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Chemical analysis *in vivo* and *in vitro* by Raman spectroscopy – from single cells to humans

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Summary

The gold standard for clinical diagnostics of tissues is immunofluorescence staining. Toxicity of many fluorescent dyes precludes their application *in vivo*. Raman spectroscopy, a chemically specific, label-free diagnostic technique, is rapidly gaining in acceptance as a powerful alternative. It has the ability to probe the chemical composition of biological materials in a nondestructive and mostly non-perturbing manner. We review the most recent developments in Raman spectroscopy in the life sciences, detailing advances in technology that have improved the ability to screen for diseases. Its role in the monitoring of biological function and mapping the intracellular chemical microenvironment will be discussed. Applications including endoscopy, surface-enhanced Raman scattering (SERS), and coherent Raman scattering (CRS) will be reviewed.

Introduction

The desire to visualize biological structure and function at micro- and macroscopic length scales *in vivo* has been a major driving force in the field of biomedical optics for the past 4 centuries. This has led to major improvements to the optical microscope since its initial development, which have significantly expanded its range of applications. The introduction of optical phase contrast allowed for the visualization of living organisms and cells at the sub-cellular organelle level simply based on differences in their local refractive index. Later, the development of fluorescent probes allowed for molecule-specific contrast and resulted in the discovery of a wealth of information about the structure and function of biomolecules. The confocal microscope, and more recent schemes for super-resolution microscopy have led to significantly improved three-dimensional spatial resolution and are now producing images approaching the resolution needed to visualize individual macromolecular complexes inside cells. Optically based biological imaging and diagnostics is, however, currently enabled almost exclusively through the use of exogenous markers that are not only

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toxic, but also have significant effects on the microenvironment and function of the molecules they label. This has led to a continued search for other contrast mechanisms, and, in particular, mechanisms that allow for chemically specific imaging through the use of intrinsic or non-perturbing extrinsic markers. The most obvious extension to fluorescence contrast is Raman scattering because of its relative similarity in the manner in which signals are being detected.

Raman scattering is the inelastic scattering of photons by molecular bonds that makes use of the fact that every chemical bond in a molecule has a characteristic vibrational energy. A Raman scattering event occurs when a photon (typically from a laser light source) is scattered by a molecular bond vibration. In this scattering event a small fraction of photons (~ 1 in 10^8) is inelastically scattered and exchanges energy with the molecular vibration. The scattered photons can either be red-shifted by providing energy to the bond vibration ("Stokes" Raman scattering) or blue-shifted by receiving energy from the bond ("anti-Stokes" Raman scattering). The difference in energy between the incident and scattered photons corresponds to the energy of the molecular vibration. Detection of these scattered photons yields a spectrum of Raman peaks, each of which is characteristic of a specific molecular bond. Collectively, these peaks provide an intrinsic "molecular fingerprint" of the sample (the Raman spectrum), resulting in a wealth of information about the chemical bonds associated with DNA, RNA, proteins, lipids, and other biomolecules present in tissue or even just a single cell. The Raman spectrum in its entirety provides not only information about the identity, composition, structure, and conformation of biological samples, but also about interactions between macromolecules, for example DNA-protein or protein-lipid interactions.

Several Raman-based techniques have been developed to address the diverse needs and situations in which biomedical samples can be found. Spontaneous Raman spectroscopy is mostly used in the near-infrared region where laser damage and excitation of autofluorescence is minimal and light penetrates deep into tissue. This technique is very suitable for applications such as monitoring changes in cellular composition as a function of malignancy, or classification of cells and bacteria. The process of spontaneous Raman scattering, however, produces a very weak signal, oftentimes orders of magnitude weaker than its fluorescence counterpart. Several techniques have been developed to address this shortcoming, including Resonance Raman Scattering (RRS), Surface-Enhanced Raman Scattering (SERS) and Coherent Anti-Stokes Raman Scattering (CARS). When the frequency of the laser used to generate a Raman signal is very close to matching the energy of an electronic transition (which is the "electronic resonance"), the Raman signal is enhanced by several orders of magnitude by RRS. Detection of the RRS signal is, however, hindered by the fact that the much stronger fluorescence signal typically overwhelms the Raman signal, and the fact that this technique only works in spectral regions where the laser matches the appropriate electronic excitation wavelength, leaving RRS with limited applicability. SERS also depends on the laser frequency matching a resonance, but in this case it is the surface plasmon resonance of a metal surface. Materials with an electronic conduction band contain electrons that essentially move freely within the material (also called "plasma"). Based on the size, shape, and electronic properties of the conducting material, standing waves of electrons ("plasmons") can be generated in the material if the frequency of the excitation laser matches the surface plasmon resonance. If Raman-active molecules are adsorbed onto the surface of these materials the surface plasmon can very efficiently couple the energy of the incoming laser light with the molecules, thereby amplifying the signal up to 10^{10} times. Although the specific details leading to this huge amplification are still not entirely understood, Raman scattering even from single molecules has been observed, making SERS ideal for label-free molecular assays. The only limitation of SERS is that it does require the presence of a relatively large metal surface and that it

decays rapidly with distance away from the surface. Coherent anti-Stokes Raman scattering (CARS) amplifies Raman signals without adding any exogenous elements. A CARS signal is generated when two lasers are focused into the sample, one acting as the source leading to Stokes-shifted Raman scattering, while the other one is tuned to the same frequency as a specific Raman peak. The two lasers interact nonlinearly within their overlap volume, simultaneously exciting the characteristic chemical vibration, and driving the amplification of the corresponding anti-Stokes peak. The resulting CARS signal is more than a thousand times stronger than the original Raman signal and can be used for chemically selective imaging without the use of fluorescent tags or dyes. By significantly reducing the exposure time required to record the signal, CARS holds great promise for imaging living cells and tissues.

In this article we will highlight recent technology developments and discuss applications of various Raman techniques to biological and biomedical problems ranging in scale from the *in vitro* analysis of single cells and molecules to the *in vivo* spectroscopy and imaging of tissues and entire organisms.

Recent technology developments

Raman, SERS, and CARS have been pursued heavily over the last several years for their potential as label-free, chemically-specific, and/or ultrasensitive analytical tools. In the past two years a number of technological key developments have been made that have the potential to expand the applicability of these Raman techniques in the biological and biomedical fields. Figure 1 briefly summarizes the principles leading to these different Raman scattering processes.

The development of Raman spectroscopy, and in particular SERS, can be considered a significant step forward in the analysis of biological samples, since Raman spectra contain more information than fluorescence spectra and can be more readily multiplexed. In addition, considering the strong enhancement of the Raman signal in the presence of metallic nanoparticles, the typical problem of low signal leading to long acquisition times can be circumvented. A significant effort has been directed towards the careful engineering of nanoprobe such as metallic nanoparticles that allow for the detection of disease-specific biomarkers. Advances in SERS probe technology have centered around two main themes: (i) the development and nanofabrication of controlled nanostructures that allow for inexpensive, reliable substrates with high enhancement factors and (ii) functionalization of nanoparticles for specific detection of molecules of interest. Small, hollow gold nanoparticles were developed by Schwartzberg et al. [1] for improved signal consistency, resulting in significant increase in sensitivity and dynamic range. Lu et al. [2] fabricated gold nanoparticles with an unusual, crescent moon-like shape that have a sub-10 nm sharp edge, allowing for a larger than 10^{10} enhancement factor offering significant potential for applications in ultrasensitive biomolecular detection. Huang and El-Sayed [3] found that the synthesis of nanorods instead of spherical gold colloids could be particularly advantageous. They observed that human oral cancer cells assembled and aligned gold nanorods that were conjugated with antibodies against the epidermal growth factor receptor. The molecules situated near nanorods yielded a greatly enhanced, sharp, and polarized Raman spectrum. The authors have shown that these nanorods have the potential to be used as cancer biomarkers. Kim and Kim [4,5] developed SERS substrates composed of silver nanoparticle-embedded silica spheres and organic Raman labels for cancer targeting of living cells. These nano-spheres were conjugated with antibodies targeting HER2 and CD10 and showed good specificity and offered potential for high throughput screening. Qian and Peng [6] describe the development of gold nanoparticles coated with polyethylene glycol (PEG) for *in vivo* tumor targeting. These probes use small molecules as Raman reporters that

are stabilized by thiol-modified PEG, to generate a signal brighter than quantum dots leading to SERS emission in the near infrared. The gold nanoparticles were then conjugated with tumor-targeting ligands such as single-chain variable fragment (ScFv) antibodies for detecting tumor biomarkers such as epidermal growth factor receptors on human cancer cells and in xenograft tumor models.

While Raman spectroscopy offers great intrinsic specificity unmatched by any other non-invasive technology, it often lacks the signal strength required by many biomedical applications for fast, real-time measurements. Several groups have addressed this problem. One such example is described by Matousek [7], who developed a method for enhancing spontaneous Raman signals in turbid media. This method uses a multilayer dielectric optical component, such as a bandpass filter, placed within the laser beam over the sample to prevent the loss of excitation photons that re-emerge from the sample. This filter acts like a unidirectional mirror and leads to increased coupling of the laser radiation into the sample, resulting in several-fold enhancement of the measured Raman signal. The technique shows promise for deep tissue Raman spectroscopy, and can be coupled with measurements where the excitation beam is spatially offset relative to the collection of the Raman signal. Other improvements to the sensitivity of Raman measurements include the development of a highly sensitive ultrabroadband CARS microspectroscope based on a white light sub-nanosecond laser source [8], the temporal separation of the CARS and fluorescence signals by time-resolved single photon counting techniques [9], the development of heterodyne CARS microscopy [10], and the use of photonic crystal fiber laser sources for CARS [11].

Another area of intense research is the development of portable, fiber-based Raman devices that enable the use of Raman spectroscopy for in-vivo endoscopies, in clinical settings or in the field. The main problems such devices face are: (i) a substantial intrinsic fluorescence signal generated in the tissue by the excitation laser, and (ii) background Raman and fluorescence signals produced by most fibers when the excitation light passes through them. While choosing excitation wavelengths in the near-infrared helps reduce the fluorescence background, this still constitutes one of the major problems for Raman tissue measurements. In the past, time-resolved measurements have been performed for temporally gating out the fluorescence, and methods that employ spatial offsetting have been developed to separate the excitation light and collected signal. This is, however, an area where significant improvements can still be made in the future. The second problem of the background Raman and fluorescence signals generated in the fiber itself requires the separation of the excitation and collection pathways, limiting the minimum size of these devices and making miniaturization difficult. One possible way to address this problem is to detect only the high wavenumber region of the Raman spectrum, where little overlap with the signal generated in silica-based fibers occurs. Nijssen et al. have shown [12] that, using this strategy, a system based on a 200 μm single optical fiber can be developed, with the possibility for further miniaturization. Other developments include a device that, using a fiber-optic array probe and photon migration through tissue, is able to perform subsurface Raman measurements [13], an improved Raman microspectrometer for dermatological applications [14], and an endoscopic Raman probe for potential *in vivo* diagnosis of lung cancers [15].

Biomedical assays

Current biomedical sensors include Enzyme-Linked Immuno-Sorbant Assays (ELISA), fluorescent antibody assays, evanescent wave and interference-based biosensors, and SERS-based biosensors. While most of the newer techniques attempt to address the limitations of sensitivity and specificity inherent to methods developed earlier, SERS has the advantage that it is sensitive down to the single molecule level. Additionally, SERS can yield molecular structural information, has the potential to be broadly multiplexed, and has the

unique ability to improve the specificity of detection by distinguishing between specific and non-specific binding events.

The development of SERS-based biomolecular sensors and assays is tightly connected with advances in nanotechnology, chemistry, and biology. Novel nanofabrication techniques, together with improvements in substrate functionalization and improved affinity of antibodies and single-stranded oligonucleotides for their targets have contributed to improved specificity, reproducibility, and stability of molecular identification. SERS assays for the detection of proteins, DNA, and other molecules of interest have been developed for the identification of disease-specific biomarkers, the detection of low-level viruses and bacteria, and the discrimination between different types and strains of pathogens.

SERS measurements can be performed in either extrinsic or intrinsic configurations. In the first case, a Raman reporter molecule is used to generate the SERS signal. Molecular beacon assays measuring combined fluorescence and SERS signals of a reporter molecule were thus developed for the detection of human viral RNA [16], while an aptamer-based SERS sensor using methylene blue as a Raman probe was developed for thrombin detection [17].

The use of Raman labels has some disadvantages as it can sometimes lead to loss of activity of the capturing molecule. In the intrinsic configuration, the analyte is either directly applied to the substrate or it is captured and immobilized by specific interactions with antibodies, aptamers, or related molecules. The SERS spectrum of the analyte itself is then measured. Much research has been devoted to the development of such assays. Folic acid in water and human serum was detected in this way by using silver nanoparticles created via ethylenediaminetetraacetic acid reduction [18]. It was also shown that very low concentrations of melamine in food could be detected using commercially available SERS-active substrates [19]. The prospect of label-free DNA detection in high-density arrays is discussed in [20] by using a SERS platform based on island film lithography combined with silver deposition by galvanic exchange. Sun et al. [21] showed that Composite Organic-Inorganic Nanoparticles (COINs) can be used for protein detection in human tissue. These particles are clusters formed by aggregating silver nanoparticles with a crosslinker such as BSA in the presence of Raman-active molecules of interest. These do not only have the advantage of high enhancement due to localizing the molecules near aggregated silver, but also enable the measurement of molecules that are not specifically bound to the metal via functional groups. Using antibody-conjugated COINs, this group was able to detect the prostate specific antigen (PSA) and cytokeratin-18 (CK-18) simultaneously and map PSA in human tissue samples. Similarly, by using small, specific oligonucleotide sequences, Pal et al. developed a SERS gene probe for breast cancer [22].

While SERS made up the majority of novel molecular bioassays, spontaneous Raman spectroscopy constituted the basis for several papers, most notably the paper by Qi and Berger [23] that describes the use of a liquid-core optical fiber for the analysis of clear biofluids such as urine and clinical blood serum, which allowed them to characterize 13 different chemicals without exogenous probes.

***In vitro* analysis of cells**

Clinically, the most accurate method for identifying cancer cells has been histopathology based on hematoxylin and eosin (H&E) staining. Among other things, H&E staining provides a clear picture of morphological differences between cell types. Such classification provides little to no information about the chemical composition of cells - therefore Raman spectroscopy stands to act as a powerful complement to H&E staining. Additionally, unlike H&E staining, Raman spectroscopy can be performed on living cells. Over the past several

years Raman micro-spectroscopy has proven to have high sensitivity and specificity for identifying cancer cells in live samples. Recently, Chan et al. [24] demonstrated better than 98% sensitivity when classifying live hematopoietic cells from immortalized cell lines as either neoplastic or normal by using a laser tweezers confocal Raman micro-spectrometer and principal component analysis of the acquired spectra. The resulting analysis was able to show that differences between normal and cancer cells could be quantified mostly based on differences in the local cellular concentration of DNA and proteins. Immortalized cell lines admittedly show less variation than samples potentially taken from patients in a clinical setting which led the same group to further demonstrate [25] that they could identify individual leukemia cells in blood samples obtained from cancer patients. The process of single cell analysis by Raman spectroscopy, together with sample spectra obtained from single cells is illustrated in Figure 2. While Raman spectroscopy is developing as a clinical tool, the acquisition times for such spectra make Raman less suitable for the study of dynamic cellular processes. Therefore in order to analyze fast subcellular processes other, more sensitive methods may be more suitable.

Among the subcellular processes of interest, revealing the cellular origins of diseases is an area where Raman based techniques are bound to make substantial contributions. For instance, the composition and movement of lipid droplets (LDs) within the cytoplasm are thought to be affected by various diseases including atherosclerosis and infection with the Hepatitis C Virus (HCV). While some studies have been conducted using fluorescent markers there is a concern that exogenous labels may critically perturb organelle behavior. CARS microscopy is a label-free imaging modality that can provide valuable information at the subcellular level. Because of the high number of CH₂ bonds in lipids and proteins, most CARS work has focused on intracellular LDs. Nan et al. [26] claim to have shown that CARS was non-perturbing to a living cell and that they were able to image intracellular organelle transport without exogenous dyes in steroidogenic mouse adrenal cortical cells. Later the same group used CARS to correlate LD density with HCV density in human hepatoma cells using a combination of fluorescence and CARS imaging [27]. More recently, another group [28] used multiplex CARS microscopy to obtain quantitative images of similar LDs and measure the composition and distribution of different species of lipids within a single droplet. Their work demonstrated that cells that took up lipids from their surrounding environment formed small LDs that later fused with the much larger LDs that already resided in the cytoplasm. This paper, as well as other similar work, has opened up an entirely new way of studying lipid interactions with cells. CARS microscopy is currently the only technique that is capable of rapidly taking such quantitative images of LDs in living cells without altering them.

At the molecular level, Raman techniques have also proven to be powerful tools for studying local chemistry and changes in the cellular microenvironment. The huge enhancements of the Raman signal induced by the presence of metal colloids make SERS ideal for non-destructive, ultra-sensitive detection of rare chemical species in live cells. The ability to reliably identify rare chemical species is key to the measurement of several important cellular parameters including enzymatic activity and pH levels. Ingram et al. [29] demonstrated the use of metal colloids coated with 8-hydroxyquinoliny azo dyes as efficient probes for local enzymatic activity within a cell with a detection limit of 0.2 ng mL⁻¹. With such high sensitivity SERS has also been demonstrated as a tool for studying neurotransmitter release [30]. Neurotransmitter release is a rapid localized process that has presented many barriers to other techniques such as fluorescence. Several groups have developed SERS based pH sensors [31–33] using coated metal colloids. These pH sensors have been shown to be accurate within a pH range between 2 and 8 and are capable of creating cellular pH maps, which is critical information in studying cellular response to various stimuli. The true strength of SERS for making these types of measurements is

neither that it can make such measurements nor that it is much more sensitive than traditional fluorescence techniques but it is the fact that the resulting Raman spectra contain very sharp distinct peaks which allows for broad multiplexing and ultra-sensitive detection of several parameters simultaneously. Admittedly, the limited reproducibility of the SERS spectra has restricted its widespread adoption in the biological sciences but as these problems become better understood and characterized, broadly multiplexed SERS probes will develop into powerful tools.

Ex-vivo tissue analysis

At the tissue level a major push among Raman spectroscopists has been the identification and quantification of chemical differences between cancerous and normal tissue. As mentioned previously, it is anticipated that Raman spectroscopy could serve as a powerful complement to H&E staining in histopathological situations and as a potential alternative when the analysis of live tissue is desired. The main strength of Raman spectroscopy is also its greatest obstacle, namely that each spectrum contains a large amount of information that can often be difficult to dissect and extract the desired information. Nevertheless, several groups have successfully demonstrated their ability to analyze cancerous tissues. By taking Raman spectra at defined points on excised parathyroid tissue, Das et al. [34] demonstrated that Raman spectroscopy could identify hyperplasia and parathyroid adenomas with a sensitivity of ~95%. Quantification of chemical differences between normal and diseased tissues is vitally important to improving the specificity and sensitivity of histopathological diagnostics. In related work, de Jong et al. [35] used Raman spectroscopy coupled with various multivariate data analysis techniques to identify similar groups of cells in bladder tissue. While identifying cancerous tissue with greater than 90% sensitivity and specificity, their work also quantitatively (and more importantly nondestructively) revealed that normal bladder tissue showed higher collagen content, while tumor tissue had higher lipid, nucleic acid, protein, and glycogen content. While the discrimination of normal and cancerous tissue is important, Chowdary et al. [36] went one step further and demonstrated that Raman spectra could also be used to quantitatively discriminate between cancerous and benign lesions in breast tissue. Quantitative tissue identification will surely improve clinical diagnosis of cancer and reduce the number of false positive diagnoses of patients.

Vascular diseases such as atherosclerosis, characterized by lipid-rich plaques forming just beneath the lumen, cause significant morphological and chemical changes in the local arterial tissue. Analyzing tissue over a relatively large area (millimeters to centimeters in size) with microscopic resolution using Raman spectroscopy, however, is very time-consuming. This obstacle can be extremely limiting for the analysis of fresh tissue samples since many medical conditions cause structural as well as chemical changes. The strong signal obtained with CARS has been utilized for imaging even at the tissue level and has proven to be a powerful alternative for studying tissue structure. The lasers used to produce a CARS signal are also ideal for other nonlinear optical techniques not discussed in this review, such as sum frequency generation (SFG), second harmonic generation (SHG), third harmonic generation (THG), and two photon excitation of fluorescence (TPEF). Therefore, CARS has recently been implemented simultaneously with these other techniques since they can all be realized using identical optical setups. Wang et al. [37] took advantage of the CARS sensitivity to CH₂ bonds and imaged carotid artery sections using a combination of CARS, SFG, and TPEF. They were able to resolve single endothelial and smooth muscle cells as well as image collagen fibrils and elastin, demonstrating that CARS can be an excellent tool for studying morphological changes associated with injury in fresh tissue samples. The same group also used this sensitivity to CH₂ modes to image myelin as an important component in neurological diseases. They demonstrated their ability to image intact myelin sheaths using CARS [38], an important step in understanding the mechanisms

of demyelination and myelin repair. Evans et al. [39] imaged an ex-vivo mouse brain using CARS and then validated their results using H&E staining, showing excellent correlation between the images. As these authors pointed out, while magnetic resonance imaging and other techniques have revolutionized how the brain is imaged, significant improvements can still be made in the analysis of biopsied tissue. These improvements include label-free imaging, chemically quantitative imaging, and 3-D sectioning of fresh samples: all of which CARS microscopy can offer.

In order to identify the presence of certain cancers or diseases, fluorescent immunostaining is often used. Spectrally, fluorescent labels produce a very broad signal that significantly limits the number of distinct signals that can be resolved simultaneously. During biopsy, this tendency for fluorescent signals to overlap limits the number of targets that can be stained against since additional tissue and time is required for each additional target. Raman spectral lines, however, are often very narrow (~ 1 nm), allowing ultra-sensitive techniques such as SERS probes to be used for broadly multiplexed immunostaining. Lutz et al. [40] demonstrated SERS probes as an alternative immunostain against the prostate-specific antigen (PSA) in tissue from patients with prostate cancer. Their analysis showed that the SERS immunostain performed similarly to the traditionally used fluorescent dyes. The authors comment that the power of this technique lies in its ability to broadly multiplex and therefore allow for staining against a wide range of proteins in a single tissue section, drastically reducing the amount of tissue required for immunohistochemical biopsy.

***In vivo* Raman spectroscopy and imaging**

A number of experimental schemes have recently been developed that have enabled the rapid in-vivo acquisition of Raman signals in both animals and human subjects. Naturally, the most straightforward approach for such studies is to target the skin. Resonance-Raman spectroscopy of pigments and antioxidant levels in the skin were conducted very early on. More recently, near-infrared Raman spectroscopy based on compact optical probe heads [41] [12] has been used to diagnose and characterize skin cancers (melanoma and carcinoma). As in most applications involving the diagnosis of cancerous lesions, the current gold standard is surgical resection and analysis by histopathology. This procedure is, however, invasive, expensive, and time-consuming, which makes the availability of a rapid, non-invasive analytical technique very desirable. Such systems require both handheld, compact devices for data acquisition and simple, automated spectral analysis and interpretation routines, which are exactly the areas where much of the work during the last two years has focused on. Results based on Raman spectroscopy of skin lesions, compared to histopathological analysis, have consistently shown high sensitivity, specificity, and accuracy during limited clinical tests demonstrating excellent feasibility for their potential clinical implementation. Of particular interest is the recent demonstration that by evaluating data only from the high wavenumber region of Raman spectra (>2800 cm^{-1}), similar sensitivity and specificity as by analyzing the fingerprint region ($\sim 400 - 1800$ cm^{-1}) was obtained. This result significantly simplifies probe design, because it obviates the need for high-performance filters to be built into the probe head [12]. Tissue analysis based on Raman spectroscopy with fiber probes is schematically illustrated in Figure 3.

As is the case for all in-vivo Raman spectroscopy applications, these studies typically require the use of near-infrared laser sources in order to minimize background contributions from tissue autofluorescence as well as additional automated fluorescence subtraction algorithms to remove residual background contributions. Data acquisition times are typically a few seconds, but recent improvements in spectrometer throughput and camera technology are expected to push this into the millisecond range, which, combined with rapid data processing and analysis, facilitates the real-time assessment of lesions [42]. For in-vivo

diagnosis inside the human body, either during surgery as surgical guidance tools, or through body cavities, narrow-diameter fiber-optic Raman probes are required. A wide range of applications of in-vivo spectroscopy have been demonstrated over the last two years, including the diagnosis of cancerous lesions in the lung, larynx, cervix, bladder, prostate, colon, esophagus, and in brain tissue. Most notably, a recent in-vivo Raman study of atherosclerotic lesions during bypass surgery demonstrated that Raman spectroscopy has the sensitivity and specificity to detect vulnerable plaques [43,44], which are highly unstable atherosclerotic lesions in arteries. Other clinical imaging techniques have failed to identify these critical tissue structures whose rupture can lead to heart attack or stroke. In a separate study, the same group also found that in-vivo Raman spectroscopy allowed them to assess the margins of breast cancer during partial mastectomy, and even find and classify cancer tissue that was initially missed during surgery - information that was used to prescribe a second procedure for the patient [45,46].

Besides acquiring direct spectra or chemical information from tissue, Raman spectroscopy has recently been demonstrated in another very interesting in-vivo application. One shortcoming of vibrational spectroscopy is the fact that most protein spectra are very similar. The main vibrational modes of most proteins that are probed by Raman spectroscopy are due to C-C, C-N, C-H and other similar organic bonds. It is very difficult, if not impossible to measure differences between different isoforms of proteins or protein mutants, especially against a background of other proteins. Also, the sensitivity of Raman spectroscopy is limited to micro- to millimolar concentrations – depending on the specific implementation. The SERS spectrum of these molecules, however, depends highly on their structure, conformation, and orientation with respect to the nanoparticle, leading to a seemingly infinite number of spectral variations. Also, SERS particles exhibit a very strong optical response approaching or exceeding fluorescence intensities, they do not photobleach, and can be designed to exhibit their optical response in any part of the optical spectrum, in particular the near-infrared. Qian et al. [6] and Keren et al. [47] have recently made use of this fact and applied it to in-vivo sensing in live animals, where the SERS response of the nanoparticle tags was detected through the skin. The specificity for tumors in this case stems from functionalizing the surface of the nanoparticles with tumor-targeting antibodies or peptides. Using this approach, Qian et al. [6] claim that they were a factor of 200 more sensitive in detecting SERS tags *in vivo* than fluorescent quantum dots, making this approach potentially very attractive for *in vivo* biosensing. SERS could become even more attractive in the future, if means of controlling the optical response upon specific binding (signal-activation or signal-shift, which allows one to distinguish specifically bound tags from nonspecifically bound tags) can be found, or if the particle emission could respond to changes in their chemical environment, making them true optical probes similar to the behavior of e.g. pH-sensitive fluorescent dyes.

The recent development of CARS microscopy has also lead to an effort to use CARS for *in vivo* imaging. Most notably, Evans et al. have recently demonstrated real-time CARS imaging of lipid structures in mouse ears and adipose tissue [48]. The group of Ji-Xin Cheng at Purdue University has further applied this to imaging myelin and modifications of myelinated tissue in spinal and nerve tissue [49]. Thus, it is only a matter of time until the first *in vivo* imaging of live brain tissue will be demonstrated in animal models. This, combined with the high speed of signal acquisition might obviate the need to inject fluorescent dyes for multiphoton microscopy into brain tissue or the development of animal models with fluorescent protein-expressing cells. Currently, however, most of the CARS imaging demonstrations are focusing on imaging “easy” targets, such as lipid modes, where CH bonds are abundant leading to the highest CARS signals. Initial approaches to combining CARS with endoscopy have also been made, but are somewhat complicated by

the requirements for short-pulsed lasers and phase-matching conditions that are necessary for generating CARS signals [50].

Conclusions and perspectives

Until recently Raman spectroscopy has been associated more with analytical chemistry than with biological and clinical applications. Significant recent developments in the field of laser technology and improvements in the sensitivity of detectors, correlated with the development of novel excitation and detection schemes, however, have motivated many research groups and companies to develop Raman-based technologies capable of approaching complex biomedical research problems. While the difficulty lies in the number and complexity of the molecules investigated, the promise of label-free, minimally-invasive molecular detection and characterization has driven the field to results unimaginable just a few years ago. While other, possibly high impact, papers were left unmentioned due to space limitations, in this paper we have reviewed those articles that we believe describe the most significant developments during the past two years. We have detailed recent improvements in and applications of Raman-based technologies that have expanded Raman capabilities beyond analytical chemistry to studies of biological function and detection of disease at all levels of organization from cells to whole organisms. Although several of these techniques, such as Raman spectroscopy of trapped cells, SERS-based bioassays, and CARS imaging systems are in advanced stages of commercialization, we anticipate several more important advances will follow in the near future. In addition to the continuous improvement of Raman-based microscopes, of particular interest seems to be the development of fiber-based Raman endoscopes that allow for true *in vivo* detection and characterization of disease in clinical settings. The pursuit of optimal combinations of Raman techniques with other techniques such as fluorescence, Optical Coherence Tomography (OCT), or opto-acoustic microscopy is another direction of extensive research. Overall, we expect that the unique capability of Raman spectroscopy to visualize biological specimens with chemical specificity will assure a steady increase in the development of novel technologies and will spur new applications by the wider biology and biomedical sciences community.

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Figure 1.

Schematics of different Raman scattering processes discussed in this article. The excitation laser beam is isotropically scattered by the vibrations of chemical bonds in spontaneous Raman scattering (top). The same objective lens can be used for the excitation and collection of the Raman signal, with a dichroic mirror serving as a means of separating the two beams. When two or more coherent laser beams with different frequencies overlap spatially and temporally onto the specimen, a coherent Raman signal on the anti-Stokes side of the spectrum is generated (middle). This CARS signal is produced by nonlinear mixing of the ultrashort excitation pulses and is several orders of magnitude stronger than the spontaneous Raman signal. Molecules adsorbed on metallic nanostructures will experience a strong

electromagnetic field in addition to chemical enhancement, due to the presence of plasmon resonances near the surface of metals (bottom). The Raman signal can be enhanced by up to 15 orders of magnitude in this way.

Figure 2.

Diagram representing a normal (top) and diseased (bottom) cell in culture with their corresponding Raman spectra. The spectra were obtained with a 785nm continuous-wave laser beam that simultaneously optically traps the cells and generates the Raman signal. Typical protein peaks can be observed around 1442 cm^{-1} and 1001 cm^{-1} and DNA peaks around 785 cm^{-1} , 1090 cm^{-1} , and 1575 cm^{-1} . Significant differences between the normal and cancerous cell can be observed in the Raman spectrum for the peaks centered on 1575 cm^{-1} , and 1090 cm^{-1} , showing the capability of Raman spectroscopy to distinguish the two types of cells.

Figure 3. Schematic of a Raman endoscope recording spectra from the lung (top) and bone (bottom). The spectra shown in this figure were obtained by a fiber-based Raman probe using a 785 nm continuous-wave excitation laser and a standard holographic grating spectrometer. The lung and bone specimens were excised mouse tissue samples. The lung spectrum shows significant similarities with the spectra of single cultured cells, reflecting the protein and DNA content of the sample. The bone spectrum, on the other hand, contains Raman peaks specific for calcium phosphate groups.