIR) spectroscopy to researchers within the region.

Biospectroscopy Approaches

Biospectroscopy is comprised of a number of approaches including Fourier-transform IR (FTIR), attenuated total reflection FTIR (ATR-FTIR), Raman and photothermal microspectroscopy (PTMS). The application of Mid-IR spectroscopy to interrogate cellular structures, considers most important, the spectral regions representative of double bonds (2000 – 1500cm^{-1}) associated with C = C, C – N and C = O and the fingerprint region (1500 – 600cm^{-1}) which identifies bending and skeletal fingerprint vibrations. Based on these, the biochemical cell fingerprint especially, provides a representation of the structure and function of the interrogated cellular specimen, using chemical bond vibrations.[3]

Fourier Transform Infrared Spectroscopy

Infrared (IR) rays as discovered by William Herschel in 1800, are absorbed by matter in the form of several bands localized in discrete frequency intervals. In recent times, basis for the widespread use of IR spectroscopy arises from the observation that many chemical groups such as C = O, absorb in a relatively narrow frequency range, irrespective of the nature of the other functional groups present. Within this frequency range, the observed frequency can be correlated to specific chemical structures. Thus, the spectral pattern may be likened to a "molecular fingerprint" particularly because similar molecules may have significantly different IR spectra, especially in the region below 1500 cm^{-1}.[7]

The IR spectra results from transitions between quantized vibrational energy states with the usual range between 4000 cm^{-1} at the high frequency range and 625cm^{-1} at the low frequency end.[8]

The introduction of interferometers, has improved the time required to acquire spectra seeing that light covering the whole frequency range, typically 5000 – 400cm^{-1}, can be split into two beams, where either one beam is passed through the sample or both beams are passed, but one beam is made to traverse a much longer path than the other. The recombination of the two beams produces an interference pattern that is the sum of all the interference patterns created by each wavelength in the beam. By systematically changing the difference in the two paths, the interference patterns change to produce a detected signal varying with optical path difference. This pattern is the interferogram and although it looks nothing like a spectrum, Fourier transform of the interferogram using a computer built into the machine, converts it into a plot of absorption against wavenumber which resembles the usual spectrum obtained by the traditional method.[9] Thus, the FTIR method of acquiring spectra is faster and provides a higher signal to noise ratio (SNR).

Following IR exposure and as the movements occur, chemical bonds within biological samples absorb IR at wavelengths correlating to the energy levels. These absorptions occur due to vibrational (bending, stretching, rocking, wagging or scissoring) movements of the chemical bonds present. Therefore IR spectroscopy can be described as the measure of this absorption, producing spectra with peaks representing the chemical bonds present in the given sample.[5]

Until date, mid-IR (MIR) spectroscopy has been more widely used in studies with biological samples. That said, near-IR (NIR, 700 - 2500 nm) spectroscopy is also commonly used. The NIR spectroscopy is a technique based on the overtones of the fundamental vibrational modes observed in the mid-IR region, and provides fast data acquisition combined with no reagent requirements and very minimum sample preparation. These characteristics make NIR spectroscopy a reliable and non-invasive approach with potential especially for rapid diagnosis of say viral/parasitic diseases and infections. Notably, when working with NIR spectroscopy in the investigation of biological samples, the most useful region is between 650 nm and 1000 nm. This region is regarded as the "optical window" due to the great absorption of haemoglobin and water occurring below 650 nm

and above 1000 nm, respectively. This absorption characteristic hinders other signals in these regions. Therefore, as per clinical diagnostic application, the use of the 650 nm - 1000 nm region is more suitable especially to analyse biochemical alterations in infectious disease studies.[10,11]

FTIR Instrumentation

The three major IR-spectroscopic sampling modes by which spectra acquisition may be carried out are: 1) Transmission, 2) Transflection and 3) Attenuated total reflection (ATR). In transmission mode, the IR beam is directed through a sample and collected by a condenser whereas, in transflection mode, the beam is directed through the sample, reflects off an IR-reflective surface [such as that found on low-emissivity (Low-E) slides] and travels back through the sample to the detector. With both measurements, the sample thickness is an important criterion, as extremely thick samples will attenuate the IR beam beyond the range where absorption is proportional to chemical concentration and very thin samples will result in low absorption where acquired spectra signal is flooded with noise.[5]

The ATR has grown into the most widely practiced technique in IR spectrometry especially because, the technique involved requires little or no sample preparation and consistent results can be obtained with relatively little care or expertise. The ATR mode of spectra acquisition involves passing the IR beam through an internal reflection element [(IRE) usually an IR-transparent element)] with a high refractive index [e.g. Zinc Selenide (ZnSe), type II diamond or Germanium (Ge)].[4] When the IRE is placed in contact with the sample and the beam passed through it, the beam is totally internally reflected, generating an evanescent wave which penetrates a few μm beyond the element into the sample.[5] The depth of penetration varies from a fraction of a wavelength up to several, depending on the index of refraction of the element and the angle of the incident radiation with respect to the interface between sample and element. It is also wavelength-dependent, increasing with increasing wavelength and has the consequence that if the sample selectively absorbs certain wavelength components of the evanescent radiation, then attenuation of the reflected beam occurs preferentially at the wavelength of absorbance bands.[4]

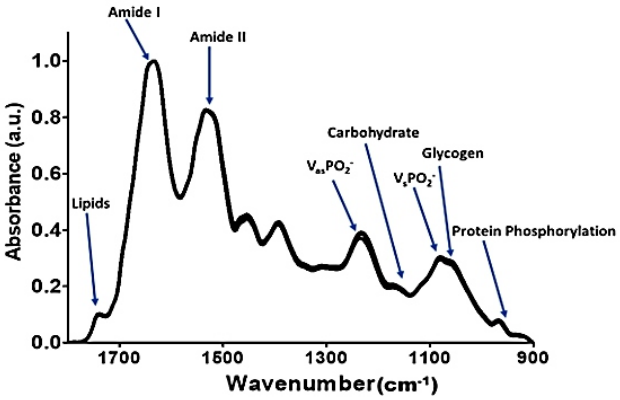

Fig. 1: A representation of an Infrared (IR) molecular/biochemical cell fingerprint, showing the peaks assignment to various biochemical molecules

Attenuated total reflection can be said to be the most versatile of all IR sampling techniques because, it requires very little sampling preparation and can be used on samples of almost all morphologies, while often maintaining the structural integrity of the sample. ATR is in large part a surface technique and the interrogation of sample is largely limited to the depth of penetration of the measurement.[8] Major advantages of the ATR-FTIR technique include 1) the ability to cover a relatively large sampling area depending on the crystal in use. 2) Dispersion effects are weaker in ATR-FTIR spectroscopy and therefore fewer spectral artefacts attributed to light scattering are generated during the acquisition of spectral measurements from biological samples.[12]

A typical spectral fingerprint of a biological sample (Fig. 1) consist of wavenumber absorbance intensities which are arbitrarily split according to the different biomolecular constituents present within a typical biological sample.[5,13] Lipids are the major contributors to peaks located at 1740cm^{-1} (C = O stretching). Proteins contribute to the Amide I peak represented at 1650cm^{-1} (80% C = O stretching, 10% C – N stretching and 10% N – H bending) and Amide II peak at 1540cm^{-1} (60% N – H bending and 40% C – N stretching). The PO^{2-} stretching vibrations of Deoxyribonucleic acids (DNA) are located at 1080 cm^{-1} (Symmetric $VsPO^{2-}$) and 1225cm^{-1} (Asymmetric $VasPO^{2-}$).[12] The position and intensities of the absorption bands are determined predominantly by the molecular or conformational structure of the various biomolecules within the sample; where intensity of peaks likely reflect underlying cellular activity and

a horizontal shift in Amide I band may point to α-helix to β-pleated sheet protein conformational changes.[14-18] The 1800 – 900cm^{-1} is regarded as the molecular/biochemical cell fingerprint region because it contains the fundamental vibrational modes of the structures present in biological specimens.[5]

Infrared techniques have been applied to several areas of research, including cancer diagnostics/screening[4,19.20] and to a wide variety of sample types.[21-25] Baker et al., (2014) documents that by using IR spectroscopy, either as an imaging tool or by classifying spectral categories, it has been possible to distinguish between benign and malignant tumours in tissue samples. A comprehensive review on the wide range of biological tissues which have been successfully interrogated using FTIR spectroscopy, exists[2] and more recently, documentation of several studies which employed the use of vibrational spectroscopy especially in cancer investigations ranging from brain, breast, lung, ovary, endometrium, prostate to skin malignancies and other non-cancerous diseases such as malaria, prenatal disorders, diabetes and Thalassemia.[26]

The use of biofluids such as urine, saliva, serum or whole blood, is desirable in a clinical setting as samples are obtained rapidly and relatively non-invasively. Readily accessible body fluids such as blood plasma/serum, saliva, or Urine have been considered ideal for diagnosing, screening or monitoring progression/regression of various diseases.[12] By using methods such as FTIR or ATR-FTIR for the study of biofluids, a spectral fingerprint of the biofluid can be obtained, allowing subsequent classification of spectra from different categories with computational methods as well as the possible identification of biomarkers.[13] Therefore, the interrogation of samples particularly with FTIR and its hyphenated approaches, allows for the generation of a spectral fingerprint which subsequently aids the discrimination of cell populations, possible alteration(s) in patients sample since the last sample collection/analysis and more importantly, the identification of possible biomarkers responsible for the observed alteration(s) in the sample.[27]

Methods within biospectroscopy are quite easy to use, although they require basic knowledge of computational analysis. The availability of easy to use benchtop FTIR spectroscopy equipment (Fig. 2 & 3) from various manufacturers (Table 1), as well as protocols[3,28] which could be optimized to suit the researcher's needs, and the vast amount of research in this field, make the technique a desirable one especially in a region/country such as West Africa/Nigeria with unique infrastructural challenges which may inhibit the use of technology equipment requiring specialized atmospheric, handling and operating conditions e.g. uninterrupted power supply and/or room temperature conditions. The elimination of the aforementioned also has implications on the operating/running costs of research on researchers.

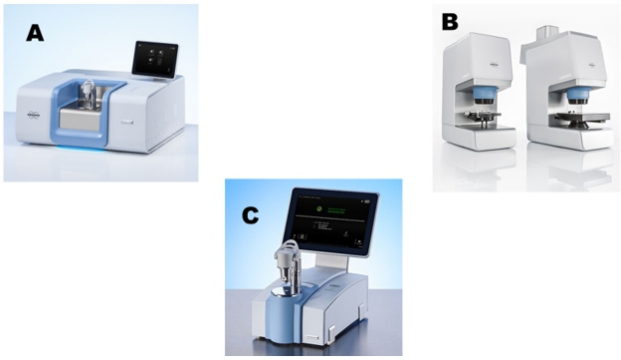

Fig. 2: A range of Benchtop Fourier Transform Infrared (FTIR) Systems: INVENIO FTIR Spectrometer (A), LUMOS II FTIR Imaging Microscope (B), ALPHA II FTIR Spectrometer (C). These are compact and could be suitable for use within developing countries. ***Images obtained with permission from the manufacturer.***

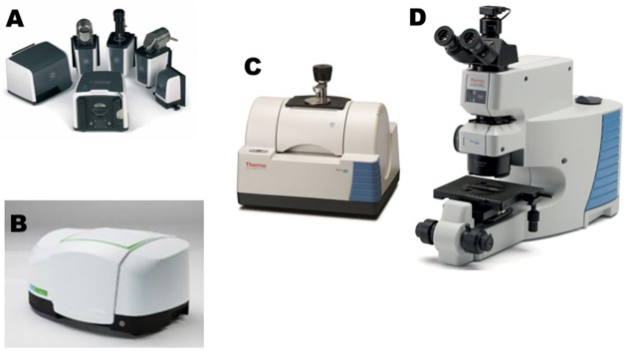

Fig. 3: A range of Benchtop Fourier Transform Infrared (FTIR) Systems; Cary 600 Series FTIR Spectrometer (A), Spectrum Two IR Spectrometer (B), Nicolet iS5 FTIR Spectrometer ©,Nicolet iN5 FTIR Microscope. These are compact and could be suitable for use within developing countries. ***Images obtained with permission from the manufacturer.***

Table 1: List of Instruments with the corresponding manufacturers and data acquisition software. Adapted from Baker et. al., (2014)

Manufacturer	**Instruments**	**Software**
Agilent Technologies	Agilent 670-IR spectrometer Cary 600 series FTIR spectrometers Agilent 600 series FTIR microscope	Resolutions Pro
Bruker Optics	Bruker Tensor 27 spectrometer ALPHA II FT-IR spectrometer HYPERION series FT-IR microscope LUMOS II FT-IR microscope INVENIO FTIR spectrometer	OPUS
JASCO UK	JASCO FTIR-4100 series JASCO FTIR-6000 series IRT-5000 FTIR microscope	Spectra Manager
PerkinElmer	PerkinElmer Frontier Spectrum Two Spotlight FTIR microscope system	Spectrum
Thermo Fisher Scientific	Thermo Nicolet iS50 spectrometer system Thermo Nicolet iN5 FTIR microscope Thermo Nicolet Scientific FTIR 5700 spectrometer with continuum microscope	OMNIC
Shimadzu	IRTracer-100 spectrometer IRAffinity-1S spectrometer	Lab Solutions IR

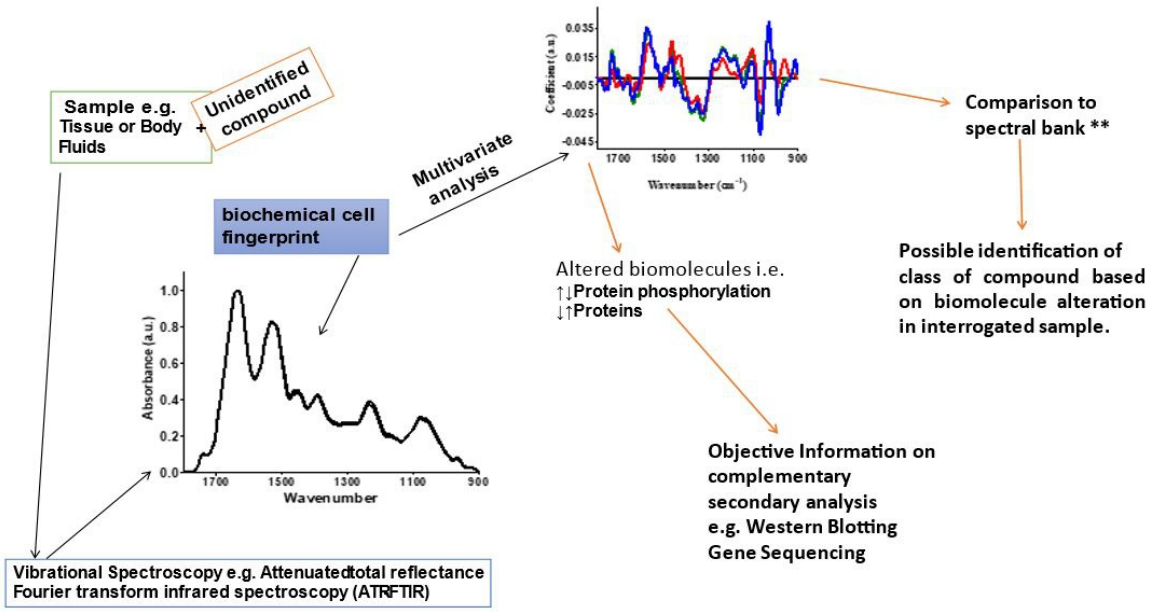

Figure 4: Illustration of the potential of how Vibrational spectroscopy aids the understanding of changes in samples exposed to unidentified compounds or substances. ** Spectral bank would typically contain the interrogated sample type i.e. Liver, collected over time and having been exposed to a wide variety of known and documented substances. A close match of the biochemical cell fingerprint of the sample under investigation would reveal the possible Class of compound and possible idea of key components of its structure.

Data Handling and Processing

Results obtained from biospectroscopy are directly dependent on the data analysis methodology being used. It is therefore necessary to apply chemometric techniques to spectral data obtained from the interrogation of samples in order to obtain reliable and chemically meaningful results.

The overview of basic data handling and processing as regards vibrational spectroscopy follows after the pattern illustrated below:

Spectral Acquisition ⟶ Data Pre-processing ⟶ Chemometric Data Analysis ⟶ Result Visualization ⟶ Interpretation.

Morais et al., (2020) provides a basic tutorial for the multivariate classification for vibrational spectroscopy in biological samples. For spectral acquisition, between 5 – 25-point spectra collection per sample is recommended[13,26] particularly for attenuated total reflection (ATR) – Fourier transform Infrared. It is noted that by increasing the number of spectra replicates, the standard deviation (SD) between measurements is reduced and with heterogeneously distributed samples such as tissues, extra caution is required to ensure that spectra replicates acquired cover the sample surface as uniformly as possible.

Spectral data Pre-processing techniques remove/reduce the contribution of signals that are unrelated to the analyte, target property or to the sample discrimination. The Pre-processing of raw data reduces chemically irrelevant variations with the goal of improving the accuracy and precision of qualitative and quantitative analyses. It is mostly essential for correcting for physical interferences such as light scattering due to different particle size, different sample thickness or different optical paths and random instrument noise.[29]

Pre-processing methods are largely divided into de-noising, Spectral Correction, Normalization and other manipulations. Much often, two or three Pre-processing methods are combined to best achieve the aims of the robustness and accuracy of the subsequent multivariate analyses, as well as increasing the interpretability of the data by correcting issues associated with spectral data acquisition.[13]

The exploratory analysis is an important tool in the data handling process because it provides an initial assessment of the data where the analyst is able to visualize clustering patterns and draw inference related to the nature of the sample outliers and experimental error.[26] Using a common tool such as Principal Component Analysis (PCA), where the original data are reduced into basic principal components (PCs) responsible for most of the variance within the data set, it is possible to determine at first glance, whether there are differences between spectra that is required from different samples and more so, if the variance observed maybe worth investigating. PCA is often the first step of the data analysis, followed by classification, cluster analysis or other multivariate techniques.[29] The main classification techniques used in most biospectroscopy investigations include 1) Linear discriminant analysis (LDA), 2) Quadratic discriminant analysis (QDA), 3) Partial Least Square Discriminant analysis (PLS-LDA), 4) K-nearest neighbours (KNN), 5) Support Vector Machines (SVM) and 6) Artificial Neural Networks (ANN).[26] These various classification techniques all allow for sample discrimination, thus allowing the analyst the ability to distinguish classes of samples on the basis of their spectral features and then make further predictions on the basis of these distinguishing features.

FTIR spectroscopy, particularly ATR-FTIR coupled with computational analysis, can be used as a first step, to explore a sample of interest within molecular research. Hypothetically, within a toxicity study particularly with regards to emerging contaminants (Ecs), the broad-spectrum effect of a substance can be observed in a sample using the various shifts in peak positions, changes in band width as well as the disappearance of peaks or appearance of peaks at specific wavenumbers. These could more effectively inform the researcher of his/her next possible line of investigation e.g. histopathology, DNA/RNA sequencing and profiling, western blotting etc, depending on the areas where the most alteration is observed. Using this technique therefore, it is possible to observe at a glance, the therapeutic or toxic effects of a substance on the various biomolecules contained within a specific sample.

For instance, in the case of exposure to an unidentified substance, as illustrated in Fig. 4, a comparison with database of previously identified contaminants may aid the identification of the

unknown substance or provide an insight regarding its possible structure.30 Obinaju et.al., (2016) has shown the possibility of identifying contaminants and contaminant-induced changes in organisms of unknown origins, based on existing knowledge of IR spectra acquired from organisms with exposure to known compounds.

Conclusion

It is no longer notion that vibrational spectroscopy plays a huge role in diagnostics and clinical research in several parts of the world. Although the vibrational spectra of biological specimens may be complex with subtle distinctions between them, over two decades of research has shown that these differences, however subtle, are more sensitive to the smallest alterations in the biomolecules and the differences between samples. With the advancement in research, protocol/equipment optimization, optimization and standardization of sample prep, including a vast library of already published and accessible literature/studies, there couldn't be a much better time for the continent of Africa including Nigeria, to harness the potential of vibrational spectroscopy as a key strategy to improve clinical research and potentially increase the chances of improving healthcare deliver.

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