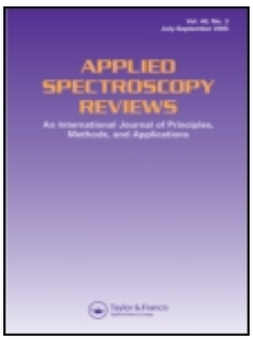

Applied Spectroscopy Reviews

ISSN: 0570-4928 (Print) 1520-569X (Online) Journal homepage: http://www.tandfonline.com/loi/laps20

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To cite this article: Andrei A. Bunaciu, Hassan Y. Aboul-Enein & Şerban Fleschin (2015) Vibrational Spectroscopy in Clinical Analysis, Applied Spectroscopy Reviews, 50:2, 176-191, DOI: 10.1080/05704928.2014.955582

To link to this article: https://doi.org/10.1080/05704928.2014.955582

Accepted author version posted online: 22 Aug 2014.
Published online: 22 Aug 2014.

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Applied Spectroscopy Reviews, 50:176–191, 2015
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ISSN: 0570-4928 print / 1520-569X online
DOI: 10.1080/05704928.2014.955582

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Vibrational Spectroscopy in Clinical Analysis

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Abstract: *Vibrational spectroscopy includes several different techniques, the most important of which are mid-infrared (IR), near-IR, and Raman spectroscopy. Raman and mid-IR spectroscopy are complementary techniques and usually both are required to completely measure the vibrational modes of a molecule. Vibrational spectrometry covers a series of well-established analytical methodologies suitable to be employed for both qualitative and quantitative purposes. In the first part of this review, we will focus on theoretical aspects related to vibrational techniques; in the second part, the most important papers, published during the period 2005–2014, related to clinical analysis performed with vibrational spectroscopy techniques will be critically discussed.*

Keywords: FTIR, Raman spectroscopy, clinical analysis

Introduction

Vibrational spectroscopy includes several different techniques, but the most important techniques are mid-infrared (IR), near-IR (NIR), and Raman spectroscopy. Both mid-IR (MIR) and Raman spectroscopy provide characteristic fundamental vibrations that are employed for the elucidation of molecular structure and are the topic of this review. Near-IR spectroscopy measures the broad overtone and combination bands of some of the fundamental vibrations (only the higher frequency modes) and is an excellent technique for rapid and accurate quantitation.

Vibrational spectroscopy is used to study a very wide range of sample types and can be carried out from a simple identification test to an in-depth, full-spectrum, qualitative and quantitative analysis. Samples may be examined either in bulk or in microscopic amounts over a wide range of temperatures and physical states (e.g., gases, liquids, latexes, powders, films, fibers, or as a surface or embedded layer). Vibrational spectroscopy has a very

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broad range of applications and provides solutions to a host of important and challenging analytical problems.

Raman and mid-IR spectroscopy are complementary techniques and usually both are required to completely measure the vibrational modes of a molecule. Although some vibrations may be active in both Raman and IR, these two forms of spectroscopy arise from different processes and different selection rules. In general, Raman spectroscopy is best for symmetric vibrations of nonpolar groups, whereas IR spectroscopy is best at the asymmetric vibrations of polar groups.

Every molecule has a unique fingerprint of vibrational frequencies, which makes Raman and Fourier transform infrared (FTIR) spectroscopy highly specific techniques for molecular identification. Both techniques can be employed noninvasively, making them ideal for biomedical applications. Raman and FTIR spectroscopy are sometimes referred to as "sister" techniques and provide complementary information about molecules, but they differ in several fundamental ways.

Raman spectroscopy arises from the inelastic scattering of ultraviolet, visible, or near-infrared light when a photon interacts with a molecule. Raman scattering is an inherently weak process and, as such, samples are typically illuminated by laser light. Light scattered by the sample is diffracted into individual wavelengths by a spectrograph and collected by a detector such as a charge-coupled device or complementary metal-oxide semiconductor sensor (1). One disadvantage of Raman spectroscopy in the biomedical arena, however, is its inherently weak signal, which can be overwhelmed by sample fluorescence. Often this is overcome by excitation in the NIR region of the spectrum where biological molecules tend not to fluoresce. Compared to infrared absorption, Raman has the advantage of having only small and easily subtracted water bands. In biological samples Raman spectra often exhibit a number of rather sharp bands, whereas infrared spectra of cells and tissue often show broader spectral features.

FTIR spectroscopy consists of the absorbance of frequencies of light by a molecule that contains the same vibrational frequencies within its molecular bonds. A beam of infrared light is passed through or reflected by a sample. Some light is absorbed by the sample's vibrational frequencies, and the remaining light is transmitted to an interferometer and then collected by a detector, such as a mercury cadmium telluride photoconductive detector or an indium gallium arsenide photodiode detector (2).

Infrared spectroscopy has emerged in recent years as the analytical method of choice in an enormous variety of applications. What brought about this revolution? The clearest advantage is that no specific reagents are required. Automated, repetitive analyses can therefore be carried out at very low cost. The appeal of these factors has spurred the development of a new generation of analytical IR spectrometers that combine high acquisition speed with superb spectral sensitivity.

The use of vibrational spectroscopy and imaging for increased diagnostic accuracy and better treatment can produce improved clinical outcomes and decreased patient morbidity, resulting in an earlier return to an improved quality of life, because we consider that the future development in the use of vibrational techniques will improve diagnostics and characterization of small changes in clinical samples and their relationships with diseases and will prove to be a valuable tool for process analysis, based on the information contained in the NIR, MIR, or Raman spectra.

Determination of clinical parameters in bodily fluids represents a very useful diagnostic tool for different illnesses and a way of monitoring the effect of medical treatments. Therefore, there is an increasing demand for clinical analysis, which obliges hospital laboratories and public health systems to make a large number of determinations and

justifies the use of powerful mechanized commercial systems. These kinds of methods involve the use of high amounts of expensive and specific reagents, which are out of the economical possibilities of many countries. Taking into account the large number of determinations required each day, the need for new and cheap alternative analytical procedures is clear, especially as screening tools, as a feasible indicator in situations where economical resources are limited and point-of-care diagnostic evidence is required.

Vibrational spectroscopy offers complete information on the chemical composition of samples regarding both major and minor compounds, which present many characteristic bands in the studied range. Additionally, the presence of trace compounds can be modelized in some cases through the multivariate treatment of the whole IR or Raman spectra of well-characterized samples based on the influence of molecules at low concentration levels on the size and shape of the bands of major compounds.

Vibrational spectroscopy is an attractive modality for the analysis of biological samples, providing a complete noninvasive acquisition of the biochemical fingerprint of the sample. It has been demonstrated that these data provide the means to assay multiple functional responses of a biological system at a spatial resolution as low as a micrometer within the sample.

The objective of this article is to review new developments in applications of vibrational spectroscopy (Raman and FTIR) in clinical diagnostics, covering the period between 2005 and 2014. Prior to a review on this subject, it is useful to give a short introduction on the concept of the vibrational spectroscopy, followed by discussion of the quantitative and qualitative biomedical investigations of the technique.

Vibrational Spectroscopy—Theoretical Aspects

Molecular vibrations can be excited via two physical mechanisms: the absorption of light quanta and the inelastic scattering of photons (3), as can be seen in Figure 1.

Direct absorption of photons is achieved by irradiation of molecules with polychromatic light that includes photons of energy matching the energy difference between two vibrational energy levels, the initial (ground state) and the final (first excited state) vibrational state. In IR spectroscopy, the vibrational transitions are induced by absorption of light quanta from a continuous light source in the IR spectral region. Vibrational Raman transitions correspond to inelastic scattering (ν_R; thin arrow) of the incident monochromatic light (ν_0), whereas the elastic scattering (ν_0) is represented by the thick arrow.

There are many reasons why scientists want to measure the Raman spectra of compounds. Firstly, many bands that are weak in the IR spectrum are among the strongest bands in the Raman spectrum. Secondly, some Raman bands are found at very characteristic frequencies. Samples for Raman spectrometry can be mounted in standard glass tubes, making sample handling far easier for Raman than for IR spectrometry. Finally, low-frequency bands are far more easily measured by Raman spectrometry than by IR.

The basic phenomena (4) involved in IR and Raman spectroscopies are outlined in Figure 2.

In IR spectroscopy, the sample is irradiated with polychromatic light, and a photon of light is absorbed when the frequency (energy) of the absorbed light matches the energy required for a particular bond to vibrate within the sample. In order for a vibration to be IR active, the molecular dipole moment must change during the vibration. The energy of mid-IR light provides a molecule with sufficient energy to vibrate but not enough energy to result in ionization or to break bonds and, consequently, there is no photodamage to

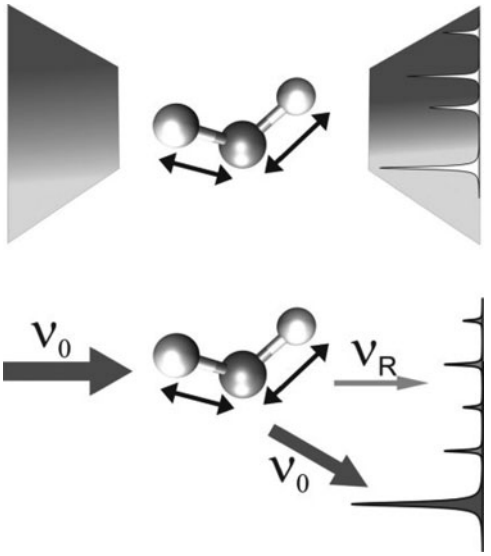

Figure 1. Illustration of the excitation of molecular vibrations in IR (top) and Raman (bottom) spectroscopy.

the sample; local heating has been found to occur when using a synchrotron source, but it was reported to be too small (0.5°C) to be a significant problem. This enables other mapping/imaging spectroscopies to be performed on the same sample after IR imaging or mapping (5). Signal (band) intensities vary with the concentration and the nature of functional groups in the molecule (primary structure) and with its conformation (secondary structure). The latter two factors also dictate the energy of the vibrational spectroscopic bands.

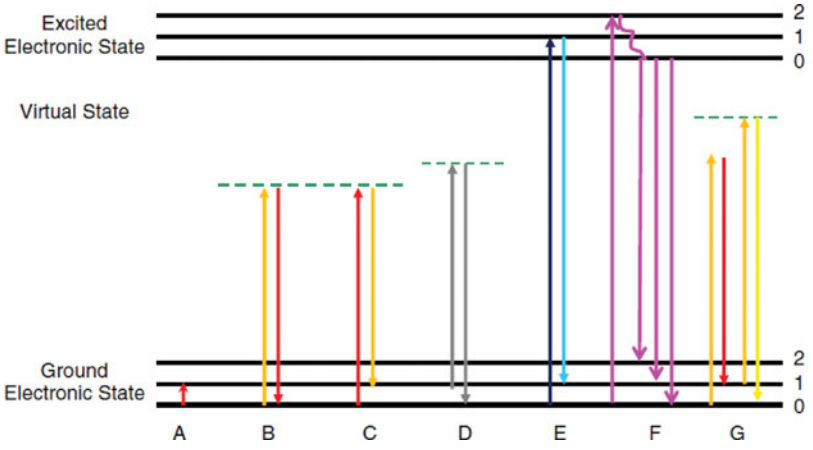

Figure 2. Schematic representations of (A) infrared absorption, (B) Rayleigh scattering, (C) Stokes Raman scattering, (D) anti-Stokes Raman scattering, (E) resonance Raman scattering, (F) fluorescence, and (G) coherent anti-Stokes Raman spectroscopy. The numbers 0–2 represent different vibrational levels (ν_{vib}) within each electronic state.

In Raman spectroscopy, the sample is irradiated with monochromatic light—that is, ultraviolet (UV), visible, or NIR excitation—and the photons are either inelastically or elastically scattered. The inelastically scattered light, known as Raman scatter, has lost (Stokes) or gained (anti-Stokes) energy during this interaction, and the emitted photon contains information about the molecular structure of the sample. The elastically scattered light has the same energy as the incident laser light and is called Rayleigh scatter. Raman scattering is a very low-probability process and relies on lasers to produce enough photons to observe the weak signals. Under ambient conditions, the Boltzmann distribution of vibrational states has most molecules in their ground vibrational states. The Raman-scattered photons from the ground vibrational state have a lower energy than the incident photons, with energy differences that correspond to those of vibrational modes (Stokes scattering). Anti-Stokes Raman scattering occurs from vibrationally excited states that are thermally populated according to a Boltzmann distribution and lead to scattered photons that return the energy to the ground vibrational state. Because the thermal population of vibrational excited states is low under ambient conditions, anti-Stokes Raman scattering results in much weaker bands than does Stokes scattering. Hence, Stokes scattering is used in most mapping experiments (5).

Figure 2 shows the quantum description of Raman scattering, fluorescence, and IR in a Jablonski energy (6) diagram. This diagram explains Raman effect quantum mechanically.

The first IR spectra were measured using dispersive instruments, glow bar sources, and mercury cadmium telluride detectors (7). On the other hand, early Raman measurements used conventional light sources, and the technique was not really widely used until the development of lasers. Due to the wavelength dependence of the Raman scattering, one would in many cases like to use the shortest wavelength possible. Early Raman instruments were dispersive and used visible lasers; for example, the Ar ion laser at 488 nm and the Nd:YAG laser at 532 nm. For many samples, these sources are fine, but for many biological and organic samples, there is a large fluorescence when one uses these lasers sources, so one can either go to even shorter wavelengths, into the UV or vacuum UV, or go to higher wavelength; for example, to use the 785 and 1,064 nm laser sources. In addition to the use of sources that do not give fluorescence, Fourier transform technology has been used (8).

Histological analyses performed by pathologists are mostly carried out on biopsies that undergo a fixation process followed by staining (9). The standard tissue processing method is the formalin-fixation and paraffin-embedding procedure (10). More precisely, the formalin-fixation and paraffin-embedding process involves a first step of fixation by formalin, followed by a second step of dehydration with increasing ethanol concentrations and finally addition of xylene to create a hydrophobic environment before paraffin embedding (11).

Clinical Applications

The function of the clinical chemistry laboratory is to perform quantitative and qualitative analyses on body fluids such as serum, blood, urine, and spinal fluid, as well as other materials such as tissue, calculi, and feces. The need for simple, noninvasive methods to diagnose or screen for important medical conditions has never been more relevant.

The ability to diagnose the early onset of disease rapidly, noninvasively, and unequivocally has multiple benefits. These include the early intervention of therapeutic strategies leading to a reduction in morbidity and mortality and the release of economic resources within overburdened health care systems. Some of the routine clinical tests currently in use are known to be unsuitable or unreliable. In addition, these often rely on single disease

markers, which are inappropriate when multiple factors are involved. Many diseases are a result of metabolic disorders; therefore, it is logical to measure metabolism directly. One of the strategies employed by the emergent science of metabolomics is metabolic fingerprinting, which involves rapid, high-throughput global analysis to discriminate between samples of different biological status or origin.

An operating room in a hospital is usually a place where bioanalytical methods are not very common. Common intraoperative imaging modalities are able to show morphological features but they do not provide information about the biochemical state of tissue or cells. On the other hand, several studies demonstrated that there is a growing need for methods and instruments to allow quick and reliable biochemical diagnosis of various medical conditions notably of cancer (12–14). This is also supported by current concepts of personalized and molecular medicine (15).

Vibrational spectrometry covers a series of well-established analytical methodologies suitable to be employed for both qualitative and quantitative purposes. An important attribute of vibrational spectroscopy is the availability of spectra–structure correlations from many tissue components. This extensive background information can provide a useful supplement for biomedical diagnostics. The past decade has seen much interest in using vibrational spectroscopy as a diagnostic tool for rapid characterization of tissues and bodily fluids (i.e., blood) nondestructively to allow in vivo interrogation. Compared to the other major vibrational spectroscopic technique, namely, IR spectroscopy, Raman spectroscopy does not suffer from the strong interference due to water (only small and easily subtractable water bands appear in Raman spectra), which is a main concern in biomedical applications.

The clinical need for analytical methods that have the capability of performing intraoperative diagnosis is due to

- limitations in preoperative diagnostic validity necessitating intraoperative diagnostics,
- long information delay in conventional intraoperative frozen section histology for single tissue pieces, and
- limitations of frozen section histology relying on tissue that has already been removed (16).

There is much interest in using vibrational spectroscopy as a diagnostic tool. It is a technique that promises to allow rapid in vivo characterization of tissue and bodily fluids in a nondestructive and less invasive way compared to methods now in general use. Raman spectroscopy is one method currently being tested as a diagnostic tool (17–20). Compared to infrared absorption, Raman has the advantage of having only small and easily subtracted water bands. In biological samples Raman spectra often exhibit a number of rather sharp bands, whereas infrared spectra of cells and tissue often show broader spectral features (19). This empirical observation is particularly important in analyzing complex biochemical systems because, whereas infrared spectroscopy is able to yield information about cellular components (e.g., proteins, lipids, nucleic acids) (21), Raman spectroscopy gives this information as well as much more information about some of the specific molecules in these groups of components (e.g., phenylalanine, tyrosine, and adenine) that is not available from infrared spectra.

Early and accurate detection of diseases permits effective intervention. It also facilitates efficacious therapy and monitoring of therapeutic progression and can reduce mortality and morbidity. New detection technologies that are reliable and of high specificity and sensitivity are therefore always being sought for disease diagnosis and severity grading.

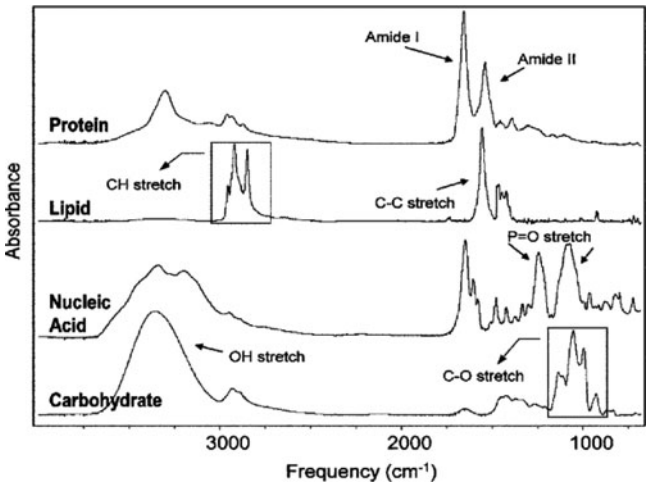

Figure 3. Various cellular components have dramatically different IR spectra: (A) lipid (palmitic acid), (B) protein (myoglobin), (C) poly-nucleic acid, and (D) carbohydrate (sucrose).

In order to perform analysis on biological samples, the analyst has to investigate the biochemical characteristics of the biological system. A biostructure is composed of several biochemicals (22), the main ones being proteins, nucleic acids, lipids, and carbohydrates. When the body has a disorder or a disease, one or more of these biochemicals is not in its appropriate condition (23), as can be seen in Figure 3.

Over the past two decades, numerous groups worldwide have started to use Raman and infrared spectral information from tissues as a means of comparing their biochemistry, and a growing body of literature now points to vibrational spectroscopy as a new and powerful method for diagnosing diseases.

A number of recent reviews (8, 24–34) have highlighted various aspects of vibrational spectroscopic of cells and tissues, as well as the use of complementary microscopic techniques. Much of the work performed thus far was done using tissue samples removed from patients. The samples then had to be sliced into thin sections and air-dried before spectroscopic analysis. This procedure is considerably less complex than many current immunohistochemistry procedures. However, for intra-operative diagnosis, the sample preparation requires far too much time to be of use during surgery. Recent advances that miniaturize optical components, and advances in flexible optical fibers, have enabled the development of Raman and infrared spectrometers that can fit within endoscopes.

The main reason for any disorder is mutation in DNA, which changes proliferation into an uncontrolled, rapid process. The initial observation could be of a benign tumor but it could develop and change to a malignant one. The main reasons for initial recognition of most cancers are the appearance of signs or symptoms, or screening. The main diagnostic method for cancer is histological confirmation that is provided by pathological examination of tissue samples (35).

Cervical cancer is the second most common cancer in women worldwide, with 80% of cases arising in the developing world. The mortality associated with cervical cancer can be reduced if this disease is detected at the early stages of development or at the premalignant state (cervical intraepithelial neoplasia, CIN). The potential of Raman spectroscopy as a diagnostic tool to detect biochemical changes accompanying cervical cancer progression

was investigated (36). Raman spectra were acquired from proteins, nucleic acids, lipids, and carbohydrates in order to gain insight into the biochemical composition of cells and tissues. Spectra were also obtained from histological samples of normal, CIN, and invasive carcinoma tissue from more patients. Multivariate analysis of the spectra was carried out to develop a classification model to discriminate normal from abnormal tissue. The results show the ability of Raman spectroscopy to classify cervical cancer and precancer with high sensitivity and specificity (99.5 and 100%, respectively, for normal tissue; 99 and 99.2%, respectively, for CIN; and 98.5 and 99%, respectively, for invasive carcinoma).

Histopathology is currently the gold standard technique for diagnosis and staging across all types of cancer. Typically, tissue samples are taken from patients and examined by pathologists using various staining techniques. This approach has several limitations, including delays in providing diagnostic results and the potential for interobserver disagreement (37, 38). To overcome these limitations, new methods are needed to allow rapid, noninvasive, and high-throughput diagnosis. Vibrational spectroscopic techniques, especially IR and Raman spectroscopy, exhibit the potential to overcome these limitations and provide an additional way of diagnosing and staging of cancer by providing a biochemical profile of the tissue that varies according to whether or not cancer is present (37). The use of vibrational spectroscopy for diagnosis and staging of cancer is extremely attractive, promising many benefits over the currently used histopathology methods. The hypothesis underlying this approach is that cancers have characteristic biochemical fingerprints that can be captured using spectroscopy (13).

Optical spectroscopy techniques such as fluorescence, Raman, and infrared, which are sensitive to biochemical composition of samples, have shown to discriminate normal and malignant tissues in oral, cervical, breast, and many other cancers (13, 39). Raman and infrared vibrational spectroscopic techniques have also been exploited to investigate eukaryotic and prokaryotic cells (40). As can be seen from the literature, among these two methods, infrared, probably due to its higher sensitivity, has been most widely used. Several successful FTIR studies for discrimination of Pap smear and other exfoliated cells have been reported (41, 42). Studies on apoptosis and identification of multidrug resistance and sensitive phenotypes in a cell line have also been described (43), showing the potentials and applications of this approach.

Biostructure disorders (e.g., uncontrolled cell division, invasive cell growth into adjacent tissue, and metastatic implantation to other body sites) are called cancer. Cancer is becoming the leading cause of death all around the world.

Rapid microbial detection and identification with a high grade of sensitivity and selectivity is a great and challenging issue in many fields, primarily in clinical diagnosis, pharmaceutical, or food processing technology. The vibrational spectroscopic techniques are noninvasive methods yielding molecular fingerprint information, thus allowing for a fast and reliable analysis of complex biological systems such as bacterial or yeast cells. Recent vibrational spectroscopic advances in microbial identification of yeast and bacterial cells for bulk environment and single-cell analysis are presented (28). IR absorption spectroscopy enables a bulk analysis, whereas micro-Raman spectroscopy with excitation in the near infrared or visible range has the potential for the analysis of single bacterial and yeast cells. The inherently weak Raman signal can be increased up to several orders of magnitude by applying Raman signal enhancement methods such as UV-resonance Raman spectroscopy with excitation in the deep UV region, surface-enhanced Raman scattering, or tip-enhanced Raman scattering. The results obtained demonstrate that spectroscopic methods (FTIR spectroscopy, micro-Raman spectroscopy with excitation in the visible or NIR and ultraviolet, as well as special Raman techniques using surface-enhanced Raman scattering) are

considered to be extremely capable methods for the characterization, discrimination, and identification of microorganisms at the genus, species, and strain level.

Vibrational spectroscopy techniques have demonstrated potential to provide nondestructive, rapid, clinically relevant diagnostic information. Early detection is the most important factor in the prevention of cancer. Raman and infrared spectroscopy enable the biochemical signatures from biological tissues to be extracted and analyzed. In conjunction with advanced chemometrics, such measurements can contribute to the diagnostic assessment of biological material. Clinical requirements are increasingly met by technological developments that show promise to become a clinical reality. Advances in fiber optics and laser technology have resulted in the development of catheter-based systems for in vivo spectroscopy use. Spectroscopy techniques show promising results for imaging vulnerable plaques and the near future will tell whether they really shine light on unstable cardiovascular disease. Clinical trials are in progress to confirm whether a vulnerable plaque identified by intravascular spectroscopy has a higher likelihood of causing cardiovascular events.

Vibrational spectroscopy techniques can be applied to identify a susceptibility-to-adenocarcinoma biochemical signature (44). A sevenfold difference in incidence of prostate adenocarcinoma remains apparent among populations of low-risk (e.g. India) compared to high-risk (e.g., UK) regions, with migrant studies implicating environmental and/or lifestyle/dietary causative factors. Samples were analyzed using attenuated total reflection FTIR spectroscopy, FTIR microspectroscopy, and Raman microspectroscopy.

The biochemical differences may lend vital insights into the etiology of prostate adenocarcinoma. The cytoscreening of cancer has been developed widely among pathologists recently (45). In this technique, the endoscopic diagnosis is used as a periodic diagnosis for the early stage cancer detection and the targeted location is enucleated partially from the biopsy of experienced pathologists. However, it takes several days in general for the diagnostic results, because the pathologist has to make histological stain (hematoxylin-eosin staining) and examine the specimen with a microscope (46). Therefore, cytoscreening is desirednot only from a biological viewpoint but also from chemical viewpoint with IR Raman spectra. The discrimination of normal and cancer cells using IR Raman spectroscopy together with analytical software using principal component analysis and linear discriminant analysis was demonstrated.

Colorectal cancer is a major public health problem, as the third most common cancer and the fourth leading cause of cancer deaths worldwide. Colorectal cancer is usually asymptomatic and is often diagnosed late, often detected after the occurrence of symptoms. Thus, a preliminary screening, which involves removal of polyps or tumors, is necessary in order to identify it early and thus significantly increase the chance of cure and hence survival (47, 48). The combination of spectroscopic imaging techniques and digital image analysis is a powerful new technique that can be used to reassemble color images of histological sections. The next great challenge is now to move from solution, to heterogeneous media like the cell, intracellular media, but in/under homeostatic conditions and in/under stress and denaturing condition, which in many cases lead to diseases, cancer being the one that we have addressed in this work (8). Hence, this method has the potential for a rapid identification of microbial pathogens against a stable database in defined application fields where only a limited number of different species and strains are present.

The wound-healing process of acute wounds such as surgical incisions is fairly well understood, so the modified wound-healing process encountered in patients with chronic wounds and some traumatic acute wounds still requires elucidation. Normal healing of an acute wound is directed by a cascade of growth factors and cell signaling that allows the wound to repair quickly. Chronic wounds and some traumatic acute wounds are much

slower to heal and behave differently for several underlying reasons. There may be a pathologic process such as infection that prevents the wound from healing normally (43). Additionally, wound healing may be complicated by a prolonged inflammatory phase that inhibits normal levels of chemical mediators and cell recruitment. Finally, the patient's general condition contributes to the rate of wound healing; malnutrition and comorbidities such as diabetes are associated with impaired wound healing (49). The application of vibrational spectroscopy to study wound healing is a developing field of interest. Both ex vivo and in vivo models of wound healing have been explored in animals and humans, but all studies published to date have focused on acute wounds versus chronic wounds. In all surgical cases, an acute wound is inflicted once a surgical incision is made.

Light interacts with tissue in a number of ways, including elastic and inelastic scattering, reflection, and absorption, leading to fluorescence and phosphorescence. These interactions can be used to measure abnormal changes in tissue. Initial optical biopsy systems have potential to be used as an adjunct to current investigative techniques to improve the targeting of blind biopsy (50). Future prospects with molecular-specific techniques may enable objective optical detection providing a real-time, highly sensitive and specific measurement of the histological state of the tissue. Raman spectroscopy has the potential to identify markers associated with malignant change and could be used as diagnostic tool for the early detection of precancerous and cancerous lesions in vivo. The clinical requirements for an objective, noninvasive, real-time probe for the accurate and repeatable measurement of pathological state of the tissue are overwhelming.

For a number of conditions, effective screening programmes have emerged that provide testing processes for diseases that have latent phases and where the natural history is reasonably well understood. Many screening programs are dependent on invasive procedures that might discourage individuals from participating. Using biofluids, specifically blood, urine, cerebrospinal fluid (CSF), or saliva (Figure 4), is significantly less invasive as a screening tool (51).

Vibrational spectroscopy has been proposed as a reagent-free, nondestructive approach toward tackling the traditional problems associated with biofluids in diagnosis and screening. The use of vibrational spectroscopy for diagnosis and staging of cancer is extremely attractive, promising many benefits over the currently used histopathology methods. Standard histological analysis of graft implants showed little or no activity in the first 10 days after implantation, but FTIR–attenuated total reflectance spectroscopy demonstrated changes within the fibrin layer of the graft that could be correlated to endothelialization of the wound (52, 53). FTIR spectroscopic maps of laminectomized tissue sections indicated a decrease in lipid and phosphate bands, which are indicators of inflammatory cells. Immunohistochemistry confirmed these results and showed a diminished number of activated macrophages in over-the-counter–treated rats. More recently, investigators successfully employed Raman spectroscopy to differentiate normal from injured tissue in rodent models of brain injury (54) and spinal cord injury (55). Vibrational spectroscopic modalities can provide an objective means of evaluation by monitoring key components of wound bed reepithelialization, such as keratin, elastin, and collagen; by identifying and quantifying bacterial load; and by detecting the hydroxyl group. These techniques have the potential to offer improved objective assessment of combat wounds, resulting in faster healing times, decreased infection rates, and decreased local and systemic complications of injury.

Vibrational spectroscopy methods are sensitive to structural variations and the amounts of biochemicals in the body, so the idea is to apply them as an inspection method in diagnostic approaches. Complexity in IR spectra makes it too difficult to provide specific information of molecular-level structures and usually these spectra are used in pattern

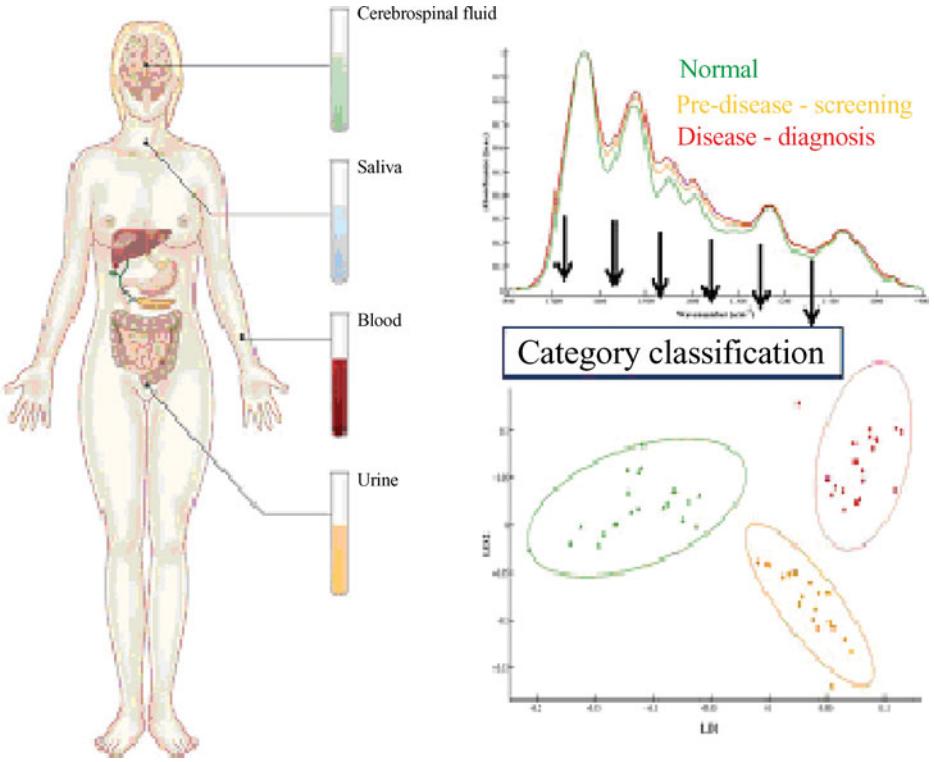

Figure 4. A variety of biofluids including blood, saliva, urine, and CSF are obtainable and applicable in a clinical setting. Other than CSF, these are relatively noninvasive. Secreted into these biofluids may be biomarkers of site-specific pathology reflecting either presymptomatic or emerging disease. Fingerprint spectra may diagnose the origin and grade of pathology based on a classification algorithm.

recognition. Investigation of biostructures by IR has gained several advantages (e.g., IR is nondestructive and the samples analyzed by IR can undergo other investigational experiments) (55).

The detection of neoplastic changes by optical spectroscopy techniques such as FTIR, Raman, and fluorescence spectroscopy has been one of the most active areas of recent research into the discrimination of oral, cervical, breast, and other cancers (56, 57). These methods are more objective, less time consuming, and have the ability to be applied in vivo.

The Raman and FTIR spectra obtained from normal and benign tissue show similarities, whereas spectra from malignant tissues are very different to these. Normal tissue spectra are characterized by higher protein contents, whereas more DNA and lipid signals are exhibited by malignant tissues (54). Among pathological tissues, malignant tissues seem to contain higher levels of lipids and DNA and lower levels of proteins compared to benign tissues. Hierarchical cluster analysis of first-derivative Raman spectra and second-derivative FTIR spectra gave good delineation of malignant from normal and benign tissues. The results obtained demonstrate the feasibility of vibrational microspectroscopic discriminarion of formalin-fixed normal, benign, and malignant ovarian tissues.

The mortality associated with cervical cancer can be reduced if this disease is detected at the early stages of development or at the premalignant state (CIN) (58). Multivariate

analysis of the spectra, obtained from proteins, nucleic acids, lipids, and carbohydrates, in order to gain insight into the biochemical composition of cells and tissues, as well as from histological samples of normal, CIN, and invasive carcinoma tissue from patients, was carried out to develop a classification model to discriminate normal from abnormal tissue. The results showed that Raman spectroscopy displays a high sensitivity to biochemical changes in tissue during disease progression, resulting in exceptional prediction accuracy when discriminating between normal cervical tissue, invasive carcinoma, and CIN. Raman spectroscopy shows enormous clinical potential as a rapid, noninvasive diagnostic tool for cervical and other cancers.

Water plays an important role in protein folding/misfolding, protein binding to specific DNA, and many other fundamental biological processes, where the balance between the flexibility of a given protein and DNA sequences and the amount of water released from the interface is essential. The internal molecular flexibility in the proteins necessary for biological activity depends on the level of hydration (59). For elucidation of the processes responsible for vibrational IR spectral properties of OH stretching modes of water involved in H-bonding with the biomolecules of human tissue, the vibrational properties of the interfacial water at the surface of noncancerous and cancerous tissues were compared. It was demonstrated that the vibrational properties of water are sensitive to the cellular environment of human tissue and are capable of distinguishing between cancerous and normal human breast tissues. These properties can be treated as hydration fingerprints to discriminate between cancerous and normal tissues, but a definite assignment of the origin and uniqueness of these bands remains and further studies are necessary.

Prospective clinical trials are required within a well-population screening service; this would prove whether biospectroscopy approaches have the capability to identify the small proportion of at-risk individuals among the large numbers that require no follow-up. It would also require correlation with gold standard endpoints such as histology for cancer diagnosis. To demonstrate its applicability toward disease screening or diagnosis, biospectroscopy analyses would likely need to be initially incorporated into an existing screening program in addition to other routine analyses.

Conclusion

Optical spectroscopy is becoming a very powerful diagnostic tool. However, to develop a cost-effective system for routine clinical uses, an enormous amount of research still needs to be conducted.

The use of vibrational spectroscopic techniques for the mapping and imaging of cells and tissues is undergoing a rapid expansion in the range of techniques, sampling procedures, and applications that span from fundamental studies to clinical applications. The research results obtained from these rapidly evolving techniques are providing many new insights into biochemical architectures and processes and are having a significant impact on the development of new treatments and diagnostics.

Probable misdiagnosis is very common with some other approaches (e.g., pathological observations) and the rate of misdiagnosis for IR analysis has been reported as being very low. IR is a rapid technique, because the spectral data are collected and interpreted within minutes. Usually, sample preparation is not obligatory prior to spectral analysis or is minimal.

The potential advantages of using IR or Raman spectroscopy of biofluids for disease detection include the following: no reagents are required, a profile of spectral

alterations can be determined, and the methods are suitable for automation. Sample preparation is minimal, techniques involved are relatively low cost, and data frameworks are available.

It is expected that vibrational spectroscopy methods will be integrated into more frequent clinical use in the near future.

We can conclude that, if the combination of spectroscopy and machine learning is transffered into clinical practice, more extensive studies are needed and researchers should routinely provide spectral data in support of their publications so that the data can be reanalyzed by other groups.

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