Surface Enhanced Raman Spectroscopy (SERS): Potential Applications for Disease Detection and Treatment

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Abstract: The implementation of Raman and surface enhanced Raman spectroscopy (SERS) for the detection of disease has increased in recent years. The reasons for their increased implementation have often been attributed to their well-known advantages, including the production of narrow spectral bands, which are characteristic of the molecular components present, their non-destructive method of analysis and the sensitivity and specificity which they can confer. This review analyses a range of diseases which can be detected by Raman or SERS, particularly those *in vitro*, *ex vivo* and *in vivo*. The sophistication of the investigated systems varied widely but the suitability of Raman and SERS for medical diagnostics and future implementation in a clinical environment is clearly demonstrated.

Highlights:

- A range of diseases can be detected by Raman and SERS *in vitro, ex vivo* and *in vivo*
- *In vivo* multiplexed detection of cancer was achieved
- SERS is also a key step in disease treatment
- Studies must be further extended to a physiologically relevant medium and *in vivo*
- The potential exists for the application of the techniques in a clinical setting

Keywords: Raman, SERS, Disease, Mapping, Imaging and *In vivo*

1. Introduction

When the pioneers of Raman spectroscopy initially conceived the technique in 1923 [1] and latterly demonstrated it in 1928 [2] it is unlikely that they had any inclination of the future impact or bio-diagnostic applications. [3-5] In particular, the use of Raman spectroscopy for *in vitro*, *ex vivo* and *in vivo* disease detection has proliferated over the last decade. This is unsurprising when considering the non-invasive nature of the technique and the provision of molecularly specific spectra which allow for the identification of disease targets. [6] However, the obvious disadvantage with its implementation is that it can be quite insubstantial, especially considering that approximately only one in every million photons are Raman scattered. [6]

This weak effect was negated by successive developments of the original technique most notably surface enhanced Raman spectroscopy (SERS) [7-9] and surface enhanced resonance Raman spectroscopy (SERRS), [10] where signal enhancements of the order of 10^{10} [8, 9, 11] and 10^{14} [10] were observed respectively. The incorporation of a roughened metal surface (metal electrodes, coatings, surfaces and nanostructures)[11] is essential for manipulation of the SERS enhancement, which is widely believed within the community to arise through a combined electromagnetic and chemical effect. [6, 11, 12] SERRS, whilst manipulating the enhancement conferred by a roughened metallic scaffold, additionally exploits excitation with a laser line that corresponds to an electronic transition of the interrogated analyte to further augment the enhancement effect. [10]

The introduction of nanostructures can render these techniques invasive however; this can be offset by the conferred enhancement. As with the original method, the employment of SERS for disease detection *in vitro*, *ex vivo* and *in vivo* has increased exponentially, again a direct consequence of the molecularly definitive spectra which allow for confident identification of disease biomarkers. However, as clinical medicine strives towards a personalised approach, there is further need to increase the sensitivity and specificity by which diseases are detected and the simultaneous requirements of comprehensive characterisation and detection of multiple pathosis. [13] SERS is ideally suited to fulfil these requirements since the observed enhancement can satisfy the sensitivity criteria whilst functionalisation with specific targeting moieties allows for disease recognition with a high degree of specificity. The sharp bands produced within a SERS spectrum lend the technique to multiplexed detection satisfying the demand to diagnose multiple aspects of a single disease or a myriad of disorders. [13]

This review will examine the application of SERS for disease detection. A range of formats will be discussed, including *in vitro* analysis of simple matrices to more sophisticated analysis *in vivo* but with the predominant focus on mapping/imaging applications. The review will additionally highlight the progression of the technique from a simple lab based analytical method to a contender for the interrogation and discernment of clinical samples. The advancement of SERS as a diagnostic imaging method is developing at a ferocious pace and if maintained at this level, there is huge potential to revolutionise the ability to detect, treat and manage disease.

2. Raman Spectroscopy and Disease Detection

While this review will predominantly concentrate on SERS it is worth highlighting the parallel advancements occurring with Raman spectroscopy. The obvious advantage of Raman spectroscopy over SERS is that it is non-invasive so there is no requirement for the introduction of nanostructures to observe a response. In terms of *in vitro*, *ex vivo* and *in vivo* analysis, this is particularly beneficial since minimal sample preparation is involved, toxicity trials are not required as adulterants have not been introduced and more importantly for *in vivo* studies there is no need to consider the distribution and localisation of nano-scaffolds for effective analysis. However, consideration must be given to the employed laser power, acquisition time and sample format to prohibit sample degradation and auto-fluorescence.

2.1 Raman Spectroscopy and *In vitro* Cell Analysis

The interrogation of single cells and cell populations may not have the same allure and glamour of *in vivo* studies but, in terms of indicators of disease, they can be equally important. The architecture of the cell can be altered considerably during processes such as differentiation [14], mitosis and apoptosis [15]. Uncontrolled division and cell death can be indicative of pathosis, including tumour growth, inflammation, HIV infection and neurodegeneration diseases such as Parkinson's and Alzheimer's. [16] Monitoring the cell composition and architecture permits a viable method for the detection of disease and, with the advent of 3D Raman imaging, the resolution of cell organelles may be more representative of the disease led changes that can occur intracellularly. [17, 18] Cell health can also be directly monitored via Raman spectroscopy and it is possible to distinguish between live and dead cells, [19] and cells that are stressed, [20] conditions which can arise as a result of infection or disease. Cells can also be readily distinguished on the basis of their phenotypic differences and this has been used to discriminate between bone [21] and lung cells, [22] and more importantly in terms of disease diagnosis between cancerous and non-cancerous cells. [23, 24] This was not completed with a clinical sample but the authors highlighted the desire to extend analysis into urine.

[23] In a clinical setting, this has the potential to provide cancer diagnosis non-invasively via an excretory product.

2.2 Raman Spectroscopy and *Ex vivo*/*In vivo* Applications for the Detection of Disease

As a diagnostic tool, Raman spectroscopy is forging ahead in the cancer field providing a method by which to detect breast [25-27], skin [28, 29], prostate [30-32], bladder [33-35], cervical [36-38], colorectal [39, 40] and oesophageal cancer [41, 42]. With bladder, [34, 35] cervical, [37, 38] colorectal [43] and oesophageal cancer [42] all being detected *in vivo*. The incidence of breast cancer in the UK accounts for the greatest proportion of all cancer diagnosis and like all forms of the disease there is demand for sensitive, selective and non-invasive detection. [44] A subsequent adaptation of the original Raman technique led to the development of spatially offset Raman spectroscopy (SORS) which has a demonstrated ability to differentiate between calcifications associated with cancerous and non-cancerous tissues and at a reported depth of ~ 8.7 mm. [26] The authors reported that this could be used to complement the current clinical method of detection, mammography, whilst simultaneously demonstrating the potential to minimise the number of invasive biopsies. [26] Successive developments and the implementation of a transmission Raman setup increased the thickness of tissue through which cancerous and non-cancerous samples could be differentiated. [27] The depth of detection was increased to 27 mm with anything above 20 mm being classified as clinically significant for *in vivo* detection, further highlighting the applicability and relevance of implementing the technique in a clinical environment. [27] A proof of concept study also investigated the possibility of detecting brain tumours *in vivo* in mice models. [45] The authors reported that the tumours could be located with an accuracy of approximately 250 μm and while principally a demonstration of what could be achieved, the authors emphasised the possibility of using Raman spectroscopy for brain analysis and tumour detection in a live subject. [45]

Raman spectroscopy is not limited to the detection of cancer and research has postulated that the method could be used for the characterisation and monitoring of atherosclerotic plaques. [46] In the UK alone coronary heart disease and the conditions which stem from it cost an estimated £19 billion. [47] This is clearly an extensive and significant problem and Raman was found to be a suitable method by which plaque deposits could be distinguished and their size determined. [46] The effects of statin drug treatment were also tracked, and in mice that were provided a cholesterol rich diet, the statins successfully minimised plaque formation. [46] Whilst this remains a proof-of-principal study there is potential for extension to studies in man and although not demonstrated remote sensing could be achieved by coupling via a fibre optic probe. [46]

Similarly, Raman spectroscopy has been implemented to monitor organ rejection in patients following transplant procedures. [48, 49] While not directly involving the detection of disease, it is possible that the processes which led to transplant were the result of disease, for example, coronary heart or chronic kidney disease. This demonstrates the application of Raman for monitoring subsequent disease effects. In the particular instances of heart and kidney transplant the confirmation of rejection requires an invasive biopsy procedure and the observation of an elevated serum creatinine level, respectively. [48] However, the rejection of heart tissues can be characterised by serotonin biomarkers, which can be detected using Raman, presenting a viable non-invasive alternative to biopsy. [49] While the methods for monitoring kidney rejection do not require the same invasive procedures, by the time detection of serum creatinine is confirmed significant deterioration of the organ can have occurred. [48] Raman spectroscopy was employed as a method to differentiate between T-cells which were produced in response to different stimuli (i.e. in response to rejection and under normal circumstances). [48] These were selected as biomarkers for monitoring the rejection response since the process is primarily characterised by T-cells. The technique was found to be suitable for differentiating between normal cells and those which are characteristic of a rejection response. [48] It should be noted that the T-cells were not directly obtained from a patient undergoing rejection but rather the cells were artificially modelled to be representative. Raman spectroscopy therefore represents a viable alternative by which rejection could be monitored.

This is in no way an exhaustive review of the methods by which Raman spectroscopy can be employed to detect disease or monitor health, however, it does emphasise the vast array of medically related applications. For further discussion regarding the employment and advancements of Raman spectroscopy in medicine, readers are encouraged to review literature from the Stone [44] and Goodacre groups [50, 51]. However, it is clear that with instrumental improvements and careful consideration of the employed laser power and acquisition time, vast amounts of information can be yielded by Raman spectroscopy. Central to all of these applications is the employment of chemometric methods. Raman spectra are often similar in appearance and discerning any differences by eye are difficult, but by implementing chemometric analysis subtle differences can be extracted and conclusions drawn with statistical significance. However, in order to make the leap from bench to body, further analysis must be completed in physiologically representative media, clinical samples and *in vivo*. [52] While diagnostic information can be extracted using chemometrics clinicians require simple yes/no answers to dispense a diagnosis or prognosis. Therefore, any

transition to a clinical environment will require coupling of the data collection and analysis so that single definitive answers can be provided.

3. Surface enhanced Raman Spectroscopy (SERS)

The advantages of SERS over conventional Raman were briefly discussed in the introduction but are worth re-iterating since these benefits in conjunction with the shortcomings of Raman are responsible for the proliferation of the technique. Although, non-invasivity is a key attraction of Raman spectroscopy, the overall outcome is that the technique is reliant on spectral signatures from the intrinsic components of the interrogated system, which in biological matrices tend to be weak, inefficient scatterers and spectrally similar, [53] such that subtle differences must be discerned by the incorporation of chemometric methods. SERS unlike Raman is not reliant on native chromophores and a vast array of efficient scattering molecules, [54-59] with large Raman cross sections, can be conjugated to metallic scaffolds for the provision of distinct, optically strong, spectral signatures. By capitalising on this conferred enhancement, through the implementation of a metal surface and superior reporting moieties, the measured spectral intensities can be significantly enhanced. [8, 9, 11] The key advantages of this enhancement are that a reduced laser power and acquisition time can be incorporated for the analysis of fragile samples with minimal loss of the spectral sensitivity whereas with conventional Raman some compromise must be made between the laser power and measurement time to obtain sufficiently distinct spectra without incurring sample damage. The sharp molecularly specific spectra produced by SERS also promote the technique for use in multiplexed scenarios including multiple disease detection and in many instances differences can be determined by eye without the need for the incorporation of chemometric methods.[13] Conventional Raman is unlikely to be employed in such a manner since the spectral differences between healthy and diseased samples are extremely subtle, let alone between multiple diseases in a single sample, and generally require the implementation of statistical methods for their differentiation. [53]

As discussed above exploitation of the SERS effect requires the incorporation of a roughened metal surface. [6, 11] The forms which this can take are vast and can include metal electrodes, [10] coatings, surfaces [60, 61] and nanostructures. [62, 63] *In vitro* examples have employed unfunctionalised nanoparticles for simple sensing applications, for example, monitoring enzyme activity [62] or the determination of intracellular components. [64] To actively image and/or target and detect disease *in vitro*, *ex vivo* and *in vivo* the sophistication of the implemented systems varies. Ranging from metallic nanoparticles labelled with reporter molecules [65-69] to more complicated

systems with mixed reporter monolayers [70], dually functionalised systems consisting of reporter molecules and membrane penetrating or targeting antibodies [71-73], peptides [65, 74, 75] or oligomers. To minimise or prohibit degradation from the surrounding environment nanostructures can be afforded a certain degree of stability by silica [13, 76] or polymer encapsulation. [71, 77, 78] The incorporation of polyethylene glycol (PEG) again offers protection from the chemical and physical environment, minimisation of non-specific binding [79] and, depending on the terminal functionality, PEG is readily amenable for bioconjugation to a range of targeting ligands. [80]

3.1 SERS and *In vitro* Applications for the Detection of Disease

SERS based nanotags have been used with a variety of different sample formats for the *in vitro* detection of disease. In some of the earliest examples, functionalised nanoparticles and nanotags were not employed for the detection of disease. Instead metallic surfaces were used to exploit the SERS effect. In one such system used for the detection of glucose, spheres were coated in a layer of silver for the provision of the SERS enhancement and they were subsequently functionalised with both decanethiol and mercaptohexanol. [60] Dual functionalisation with molecules of varying chain length results in hole formation between the different chains actively providing a region into which the glucose molecules can enter and the response can be measured via SERS. [60] With the implementation of the system it was possible to quantitatively detect glucose and the majority of the measurements were detected within levels which were considered to be medically relevant. [60] The authors also reported that the system worked effectively even when interfering analytes were present. [60] Detecting glucose in real-time is of huge importance for diabetes sufferers who currently check their levels via pin prick blood tests. With this method of monitoring huge variations in glucose levels are often missed and in order to improve disease management, there is a real need for accurate continuous monitors. [60] Later work by the same group has strived to further develop this method for *in vivo* sensing. [81-83] In the initial stages the sensors (as described above) were implanted into animal models and the glucose levels were successfully measured via SERS and surface enhanced spatially offset Raman spectroscopy (SESORS) respectively. [81-83]. (SESORS shall be discussed further in section 3.3). In the most recent developments, the same sensor has been investigated via animal implementation. [83] The device was found to actively work over 17 days and more importantly the authors reported that between days 6 and 17, the glucose levels measured were found to lie within the clinically relevant levels. [83] This elegant and sophisticated example is a significant step forward in the development of a method by which diabetes sufferers could monitor and control their condition more accurately. [83] A similar format was also used to monitor lactate quantitatively. [61] Variations in lactate levels can be indicative of trauma in a number of medical

conditions and as with glucose monitoring the ability to measure lactate in real time would further improve the clinical care offered to patients suffering from a range of conditions. [61]

In other examples, the SERS enhancement was achieved using metallic nanoparticles. Using this format and a nanofluidic device Chou *et al.* demonstrated the detection of the β-amyloid peptide, which is a key component in plaques in sufferers of Alzheimer disease. [63] β-amyloid detection was achieved by concentrating the nanoparticles and target molecules within the device, irradiating with a laser and monitoring the SERS signal. [63] To demonstrate the sensitivity of the detection system the author's added additional proteins of similar and different conformations. Analysis of the SERS signals found that it was possible to discriminate between the different protein forms, [63] in particular, α-helices could be discriminated from β-sheets and between the different β-sheet forms discrimination was also possible. [63] Having a method by which to characterise the disease process is hugely beneficial and it could be used as a method to monitor disease development. [63]

Unfunctionalised nanoparticles have also been used to monitor enzyme activity intracellularly. [62] This is relevant in terms of disease detection since some notable diseases, including cystic fibrosis, Parkinson's and Alzheimer's are all characterised by some form of abnormal enzyme activity. [62] In the study Au nanoparticles were incorporated by cell populations alongside the colourless substrate X-Gal, where upon internalisation it was enzymatically transformed by β-galactosidase enzymes to 5,5'-dibromo-4,4'-dichloroindigo. This transformation was characterised by the appearance of a peak in the SERS spectra at 598 cm^{-1} and a blue colour which was indicative of the turnover of the substrate by the enzyme (Figure 1). [62] The conversion was specific to the enzyme as confirmed when known enzyme inhibitors were introduced (Figure 1) and the corresponding reduction in the measured SERS signal. [62] High resolution analysis of single cells and cell populations suggested that enzyme action was localised in specific cellular compartments which were proposed to be endosomes. [62] The authors expressed a desire to quantify enzyme levels within these compartments, particularly in response to different disease stimuli and states. It is hoped that such knowledge will facilitate the development of suitable treatments. [62]

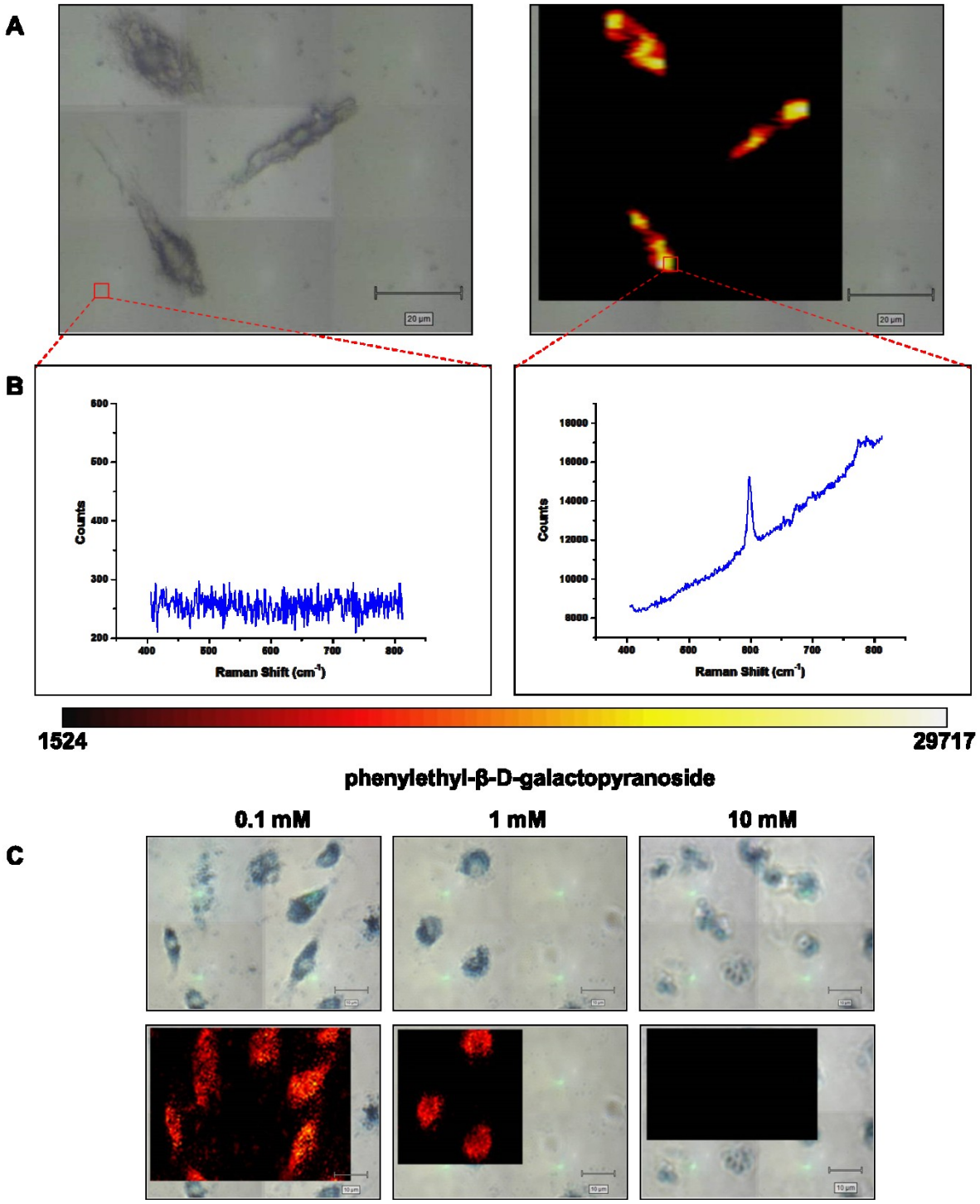

Figure 1: Intracellular conversion of X-Gal to the blue coloured product 5,5'-dibromo-4,4'-dichloro indigo by the enzyme β-galactosidase – A) white light and false colour image based on the 5,5'-dibromo-4,4'-dichloro indigo peak at 598 cm^{-1}, B) corresponding spectra from the highlighted areas and the associated false colour LUT bar and C) the effect of an enzyme inhibitor on the measured SERS signals. [62] Modified and reproduced by permission of the RSC.

More recently systems for disease detection have looked at functionalised nanotags. [84-86] In one immunoassay, the detection of the carcinoembryonic antigen (CEA), a marker frequently used for the detection of lung cancer, was achieved using a combination of hollow gold nanospheres (HGNs) and magnetic beads. [86] Porter *et al.* also implemented an immunoassay format for the detection of the pancreatic cancer marker, MUC4. [84] This marker is suitable for detecting cancer since it appears to be absent in healthy and pancreatitis-suffering populations. [84] Detection was achieved using SERS via gold nanoparticles which were labelled with a reporter molecule and an antibody specific for the marker. SERS was critical for the detection of disease, especially as the authors noted that conventional immunoassay formats had failed to detect the protein in human sera. [84] In an extension of this initial work Porter *et al.* developed an immunoassay system which could simultaneously detect two markers of pancreatic cancer. [85] The detection of multiple disease markers is a key step in the future of disease diagnosis since it is rare for disease processes to be represented or characterised by a single indicator. [13] In addition, the detection of multiple aspects of a disease will undoubtedly lead to better personalised medicine and immunoassays could be extended to detect a plethora of diseases simultaneously.

Cell based detection is a vast field and in particular research has centred on detecting breast cancer. [87, 88] One of the earliest studies employed SERS dots (a form of silica encapsulated nanotags) functionalised with breast cancer (HER2) or leukaemia specific antibodies (CD10). [87] Antibody functionalisation was required so that the dots would bind specifically to the corresponding cell line and when the conjugates were incubated with different cell populations this specificity was observed. [87] HER2-functionalised dots only bound to the breast cancer cells and similarly the CD10 functionalised dots bound only to the leukaemia cells. [87] SERS signals were not observed when either of the antibody functionalised nanotags was incubated with the control cell population. [87] This initial study highlighted the possibility of screening cell populations for cancerous and non-cancerous cells and, provided that each subset of dots was labelled with a distinct reporter and an antibody, a range of disease could be detected simultaneously. The authors also reported that the method could potentially be implemented as an alternative to the radioactive tagging of cells and tissues. [87]

Later, studies took advantage of the same binding relationship between HER2 antibodies and the corresponding receptors on cell membranes. [89, 90] However, rather than focusing on spherical shaped nanostructures rods and HGNs were investigated as alternative imaging scaffolds. [89, 90] In both instances, recognition via the specific antibody-antigen interaction was achieved and brighter

images were observed (Figure 2). [89, 90] The increased SERS intensity in the false colour images were not the result of any improvement in the binding efficiency but were a direct result of the enhanced optical properties demonstrated by both the rods and HGNs (Figure 2). [89, 90] The authors suggested that both systems are extremely sensitive imaging agents and when designing a disease detection system consideration should also be given to the imaging scaffold especially if the disease target is present at low concentrations. [89, 90]

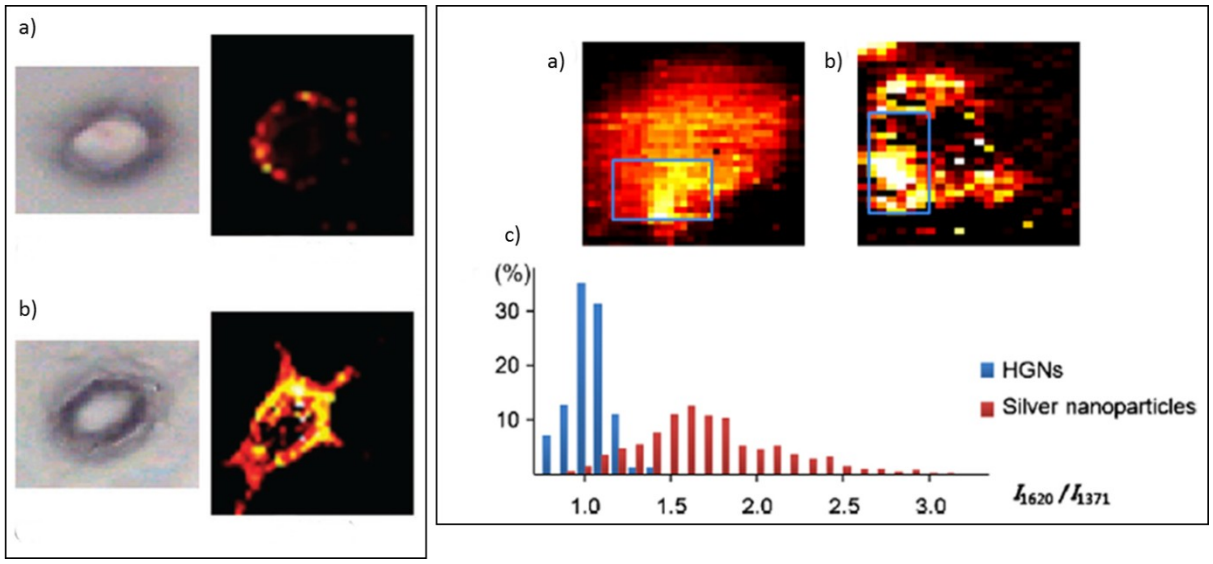

Figure 2 : Left panel – differential levels of binding between a) gold nanospheres and b) rods functionalised with HER2 antibody and exposed to MCF7 cells overexpressing HER2. [90] Modified and reproduced from [90] with permission from the PCCP owner societies. Right panel – differential levels of binding between a) HGNs and b) silver nanoparticles functionalised with HER2 antibody and exposed to MCF7 cells overexpressing HER2, c) intensity ratios for the peaks at I_{1620}/I_{1371} for the crystal violet reporter.[89] Reprinted with permission from Elsevier.

In a recent study antibody-antigen interactions were studied as means for differentiating between disease states. [72] The development of the nanostructures involved the labelling of silver nanoparticles with reporter molecules, and HER2 antibodies were subsequently conjugated via the terminal carboxylic acid group on the reporter. [72] The functionalised nanostructures were then exposed to two cell lines (SKBR3 and MCF7) which demonstrated a differential level of HER2 expression. [72] In the SKBR3 cell line which exhibits a high level of HER2 expression the SERS signal was observed by the authors to be 3-4 times greater than in MCF7 cells, which have a low level of expression. [72] Although significantly greater levels of data would need to be collected from cells, at a number of different disease stages, this preliminary study highlights the implementation of SERS nanotags for the categorisation of cancerous disease states. The principles of the study are not

limited to cancer and any other condition which exhibits differential levels in the expression of disease markers could be analysed and graded in an analogous manner.

In the most recent study, implementing breast cancer cell lines, the cell phenotype was determined from specific antibody antigen interactions occurring between nanostructures labelled with antibodies and antigen receptors present on cell surfaces. [91] This was achievable because each of the nanostructures was labelled with a different reporter molecule and thus different SERS signals were measured (Figure 3). [91] The false colour SERS maps were subsequently used as a method to quantify the level of biomarker expression since the SERS intensity is a direct measure of the concentration of biomarker. [91]

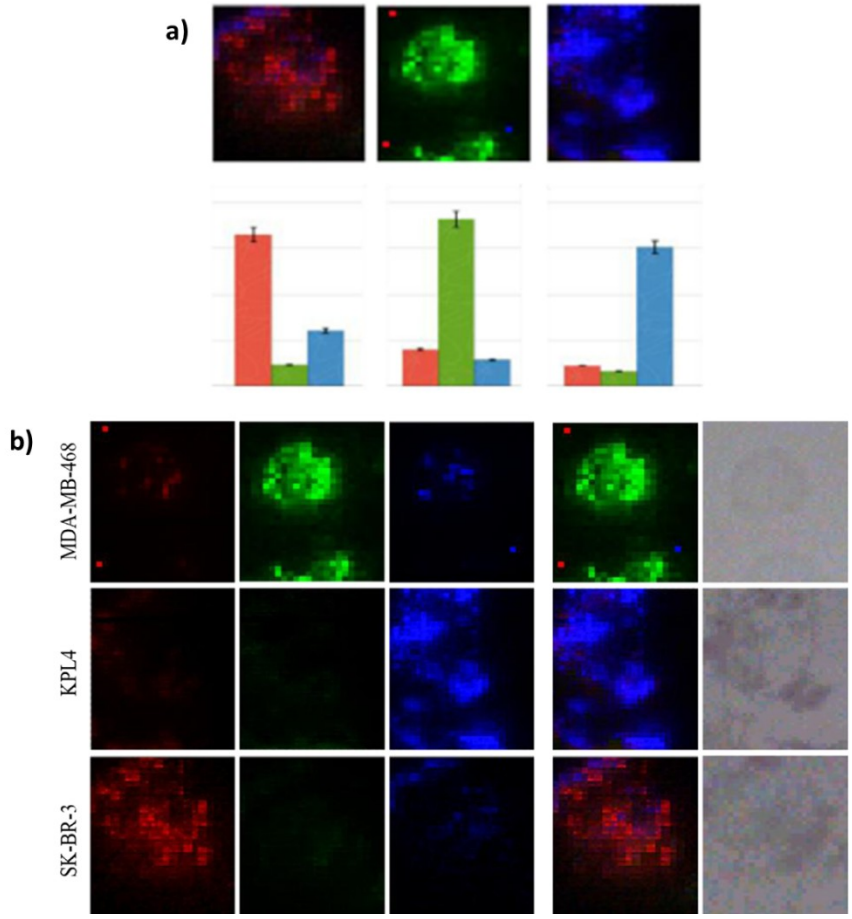

Figure 3: Silica encapsulated hollow gold nanoshells (SEHGNs) used to phenotypically identify cancerous cell lines and determine the level of antigen expression in each cell line – a) SEHGNs were applied to the different cell lines and each of the cell types were identified based on the signal from the specific reporter molecules and b) SERS mapping images of the corresponding cell lines were measured at 1650 cm ^{1} (RBITC), 1619 cm^{-1} (MGITC) and 1490 cm^{-1} (RuITC) for the SK-BR-3, KPL4 and MDA-MB-468 cell line specific reporter molecules. [91] Modified and reprinted with permission from Elsevier.

Biomarker or protein expression can also be used to differentiate cancerous from non-cancerous cells. [92] In prostate cancer cells neuropilin-1 is overexpressed and, on the basis of this knowledge, selective SERS nanotags were constructed for differentiating between cell lines. [92] To actively target cancerous cells or more specifically the receptors, nanotags were functionalised with a peptide with a specific affinity for neuropilin. [92] Control nanotags were functionalised with the HIV transactivator of transcription (TAT) peptide sequence and these nanotags had the ability to bind to both cells lines. [92] In order to positively identify the cancerous cells, the ratio of the SERS signal from each of the reporters was calculated. [92] The cancerous cells should have a high contribution from the reporter on the specific targeting nanotags whilst the non-cancerous cells should have a high contribution from the reporter on the control system. [92] This was found to represent a viable method for differentiating between the two cell lines. [92] Prostate cancer cells can also be positively identified from interactions between imaging agents and surface carbohydrates. [93] For example, PC3 cancerous cells exhibit elevated levels of sialic residues on their surface. [93] These can be actively targeted with nanostructures functionlised with the lectin, *Triticum vulgaris*, which is known to have a specific affinity for these glycans. [93] Craig *et al.* demonstrated this lectin-glycan interaction and in doing so successfully distinguished cancerous PC3 cells from the non-cancerous PNT2A cells. [93]

Further applications involving the detection of cancerous cells have centred on circulating tumour cells (CTCs). Unlike other applications, which might be concerned with the primary detection of cancer, CTCs are particularly aggressive and are associated with the initiation of further cancerous growth. [73, 94] During an initial study the CTCs were exposed to antibody functionalised nanotags and magnetic beads. The nanotags whilst simultaneously binding to the CTCs acted as the reporter component and similarly, the magnetic beads whilst capable of binding to the cancer cells were incorporated so that the samples could be concentrated via a magnet. [73] The implementation of this method has resulted in successful detection of CTCs via SERS. [73] Detection was also achievable with high sensitivity and specificity when whole blood was employed. [73] In a subsequent study by Wang *et al.* a similar method was employed but the incorporation of magnetic beads was substituted for a physical separation step. [94] Initially, the blood was separated by centrifugation and, because of their low density, CTCs were removed from the upper portion of the container with relative ease. [94] CTCs were then incubated with nanotags for a specified period and after this any unbound nanotags were also removed by centrifugation prior to the SERS analysis. [94] Both methods allow for sensitive and selective detection of CTCs. [73, 94]

In a final example of SERS based disease detection *in vitro*, targeting of stem cells was investigated. Although this is not strictly a method for the detection of disease, stem cells have an important role in disease research. [95] In this study nuclear targeting SERS nanotags were deployed to ascertain the differences between differentiated and undifferentiated stem cell populations. [95] The cells were analysed by SERS and spectral information was extracted. Principal component analysis (PCA) was employed and it revealed that there were distinct differences between the two stem cell forms with regards to the organisation of the nucleus. These changes were suitably distinct and allowed for cell identification. [95]

3.2 SERS and *Ex vivo*/*In vivo* Applications for the Detection of Disease

3.2.1 Tissue Imaging and Disease Detection

The use of SERS for disease related tissue imaging was initially performed by Schlucker *et al.* in 2006. [96] The SERS based detection system consisted of gold nanoparticles conjugated to an antibody via a small molecule reporter and this was used to actively detect prostate specific antigen (PSA) in tissue samples. [96] In a later study the same group extended this to monitor PSA expression in tissues. [80] This predominantly occurs in the epithelium of the prostate and, when incubated with functionalised nanoshells, the localised expression was confirmed by SERS signals arising from the specific nanoshell antibody-tissue antigen interaction and it was particularly evident in the false colour SERS maps (Figure 4). [80] Determining the localisation of other components within the tissues is not isolated to biomarkers of disease and the group successfully determined the localisation of the tumour suppressor p63, in non-cancerous tissue. [97] Elucidation of this was again determined by SERS signals arising from the specific interaction between the detection system and tissue. [97]

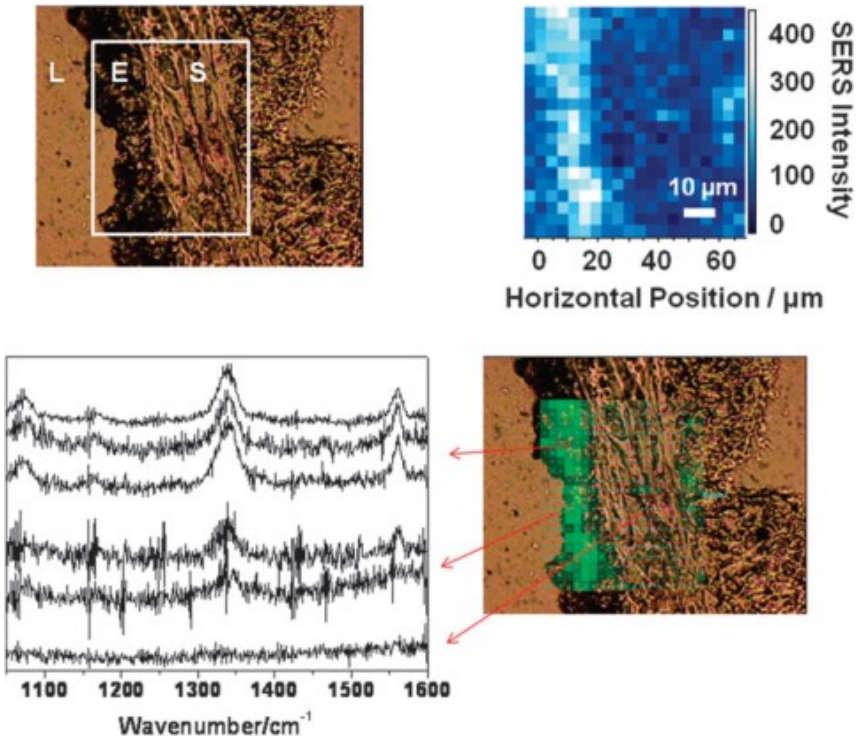

Figure 4: Detection of PSA *ex vivo* – top: bright field microscope image of a prostate tissue section. The grid shows the locations at which Raman spectra were acquired in a point mapping experiment. The false colour SERS image shows that the characteristic signal of the SERS-labelled antibody is observed selectively in the epithelium. Reproduced from [80] with permission from the PCCP Owner Societies.

Whilst detection systems like this might seem trivial, they are actually of huge importance for the characterisation of disease in tissue. In all of the examples discussed, the measured signals arise from the SERS reporters. However, a second laser line could be employed at a frequency where the nanotags are not SERS active but where intrinsic Raman signals from the tissues dominate. This would allow biochemically characteristic information to be obtained from the tissues and the false colour SERS images could be used to delineate the boundaries between the regions where, for example, PSA expression is high and low. This would provide further information which is biochemically representative of the disease biomarkers. Whilst areas of protein expression can be imaged using traditional immunohistochemical methods, no biochemical information is obtained. Additionally, conventional fluorophores have broad absorption and emission bands so the staining of multiple areas is difficult. However, when employing either Raman and/or SERS imaging, the spectral bands are narrow and not limited in the tissue regions or receptors which could be targeted simultaneously. This is particularly important as the documentation of disease processes progresses towards the detection of multiple markers. [13]

Manipulation of the antibody-antigen interaction has also been exploited for the determination of nasopharyngeal cancer in tissue samples. [98] As with most of the discussed samples, antibody functionalised SERS nanotags were exposed to tissues. [98] Clinical samples were analysed and directly compared with the tissue, which had been analysed via a traditional immunohistochemical method. SERS analysis was found to considerably exceed the conventional method. [98] In total, there were 34 cancerous and 20 non-cancerous tissue samples and the SERS detection method correctly identified 33 of the cancerous samples whilst the traditional method only identified 22. [98] Negative samples were correctly identified by both methods. [98] As with the previous examples all of the above discussed advantages of the SERS method are equally applicable to this study. [98]

SORS has been previously discussed as a method for disease detection in section 2.2. SORS has also been developed to incorporate a surface enhanced method known as surface enhanced spatially offset Raman spectroscopy (SESORS). [99] The key feature and main advantage of this technique is that it can measure Raman or SERS signals through medically relevant tissue depths. [99] This is of huge importance since it actively demonstrates non-invasive detection of disease. [99] In one of the latest studies cumulative SERS signals were measured from four different SERS nanotags which were located within tissue at a depth of 20 mm. [99] Even more significantly, it was possible to measure SERS signals through 50 mm of tissue. [99] This study highlights the possibility of implementing SERS and their nanotags for use *in vivo.*

In a subsequent study the method was investigated in specific relation to a disease process. [100] Osteoporosis is a progressive bone disease which can be treated with drugs known as bisphosphonates. [100] The disease itself was not investigated but rather the study was designed to replicate drug distribution and investigate its localisation. [100] In order to do this, the drug was conjugated to SERS nanotags and subsequently incubated with representative bone samples. The bones were then transplanted into a tissue mass and the samples were analysed. [100] It was possible to detect the drug-nanotag conjugates at a depth of 20 mm. [100] Although this study did not involve the direct detection of the disease it is nonetheless important because it actively demonstrates the ability to monitor drug distribution non-invasively. [100] This therefore highlights the suitability of SERS as a key process in the treatment of disease.

3.2.2 *In vivo* Imaging and Disease Detection

One of the most recognisable and well documented examples of *in vivo* SERS imaging is undoubtedly the study by Qian *et al.*, [101] where tumours were successfully targeted with an antibody functionalised gold nanoparticle system *in vivo*. [101] The nanotag conjugates were introduced into the mice model via tail injection and they were successfully translocated to tumour locations. [101] Successful targeting was demonstrated via monitoring of the SERS signals and those measured from the tumour regions of the mouse were found to be representative of the reporter from the SERS nanotags (Figure 5). [101] This study actively demonstrated that the nanoparticles were able to travel through the animal model and reach their targets. [101] It was also critical for demonstrating the success and application of SERS for monitoring such interactions *in vivo*.

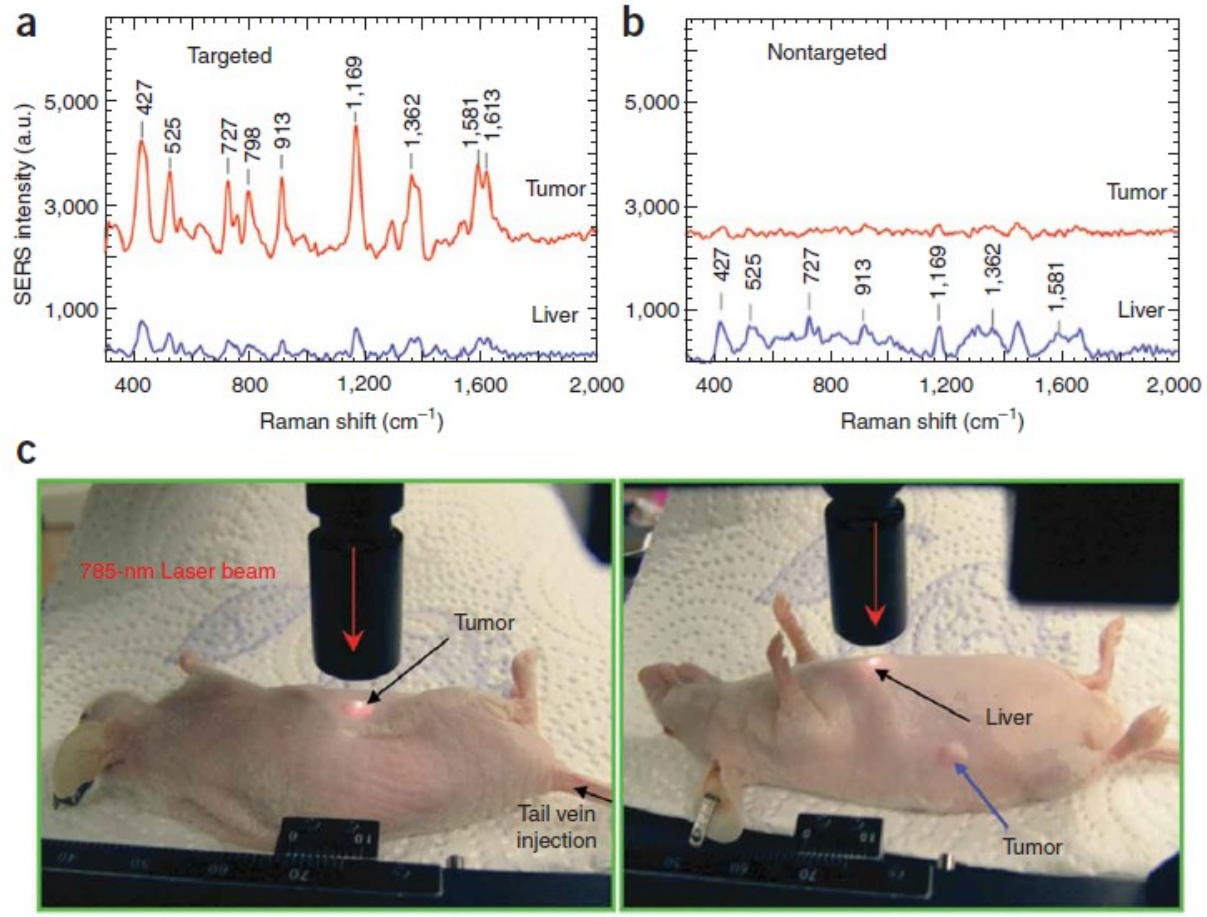

Figure 5: *In vivo* cancer targeting and surface enhanced Raman detection by using ScFv-antibody conjugated gold nanoparticles that recognise the tumour biomarker EGFR. (**a,b**) SERS spectra obtained from the tumour and the liver locations by using targeted (**a**) and nontargeted (**b**) nanoparticles. Two nude mice bearing human head-and-neck squamous cell carcinoma (Tu686) xenograft tumour (3-mm diameter) received 90 μL of ScFv EGFR-conjugated SERS tags or pegylated SERS tags (460 ρM). The particles were administered via tail vein single injection. SERS spectra were taken 5 h after injection. (**c**) Photographs showing a laser beam focusing on

the tumour site or on the anatomical location of liver. *In vivo* SERS spectra were obtained from the tumour site (red) and the liver site (blue) with 2-s signal integration and at 785 nm excitation. The spectra were background subtracted and shifted for better visualisation. The Raman reporter molecule is malachite green, with distinct spectral signatures as labelled in **a** and **b**. Laser power, 20 mW. Reprinted by permission from Macmillan Publishers Ltd. Nature Biotechnology, [101] (2008).

In vivo imaging of SERS nanotags has also been applied for the observation of inflammation and again, while not a disease process, it is of relevance since any change can be indicative of infection and underlying disease conditions. [102] Nanotags were functionalised with antibodies which have a specific affinity for the identified inflammation biomarker, intracellular adhesion molecule 1 – (ICAM-1-). The nanotags specifically targeted inflammation *in vitro*, *ex vivo* and *in vivo*. [102] When the effectiveness of the SERS approach was directly compared with the conventional fluorescence method it was found to be significantly more sensitive. [102]

Whilst the previous examples have demonstrated active *in vivo* disease targeting, a multi marker approach is considered to be beneficial, especially if multiple disease aspects are to be detected simultaneously. [13] It is hoped that by analysing disease processes or even multiple different diseases it will lead to better characterisation and personalised medicine.

With the advent of superior nanotag systems, which display excellent stability and are optically 'hot', the possibility of multiplexed imaging *in vivo* has become a real possibility. [13, 76] In the initial stages it was possible to simultaneously detect two different nanotags within a live subject but this was latterly extended to include the simultaneous detection of ten different nanotags (Figure 6). [13, 76] This was a crucial step forward for multiplexed detection *in vivo*, actively demonstrating the potential to detect multiple pathosis or different elements of disease simultaneously. [13] In a further extension of the work, in terms of true multiplexing and the confident identification of nanotags present within a specified location, it was possible to positively identify four nanotags in a multiplexed scenario. [13]

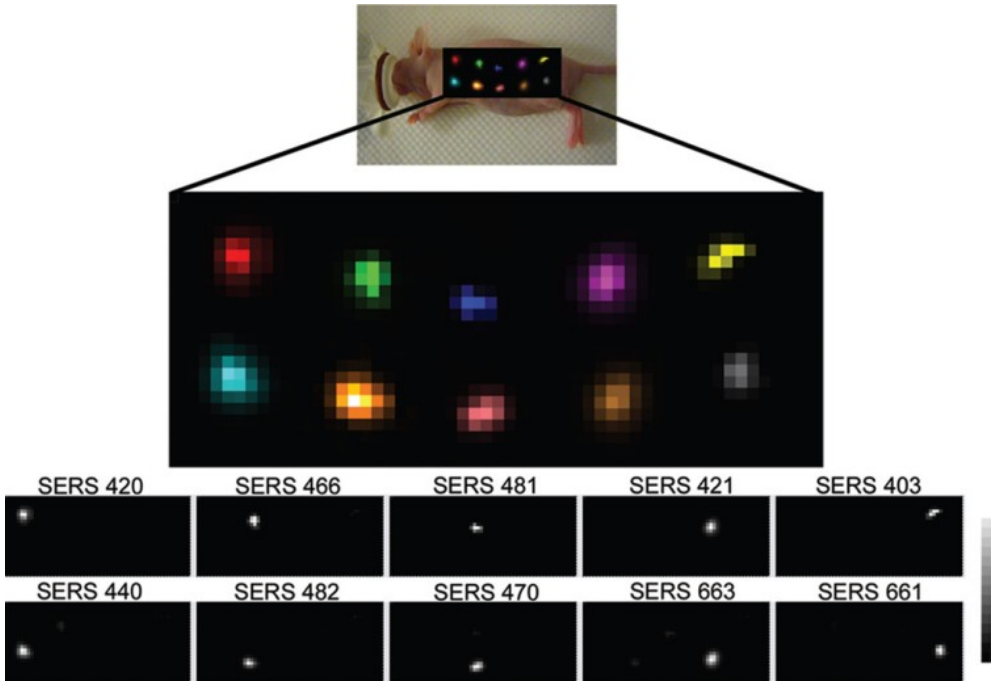

Figure 6: Evaluation of multiplexing 10 different SERS nanoparticles *in vivo*. Raman map of 10 different SERS particles injected subcutaneously in a nude mouse. Grayscale bar to the right depicts the Raman intensity, where white represents the maximum intensity and black represents no intensity. [13] C.L. Zavaleta, B.R. Smith, I. Walton, W. Doering, G. Davis, B. Shojaei, M.J. Natan, S.S. Gambhir, Proc. Natl. Acad. Sci. USA., 106 (2009), 13511-13516. Copyright (2009) National Academy of Sciences of the United States of America.

Further *in vivo* applications have been demonstrated by Maiti *et al.*[103-105] In the first of their *in vivo* experiments nanotags were injected into a mouse model and successfully imaged using SERS.[103] The only difference being that the antibody functionalised nanotags were actively bound to cancerous cells prior to their introduction, highlighting the opportunity to successfully image the nanotags *in vivo* even after participation in a disease recognition event. [103] In an extension to this work nanotags functionalised with antibodies which had a specific affinity for the receptors in the xenograft were found to translocate to the xenograft site following introduction into the animal. [104] Thus the conjugates participated in antibody-antigen recognition and signals representative of the reporter molecule were measured at the xenograft site. [104] However, when nanotags were introduced into mice bearing xenografts of the non-cancerous cell population SERS signals were not measured in accordance with the absence of a specific binding event. [104]

In the most recent of their work, the group elegantly demonstrated the selectivity of their targeting nanotags. [105] In this study the xenograft was composed of oral squamous cell carcinoma (OSCC) cells which exhibit a differential level of expression of EGFR and HER2. EGFR receptors are present at a much higher level than HER2 receptors on the surface of the OSCC cells. [105] In accordance with

the expressed receptors three nanotags were injected into the tail of the animal subject, two of which had been functionalised with the EGFR specific antibody and the remaining nanotag was functionalised with the HER2 antibody. [105] The signals which were measured from the site of the xenograft were in accordance with the two nanotags functionalised with the specific antibody and two different reporter molecules. There was no contribution from the nanotag functionalised with the HER2 antibody (Figure 7). This study represents one of the first true targeted *in vivo* multiplexing examples and this is an important step forwards in terms of striving towards the deliverable of comprehensively characterising and detecting multiple aspects of disease. [105]

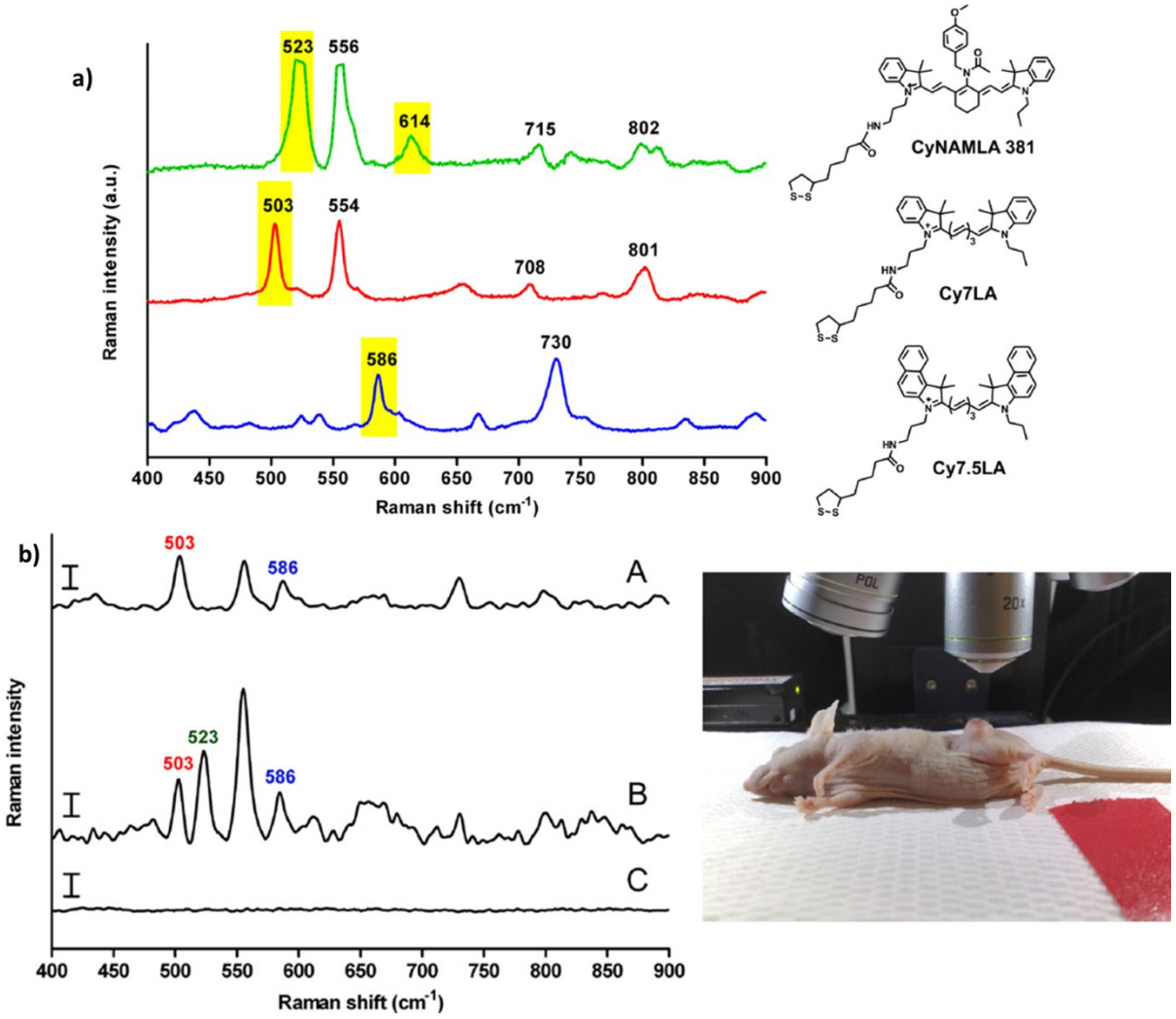

Figure 7: *In vivo* multiplex detection in xenograft tumour – a) Normalised SERS spectra of CyNAMLA-381, Cy7LA and Cy7.5LA after chemisorption of AuNPs and b) – A: SERS spectra from tumour site (peaks obtained at 503 and 586 cm^{-1} from two EGFR positive nanotags, Cy7LA and Cy7.5LA), B: SERS spectra from liver site (peaks obtained at 503, 523 and 586 cm^{-1}) from two EGFR nanotag Cy7LA, Cy7.5 and anti-HER2 nanotag CyNAMLA-381) and C: SERS spectra from dorsal region. [105] Modified and reprinted with permission from Elsevier.

4. SERS as a Method for Disease Treatment

The idea of implementing nanoscaffolds for disease treatment is not a new concept and nanostructures in many different forms (i.e. nanorods, nanoshells and nanoparticles), predominantly gold based, have been utilised for the destruction of cancerous cells. [53, 106-111] This occurs because nanostructures absorbing the interrogating laser light results in localised heating, which is subsequently dissipated to the cell resulting in destruction. [53, 106, 107, 112, 113] It is widely believed that an increase in temperature is responsible for this effect but recent studies have shown that cellular damage can result without a significant temperature increase. [114] The method must therefore elicit some of form of damage which can ultimately result in cell death. [114, 115] While there are many examples highlighting the use of nanomaterials for therapeutic purposes, there are no demonstrated applications where SERS has been employed as a treatment method. However, its use has recently been critical in determining the localisation of nanostructures so that treatment can be targeted to a specific position. SERS is therefore becoming a key step in treatment processes.

The detection of bacteria which are drug resistant is one such field where this method might be beneficial. Fan *et al.* described the generation of popcorn shaped magnetic nanoparticles which consisted of an iron core and a gold shell. [116] The nanostructures were further labelled with an antibody with affinity for *Salmonella* DT104. [116] In the study the nanoparticles were found to bind to the *Salmonella* cells with high affinity and, because of their magnetic properties, cells could be extracted from the bulk solution. [116] Cells were then destroyed by illuminating with a therapeutic light source. [116] As with several of the studies which will be discussed, SERS was not directly involved in the treatment process, but it was essential for determining that the nanoparticle conjugates had successfully bound to the target cells and without these conjugates treatment would not have been successful. [116] While this remains a preliminary study the method is postulated as an alternative to drug based treatment methods for bacterial infections and disease, and this has important consequences since drug resistance continues to increase. [116]

Implementation in the detection of lung cancer is another field where these SERS based detection methods might be beneficial, especially since lung cancer is often diagnosed beyond a stage of effective treatment. [113] In a study by Wu *et al.* aptamer conjugated silver shell gold core nanoparticles labelled with rhodamine 6G were used for the specific targeting of A549 lung cancer cells. The specificity of the system was tested against six cell lines (two generic cancer and four lung cancer cell lines) and the aptamer conjugates were not found to give a significant SERS response, suggesting that the conjugates did not bind to any other cell lines except those which were

specifically targeted. [113] Implementation of the aptamer system allowed for considerable levels of aggregation between the cancer cells to occur since aptamers on a single shell could bind to different cells within the exposed population. [113] This was beneficial since it created concentrated areas of the conjugates and cells, ideal preparation for SERS detection and treatment with the irradiating laser. During the study it was found that a laser power of 0.2 W cm^{-1} applied for 60 min was sufficient to kill the cancerous cells. [113] The SERS response was also observed to decline following application of the treatment laser and it was postulated that this could be used as a measure of treatment efficacy. [113] When the same procedure was performed with the conjugates and the remaining cell lines, cell damage or death was not observed. The authors highlighted that this method could be applied for the medical treatment of cancer since differentiation between the cancerous cells was both selective and specific. [113] In this particular application SERS has been implemented to confirm the binding reaction and the possibility exists for it to be used as an indicator of treatment progression. Although not directly responsible for the treatment the implementation of SERS was critical to the overall therapeutic procedure and success of the system.

Such procedures are not isolated to the treatment of lung cancer and a similar scenario was developed for breast cancer cells (BT-549). [117] In this procedure gold nanostars which had been previously pegylated were labelled with the reporter molecule 3, 3'-diethylthiadicarbocyanine (DTDC) before silica encapsulation and further labelling with the phototherapeutic drug protoporphyrin IX (PpIX) and the cell penetrating peptide TAT. [117] In the study SERS was employed to monitor the uptake of the nanostars by breast cancer cells. When functionalised with the TAT peptide considerable SERS signals were observed. However, when the peptide sequence was removed from the conjugate, SERS signals were significantly reduced or not detected. [117] This is consistent with observations that functionalisation with the TAT peptide aids in the cellular uptake of nanomaterials. [117-119] In this particular study, TAT functionalisation was critical for the successful treatment of cancerous cells using PpIX. PpIX is used as an initiator for the production of damaging oxygen species which in turn continues the cascade of reactions initiating cellular damage and death. [117] Following exposure to the therapeutic light source, cell death was apparent in those cells which had been subject to the TAT functionalised conjugates whilst no significant effect was observed in those exposed to the TAT or PpIX free nanostructures (Figure 8). [117] The use of SERS in this study was again critical for determining the successful targeting of the conjugates, and whilst it is not directly responsible for the therapeutic effects observed, without it the delivery of the treatment could not be confirmed. [117]

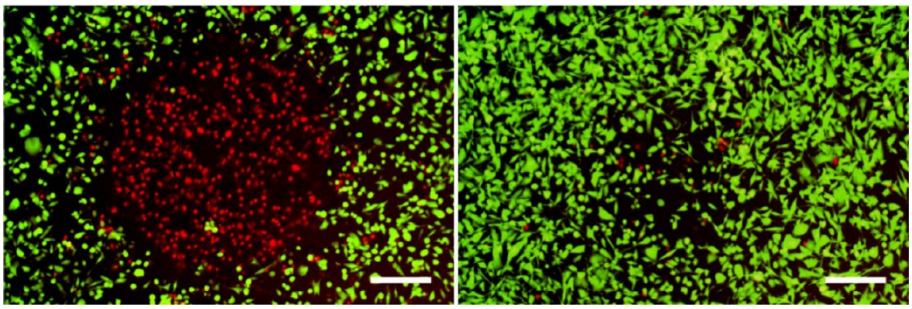

Figure 8: Viability staining of cells incubated with AuNS-DTDC@SiO_2-PpIX-TAT (left) and AuNS-DTDC@SiO_2-TAT (right) after 30 s of light irradiation. Live cells are stained green and dead cells are stained red. Scale bars are 250 µm**.** Reprinted with permission from [117]. Copyright (2013) American Chemical Society.

In a further example silver nanotriangles encapsulated in chitosan and labelled with a reporter molecule and folic acid were used for the treatment of ovarian cancer cells (NIH-OVCAR-3). [120] Folic acid was essential for transporting the conjugates across the cell membrane via a receptor mediated targeting strategy. [120] SERS was again implemented to confirm the transport of the nanostructures to their specified targets. Treatment with the therapeutic laser lead to cell death and the levels of cellular destruction were correlated with nanostructure concentration. [120] This method could be used to deliver therapeutics specifically to a diseased cell population since nanotriangle uptake between the cancerous and non-cancerous cells should vary in accordance with the expression of cancer related receptors. [120] SERS was again a vital step in the overall treatment process.

While cell based examples are undoubtedly interesting and stimulate further research this ultimately must be translated to *in vivo* applications. Conde *et al.* developed gold nanoparticle conjugates which were labelled with a reporter molecule and the antibody-drug conjugate Cetuximab (Erbitux®) for targeted detection and treatment of tumours *in vivo*. [121] Mice models were implanted with cancerous cells to generate xenografts [121] and mice were then treated with nanostructures via tail injection, while the tumours were located in the right leg. [121] The conjugates were successfully translocated to the tumour site and resulted in a reduction of further tumour development by the inhibition of cellular division. [121] Active targeting of the tumour was monitored via SERS and large SERS signals were observed in mice treated with the drug conjugates whilst minimal signals were observed from those treated with drug-free nanostructures (Figure 9). [121] The authors reported that the signals measured from drug labelled nanoparticles at the xenograft were 4.5 times higher than those of the drug free system as measured from a key identification peak at 508 cm^{-1}. [121] The variation in the SERS signal between the two sites and samples presumably arises because the drug-free conjugates are not specifically targeting the tumour site. [121] The SERS studies were

performed *ex vivo* but the method further highlights the implementation of SERS based conjugates and SERS as a method for the successful targeting and treatment of disease *in vivo.* [121]

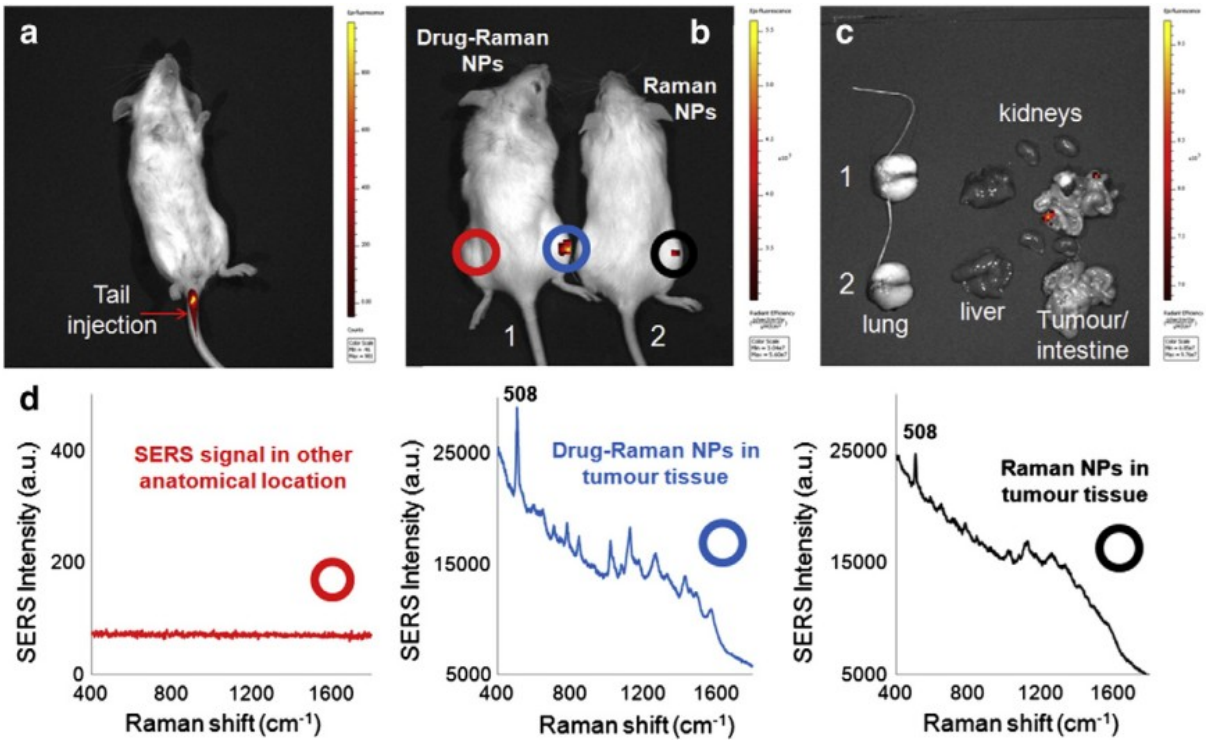

Figure 9: Drug–Raman NP uptake and bio-distribution in whole body mice. Representative imaging of individual mice from each group (n = 10 animals) is shown, with the same scale of epi-fluorescence (a,b) indicating emission signal from the Raman reporter, DTTC (Emission = 780–800 nm). (c) Evaluation of nanoparticles distribution in mice organs treated with Raman- and drug–Raman NPs. (d) SERS spectra of the tumour site, measured through the tumour tissues excised from the mice, of drug-Raman NPs (blue line), Raman NPs only (black line) and other anatomical locations (*i.e.*, the opposite leg, red line). Characteristic SERS peak at 508 cm^{-1}. [121] Reprinted with permission from Elsevier.

5. Conclusion

The rich and varied applications of Raman and more significantly SERS for the detection of diseases are direct consequences of the numerous advantages that they confer. Unlike their fluorescent counterparts, sharp molecularly specific spectra are obtained, which instantly and readily lends the techniques to multiplexed disease detection opportunities. As demonstrated in some of the examples discussed, detection via these methods is often more sensitive and selective. In addition, the possibility exists for combined Raman and SERS imaging of cells and tissues so that in the advent of implementing SERS active nanotags, biochemical and characteristic information relating to the disease process can be simultaneously obtained. In order for SERS to achieve and remain at the

forefront of disease diagnostics further studies are required in physiological representative media, clinical samples and *in vivo*. When these applications fully demonstrate their robustness, reliability, repeatability, sensitivity and selectivity *in vivo*, the technique will make the full transition from the laboratory to a clinical setting. Presently, the techniques are strong contenders for revolutionising our ability to detect disease.

Acknowledgments

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