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**Raman spectroscopy in nanomedicine: current status and future perspectives**

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Keywords: Spontaneous Raman Spectroscopy, Surface Enhanced Raman Spectroscopy, Tip Enhanced Raman Spectroscopy, Coherent Anti-Stokes Raman Spectroscopy, Nanotechnology, Nanomedicine

**Abstract:**

Raman spectroscopy is a branch of vibration spectroscopy which is capable of probing the chemical composition of materials. Recent advances in Raman microscopy have added significantly to the range of applications which now extend from medical diagnostics to exploring interfaces between biological organisms and nanomaterials. In this review, Raman is introduced in a general context, highlighting some of the areas in which the technique has found success in the past, as well as some of the potential benefits it offers over other analytical modalities. The subset of Raman techniques which specifically

probe the nanoscale, namely Surface Enhanced and Tip Enhanced Raman Spectroscopy, will be described and specific applications relevant to nanomedical applications will be reviewed. Progress in the use of traditional label-free Raman applied to investigation of nanoscale interactions will be described, and recent developments in Coherent Anti-Stokes Raman Scattering will be explored, particularly applications to biomedical and nanomedical fields.

**Keywords**: Spontaneous Raman Spectroscopy, Surface Enhanced Raman Spectroscopy, Tip Enhanced Raman Spectroscopy, Coherent Anti-Stokes Raman Spectroscopy, Nanotechnology, Nanomedicine

**Introduction**

Nanomedicine can be defined as the medical applications of nanotechnology[1], ranging from the use of nanomaterials in regenerative medicine, drug delivery strategies, medical diagnostics and therapeutics and including potential negative impacts of nanomaterials to human health, commonly encompassed under the term Nanotoxicology. In the context of this review article, nanomedicine is viewed from the perspective of how Raman spectroscopy (and its variants) can be used in the assessment of the beneficial as well as the potential negative impacts of Nanomaterials on human health. Nanomaterials have already found uses in a wide range of applications, including anti-microbial paint coatings[2], textile finishing[3], and novel applications in the electronics industry[4]. Notably, biomedical applications are rapidly emerging, ranging from nanoparticle coated stents for angioplasty[5], contrast agents for diagnostic imaging[6,7] and also potential drug and gene delivery vehicles[8–10]. These applications are largely dependent on the particular characteristics which nanomaterials and nanoparticles possess. These include properties such as increased surface to mass ratio which in turn results in an increase in surface reactivity, while novel optical properties associated with some classes of nanoparticles are important for applications in theranostic imaging and subsequent monitoring of drug delivery. However, whilst these technologies show promise, it is important to be able to visualise how the materials behave *in situ*, and particularly in the biological context, to be able to characterise their interactions and toxicological effects, be they *in-vitro* or *in-vivo*. While it has been highlighted that comprehensive characterisation of the physico-chemical properties of nanoparticles is imperative, changes to these properties, such as aggregation state and effective surface chemistry, can play a critical role in their modes of interaction and action [11]. Equally, to understand the modes of action and optimise efficacies, monitoring and understanding changes to the biological environment is critical, not only on a cellular level but also when considering the systemic responses.

Considering the system as a whole, one must be able to track a particle or material from initial exposure or administration through to the site of action and on to assimilation, degradation or excretion. At each step in this process, one must be able to access and visualise the efficacy by which the particles can overcome certain barriers to successful administration. These can vary from the route of exposure, assessing whether the particle causes toxicity, particle retention (e.g. via the enhanced permeability and retention (EPR) effect), or removal for circulation via uptake by the reticuloendothelial system (RES), accumulation of the nanoparticles over time, non-specific interactions, the efficacy with which the particle reaches its desired location etc..

Ideally, what is required is a method which can successfully characterise these processes, firstly in fundamental *in vitro* cytological and *ex-vivo* histological studies and ultimately in more realistic *in-vivo* applications. This method should be capable of identifying the particle or material of interest while simultaneously being able to access the surrounding environment while measuring the efficacy of the probe or nanocarrier and/or the physiological response of the organism.

There exists a large range of analytical methods which can be used in the classification and characterisation of nanomaterials. These include scanning and transmission electron microscopy (SEM and TEM), atomic force microscopy (AFM), other label free optical methods such as differential interference contrast (DIC) and dark field microscopy and fluorescent microscopy methods based on intrinsic nanoparticle or external label fluorescence, to name but a few. However, these methods are not without certain drawbacks which limit to some extent their applicability and effectiveness.

Firstly, both AFM and SEM can be considered as primarily surface sensitive techniques, while, when TEM is coupled with serial sectioning and ultra-microtomy, it has been used for 3D reconstructions and tomography[12,13]. However, these processes are time consuming, costly and laborious. In addition, EM requires a particle to have contrasting electron density compared to its environment to

allow for a particle to be visualised, which renders it ineffective for many “softer” polymeric nanoparticles. EM does not allow live cells to be imaged and, as it requires extensive sample processing, it provides only a limited scope for rapid or routine investigation of nanomaterials *in-vitro*. What EM and AFM do provide is the capability of imaging beyond the optical diffraction limit. More recently developed optical based methods, so-called super resolution microscopy, have become available that allow for imaging beyond this limit [14–16]. However, their use has been limited in the field of nanomedical sciences as of yet.

In contrast, standard fluorescent based microscopy has been used extensively in nanoparticle studies[16–19]. Confocal Laser Scanning (fluorescence) Microscopy (CLSM) has become a standard in the toolbox of techniques for *in vitro* cytommetry [21]. Although the technique is limited in resolution to hundreds of nanometers, it can potentially detect fluorescence emission from, and therefore the location of, individual nanoparticles. Penetration depths *in vivo* can be extended through two photon excitation techniques and/or NIR fluorophores [22,23]. In the visible region, a range of fluorescent assays and labels are commercially available to probe a range of physiological processes *in vitro,* such as lyso and mitotracker used for labelling lysosomes and mitochondria [101] Intrinsically fluorescent nanoparticles such as inorganic semiconductor quantum dots have been developed for similar applications [102] and surface functionalisation of these types of materials has contributed to understanding the dependence of uptake and intracellular trafficking on surface chemistry [24]. Many similar studies have been performed with fluorescently labelled nanoparticles [25,26] which are commercially available in a range of sizes and surface functionalities. However, not all nanoparticles can be easily fluorescently labelled. Furthermore, it is not clear that the transport mechanisms of smaller nanoparticles, fluorescently labelled with anionic moieties, are the same as their unlabelled counterparts [27]. Critically, there have been reports that labelled nanoparticles can release the dye into the surrounding biological environment, and so the distribution of fluorescence within the cell does not

necessarily represent the presence or subcellular distribution of the nanoparticles[28–30]. Other label free optical microscopy techniques are also limited by the type of particle which can be visualised i.e. only metal based particles are effective for dark field and DIC microscopy [31].

Raman spectroscopy has been proposed as a method for monitoring nanomaterials in biological systems, as it potentially provides a label free, non-invasive probe of the nanoparticle itself, the local environment and the physiology of the organism [32] . Over the past decade, Raman spectroscopy has been applied to a range of biomedical areas, including cancer diagnostics[33], toxicity studies[34], atherosclerosis[35] and investigation of skin[36,37]. Importantly, what Raman provides is not just a method for differentiation between a diseased and non-diseased state, it is based on characterisation of the (bio) chemical nature of a sample, based on the characteristic vibrations of the molecular bonds of the constituent components. Raman is a form of vibrational spectroscopy, which in itself is a subset of a more general umbrella term of spectroscopy. The vibrations are characteristic of the molecular structure and, in polyatomic molecules, give rise to a spectroscopic "fingerprint". The spectrum of vibrational energies can thus be employed to characterise a molecular structure, or changes to it due to the local environment or external factors. The Raman spectrum is thus a truly label free signature of the nanoparticle. Vibrational energies typically fall in the mid Infrared (IR) region of the electromagnetic spectrum and are quite commonly probed using IR absorption spectroscopy. Raman in many ways can be viewed as a complementary technique to IR spectroscopy; whereas IR involves absorption of radiation, Raman is an inelastic scattering technique whereby the incident radiation couples with the vibrating polarisation of the molecule and thus generates or annihilates a vibration. For a vibration to be active in IR spectroscopy, a change in dipole is required, whereas to be Raman active, a change in polarisability is required. As a rule of thumb, vibrations of asymmetric, polar bonds tend to be strong in IR spectra, whereas Raman is particularly suitable as a probe of symmetric, nonpolar groups. Importantly, this results in the O-H bonds of water being strong absorbers in IR spectroscopy, whereas

they are relatively weak Raman scatterers. This allows for samples to be investigated in an aqueous environment and thus the technique of Raman spectroscopy more readily lends itself to live cell *in vitro*[38] or *in vivo*[39] measurement. As the vibrational spectrum is measured as a frequency (or energy) shift from that of the incident radiation, Raman spectroscopy can be performed across the UV, visible or near infrared spectral regions, and thus can benefit from the technologies available and advances made for confocal optical microscopy.

A number of variants which are based around the physical principal of Raman spectroscopy exist. Spontaneous Raman can take the form of Stokes Raman scattering and anti-Stokes Raman scattering, the former resulting from the creation of a vibration in a material, characterised by a decrease in the incident photon energy (frequency), the latter from the annihilation of vibration, characterised by an increase in the incident photon energy. If the incident radiation is resonant with an electronic absorption of the analyte, the Raman signal can be resonantly enhanced by several orders of magnitude. The use of Resonant Raman Spectroscopy (RRS) in biomedical systems has been limited, however, due to associated photochemical degradation phenomena and the generation of fluorescence which can swamp the Raman signal of the overall sample.

Other variants of these two techniques with increased sensitivities for more molecularly specific characterisation have been developed. These include resonant Raman spectroscopy, coherent anti Stokes Raman spectroscopy (CARS), tip enhanced Raman spectroscopy (TERS) and surface enhanced Raman spectroscopy (SERS). The majority of these techniques have been applied to nanomedical applications; however, two of these methods deal inherently with the nanoscale, namely TERS and SERS. Although Raman is fundamentally an optical technique and is thus similar to confocal optical microscopy, limited to spatial resolution of the order of hundreds of nanometres, nanometre resolution can be obtained through localised enhancement processes. This localised enhancement led to the initial

interest in the prospect of the use of Raman spectroscopy to probe the specific environment of the nanoparticle.

This article will outline the applications of the various Raman spectroscopy based technique in the broad area of Nanomedicine. As they are nano-specific, the use of SERS and TERS techniques will be presented initially, while the increasing interest in the use of truly label free spontaneous Raman and Coherent Anti-Stokes Raman Spectroscopy (CARS) in nanomedical applications will then be explored. In Raman spectroscopy, the sensitivity, spatial resolution and penetration depth and required scan rates depend on technique employed, resonance conditions and even the instrumental set-up (microscope objective, grating, laser power). In the respective section describing each modality, examples of the state of the art in nanomedical applications are provided. The future perspectives attempts to address routes beyond the current state of the art. A more detailed description of the historical origin and basic principles of the Raman scattering process can be found in numerous excellent text books[40–44] and review articles[45–47]. A comparison of Raman and IR spectroscopies for biomedical applications can be found in [48].

**SERS**

The phenomenon of surface enhanced Raman spectroscopy was described as early as 1974 [49,50], and is understood to arise from a localised increase in the coupling between the electromagnetic field of the incident radiation and the polarisation of the analyte in the presence of optically induced surface plasmons on a metal surface. Increases of Raman intensities as high as $10^{10}$ have been reported [51], although the spatial range of enhancement is only of the order of tens of

nanometers. The enhancement process can be achieved using a number of substrates including roughened metallic surfaces, structured metal arrays and specially imprinted surfaces.

Notably, the SERS effect can be induced through the use of metallic nanoparticles and nano colloid aggregates. SERS is a direct enhancement of the Raman signal and in the case of nanoparticles this occurs in the immediately surrounding local vicinity. The true principal that governs SERS enhancement is not fully understood, although the effect has largely been attributed to an electronic enhancement due to local fields generated by surface plasmon resonances at the metal surface. Alternatively, the enhancement has been attributed to a charge transfer process between the analyte and the surface, although it is probable that the processes act in tandem[52]. The technique of SERS in a biomedical context is reviewed in greater detail in the following papers[53–55].

Nanoparticles and aggregates which are used for SERS enhancement typically consist of a metallic nanoparticle, most commonly gold and silver. Quite often, these particles are subsequently modified via surface functionalisation which can include targeting moieties designed for specific applications, especially as nanosensors. The particle may also be labelled with a Raman reporter moiety which allows for identification of the particle in the biological milieu. Using these particles, it has thus been possible to apply SERS to a number of biological scenarios, which include diagnostic studies *in vitro*, *ex-vivo*[56,57] and *in-vivo*[58,59] , novel bio assays[60–62] as well as cellular studies.

SERS has been proposed as a method for understanding how nanomaterials behave in a cellular environment, important in the study of the fundamental interactions of nanoparticles in the context of toxicology, drug delivery or contrast agents for diagnostics. In 2003, Kneipp et al. proposed that by using SERS it would be possible to probe the chemical nature of the subcellular environment and the intracellular distribution of biomolecules. This work was extended by incorporation of Raman reporters which allowed for localisation of the SERS probe within the cell, leading to chemical probing of sub

cellular nanostructures[63–66]. For example, in 2010, the group showed how a SERS nanosensor was capable of investigating pH changes in a cell throughout the stages of the endocytic pathway of the nanoparticle probe. The study was based on changes in the pH of the local environment in different cellular organelles which can be monitored via changes in the pH sensitive nanoprobe over time[67].

Other cellular studies have also investigated the possible use of SERS in the investigation of cell surface receptors associated with cancer. In one such study, Kong et al 2012 used organometallic SERS active nanoparticles which were targeted to live cells expressing the EGFR (epidermal growth factor receptor). The SERS nanoparticles were shown to be capable of specific targeting to the cell surface and offered increased sensitivity in comparison to other imaging modalities[68]. Figure 1 a-e, shows oral squamous cell carcinoma (OSCC) cells expressing the epidermal growth factor receptor (EGFR), c and e show the SERS image generated by CO at $2030cm^{-1}$ and protein at $1600cm^{-1}$ respectively. The targeting is verified in Figure 1 f – j in a non-EGFR expressing cell line SKOV3 (ovarian carcinoma) and in Figure 1 k-p by blockage of the EGFR using an EGFR antibody.

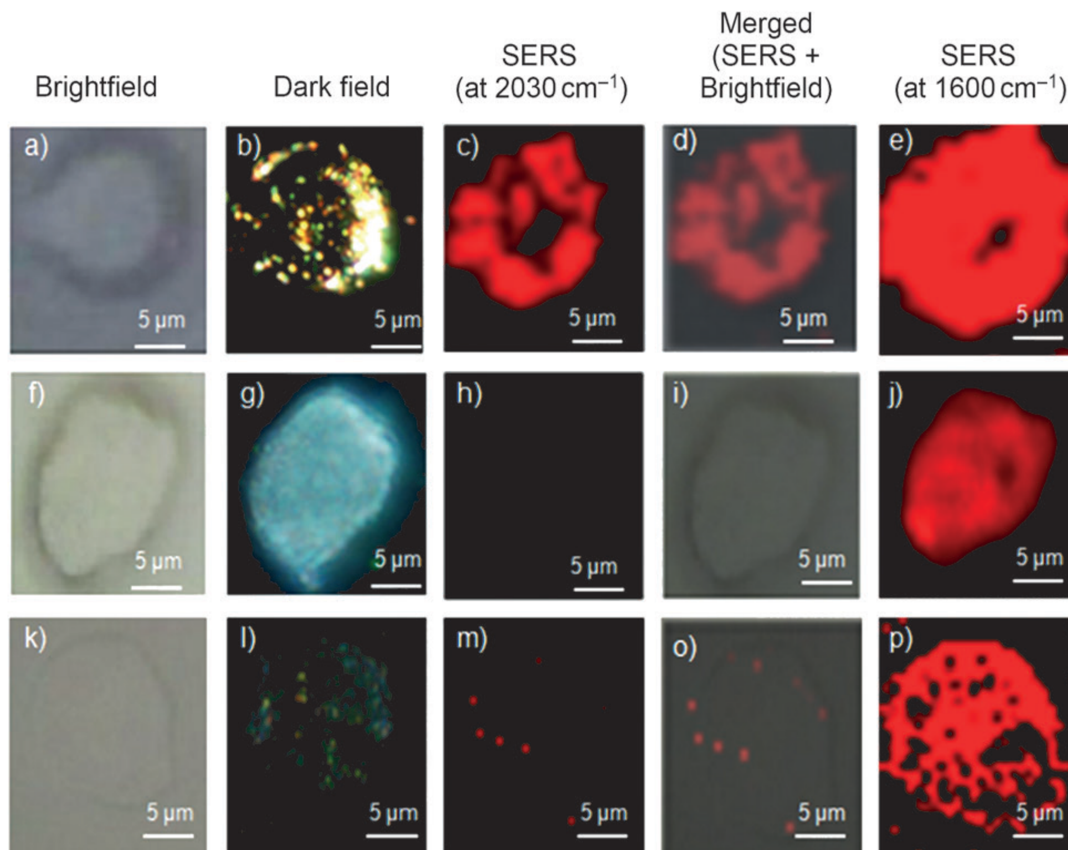



Figure 1 shows (a, f, k) the brightfield image, (b, g, l) the darkfield image of the nanoparticles, c, h, m) the SERS image of CO at 2030cm$^{-1}$, (d, I, o) merged SERS and brightfield, and (e, j, p) the SERS image generated using the protein band at 1600cm$^{1}$. a – e shows OSCC cells, f – g SKOV3 cells not expressing EGFR and k – p OSCC cells treated with anti-EGFR. Reproduced from [68]

Another demonstrated application of SERS in is the analysis of human serum. Lin et al., in 2011, demonstrated the power of SERS coupled with multivariate analysis to distinguish in a non invasive way between patients previously diagnosed with colorectal cancer and control patients with 100% diagnostic sensitivity and specificity[69].

*In-vivo* SERS is also possible, and has been demonstrated as a potential labelling method for a number of applications. SERS has been used *in-vivo* to investigate how enhancement of the Raman signal can be used as a method for tumour detection. Qian et al. showed how EGFR targeting PEGylated

gold nanoparticles labelled with a Raman reporter where capable of >200 times greater signal generation in the infrared compared to that of near infrared fluorescent quantum dots, which allowed for the possible identification of small tumours at penetration depths of ~ 1-2cm[58]. Other *in-vivo* applications of SERS have also been explored, including an *in-vivo* study of inflammation in mice[70], demonstrating improvements over fluorescent based methods. SERS has also been shown to be capable of single molecule detection *in-vitro,* a sensitivity which sets it apart from spontaneous Raman spectroscopy [71].

More complex Raman based investigations have also taken advantage of the surface enhancement process. Techniques such as deep penetrating spatially offset Raman (SORS) have been combined with nanoparticle based SERS in SESORS[72,73]. In brief, in the SORS technique, introduced in a paper by Matousek *et al,* the Raman spectra are collected at positions spatially offset from the point of incidence of the probe laser beam. Rather than using microscopic objectives for delivery and collection, fibre probes are used. By moving the collection point away from the probe launch site, contributions from the surface Raman photons are diminished and those of Raman photons from deeper within the sample are increased. Using multivariate statistical methods, it is possible to reconstruct spectra from the different layers with a much greater depth of penetration than a traditional confocal microscopy setup[74]. Depth sensitivities of up to several millimeters are now achievable and examples of emerging applications include non-invasive diagnosis of bone disease, cancer and monitoring of glucose levels[75] SESORS uses this same principal, taking advantage of the surface enhancement of the Raman signal from metallic nanoparticles embedded within the sample. In a recent publication by Xie et al, SESORS was used to identify bisphosphonate-functionalized nanotags on bone through 20mm of porcine tissue[76]. This study highlights the increasing potential for *in-vivo* applications which SORS and SESORS may have, in the field of nanomedicine.

SERS has enjoyed increasing popularity over the past decade, particularly since the emergence of as increasing range of nanoprobes. However, the uptake rates and mechanisms as well as the subsequent trafficking may be specific to the nanoparticle type, size and surface chemistry. Most SERS probes are specifically designed for a target application and so are labels themselves for the SERS signal. Furthermore, the molecular specificity of the surface enhancement process is not well understood. Therefore, a truly label free method for generic monitoring and characterising the cellular uptake and subcellular localisation of nanoparticles in general is still required.

TERS another method for generating enhancement of the Raman signal. Like nanoparticle based SERS, this method is also based on probing of the inherent nanoscale environment of the sample in close proximity to a nanoprobe and will therefore be discussed.

**TERS**

Tip Enhanced Raman spectroscopy, or TERS, is a method which combines Raman spectroscopy and scanning probe microscopic techniques such as AFM. TERS, like SERS, is a method to enhance the Raman signal and, in principle, the mechanism of enhancement is the same. Scanning probe tips have dimensions of the order of tens of nanometers or less, and when metal coated, surface plasmon resonances can be optically induced, similar to the case for metallic nanoparticles. In TERS, the topography of the nanoscale environment of samples can be probed by bringing the tip into close proximity with the area of the sample to be probed, but the Raman signal from the environment being probed by the tip is selectively enhanced by several orders of magnitude, swamping the spontaneous Raman from the remainder of the illuminated spot. Therefore TERS is a method which allows for very small areas or even individual molecules to be probed in a label free manner.

TERS has been used to investigate viral cell interaction[77], cytochrome-c states in isolated mitochondria[78], lipid and protein organisation in artificial cell membranes[79], as well as hemozoin crystal formation inside malaria infected red blood cells, as shown in Figure 2 [80]. Figure 2 A –C show AFM images of infected red blood cells, highlighting the hemozoin crystals inside the cellular vacuole in C. Figure 2 D shows the TERS spectrum from the edge of the crystal deposits showing characteristic peaks associated hemozoin and the profile is compared to the SERS and RRS spectra of β-hematin in F and G . This study highlights TERS as a nanoscale technique with can be used to probe very specific areas which may have implications in disease. In this instance, TERS provides a potential method to study the interaction of quinoline anti-malarial drugs which are believed to preferentially bind to the edge of hemozoin crystals.

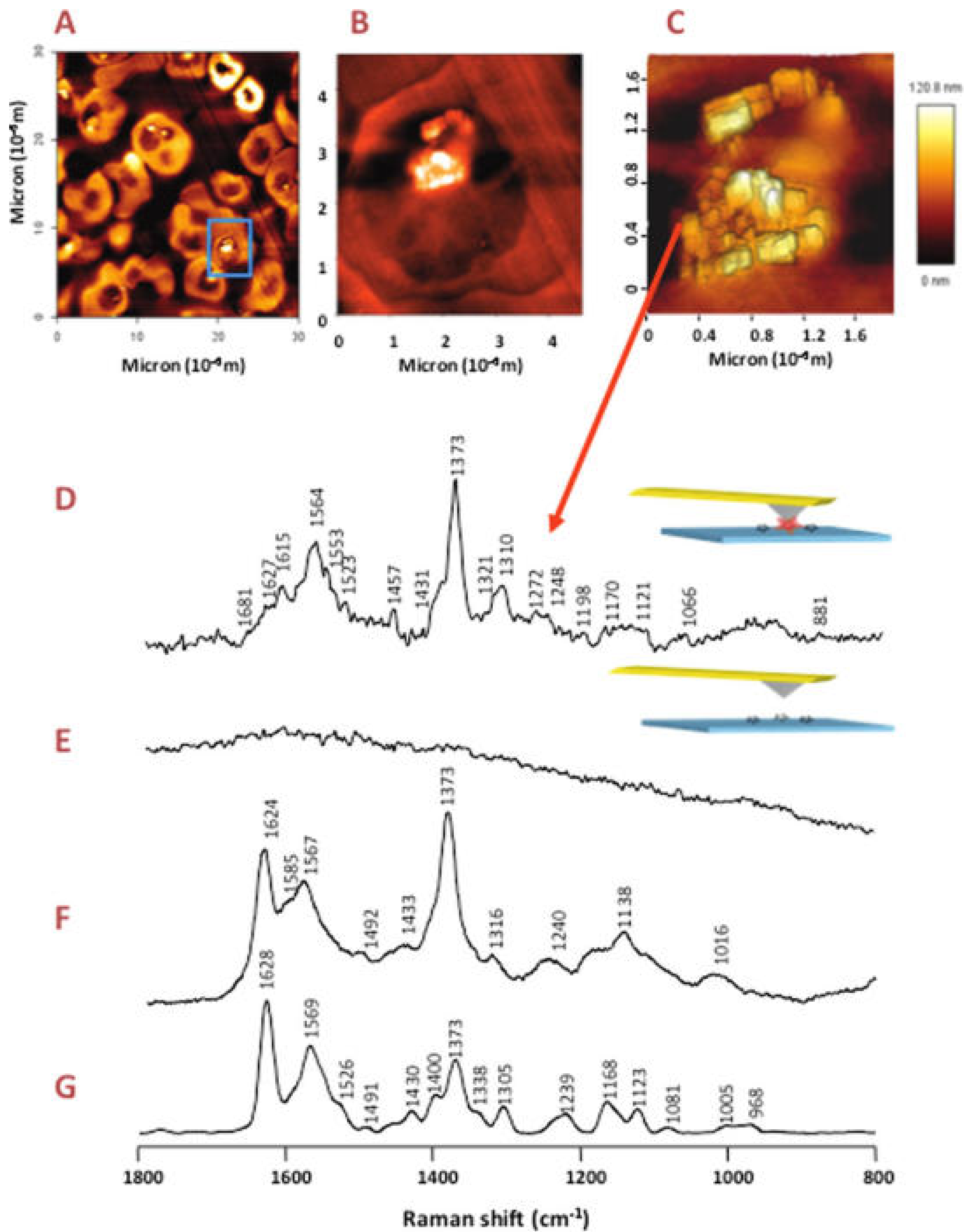



Figure 2. TERS probing hemozoin crystal formation inside malaria infected red blood cells. A – C show AFM images of infected red blood cells. D shows the TERS spectrum for the edge of the hemozoin crystal deposit, E is the spectrum of the tip following retraction from the cell, F SERS spectrum of β-hematin, G resonance Raman (RR) spectrum of β-hematin. Reproduced from [80].

TERS has also been used in the investigation of the interaction between cells and nanoparticles. Alexander and Schultz (2012) were able to show the interaction of individual antibody conjugated nanoparticles and cell surface bio molecules using TERS, with a similar sensitivity to SERS[81].

However, as TERS requires the use of AFM tips to enhance the signal, the method is restricted to being a surface classification technique and thus is of limited use for intracellular or indeed *ex-vivo* or *in-vivo* tissue analysis. While surface enhanced methods provide promise in a number of nanomedical areas, there are some caveats associated with these methods. Firstly, the probe must be capable of generating a surface enhancement of the Raman signal; this is only applicable to certain types of gold and silver particles or coated tips, as well as nanoaggregates of these metals. Additionally, these techniques require a considerable expertise in synthetic chemistry and design of probes or tips for specific target applications. Furthermore, reproducibility of the enhancement is also a concern, in particular with TERS, were the reproducibility of the tip characteristics is important in gathering reproducible spectra. Therefore it is important to consider that, while surface enhanced methods have been shown to be capable of nanoscale accuracy, these methods are heavily reliant on specifically designed nanoparticle sensors or probes and tips which in some way dilutes the label free aspect which spontaneous Raman spectroscopy provides.

**Spontaneous Raman Spectroscopy**

To differentiate it from the numerous variants of Raman spectroscopy which have emerged over the past decades, including SERS and TERS, the originally named phenomenon of Raman spectroscopy is now frequently called spontaneous Raman spectroscopy. Spontaneous Raman spectroscopy has been used extensively over the past decades for a range of biomedical applications and is emerging as a viable alternative to gold standard protocols in medical diagnostics. Other uses include investigations in

blood[82] and serum samples[83], investigations of human skin[36,37], cellular investigations[84–86], *in-vivo*[39] and *ex-vivo*[33] characterisations as well as studies of interaction of nanoparticles[34].

Importantly, these applications using Raman spectroscopy rely on the use of data analytical methods which aid in the classification and understanding of the data which has been acquired. This may entail the use of chemometric methods to cluster a data set so that one can see a cell or tissue as a distribution of similar spectra in a map. Multivariate statistical methods can be employed for the separation of two different classes of spectra e.g. a diseased and non-diseased state. A full description of such analytical methods is beyond the scope of this review. However, it is important to highlight how Raman spectroscopy and multivariate data mining approaches are commonly used together to investigate the biochemical nature of samples. Some good examples of where these statistical methods have been applied to Rama hyperspectral datasets can be found here[85,87,88].

Despite the extensive development of Raman spectroscopy for biomedical applications and the specific use of SERS using nanoprobes, not many studies have explored the use of spontaneous Raman spectroscopy for nanomedical applications. Of the reports that exist, some have aimed to look at probing cells for a toxic response [34,89], others have aimed to look at how nanomaterials behave in a cellular environment[32,90] and some have looked at degradation patterns of potential nanoparticle drug carriers[91].

The potential of Raman spectroscopy as a toxicological screening method has been demonstrated for the case of carbon nanotubes and their effects on human cells *in vitro*. Kneif et al 2010, showed how the cellular spectral signatures differed between control and exposed cells due to changes in specific Raman spectral peaks of the cell nuclei. This method provided a way of investigating the toxic response of cells to nanomaterials in a truly label free manner, compared to more typical dye based cytotoxicity testing. In addition to detecting differences in response due to nanoparticle exposure,

it was also possible to statistically compare the dose dependent responses of the Raman signatures with other gold standard toxicity tests, demonstrating the potential of the technique as a quantitative high throughput screening assay[34].

In a different type of study by Dorney et al 2012, the aim was to demonstrate the potential of Raman spectroscopy to visualise and investigate the interaction of polystyrene nanoparticles in cells. The purpose was to use these particles, which are often used as a standard in toxicity studies, as a model particle for further applications using Raman spectroscopy. In brief, the Raman spectroscopic signatures of the cells were mapped with a step size of 0.75μm over a region which contained both nuclear, perinuclear and cytoplasmic regions of the cell. Using a combination of K-means clustering and principal component analysis, it was possible to identify the localisation of the particles inside cells based on the intrinsic polystyrene signature and also to probe the chemical characteristics of the local subcellular environment[32]. A highlight of the results in shown in Figure 3 for cells incubated for 24hrs with polystyrene nanoparticles. The image in Figure 3 (i) shows the brightfield image (A) and the K-means image constructed for the Raman hyper spectral dataset (B). The polystyrene nanoparticles are shown as green pixels in the image and the K-means average spectra are shown in Figure 3 (ii) A-D. The cluster associated with the green pixels clearly shows characteristic peaks associated with polystyrene when compared to a pure sample spectrum, Figure 3 (iii) A and B. The light blue and green clusters were then compared using Principal Components Analysis showing that the nanoparticles are located in lipid rich regions of the cell, which, by comparison with confocal fluorescence microscopy, was demonstrated to be the endoplasmic reticulum.

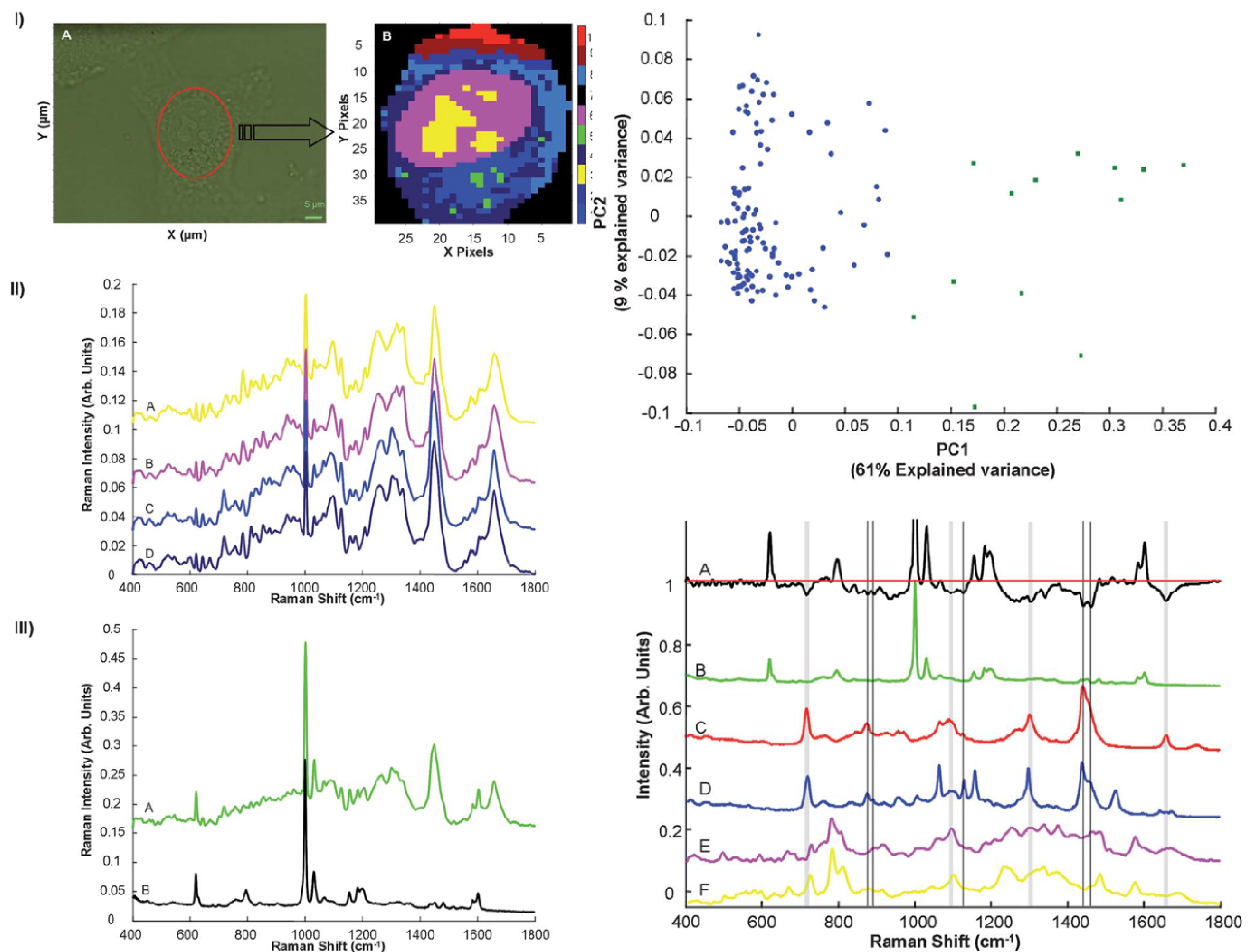
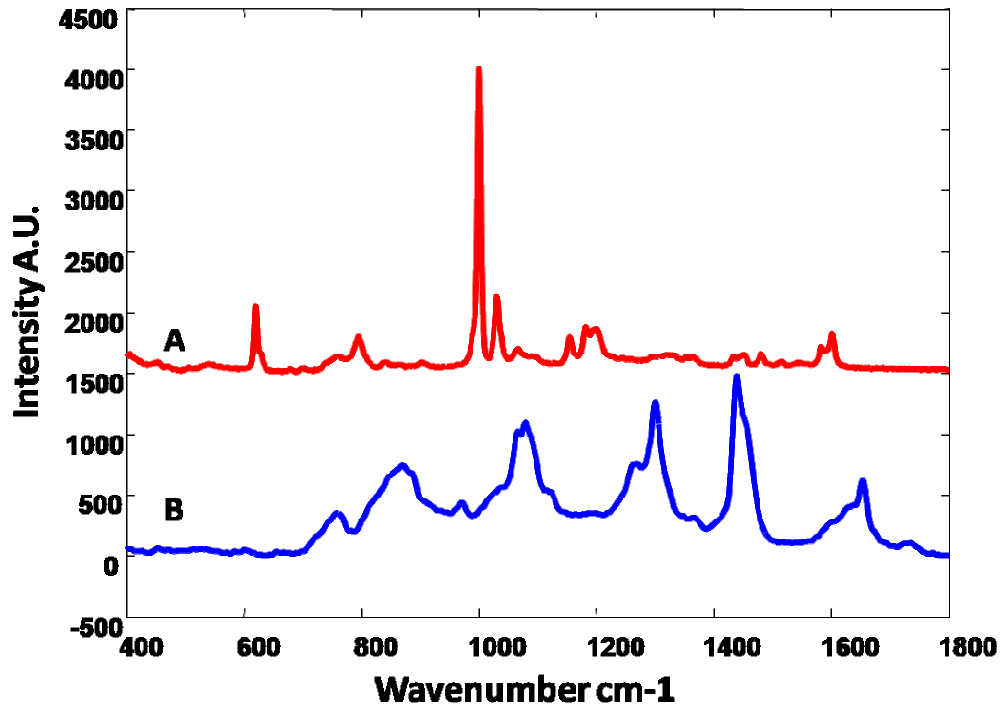



Figure 3. Identification of intracellular distributions of polystyrene nanoparticles using Raman spectroscopy. (i) A shows the brightfield and (B) K-means image of the cell. (ii) shows the K-means cluster average spectra associated with the clusters in the K-means image in the panel above, (iii) shows the K-means cluster spectrum associated with polystyrene nanoparticles (A) compared with a pure spectrum of polystyrene (B). The Right panels show a Principal Component Analysis scatter plot (top), differentiating the green (nanoparticle) and light blue clusters (cytoplasm), and the loading of Principal Component 1 (Bottom, A), suggesting the local environment surrounding the nanoparticles is lipid rich. Reproduced from [32].

Additionally, in a follow up paper by Keating et al 2012, the supervised approach of spectral cross correlation analysis (SCCA) for localisation of nanoparticles was used on the same spectral dataset.

It was thus possible to identify all the regions within the cell that contained polystyrene nanoparticles, accounting for the misidentification of some clusters in the dataset that were apparent with K-means cluster analysis. The localisation of the nanoparticles to a lipid rich region of the cell is highlighted in Figure 4. Figure 4A and B show a pure polystyrene and lipid spectrum which have been cross correlated against the dataset to produce the pseudo colour images in Figure 4C, showing the lipid distribution, Figure 4D the distribution of polystyrene and the merge of the two in Figure 4E. This method provides a clear analytical approach for identification of nanomaterials and separation of different regions of the cell with a particular biochemical distribution[92].



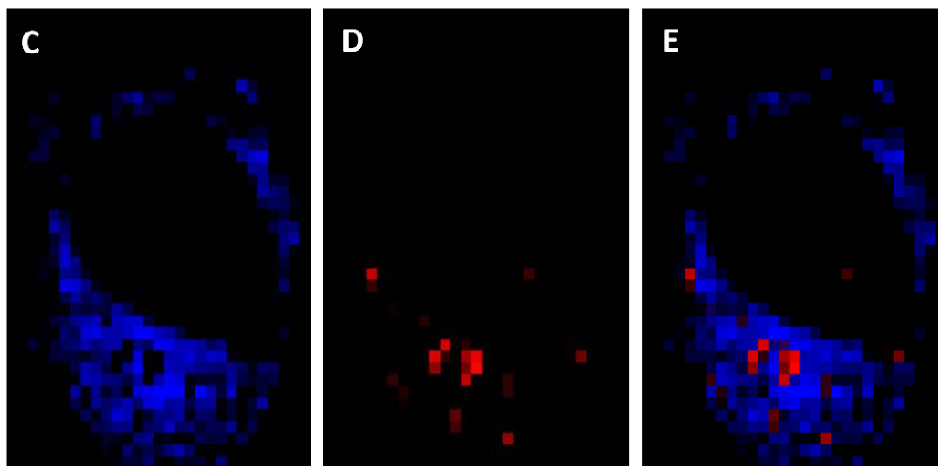




Figure 4. Verification of identification of intracellular nanoparticles using spectral cross correlation analysis (SCCA). (A) and (B) show a pure spectrum of polystyrene nanoparticles and 3-sn-phosphatidylethanolamine. (C) and (D) show the pseudo colour image of lipid and nanoparticle distribution following SCCA with 3-sn- phosphatidylethanolamine and polystyrene. The merged image is shown in E, indicating the particles in a lipid rich environment. Adapted from [92].

Another recent study by Chernenko et al[93] aimed to investigate how different types of deuterated liposomal nanoparticles are distributed in cells. More specifically, it aimed to investigate how different chemical compositions affected how the liposomes associated with the mitochondrion. Notable in this study is the use of deuterated liposomes to enhance the ability to differentiate liposomes from endogenous lipids in the cell, based on the fact that the C-D vibrational frequency is significantly down shifted from that of the C-H stretch of the very abundant intrinsic macromolecules of the cell. Another paper by the same group also looked at the degradation of polymeric nanoparticles over time in cells and concluded that poly lactic-*co*-glycolic acid (PLGA) and polycaprolactone (PCL) drug delivery systems are degraded and incorporated into the late endosomes of the Golgi system, based on spectral changes associated with the specific degradation patterns of the nanocarriers[91].

Spontaneous Raman spectroscopy has therefore already been demonstrated to be a chemically specific method for investigating nanoparticle interactions and also to probe the biochemical nature of cells. Notably, a number of biochemical features can be accessed simultaneously without the need for fluorescence or other labelling methods, or for costly cytotoxicological assays. It should be noted, however, that, based on current technologies, spontaneous Raman is a relatively weak effect, thus highlighting the attention which surface enhanced techniques such as SERS and TERS have received. Relatively weak signals can be compensated for by longer acquisition times, with maximum 2D scan times of the order of 40-80 mins for a 50μm*50μm area with a step size of 500nm for cellular data[94]. However, these scan speeds are largely dependent on the required signal to noise ratio and the step size used in image acquisition. For these reasons real-time imaging has not been realised to date.

Ultimately, for *in vivo* applications, penetration depth is also an important consideration. In Raman microscopy, sensitivities are optimised by choice of objective, providing optical spatial resolution but limited penetration depth (~1-50μm). As Raman spectroscopy is an optical technique, the

penetration depth is largely determined by the choice of wavelength of the source laser, and optimally this can be chosen in the near infrared region where tissue has a transmission window. Absorption is largely governed by that of chromophores such as melanin (in skin) or haemoglobin across the visible, and by the overtones of OH vibrations in the near infra red regions. Scattering is an additional loss mechanism, but the development of Spatially Off-Set Raman Spectroscopy [75] using fibre probe rather than microscope objective delivery and collection optics, has exploited the fact that the signal from the deeper layers is scattered to a greater extent, to improve penetration depth sensitivities. CARS is another label free type of Raman scattering which can be used to probe bio and nanomedical scenarios and in recent years has seen a growth in applications in cells, tissues and *in-vivo* imaging. Using single wavelengths, imaging of large areas can be achieved at video rates.

**CARS**

Coherent anti-Stokes Raman spectroscopy (CARS) is a form of Raman spectroscopy whereby the anti-Stokes shifted Raman signal is used to probe the molecular bonds within a sample. The coherent process takes advantage of a third order non-linear optical phenomenon by which three beams are used to probe the sample. A fixed pump laser beam, a tunable probe beam are set at a frequency difference which is exactly equal to the frequency of a specific molecular vibration, resulting in the coherent build up of a scattered signal on the anti-Stokes side of the pump laser frequency [95,96]. The signal can be orders of magnitude larger than a spontaneous Raman signal. Thus, CARS can be used to rapidly generate images of a particular biochemical distribution and therefore can be used in the generation of video rate image sequences of cells and tissues. To generate a full spectroscopic signature, however, the pump beam has to be tuned such that the difference frequencies scan the vibrational spectrum, a process which can take considerable time, under current technological constraints. The nonlinear

process is furthermore intensity dependent, requiring costly and notoriously temperamental short pulse lasers, whereas spontaneous Raman can be conducted with conventional steady state lasers.

In a biomedical context, the technique has been used to investigate a number of phenomena, also in conjunction with other methods such as immuno fluorescent labelling. Primarily, CARS has been used in the study of the C-H stretch region which is most commonly associated with lipids in living organisms. Examples include the use of CARS for the study of atherosclerotic lesions[97], intracellular trafficking[98], drug delivery[99], cancer metastasis[100], quantitative imaging of lipid distributions in living *Caenorhabditis elegans*[101] as well as imaging of the axonal myelin both *in-vivo* and *ex-vivo*[102,103] CARS has also been used in the assessment of nanomaterials. Notably ,the technique has been used to study particle interaction in biological organisms, receptor mediated particle uptake[104] as well as the effects of particle size and coating on zebra fish embryos[105]. Moger *et al*[106] used CARS to investigate the interaction of metal oxide nanoparticles within the gills of rainbow trout, *Onchrhynchus mykiss*. They were able to show in a label free manner the translocation of $TIO_2$ particles across the epithelial membrane and into the capillaries in fish gill tissue. This is shown in figure 5, which illustrates the forward (a) and epi-CARS images (b) of exposed fish gills. The merged image shows the localisation of the particles in the gill tissues, revealing particle clumps in green.

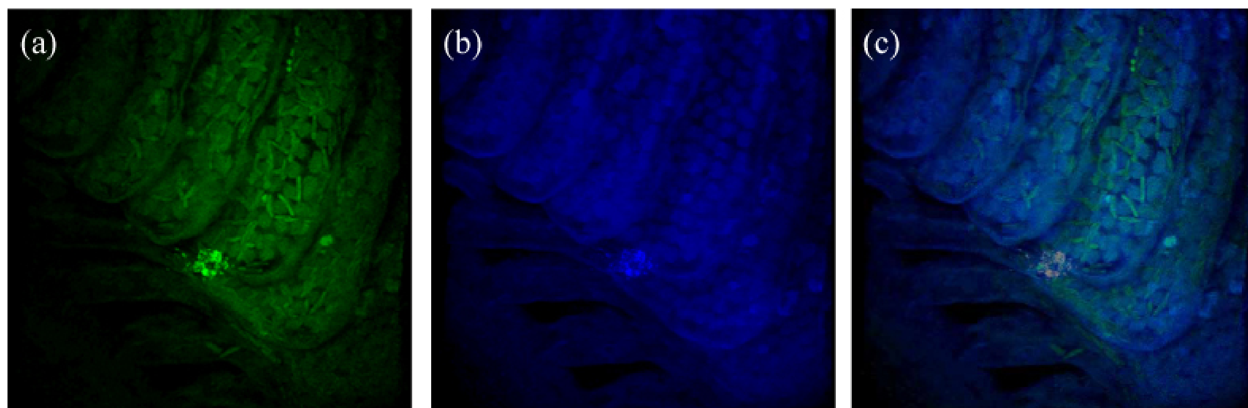


Figure 5. CARS images of the $TiO_2$ nanoparticle distribution in *Onchrhynchus mykiss* gills, (a) forward CARS image showing the nanoparticles, (b) epi-CARS image of the gill tissue and (c) merged forward and epi CARS image. Images reproduced from [106].

The method has also recently been used to investigate the mechanisms of oral uptake of Quaternary Ammonium Palmitoyl Glycol chitosan (GCPQ) nanoparticles. In this study, the particles were deuterated to shift the $CH_2$ stretching vibration located at 2840cm$^{-1}$ to a $CD_2$ stretching vibration of 2100cm$^{-1}$. This allows for CARS to be carried out in the so called 'silent region' of the cell. Additionally second harmonic generation and two photon fluorescence were used to image the tissue containing nanoparticles. In doing this, Garrett et al. were able to examine chitosan uptake and recirculation in the gut by being able to target the nanoparticles with cellular precision to the gastrointestinal tract, liver and gall bladder, providing novel insights in the role of enterocytes and bile recirculation regarding chitosan nanoparticles [99,107]. Figure 6 shows the identification of the deuterated nanoparticles in green (2100cm$^{-1}$), which are highlighted by the arrows. Figure 6A and 6B show liver and stomach tissue respectively, with the C-$D_2$ resonance being used to identify the deuterated nanoparticles (2100cm$^{-1}$) in green and the C-$H_2$ (2845cm$^{-1}$) in red. Figure 6C shows a multimodal label free imaging approach combining CARS imaging (green), second harmonic generation (SHG) and two photon fluorescence (TPF) in imaging nanoparticle interaction with jejunum tissue. Figure 6D and 6E show the use of a combination of CARS and TPF to image the ileum and duodenum respectively, while Figure 6F shows a combination of CARS, SHG and TPF of the gall bladder. These approaches show not only how CARS can be used to probe nanoparticle interactions, but also highlight how multiple imaging approaches can be combined in multimodal approaches to give different types of information building towards a more complete picture.

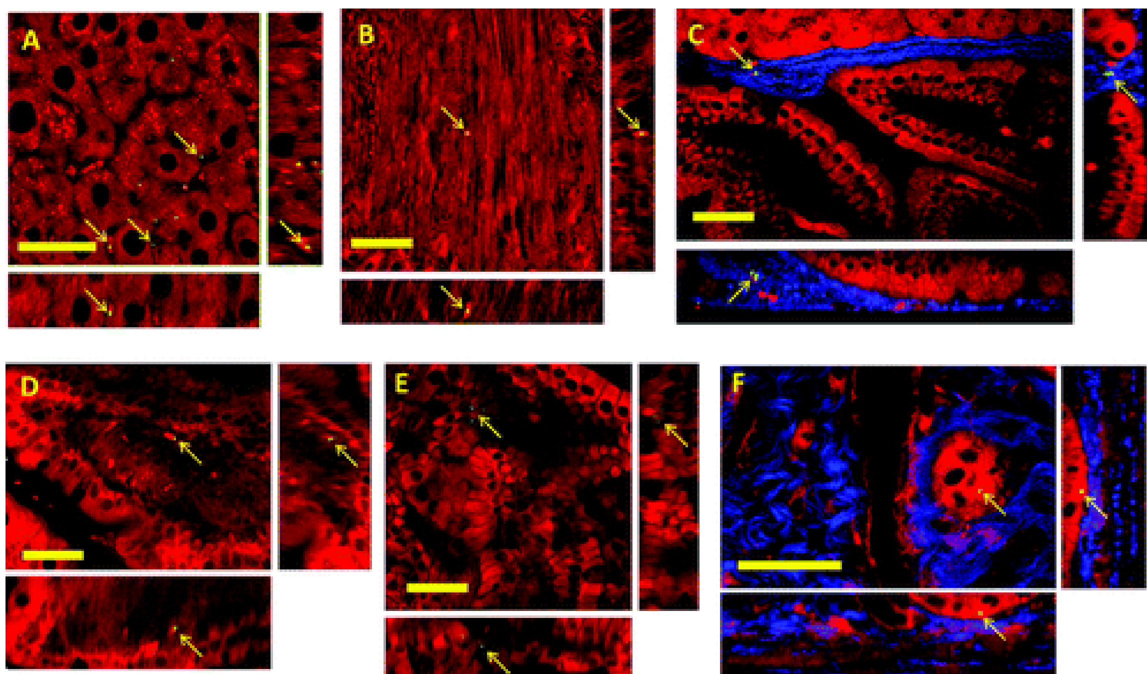



Figure 6: Epi-CARS images with contrast derived from $CD_2$ and $CH_2$ resonances in GCPQ nanoparticles at 2100 $cm^{-1}$ (green) and 2845$cm^{-1}$ (red) respectively. (A) Liver tissue. (B) Stomach tissue samples. (C) shows Jejunum tissue imaged with epi-CARS with contrast derived from the $CD_2$ resonance (green), SHG contrast derived from collagen (blue) and TPF contrast derived from endogenous fluorophores. (D) Ileum tissue imaged with epi-CARS with contrast derived from the $CD_2$ and TPF (red) (E) Duodenum imaged with epi-CARS with contrast derived from the $CD_2$ and TPF (red). (F) Gall bladder imaged with epi-CARS with contrast derived from the $CD_2$ resonance (green), SHG (blue) and TPF (red). Reproduced from [99]

Surface enhancement can also be exploited in the CARS format. Surface enhanced CARS (SECARS) has been used in conjunction with nanoparticles and has been shown to be capable of achieving greater signal enhancement than that of SERS or CARS alone. For biomedical applications, it has also been used for the detection of single molecules of deoxyadenosine and deoxyguanosine

monophosphate (dAMP and dGMP) [108] and has also been used in immuno-histochemistry studies[109].

**Conclusions and Outlook**

This article has attempted to provide an overview of the current state of the art of the developing applications of Raman spectroscopic techniques in Nanomedicine. A recent review has dealt more broadly with the applications of these techniques in the investigation of the interaction of nanomaterials with complex biological systems [110]. The development of biomedical applications of vibrational spectroscopy, both Raman and IR, has been extremely active for the past two decades and more and the challenges to nanomedical applications are intrinsically linked, as indeed they are to those of the fundamental understanding of nanobio interactions in general.

As a molecular specific tool, Raman spectroscopy can potentially aid significantly to the understanding of nanobio interactions *in vitro*. Even before interaction with the cell, it has been argued that the biological identity of the nanoparticle is determined by the surface coatings of the dispersion medium, the co-called protein corona [111]. While SERS active nanoparticles can be employed to probe this interaction acellularly, there is evidence that the nanoparticle medium interaction is very specific to the surface characteristics and size, and thus the use of truly label free spontaneous Raman spectroscopy may lead to broader insights. In this context, the increased sensitivity of TERS may be of significant benefit. SERS has however demonstrated that the surface coating can evolve significantly after endocytosis of the nanoparticle [110], and this is a critical consideration in the bioavailability of surface functionalities, including release of active ingredients, which have been specifically designed for nanomedical applications.

As an confocal optical microscopic technique, Raman holds all the benefits of confocal fluorescence techniques, but has the potential advantage of being truly label free, adding the promise of reduced cost and sample processing requirements. SERS probes have demonstrated the potential to probe nanoparticle uptake, trafficking as well as the local environment, but these probes need to be specifically chemically tailored for the given application can so the technique cannot be considered to be truly label free. Spontaneous Raman spectroscopy is, on the other hand, an intrinsically weak phenomenon and cellular mapping is often a prolonged processes. Nevertheless, a number of cellular studies have been conducted which, although not specifically probing nanoparticles, may have implications in future nanomedical applications. For example, some studies have shown the application of Raman to drug delivery investigations [112,113] while other studies have identified sub cellular structures such as the mitochondrion as well as lipid rich regions which may be associated with the Golgi and endoplasmic reticulum [86]. Klein et al. used image registration and immuno fluorescence to verify the locations of cellular organelles and also as a means of extracting the spectra which were specifically associated with the organelle[114]. These studies could be extended to look at nanoparticle trafficking studies, colocalizing the particle to an organelle in a label free manner, without using fluorescently labelled nanoparticles or organelle stains. Although spontaneous Raman studies are commonly conducted on fixed cells, live cell spectral profiling has been demonstrated [37]. Image analysis is ultimately dependent on the reliability of multivariate chemometric techniques and simulated model systems can prove invaluable in validating their accuracy [81]. Increased acquisition rates can be achieved by systems custom designed for biological applications, and CARS potentially offers a route towards routine *in vitro* screening of intracellular nanobio interactions, although its ability to rapidly screen the full spectrum is currently limited by the (tuneable) laser source technologies and applications are thus restricted by the need to identify specific spectral marker bands.

In terms of disease diagnostics, *ex vivo* applications of Raman spectroscopy have received much attention. For the range of Raman modalities, however, mapping of large areas of tissue biopsies also suffer from issues of weak signals (spontaneous), specifically targeted probes (SERS), surface sensitivity (TERS) or the need for specific spectral markers (CARS). As a chemically specific probe, however, Raman techniques are particularly suitable for analysis of biomarkers of disease in biological fluids [71,72] and this suitability is readily extended to applications in nanomedicine.

Raman scattering is fundamentally an optical technique and *in vivo* applications are thus limited by the ability to access the area of interest. For dermal analysis, custom designed systems are commercially available which exploit the near infrared transmission window of skin, although, in a microscopic format, the penetration depth is further limited by the delivery optics, typically to some hundreds of microns. Advances in SORS have increased the depth resolution, and such technologies could prove invaluable tools for analysis of transdermal nanodrug delivery or environmental exposure to nanoparticles. As an optical technique, Raman spectroscopy readily lends itself to endoscopic probes [115], however, and recent advances in such *in vivo* probes may significantly impact on biomedical applications of Raman spectroscopy, including, inevitably Nanomedicine.

**Future Perspectives**

The field of nanotechnology is set to grow ever rapidly as new applications and avenues of research are explored over the coming decade. Crucially, characterisation and visualisation methods in a medical setting must develop in tandem, to access the applicability of such nanotechnology. Raman spectroscopy represents a method proven in the field of disease diagnostics and biomedical imaging and thus by extension holds the capability to progress the field of nanomedicine.

Spontaneous Raman spectroscopy provides a versatile and truly label free method which has seen success in a number of different medical applications, most notably in disease diagnostics. Key enabling

technological developments in this context include endoscopic and other *in vivo* probes. Relatively Low signal strengths currently limit the technique to small areas and/or long scan times, however, and continuing improvements in signal throughput and detector sensitivities are important. EU Directives limiting the use of animal models will put increasing emphasis on the development of in vitro screening methods and Raman is a potential candidate for high content analysis of, for example, the efficacy and mode of action of novel chemotherapeutical agents of toxicants. The high optical resolutions obtainable make Raman particularly suitable for acellular or subcellular studies of nanobio interactions. As the sensitivity of the Raman technique is intimately linked with the multivariate statistical data analysis methods, the quantitative specificities of these methods must be established. This can only be done if the true result is known, and in this context the use of specifically constructed model datasets may provide a quantifiable insight into how far Raman spectroscopy can be pushed in both a medical and nanomedical context.

SERS provides increased sentitivities to probe the nanoscale environment surrounding metallic nanoparticles. Although the technique is not truly label free, with the increased sensitivities achievable as well as the targeting potential of such probes, SERS may provide alternative imaging strategies for disease diagnostics *in-vivo,* as well as provide enhanced methods for the monitoring of human fluids such as serum and other metabolic excretions *ex-vivo*. SERS *in-vitro* may also prove a useful tool in probing the nature of the so called protein corona of nanoparticles in biological media and thus provide valuable insights into the surface behaviour of nanomaterials in a biological setting. Other enhancement methods such as TERS also provide novel insights into the nanoscale environment although they are limited by being mainly a molecular or surface specific technique.

Coupling these advances in spontaneous and surface enhanced Raman with the development of SORS and SESORS, some of the shortcomings in signal generation and depth penetration of Raman

spectroscopy *in-vivo* may be overcome. In addition to the development of endoscopic and needle based probes which will increase access to the point of interest, realistic applicable *in-vivo* Raman studies in nanomedicine may not be too far away. CARS provides a method which is capable of video rate scan speeds. However, as of yet the technique is not a spectroscopic imaging technique as it only allows for the probing of one particular wave number or vibrational marker at a time. The technique therefore requires a clearly identifiable biomarker for imaging, which may not be the case for all biomolecules. A CARS system that could provide a spectrum of the finger print region of the sample with similar real time imaging capabilities would be ideal. Specifically for CARS to progress as a spectroscopic imaging modality, advances in laser technology such as rapidly tunable lasers will need to develop in tandem. These advances would then open a myriad of applications for CARS imaging along the lines of spontaneous Raman imaging.

**Executive Summary**

**Raman Spectroscopy:** Raman spectroscopy is a well established chemical analysis technique finding increasingly broader applications, particularly in biochemical analysis and disease diagnostics.

**Surface/Tip enhanced Raman Spectroscopy:** The techniques of SERS and TERS specifically probe the nanoscale and, although TERS is a topical/surface technique, SERS probes have already been used extensively for *in vitro* and *in vivo* studies. SERS probes are normally chemically functionalised according to the specific target, and so the technique is arguably not truly label free.

**Spontaneous Raman Spectroscopy:** As a truly label free technique, (spontaneous) Raman spectroscopy, coupled with multivariate analytical techniques potentially provides a probe of nanoparticles in

cells/tissue, their nature of their local environment, and physiological changes. Unenhanced, the signals are however relatively weak, and large scale mapping can be time consuming.

**Coherent anti-Stokes Raman Spectroscopy:** CARS is a nonlinear optical technique which is increasing in prominence for biomedical applications. Tuned to a specific vibrational frequency, it can scan large areas ($cm^2$) at video rates. Currently, however, it is not a spectroscopic technique and does not avail of the full biochemical information available, but relies on the presence of a specific spectral marker.

**Outlook:** The range of modalities of Raman spectroscopy potentially hold great promise for biomedical and nanomedical applications, although many technical challenges remain.

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