Raman spectroscopy in chemical bioanalysis
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Advances in instrumentation are making Raman spectroscopy the tool of choice for an increasing number of (bio)chemical applications. Raman is an interesting option for several reasons, including the sensitivity to small structural changes, non-invasive sampling capability, minimal sample preparation, and high spatial resolution in the case of Raman microspectroscopy. Herein we discuss the most recent technical approaches employed, from the well-known surface enhanced resonance Raman spectroscopy (CARS) and related techniques. Relevant applications of Raman spectroscopy in the fields of clinical pathology, in vivo and ex vivo imaging, classification and detection of microorganisms and chemical analysis in the past three years are also included.

Different approaches in Raman spectroscopy
Two major instrumental approaches used to collect the Raman spectra, Fourier transform (FT)-Raman [1] and charge-coupled device (CCD)-based dispersive Raman, [2] are responsible for this unmistakable resurgence of interest in Raman spectroscopy. The technologies differ in the laser that is used and the way the Raman scattering is detected and analysed [3]. More recently, the development of important technological advances has made possible the application of further approaches which were known to be theoretically feasible but not yet applicable. These include resonance-based enhancements of the Raman effect, the use of confocal arrangement for microscopy and, more recently, the application of non-linear Raman techniques. In this article, we review the recent advances in Raman spectroscopy, briefly introducing the most recently developed techniques and presenting the new trends in the field. A summary of the latest applications of biological relevance is also included.

Resonance Raman spectroscopy (RRS) occurs when the excitation laser frequency is coincident (or near coincident) with an electronic transition of the analyte. This can result in a $10^2$–$10^6$ increase in the Raman signal compared with dispersive Raman spectroscopy [3]. However, because visible or ultraviolet excitation must be used to achieve resonance, interference from fluorescence is often a problem with RRS.

Surface enhanced Raman spectroscopy (SERS) has become a powerful tool for the characterisation of a wide range of inorganic and biologically relevant analytes [4,5]. The main advantage of SERS is a $10^3$–$10^7$ enhancement of the Raman signal of an analyte when it is adsorbed to or near to the surface of certain noble metal structures with nanoscale features [5,6]. The enhancement mechanisms even permit single molecule detection [7,8]. Continuous irradiation of the laser beam over the SERS substrate can promote the decomposition of the sample analytes and significantly broaden and diminish the intensities of observed spectral bands. Chemical or morphological changes in the SERS substrate are also possible. These effects can be minimised using a sample translation technique; by spinning the sample rapidly, the residence

Abbreviations
CARS coherent anti-Stokes Raman scattering
CCD charge-coupled device
FSRS femtosecond stimulated Raman spectroscopy
FT Fourier transform
RRS resonance Raman spectroscopy
SERS surface enhanced Raman spectroscopy
SHG second-harmonic generation
SFG sum frequency generation
THG third-harmonic generation

Introduction
Recent years have seen an explosion in the use of Raman spectroscopy for biological purposes such as tissue diagnostics, blood analyte detection and cellular examination, among others. The greatest benefit of this technique lies in its high sensitivity to subtle molecular (biochemical) changes, as well as its capability for non-invasive sensing. In addition, the Raman technique offers generally narrow bandwidths, minimal sample preparation and is easily interfaced to fibre-optics for remote analysis; therefore, interfacing to separation techniques or remote sensing is usually straightforward. Furthermore, the spatial resolution of Raman microspectroscopy in the low micrometer scale and its ability to probe samples under in vivo conditions allow new insights into living single cells without the need for fixatives, markers or stains.
time of both analyte and substrate within the irradiated zone is dramatically decreased [9].

Confocal Raman microscopy is the usual technique of choice when dealing with microscopic samples. The major problem in these samples is obtaining a good spatial resolution perpendicular to the optical axis (lateral) as well as along the optical axis of the microscope. Confocal microscopy uses a pinhole placed in the back image plane of the microscope objective so that the light originating from a small region of the sample coincident with the illuminated spot is isolated and the contributions from out-of-focus zones are efficiently eliminated, obtaining the benefit of spatial filtering by an optically conjugated pinhole [10,11]. Confocal Raman microspectroscopy has been widely applied in several fields, imaging microscopy being one of the most extended applications, because it provides 3D spatial resolution. There are several imaging methodologies, classified as direct or wide-field and serial imaging techniques (point and line mapping). It has been experimentally found that line mapping is the fastest method for acquiring spectral information at a reasonable spatial resolution (approximately 1 µm) [12*]. Even with that method, it takes several hours to acquire a Raman image of cells and tissues [13], which can pose a severe drawback for the study of dynamic living systems.

Nonlinear (coherent) Raman techniques
Analytical applications of nonlinear Raman techniques have been slow in coming, due largely to the complexity of both the instrumentation and the underlying theories.

Coherent anti-Stokes Raman scattering [14] (CARS) has become the most extensively used nonlinear Raman technique; it has several features that make it an excellent tool for non-invasive diagnostic in reacting systems. The technique produces a laser-like signal beam that is many times stronger than spontaneous Raman scattering. In CARS microscopy, the temporally and spatially overlapped pump and Stokes laser pulses are tightly focused into a sample to generate a signal in a small excitation volume (<1 µm³), thus providing a high 3D sectioning capability. In addition, the coherence of the signal beam results in a large and directional signal, which permits low excitation powers and fast scanning rates; the signal frequency is blue-shifted from the excitation frequencies, so that as a consequence the CARS signal can be easily detected in the presence of fluorescent background. However, the use of CARS has only become possible after the development of technical advances to overcome the original problems of the technique. Various schemes including picosecond excitation, epi-detection, phase-matching techniques [15], polarization-sensitive detection and the development of time-resolved CARS significantly improved the ratio of the resonant signal to the non-resonant background. The imaging speed was boosted by the use of a laser-scanning microscope (multi-plex CARS microspectroscopy). Developments in laser technologies including jitter reduction and the picosecond amplifier increased the imaging sensitivity even further. The theoretical understanding of its mechanisms, critical for image interpretation, was finally developed. The description of these recent advances in CARS microscopy and its fundamental properties and applications can be found in an extensive and comprehensive review published recently [16**].

In addition to CARS microscopy, other nonlinear optical processes, such as second-harmonic generation (SHG) [17], sum frequency generation (SFG) [18], third-harmonic generation (THG) [19] and femtosecond stimulated Raman spectroscopy (FSRS) have also been incorporated to scanning microscopy. High harmonic microscopy (SHG and THG) is less informative than CARS, but it is also used for biological applications. SFG, by contrast, is more surface-sensitive instead of bulk sensitive. FSRS offers the advantage versus CARS of being self-phase matched, avoiding the need for phase-matching methods.

Ongoing research and applications
Clinical pathology
The ability of Raman spectroscopy to detect small structural changes has been useful for the development of methods to discriminate between healthy and unhealthy tissues, or to determine the degree of progress of a certain disease, mainly focused on ex vivo targets in the form of biofluids or tissue samples [20]. The recent technological improvements have made it possible to utilize Raman spectroscopy in medicine [21**], aiming to the development of new therapeutic drugs (see Update) and particularly in the diagnosis of arteriosclerosis and cancer. In the study of arteriosclerosis, quantification of cholesterol and cholesteryl esters was studied in the coronary artery in vitro. Researchers were able to correctly identify lesions and to differentiate the normal artery, atherosclerotic plaque without calcification, and atherosclerotic plaque with calcification [22]. The goal of Raman spectroscopy in this field is real-time catheter-based examination in vivo. In cancer research, studies have mainly investigated intestinal, breast, uterine, laryngeal, skin and brain malignancies. They revealed specific Raman spectral changes in each cancer. The ultimate objective in cancer research is the replacement of biopsy with fiber-optic Raman spectroscopy.

FT-Raman has been used to extract information about cancerous tissue of the stomach, which can be used to differentiate malignant tissue from normal tissue [23]. Also aiming at the detection of cancerous tissue, in this case in Barrett’s epithelium (oesophagus), Boere et al. [24] employed flexible fibre optic probes to record ex vivo Raman spectra of a rat oesophagus, as this particular animal model is frequently used for studies on Barrett’s oesophagus. The spectra of normal epithelium and...
Barrett’s epithelium were used to build a database and a classification model that was correct in >93% of cases. Unfortunately, the lack of reproducibility of the fibre optic used and the relatively long integration time (10 s) needed for signal collection preclude the method from direct application to *in vivo* surveillance of human Barrett’s oesophagus.

For the development of new diagnostic methods for detection of tissue states *in vivo*, it is important to understand the spectral features of pure individual components of tissues; that means cells and subcellular components. Raman microspectroscopy has been applied to record complete spectral maps of freeze-dried cells as well as living cells in media without the need for any fixing technique before the mapping. The resulting spectra (Figures 1,2) showed the localization of the nucleus, organelles and the membrane [25**].

CARS microscopy has been used for selective imaging of lipid droplets in unstained living cells with a very high contrast [26,27]. In a recent study, Nan *et al.* [26] applied CARS microscopy to monitor the growth of triglyceride droplets during the differentiation process of 3T3-L1 cells. In addition to the traditional picture of lipid accumulation in the differentiation process, the images indicate an intermediate stage (i.e. the removal of cytoplasmic lipid droplets after addition of the induction medium). This reduction of lipid droplets was attributed to an increased activity of hormone sensitive lipase, the enzyme responsible for hydrolysing intracellular triglyceride and sterol esters.

**In vivo Raman spectroscopy**

One of the greatest appeals of Raman spectroscopy lies in its potential for *in vivo* measurements. For a long time, tissue fluorescence and the lack of appropriate and sensitive instrumentation were important obstacles that delayed the development of *in vivo* Raman applications. Nevertheless, the use of instruments working in the near-IR region of the spectrum easily overcomes this problem. Rapid spectra collection is currently achieved by a setup consisting of a near-IR solid state laser coupled to a suitable fiber-optic probe for delivering the laser beam and collecting the scattered radiation, and a high numerical aperture single-stage spectrometer equipped with a CCD camera that are both optimised for the near-IR region [28,29]. More recently, confocal Raman spectroscopy, as well as confocal scanning laser microscopy has been employed as noninvasive methods to obtain detailed information about the molecular composition of different tissues with high spatial resolution, providing optical sections of the tissue without physical dissection. A combination of both techniques in a single instrument has also been recently described [30*], with the conclusion that the technique has great potential for fundamental skin research, with direct applications in pharmacology (percutaneous transport), clinical dermatology, and cosmetic research, as well as for noninvasive analysis of blood analytes, including glucose. The more recent applications of Raman spectroscopy for skin studies consist of its use as a highly specific, sensitive and precise method that allows the rapid assessment of dermal carotenoid content in large populations with excellent correlation to serum level [31], the identification of epithelial cancers [32] or the *in vivo* study of the human stratum corneum composition [33].

**Microorganisms**

Accurate and rapid bacterial identification is necessary in many areas, and in particular, it is crucial within clinical
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Raman spectroscopy has been used by Esposito et al. [35] to describe the analysis of individual bacterial endospores from four species in the genus Bacillus by confocal Raman microscopy. The broad fluorescence background observed was reduced by photobleaching of each spore for approximately 1 min by the excitation laser before the Raman spectrum was collected. The combination of confocal Raman microscopy with optical trapping techniques such as optical tweezers enables the manipulation of particles to allow the acquisition of Raman spectra of isolated cells in aqueous media. Recently, Chan et al. [36*] obtained rapid detection and identification of bacterial spores with no false positives using the above-mentioned combination.

The study of bacterial components has been also described employing SERS [37]. To achieve the necessary enhancement, the silver colloids are targeted to specific regions within the bacterial cell so that, depending on the methods used for their preparation, sensitivity to different cell components is obtained. Thus, a very high sensitivity and specificity to flavins is found in the cell wall, while the inside of the cells show mostly carboxylate and phosphate bands.

**Chemical analysis**

Raman spectroscopy is more often associated with the determination of molecular structure and with qualitative analysis; as an example, the conformational characterisation of natively unfolded proteins through a simple band fitting of the amide I region has been recently described [38]. Nevertheless, there are also numerous applications of Raman spectroscopy for the quantitative analysis of sample composition [39], the most relevant application fields being clinical [40,41], pharmacological [42*,43] and food analysis and quality control [44].

A representative example of the suitability of Raman for analytical measurements is the quantitative analysis of mitosantrone (an anticancer agent that must be controlled during therapy) by SERS using a silver colloid and a flow cell [40]. The limit of detection in serum was $4.0 \times 10^{-11}$ M (0.02 ng/ml) mitoxantrone. The reproducibility ranged from 0.6 to 19.2%, with the greatest variability in signals (19.2–9.4%) at the lowest concentrations. The method was validated by comparison of the results with those obtained by HPLC.

**Conclusions**

Raman spectroscopy has proven to be a very powerful technique that is currently experiencing a renaissance. In addition to well-known techniques such as FT-Raman spectroscopy and SERS, the technological advances now open the field to new possibilities, exploiting the
achievements in theory developed during the past few years. Well-known problems such as fluorescence, poor sensitivity or reproducibility, are being overcome by new techniques and approaches. Raman spectroscopy has been applied to several fields, with the role played in chemical biology being remarkably relevant. Single molecule detection, single cell detection and tissue imaging, are currently the state of the art; the combination of Raman spectroscopy with powerful chemometric tools enables reliable quantitative analysis of target molecules, cluster classification and identification and differentiation of cells and bacteria for clinical pathology and microorganisms study. With the current involvement of the scientific community in the development and use of this technique, new advances and applications are soon to be expected, such as the establishment of in vivo measurements as routine technique for clinical diagnosis, or even more sensitive and reliable detection methods.

**Update**

Recently, a new insight into the electronic structure of β-hematin and therefore hemozoin (malaria pigment) has been provided by a detailed study using resonance Raman spectroscopy [45]. The Raman excitation profiles of β-hematin show a dramatic enhancement compared with monomeric hemein, demonstrating that the stacking of hemes results in a strong $z$-polarized charge transfer. These results have important implications in understanding the structure and formation of malaria pigment, which is very valuable information for the required development and testing of novel therapeutic agents for the treatment of prophylaxis of human malaria infections.

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**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

● of special interest
●● of outstanding interest


21. Choo-Smith LP, Edwards HGM, Endtz HP, Kros JM, Heule F, Barr H, Robinson JS Jr, Bruining HA, Puppels GJ: Medical applications of Raman spectroscopy: from proof of principle to clinical implementation. Biopolymers 2002, 67:1-9. Taken from the viewpoint of clinicians and medical analysts, the potential of Raman spectroscopic techniques as new tools for biomedical applications is discussed and a path is proposed for the clinical implementation of these techniques.


A very interesting paper in which Raman mapping data sets of human living cells were recorded without the need for fixatives, markers or stains. Using PCA for data evaluation, bands were assigned to proteins, lipids, cholesterol and nucleic acids, allowing for the identification of the main cellular constituents of freeze dried and living cells.


Combining optical trapping with confocal Raman spectroscopy using a single laser source, the rapid identification of micrometer-sized bacterial spores in an aqueous environment is demonstrated. The optical tweezers immobilise the bacteria while maintaining it in the center of the laser beam path and within the laser focus, maximising the collection of the Raman signals.


This article presents a short introduction to Raman spectroscopy and several applications in the pharmaceutical field, including a complete list of pharmaceuticals and related chemicals studied by Raman spectroscopy, with the corresponding references.

