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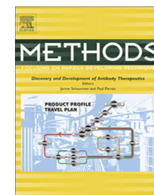

State of the art Raman techniques for biological applications

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ABSTRACT

Raman spectroscopy is a powerful tool for the elucidation of qualitative and quantitative information from biological systems and has huge potential in areas such as biotechnologies, drug discovery, agro-chemical research and clinical diagnostics. This report summarises the principal Raman techniques applied to biomedical systems and discusses the challenges that exist to the wide spread adoption of Raman spectroscopy.

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1. Introduction

Optical techniques such as bright field and differential interference contrast microscopy have long played an important role in our understanding of biological systems [1]. However, these microscopy tools provide no chemical information about the system under investigation. Techniques such as fluorescence microscopy allow the identification and localisation of specific chemical species by detecting light emission from fluorophores. However, the number of intrinsic fluorophores, such as green fluorescence protein (GFP) is limited, and extrinsic labels often perturb the system or alter the physiochemical properties of the substance under investigation [2]. A range of label-free techniques along with the required measurement environments, spatial resolutions, sensitivities, costs, ease of use, comparability and traceability to the SI are listed in Table 1. Raman microscopy is a powerful tool for the non-destructive, *in vivo*, label-free extraction of chemical information from biological materials with lateral resolution at the micrometre length scale [3]. Raman scattering is an inelastic scattering process in which photon energy is altered, however, the momentum is conserved [4]. When light is incident on a sample, the majority of the photons are scattered elastically without energy loss which is called Rayleigh scattering. However, a small number of photons, around 1 in 10^6, go through the Raman scattering process. These inelastically scattered photons contain information about the vibrational motions of the covalent bonds present in the chemical species. Collection and analysis of these inelastically scattered photons by a spectrometer can be used to identify, locate and quantify the concentration of the chemical species.

Although Raman spectroscopy has long been applied to fields ranging from forensic art identification to industrial scale quality control, it is only recently that it has seen wide spread application to biological systems [5]. This has largely been the result of the advances in laser technology, availability of the fibre probes, improvements in detector technologies, and an increase in demand for label-free chemical analysis for the study of biological systems. One emerging area is the use of Raman chemical signatures as *in vivo* and non-destructive probe of diseases. As the progression of a disease is inevitably accompanied by chemical changes, a chemically specific technique, such as Raman microscopy, has the potential to become a compelling alternative to the almost universally applied method of biopsy and histopathology, which delays diagnosis and increases patient suffering. Additionally, biochemical changes are expected to precede the architectural and morphological changes and can facilitate earlier detection and improved prognosis. Raman spectroscopy has already been applied to the diagnosis of several diseases [6–8], and has enormous potential as a routine clinical tool. It is not only confocal Raman spectroscopy that can be applied to the study of biological systems, but a number of new Raman scattering based techniques have also emerged in response to the specific problems within various fields. This review summarises the foremost of the Raman

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Table 1
Comparison of existing label free chemical analysis techniques.

	Technique	Measurement environment	Spatial resolution	Sensitivity	Cost	Ease of use	Comparability	Traceable
	Spontaneous Raman	Ambient	1 µm	4×10^{10} Oscillators	<$50K	Very easy	Medium	No
	FT-IR	Ambient	>3 µm	–	<$50K	Very easy	Medium	No
	CARS	Ambient	≈300 nm	1×10^{5} Oscillators [103]	<$250K	Difficult	Poor	No
Vibrational	SRS	Ambient	≈300 nm	1×10^{3} Oscillators [55]	<$250K	Difficult	–	No
	Offset Raman	Ambient	>1 µm	4×10^{10} Oscillators	<$50K	Easy	–	No
	TERS	Ambient	≈15 nm	Single molecule [104]	<$ 250K	Very difficult	Poor	No
	ULRS	Ambient	≈300 nm	5×10^{2} Oscillators [105]	<$250K	Difficult	–	No
	SIMS	UHV	100 nm	Attomole	>$1M	Difficult	Medium	No
	MALDI	Ambient/UHV	5 µm	Attomole	<$500K	Intermediate	Poor	No
Mass spectrometry	Ambient MS	Ambient	40 µm	Femtomole	<$500K	Intermediate	–	No
	Electrospray-MS	Ambient		–			–	No
	Confocal	Ambient	≈300 nm	SM [106]	<$50K	Easy	Medium	No
Fluorescence	Super-resolution	Ambient	≈50 nm	SM [107]	<$50K	Intermediate	–	No
	Two-photon fluorescence	Ambient	≈300 nm	SM [108]	<$250K	Intermediate	–	No
	EDAX	UHV	>2 µm	≈200 ppm [109]	<$1M	Easy	–	No
Electron	EELS	UHV	0.1 nm	≈10 ppm [110]	<$1M	Easy	–	No
	XPS	UHV	≈100 µm	Highly sample dependant [111]	<$1M	Easy	Good	No

scattering based techniques applied to biological systems, and discusses the barriers to making Raman scattering techniques robust and routine biomedical tools.

2. Challenges in bioanalytical measurement

Although Raman scattering tools have many desirable advantages, traceable quantification is still one of the major barriers to its widespread adoption in clinical and biomedical diagnostics. Previously, studies have focussed primarily on using Raman spectroscopy for qualitative characterisation of biological samples. Studies, in which quantitative information has been obtained, are often performed *ex vivo* with measurements carried out on carefully designed research instruments. However, many clinical and biomedical applications require the *in vivo* determination of quantitative chemical information. For example, the quantitative analysis of transdermal drug delivery is essential for pharmacological studies [9]. Currently, this requires a long process of destructive sectioning and subsequent analysis. Here, the benefits of a quantitative, *in vivo*, non-destructive alternative are obvious. The transdermal delivery of several substances has been investigated *via* Raman spectroscopy [10–13]. However, little work has been done towards robust quantification of the results. More generally, the quantification of analyte concentrations in blood and tissue is predominant for the diagnosis and monitoring of most diseases. Tissue biopsy or the drawing of blood samples, followed by lengthy extraction and measurement procedures delays diagnosis, and a non-invasive technique, such as Raman spectroscopy, is highly beneficial. In cases where the sensitivity of conventional Raman scattering is insufficient to measure the physiological concentrations of biomarkers, typically found in human serum e.g., creatinine and uric acid, the detection limit can significantly be improved with the aid of surface-enhanced Raman scattering (SERS). When combined with isotopically labelled internal standards, the resulting approach (isotopically diluted SERS or ID-SERS) unites high detection sensitivity and molecular specificity with the advantages of reliability, accuracy, and repeatability during quantification [14–16].

The primary challenge in quantitative Raman spectroscopy lies in the fact that the absolute Raman response is difficult to measure reproducibly [5]. Typically, this is due to a combination of factors such as scattering of the incident beam by the sample, inhomogeneity in the optical properties of the sample, and calibration of the optical system employed. Variations in instrumentations can be overcome through the use of internal standards to normalise the Raman signal. In simple systems, the overall Raman intensity is then assumed to be a linear superposition of all of individual spectral contributions. This method has been applied to the *in vivo* determination of blood glucose concentrations, using both haemoglobin [17] and water [18] as intrinsic internal standards. However, these measurements are susceptible to the compositional inhomogeneities common to biological systems. *In vivo* determination of blood glucose concentration in whole blood samples has also been achieved using a non-imaging instrument, with quantitative data extracted *via* a partial least squares approach [19]. Although these studies have shown success in the quantification of analytes using individual calibration schemes, their application to larger populations has not shown adequate quantification. This difficulty in calibration transfer and by far the highest barrier to quantitative *in vivo* Raman spectroscopy comes down to the highly variable optical properties of biological media.

Turbid media contain inhomogeneities at the length scale of the wavelength of light and therefore, scatter strongly. Biological samples, such as tissue, can almost universally be classified as turbid [20]. It is this turbidity that severely hinders the reliable and routine acquisition of quantitative Raman data. Variations in optical properties both within and between samples manifest as probe volume uncertainties. As the probe volume is intimately tied to the number of molecules sampled, this is a critical parameter in quantitative measurements. This is compounded by the fact that the wavelength dependant optical properties of turbid media distort the shape and intensities of the observed Raman spectrum. These factors serve to introduce analyte independent variance into calibration plots [21], increasing uncertainty in quantitative measurements and making inter-sample calibration transfer unrealisable. Samples bearing the extremes of these effects can be incorporated into multivariate calibration models; however, this comes at the expense of increased complexity and reduced accuracy. Formalisms exist for the correction of distortions to spectral shape and intensity as a function of the optical properties of the sample [22]. However, these models assume that the sample scatters isotropically and are thus more applicable to inorganic solids and powders than anisotropically scattering biological media. The realisation of reliable and quantitative *in vivo* Raman measurements hinges on the development of methods to determine robust calibration models in turbid media. One potential approach for the

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