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MINIREVIEW

Surface enhanced Raman scattering for multiplexed detection

Jennifer A. Dougan and Karen Faulds*

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The multiplexed detection of biological analytes from complex mixtures is of crucial importance for the future of intelligent management and detection of disease. This review focuses on recent advances in the use of surface enhanced Raman scattering (SERS) spectroscopy as an analytical technique that can deliver multiplexed detection for a variety of biological target in increasingly complex media. The use of SERS has developed from the multipelxed detection of custom dye molecules to biomolecules such as DNA and proteins. Recent work has also shown the capability of SERS multiplexing for *in vivo* as well as *in vitro* applications.

Introduction

The Oxford English Dictionary defines the word "multiplex" as a verb "to enable a line to carry several signals simultaneously."[1] From an analytical detection point of view this descriptor applies to the concept of multiplex species detection *i.e.* the simultaneous detection of more than one analyte from a single sample without the need for separation and using a single sample readout. There are a number of analytically significant situations in which multiplexing is an attractive goal. One of the most desireable applications is for the detection of biomolecules where it is often required that several species be detected simultaneously and prefereably without separation from the biological matrix, reducing time consuming separation steps. This review will therefore focus on the multiplex detection of biomolecules.

The vibrational technique of surface enhanced Raman scattering (SERS)[2] is ideal for the detection of multiple analytes due to the sharp fingerprint spectra which are obtained although other vibrational spectroscopies have previously been used for multiplexing.[3,4] Vibrational techniques are ideal for multiplexing due to their sharp peaks which can be used to differentiate multiple analytes in a mixture when the analytes have distinct enough spectra to allow spectral separation of components. SERS involves the adsorption of an analyte molecule onto, or close to, a roughened metal surface which can result in enhancements in the order of 10^6 over normal Raman scattering.[5–7] A wide range of metallic substrates have been studied. The coinage metals Au and Ag are of the greatest practical use since their surface plasmons lie in the visible region of the electromagnetic spectrum which coincide with the common Raman excitation wavelengths. The most commonly used SERS

Centre for Nanometrology, Department of Pure and Applied Chemistry, WestCHEM, University of Strathclyde, 295 Cathedral Street, Glasgow, G1 1XL, UK. E-mail: Karen.faulds@strath.ac.uk

Jennifer A. Dougan

Jennifer Dougan obtained her BSc (Hons) in 2004 from the University of Strathclyde in Pure and Applied Chemistry. Having researched bio-functionalised nanoparticles of enhanced stability for bio-analytical detection, her PhD in Chemistry was completed in 2009 under the supervision of Professor Duncan Graham. She then moved to a postdoctoral position with Dr Karen Faulds, focusing on bioanalysis using SERRS and plasmonic based detection strategies.

Karen Faulds

Karen Faulds is a senior lecturer within the Centre for Nanometrology at the University of Strathclyde. Her main interests are in creating new methods of bioanalysis using SERRS and in particular, the development of molecular diagnostic SERRS based assays and nanoparticle biosensors. She was awarded Nexxus young life scientist of the year and is a member of the Royal Society of Edinburgh Young Academy of Scotland and is a member of Analyst editorial advisory board.

substrates are colloidal suspensions of the desired metal.[8] Colloids are attractive as they are readily prepared, relatively stable and reproducible as well as being compatible with solution based assays for biomolecules.[9–12]

A further advancement is surface enhanced resonance Raman scattering (SERRS).[13] For SERRS to occur a molecule with a chromophore coincident or close to the laser excitation frequency is required and it must also have the ability to adsorb onto a metal surface. If the molecule does not naturally have these properties then they can be achieved by attaching a SERRS active label to the analyte of interest.[14] These labels can either be specifically designed for SERRS[15,16] or commercially available fluorescent labels since the use of a metal surface quenches any fluorescence emitted by the label allowing common fluorophores to be used.[8,17–19] The added advantage of using labelling strategies to tag the analyte of interest means that it will have a strong signal that will be greater than any components of the sample. This will ensure that the SE(R)RS signal from the analyte is always greater than any background signal from non-specific SERS signals from components within the biological matrix.

As already stated, as well as its inherent sensitivity, one of the main advantages of SERS is the ability to multiplex. The mutliplexing capability of SERS is often stated by researchers, however this is often not demonstrated experimentally. This review article highlights where genuine multiplexing by SERS for the detection of multiple biomolecules has been reported.

The bio-diagnostics field is concerned with the detection of biomolecules, such as DNA, proteins and enzymes from complex biological matrices. Current methods tend to require separation and isolation of the components, both from the matrix *e.g.* serum and from each other before analysis can be carried out. This is a time consuming and often complex process which can result in loss or dilution of the analyte components. Successful multiplexing of multiple diagnostically significant disease markers within the same sample would allow for unambiguous diagnoses with fewer separate measurements.

Detection is normally carried out using a spectroscopic method, most commonly fluorescence spectroscopy, in which case a fluorescent label will be attached to the biomolecule of interest or a change in fluorescence will be observed due to interaction with a biomolecule. However, there are several drawbacks to using fluorescence as a detection technique. The main problem is the nature of the fluorescence emission spectrum which is broad and gives limited characteristic information about the target analyte. This makes the detection of multiple analytes in a mixture difficult due to the large spectral emission overlap that occurs from more than one fluorophore. In practice, using a single excitation light source, only four labels are generally detected at once, three if an internal standard is used unless some sort of physical separation method is employed. SERRS has also previously been shown to be generally 3 orders of magnitude more sensitive than fluorescence for the detection of dye labelled oligonucleotides.[20] Thus, to increase the amount of data obtained per experiment and reduce the number of separate measurements required, it is desirable to increase the number of biomolecules that can be detected simultaneously, without separation, in a single experiment. This will result in a cost reduction since less separate tests will be required and more information per test will be available.

Multiplex SERS of reporter molecules

As already stated SERS, as a vibrational spectroscopy technique, lends itself to mutliplex analysis by virtue of the sharp (nm width), molecularly specific bands that are produced by the analyte. SERS is a surface-based technique since the analyte must be adsorbed onto a suitably roughened metal surface, therefore dyes which comprise both a chromophore and a surface seeking component have been specifically developed for use in SERS analysis.[21–23] The inclusion of a surface complexing moiety facilitates adsoprtion of the chromophore (or reporting group) to the enhancing metal surface. Smith *et al.* reported the synthesis of such dyes and evaluated their suitability for multiplex analysis.[22] Azo dyes were prepared with different surface seeking functionalities, benzotriazole, hydroxyquinolyl or pyridyl ring systems. Each of the dyes were analysed separately and then as part of a composite mixture. By reference to the "pure" dye spectrum, peaks characteristic of each dye could be identified within the mixed sample thereby indicating the presence of that dye. In this case, a 4-plex was reported by use of 3 specifically designed SERRS dyes and a HEX-labelled oligonucleotide, Fig. 1. An important factor was highlighted in this study; that the SERRS cross section of the individual reporters within the multiplex must be taken into account. The effective response from a SERRS reporter may be significantly different depending upon a number of factors including differences in surface adsoprtion characteristics, absorption maxima of the analyte at the laser excitation wavelength and the relative SERRS cross-section of each molecule. Thus, in order to obtain SERRS responses of similar intensities a range of concentrations of dye label had to be used (0.1 mM to 10 μM). By carrying out the multiplexed experiment with the components mixed across that concentration range, peaks relating to each of the dyes could be identified. Had each of the analytes been interrogated at the same concentration, SERRS responses from the best reporters would have "swamped" the responses from the weaker reporters. Therefore, for successful multiplexing the relative SERRS response of the analytes must be taken into account.

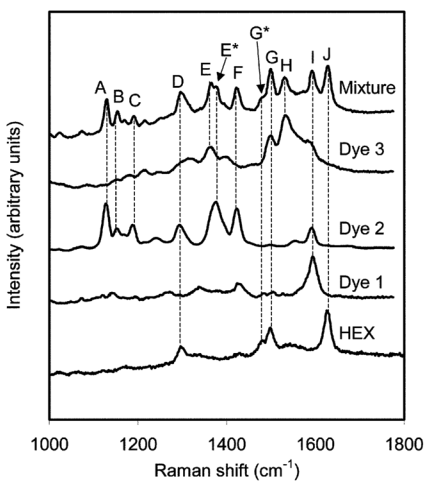

Fig. 1 SERRS spectra recorded from individual dyes and from a mixed sample, where the concentration of analyte has been varied to produce similar SERRS intensities.[22]

A recent report has investigated the use of SERS labels with the aim of preparing multiplexed reporter particles. Schlücker *et al.* have used Raman active reporter molecules with the ability to form self assembled monolayers (SAMs) to generate "bar coded" plasmonic particles.[24] Three Raman reporters, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2-bromo-4-mercaptobenzoic acid (BMBA) and 4-mercaptobenzoic acid (MBA) were directly adsorbed onto the nanoparticle substrates used in the study. SAMs of each individual reporter as well as mixed monolayers of each possible combination of the 3 reporters were prepared on the surface of gold nanoparticles. The spectrum from each individual reporter was used as a reference which then allowed the combination of the different reporters to create a barcode system, Fig. 2. This was possible due to the similarity in the molecular structure of all three reporters which reduced potential differences in signal intensity due to the surface adsorption characteristics. The labels were not in resonance with excitation wavelengths in the visible region, therefore there was no additional enhancement from any of the labels due to resonance. Instead, the appearance, or lack of appearance, of peaks indicated which reporters were present or absent and therefore the identity of the barcoded particle. It is speculated that through this method, with less than 50 unique Raman labels, millions of identifiable unique barcoded particles could be produced.

SERS for multiplexed deoxyribonucleic acid (DNA) detection

One important field of bio-analysis is the sensitive and selective detection of sequences of DNA. Spatial multiplex analysis of DNA has previously been achieved by array-based fluorescent and electrochemical techniques.[25] In solution, fluorescence multiplexing is limited since the technique suffers from broad, overlapping spectra of molecular fluorophores, photobleaching and the requirement for multiple excitation sources. Advances have been made in the field of fluorescence detection by the development of quantum dots which produce a relatively narrow fluorescence emission profile coupled with a broad excitation profile.[26] However, issues of toxicity limit the use of quantum dots in a broad sense.

Electrochemical techniques require spatial separation *i.e.*, in an array format, for multiplexed detection. Spatial resolution in this manner can also be applied to optical spectroscopies and, indeed, allows for unparalleled multiplexing capabilities. Nevertheless, for some applications dynamic solution based analyses are required. This review focuses on the use of solution based SERS multiplexing techniques and the associated spectral, rather than spatial, resolution of analytes.

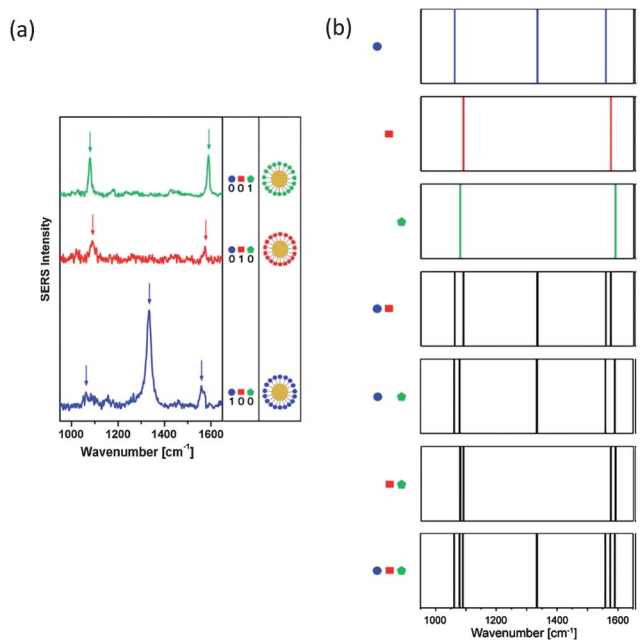

Fig. 2 (a) SERS spectra of individual reporters, MBA (green), BMBA (red), DTNB (blue) [The presence or absence of a reporter is indicated by the digital "1" or "0" nomenclature] and (b). bar code style representation of SAMs (single and mixed).[24]

Single stranded DNA (ssDNA) – proving the concept. An interesting example highlighting the power of SERRS multiplexing involves the discrimination of two phthalocyanine labelled oligonucleotides by Graham *et al.*[27] Phthalocyanines have porphyrin structures with metal centres and by varying the metal centre the optical properties of the phthalocyanine can be altered to change their absorbance properties or to become fluorescent or non-fluorescent. Oligonucleotides were functionalised with 3 different phthalocyanines and using SERRS, researchers were able to discriminate between the oligonucleotides based upon the differing spectra due to the metal ion complexed at the centre of the phthalocyanine ring system – whether Co or Zn. This relatively trivial change in the label afforded vibrational changes within the ring system, and therefore the SERRS response. Much work has also been carried out on the ability to reproducibly detect quantitative SERRS signals from DNA which has been labelled with commercially available fluorescent dyes.[17–19]

ssDNA sequences possess a polyanionic backbone and this, coupled with the anionic surface charge left on the nanoparticle post-synthesis requires the use of spermine hydrochloride to facilitate surface adsoprtion (in the absence of functionalising the DNA with a surface seeking dye moeity).[28] The spermine is thought to form electrostatic layers between the negatively charged oligonucleotide and nanoparticle; bringing the DNA and hence the fluorescent dye label close enough to the surface to generate a SERRS response. It should be noted that spermine acts both to aid surface adsorption of the oligonucleotides to the surface and to cause aggregation of the nanoparticle substrates – generating the hotspots that are required for SERRS. When oligonucleotides are designed and analysed using the conditions described above it is possible to achieve extremely sensitive detection of labeled oligonucleotides using metal nanoparticles.[17] It is possible to achieve quantitative, linear SERRS responses when the concentration of the labelled oligonucleotide is kept below monolayer coverage of the nanoparticle surface.

The simultaneous detection of 5 labelled sequences of ssDNA has been achieved without the need for chemometric analysis, Fig. 3.[29] This was achieved by judicious selection of dye labels and the employment of two wavelengths of excitation. Faulds *et al.* made use of the differential SERRS response of dye labels as a result of their differing absorption maxima, and hence their differing resonance contributions with the two excitation

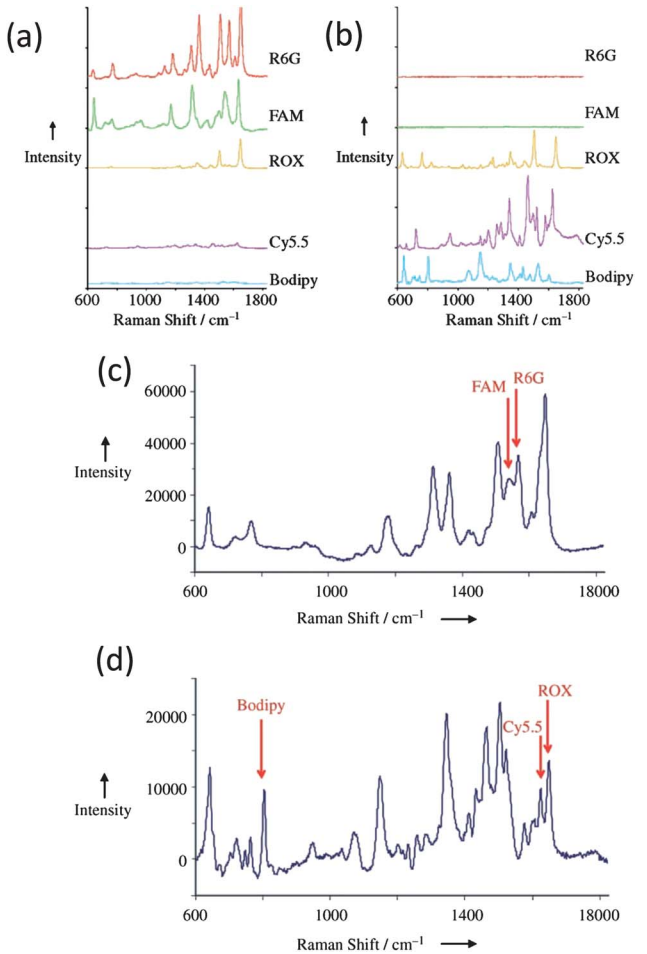

Fig. 3 Separate SERS analysis of each of the 5 labels at 514.5 nm (a), and 632.8 nm (b). Simultaneous analysis of the 5 labels at 514.5 nm (c), and 632.8 nm (d).[29]

wavelengths. By this method, five labelled oligonucleotides were mixed together in one sample and two labels (FAM and R6G) were detected due to their resonance contribution at 514.5 nm and the remaining three labels (BODIPY-TRX, ROX and Cy5.5) were identified by use of a 632.8 nm wavelength laser, Fig. 3. All other experimental paramaters were kept constant. To prove that the detection of each of the labelled oligonucleotides within the multiplex was still quantitative, a dilution series of the mixture of the five oligonucleotides was performed and the detection limits determined (Table 1). The detection limits obtained for all of the labelled probes in the multiplex were very close to values

Table 1 Labelled oligonicleotides and the detection limits achieved within a multiplex and when each oligonucleotide was analysed individually

Label	λ_{max} (nm)	L.O.D. (mol dm^{-3})	L.O.D. Multiplex (mol dm^{-3})
FAM	494	7.73×10^{-12}	2.91×10^{-12}
R6G	524	1.17×10^{-12}	3.22×10^{-12}
ROX	585	3.30×10^{-11}	1.25×10^{-11}
BODIPY TR-X	588	7.85×10^{-12}	3.08×10^{-11}
Cy 5.5	683	5.52×10^{-12}	6.70×10^{-12}

obtained from SERRS detection of the isolated labelled oligonucleotide probes. This clearly indicates that there is no reduction in the sensitivity of detection of these probes when used in a 5-plex and that the data obtained is still quantitative. This study clearly showed that through careful choice of the labels and excitation frequencies quantitative multiplexing at ultra low concentrations is possible. This further suggests that the multiplexing of different analytes by using SE(R)RS does not necessarily compromise the sensitivity of the detection and that the presence of multiple analytes does not affect the ability to quantify the results as long as the experimental conditions are carefully controlled.

However, moving to a single wavelength multiplex system and increasing the number of spectral compnents that can be identified from a single experiment required the use of chemometric analysis. In the first reported example of multivariate analysis for SERRS multiplex detection, each labelled sequence of ssDNA could be identified from within a mixture of six with high sensitivity, specificty, accuracy and precision.[30] Every possible combination of the six labelled oligonucleotides, whether present or absent, could be successfully detected. This was achieved by use of partial least squares regression analysis. This is a significant advance in the use of SE(R)RS for multiplexed detection as often, authors indicate the suitability of SERS to multiplexed analysis by showing stacked SE(R)RS spectra, rather than carrying out the true mixed sample experiment. Recently, the same 6-plex sample has also been analysed using Bayesian methods to resolve the multiplex components.[31] Furthermore, these techniques offer an advantage over the higher order multiplexing by Irudayaraj *et al.* where peaks were picked out from a known mixture. The 8-plex reported produced spectra which would be hard to distinguish "blindly" which may limit its applicability for "real world" analyses.[32,33]

DNA assays. DNA in its native state does not posess a chromophore that readily enables a uniquely identifiable SERS response. It should be noted that although SERS of the nucleobases can be observed,[34] these would not vary in a sequence specific manner that would be required for disease diagnosis.[35] Instead, it is necessary to introduce a dye label that provides a characteristic SERRS response. In a diagnostically useful situation it is not possible to specifically label a target strand of DNA. Instead the fidelity of DNA duplex formation *via* Watson–Crick base pairing may be employed to allow identification by labelling of the sequence complementary to the target.

Initial work on the duplex detection of fluorescently labelled oligonucleotides was carried out using HEX and R6G labelled oligonucleotides where it was possible to discriminate the ratio of each oligonucleotide sequence present based on the SERRS signal from the labels.[36] A 3-plex of fluorescently labelled oligonucleotide sequences has also been detected using a lab-on-a-chip format. In this approach microfluidics chips were generated from PDMS and DNA sequences labelled with Cy3, FAM and TET were introduced into the chip. The SERRS signals were then measured at a point further down the channel. This approach allowed detection of three different DNA sequences corresponding to different strains of *Escherichia coli* bacterium.[37]

In 2002, Graham *et al.* reported the first SERS multiplexing for DNA genotyping.[38] This was achieved by the combination of

an amplification refractory mutation system (ARMS) approach with SERRS for the detection of the cystic fibrosis transmembrane conductance regulator (CFTCR) gene. This gene can be present in three formats – the wild type, where both alleles are normal, the heterozygote, where one allele is mutated and the homozygote mutant where both alleles are mutated. HEX and rhodamine labelled primers were incorporated into PCR product and any unincorporated dye removed by a streptavidin-biotin wash step. This allowed the successful identification of the mutational status of these particular samples to be determined, Fig. 4.

Recently an assay was reported which allowed multiplex detection of DNA sequences relating to MRSA in a closed-tube, homogeneous assay format.[39] The assay is based on the observation that double stranded DNA has a lower affinity for the surface of silver nanoparticles than single stranded DNA. When a single stranded probe sequence labelled with a SERRS active fluorophore is added to silver nanoparticles a strong SERRS signal is obtained. However, when the complementary target sequence is present, it will hybridise to the labelled probe sequence resulting in a duplex which has a lower affinity for the metal surface, resulting in a much reduced SERRS signal. Therefore, an increased SERRS signal is obtained when no target or non-complementary target is present compared to when target is present. The lower affinity of the dsDNA for the metal surface is thought to be due to the increased electrostatic repulsion of the exposed negative phosphate backbone of dsDNA compared to ssDNA.

This assay was used for the detection of three genes which are associated with methicillin-resistant *Staphylococcus aureus* (MRSA) and labelled with three different fluorophores namely FAM, HEX and TAMRA.[39] Using this approach it was possible to detect not only complementary synthetic DNA, but also PCR product. It was also possible to detect every possible combination of the three sequences, present or absent, within a mixture demonstrating the multiplexing capability of SERRS in a homogeneous molecular diagnostics assay, Fig. 5.

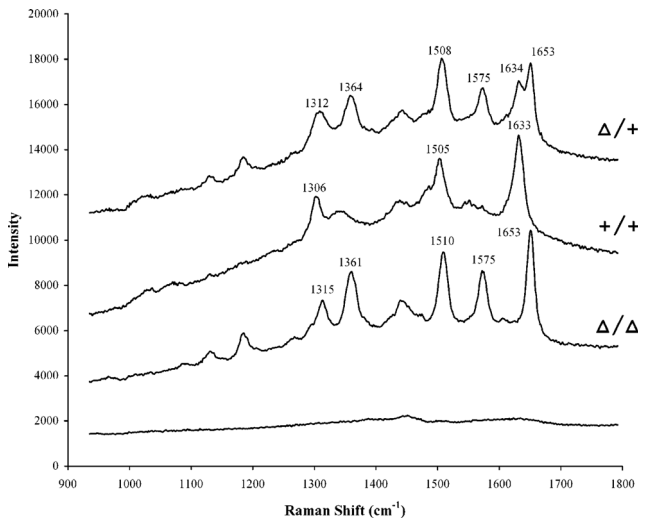

Fig. 4 Multiplexed SERRS analysis of CFTCR gene; wildtype (+/+), heterozygote (Δ/+) and mutant homozygote (Δ/Δ). With each allele separately labelled (HEX = +, rhodamine = Δ) it was possible to see the spectra corresponding to each dye in both the wild type and homozygotic samples. The heterozygote could be easily identified by eye as a composite spectra of both dyes.[38] Reprinted with permission from ref. 39. Copyright 2009 American Chemical Society.

A "molecular sentinel" approach to multiplexed DNA detection was reported by Vo-Dinh *et al.*[40] The molecular sentinel is comprised of looped DNA with a region complementary to the target DNA sequence and a self-complementary stem that holds the sentinel in a closed, hairpin loop conformation. One of the stems of the hairpin was functionalised with a thiol group to allow it to be attached to the surface of a silver nanoparticle while the other end was functionalised with a Raman reporter. In the 'closed' conformation, an intense SERS signal was observed due to the close proximity of the Raman reporter to the metal surface. However, in the presence of the target DNA sequence, the sentinel 'opened' resulting in the Raman reporter becoming distal to the surface of the metal nanoparticle. This approach therefore resulted in a reduction in the SERS signal obtained upon binding of target DNA. The molecular sentinels have been used to achieve the multiplexed detection of two genes which were biomarkers for breast cancer by creating two sentinels, one labelled with Cy3 and TAMRA. Both the molecular sentinel and the ssDNA *vs.* dsDNA assay have the distinct disadvantage that they are negative assays *i.e.* the presence of the target DNA sequence results in a decrease rather than an increase in signal. Although, not yet multiplexed, as a means of producing a positive assay, *i.e.* signal increase upon incubation with target, van Lierop *et al.* have designed a SERRS primer approach.[41] The SERS primer consists of a double stranded DNA loop that opens upon hybridisation with a target piece of DNA rendering a single stranded dye-labelled portion of probe free and able to adsorb more efficiently onto the nanoparticle surface thereby producing an increase in SERRS response.

A triplex genotype detection system, capable of detecting single nucleotide polymorphisms (SNPs) has been reported by Batt *et al.*[42] This method involved the use of a ligase detection reaction to bind a fluorophore labelled probe to a probe bound to a Raman enhancer (silver nanoparticle). This reaction only occurs in the presence of fully complementary target, thereby allowing discrimination of SNPs.

Non-fluorescent Raman reporters have been used in the 4-plex detection of alternative splicing junctions for the breast cancer susceptibility gene 1 (BRCA1).[43] Here, the four nanoparticle enhancers were each functionalised with a non-fluorescent reporter and a specific target sequence. Only in the presence of both target and capture strand would the Raman active NP be captured onto a surface *via* a sandwich hybridsation assay. This surface was then interrogated and produced distinct SERS spectra that could be discriminated without the need of deconvolution techniques. This methodology was further extended by the same group who reported PCR-free duplex detection of gene splicing variants by use of an enzymatic approach.[44]

Another approach to solution based SERRS detection of DNA makes use of DNA functionalised nanoparticles and a sequence specific hybridisation event to 'turn on' the SERRS signal through controlled assembly of nanoparticles into aggregates.[45] Three batches of nanoparticles were prepared, each functionalised with a different sequence of DNA and coded with two different dye labels, dye 1 and dye 2. All of the ssDNA probes were non-complementary to one another. Addition of

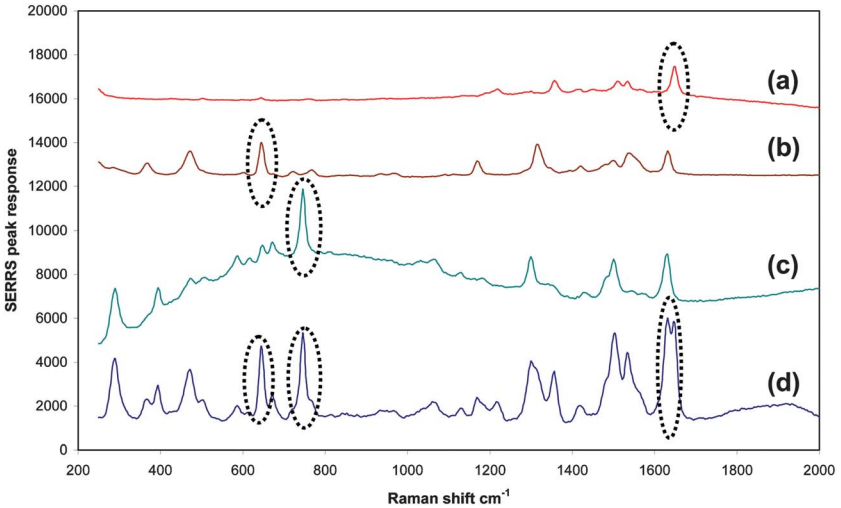

Fig. 5 The SERRS spectra of TAMRA, FAM and HEX labelled sequences (a), (b) and (c) coding for *femA*-SE, *femA*-SA and *mecA*, respectively and the triplex spectrum containing all probes (d).[39]

a target sequence complementary to two of the nanoparticle bound probes caused hybridisation driven aggregation of the nanoparticles. This aggregation event caused a large increase in the electromagnetic field strength between particles resulting in SERRS of the corresponding dye to be observed. By this method two different target sequences could be distinguished by the presence or absence of the spectra relating to the SERRS dye incorporated within that conjugate. Two different target sequences were used to assemble the three sequences such that one hybridisation event resulted in SERRS signals being obtained from one dye label only, dye 1, whereas the second hybridisation event resulted in SERRS spectra being obtained from both dye 1 and dye 2, Fig. 6. This was the first report of nanoparticle assemblies having been used to turn on the SERS effect due to a biological interaction as well as demonstrating the potential to use this effect to simultaneously detect multiple target sequences in one analysis. However, a relevant sequence of social interest is still to be targeted by this methodology.

SERS for multiplexed protein detection

The multiplexed detection of protein biomolecules predominantly falls broadly into two categories – that of immunoassays and of enzyme analysis.

Immunoassays. Immunoassay approaches for the detection of target protein molecules have been developed using SERS as the detection method. Researchers investigating multiplexed immunoassays have developed a SERS based approach that is capable of a 4-plex discrimination (although only a 3-plex is demonstrated explicitly).[46] In this experimental set-up, nanoparticles were functionalised both with an antibody and a unique Raman reporter, such that each type of antibody was conjugated to one SERS reporter. These were prepared separately and then mixed together. A "capture substrate" was then functionalised with equimolar amounts of antibodies for four different antigens. The SERS response from the specific label was generated only if the conjugate was captured onto the "capture substrate" through

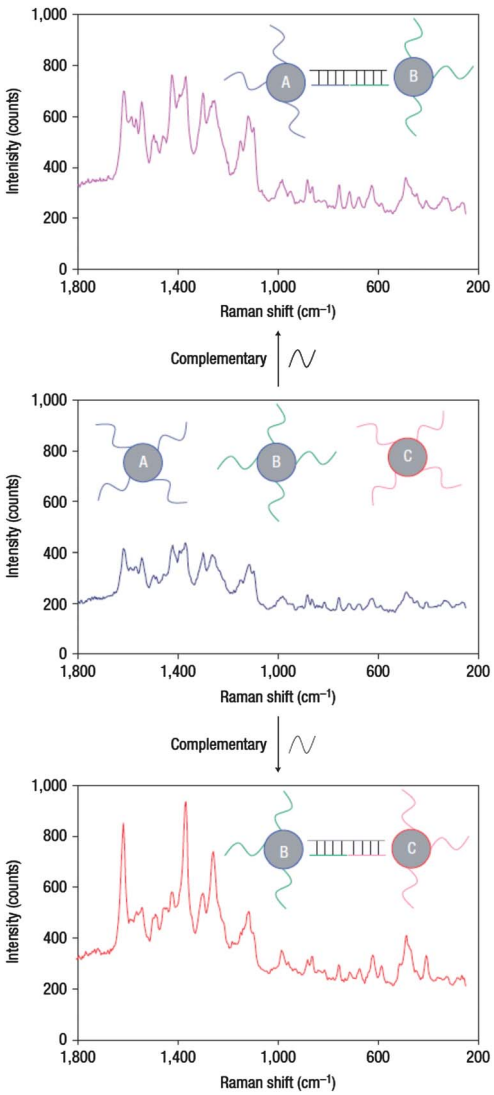

Fig. 6 SERS spectra of different dye coded particles which pertain to the differing complementary target introduced to the system.[45]

a biorecognition sandwich event *via* a target antigen. Using this method, it was possible to detect four different antigens simultaneously, although representative spectra were only shown for three and the tetraplex was reported with peak listings, Fig. 7. The authors noted that higher order multiplexing was, at this stage, limited by the cross-reactivity of the antibodies, rather than being compromised by spectral overlap from the SERS reporters.

Ozaki *et al.* have used a different approach to detect proteins *via* SERS detection.[47] Labels were not added to the nanoparticle surface as was the case in the previous assay. Instead, Coomassie dyes were used which bind to proteins *via* physisorption to the aromatic amino acids as well as arginine and proline. In this case the analyte antigen was dye labelled simply by incubation with the Coomassie dyes, brilliant blue G (BBG) and brilliant blue R (BBR). BBG was mixed with mouse IgG and BBR with avidin as a convenient means of labelling the analyte with a SERS reporter. Thereafter, incubation with nanoparticle bound anti-IgG or biotin resulted in signal from the BBG or BBR, respectively. Peaks corresponding to either, or both, labels could be observed depending upon the ratio of antigen added.

Enzymes. The basic concept of enzymatic SERS analysis is the conversion, by enzyme action, of a SERS-inactive precursor into a strongly SERS active product. This can be achieved by the production of a dye as a result of enzymatic action upon a substrate and subsequent SERS detection.[48,49] Masked dyes were synthesised by Ingram *et al.* that produced no SERRS response until enzymatic action cleaved the masking group allowing the surface seeking moeity to be revealed.[50,51] Surface adsorption was then possible and SERRS analysis confirmed the presence of the enzyme due to its activity. This methodology was multiplexed in the sense that two masked dyes were mixed together, each with the masking group as a substrate for a different enzyme.[52] No SERRS signal was observed until an enzyme was added that would act upon one of the masked dyes. Alkaline phosphatase and β-galactosidase were detected in this way from a mixed solution of masked dyes.

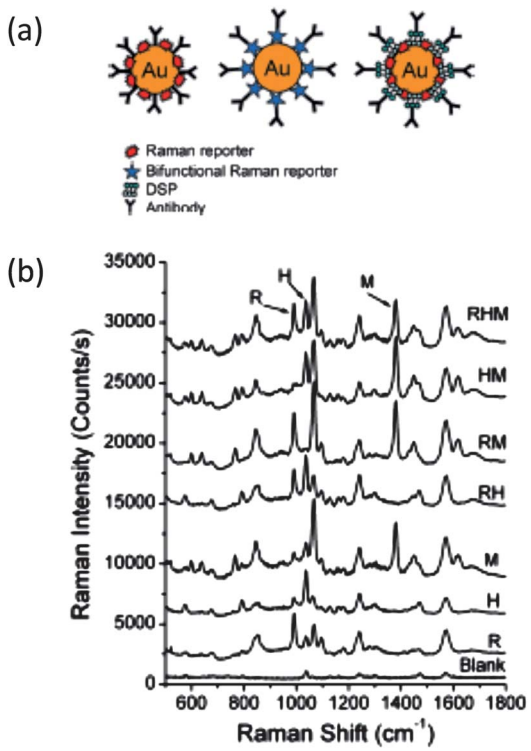

Fig. 7 (a) Representation of nanoparticle functionalisation (b) Single spectra for SERS tags (R, H, M) relating to rabbit, human and mouse IgG (Reporter molecules 3-MeOBT, 2-MeOBT and 4-NBT, respectively). The three possible duplex and the triplex multiplex analysis are shown (RH, RM, HM, RHM, respectively).[46] Reprinted with permission from ref. 46. Copyright 2009 American Chemical Society

Extra- and intra-cellular multiplexed detection

Recent work has focussed on utilising the benefits of SERS for carrying out analysis within cells and tissue as well as *in vivo*. Pezacki *et al.* have demonstrated a 2-plex detection of cell surface proteins by use of non-fluorescent reporters encoded silver nanoparticles.[53] Four batches of Raman reporter nanoparticles were mixed together to prove their multiplexing capability. The corresponding spectrum was deconvoluted using direct classic least means squares analysis to show the relative contribution from each reporter. Two of these reporters were then applied to cell surface protein detection and duplex detection of β_2-adrenergic receptor and caveolin-3 were achieved by SERS mapping.

Irudayaraj *et al.* developed a sandwich assay for the detection of bacterial cells using both antibodies and aptamers.[32] Aptamers are single stranded pieces of DNA that are raised to bind to a target with high specificity and selectivity. Two nanoparticle systems were separately functionalised with antibodies and the third with an aptamer, raised against pathogenic targets *S. typhimurium*, *S. aureus* and *E. coli*. The nanoparticles were also each labelled with a unique Raman reporter. Exposure to pathogenic threat species caused aggregation of the nanoparticles on the bacterial cells which were then collected by filtration through a filter membrane and subjected to SERS interrogation. In the presence of three threat agents, peaks arising from the three reporters could clearly be discriminated by eye in the multiplex spectrum.

Culha *et al.* have developed label free mutliplexed detection for different bacterial pathogens; *S. sonnei*, *P. vulgaris and E. amylovara* and also to discern between three strains of *E. coli*; BFK13, BHK7 and DH5 α.[54] Chemometrics was used to process the spectra obtained and indicated the presence of all three species from distinct differences in their individual spectra.

The ability of multiplexed SERS to detect two different, co-cultured cancer cell lines has been shown by Chang and coworkers.[55] SERS tags with different reporter dyes were prepared, each with a different antibody. Epidermal growth factor receptors (EGFR) were targeted as they are known to be over-expressed in cancer cells such as OSCC cells, whereas HER2 is a known biomarker for breast cancer cells with a high expression in SKBR-3 cells. Nanoparticles were functionalised with anti-EGFR and anti-HER2 antibodies and treated, separately, with a different dye. Incubation with either cell line or co-cultured cells clearly indicated which species was present in the sample with visibly discriminatory multiplexing achieved, Fig. 8.

Although not the first to use multivariate analysis for the discrimination of multiplex SERS,[30,56] Kneipp *et al.* have applied multiplex imaging to biological systems, achieving a 5-plex in

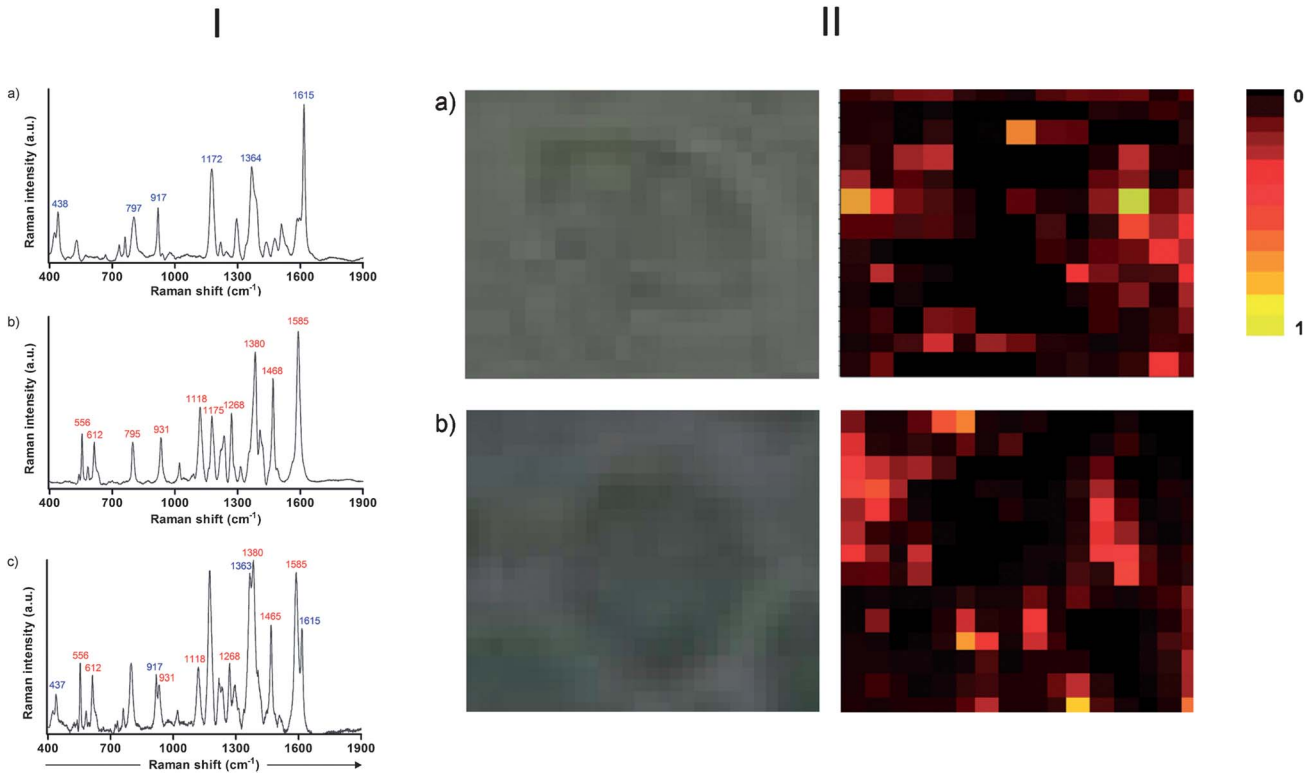

Fig. 8 I- The SERS spectra obtained from OSCC cells (a), SKBR-3 cells (b), and co-cultured cells (c) when exposed to specific antibody and Raman labelled reporter tags and II- Bright field and SERS mapping images of: (a) B2LA anti-EGFR nanotag-treated OSCC cells (1615 cm^{-1}), (b) Cy3LA anti-HER2 nanotag-treated SKBR-3 cells (1468 cm^{-1}).[55]

a cellular environment by use of chemometrics.[57] Principal components analysis was used to spectrally resolve the responses from 5 different SERS labels incubated within a cell. Furthermore, the toxicity of the thiolated reporter modified nanoprobes were assessed and found to be non-toxic to the cell lines studied.

Multiplexed detection has also been carried out on tissue samples. Antibody-conjugated composite organic-inorganic nanoparticles (COINs) have been evaluated for their ability to detect PSA in prostate tissue.[56] COINs consist of silver nanoparticles which have been aggregated in the presence of organic Raman active reporters before being coated with BSA to encapsulate the tags. Initially a simple and effective spectral fitting method was developed to resolve a 4-plex using a plate binding assay and 4 different COIN SERS labels conjugated to anti-PSA. This was then extended to detecting a triplex consisting of two different antibody functionalised COINs to detect prostate-specific antigen and cytokeratin-18 and a fluorophore stain to detect DNA in a fixed sample of human prostate tissue. This work demonstrated the advantages of using enhanced Raman to detect multiplex signals in a sensitive and quantitative manner. In a subsequent paper by Schlucker *et al.* SERS active silica encapsulated gold/silver nanoshells with two different labels, but functionalised with the same antibody, were developed and subsequently used for tissue imaging for prostate specific antigen.[58]

In Vivo multiplexing

Recent work has been published on the use of SERS reporters for *in vivo* analysis. In 2008 Shuming Nie published work on the *in vivo* targeting of tumours in live mice.[59] The SERS particles consisted of 60 nm gold nanoparticles functionalised with a Raman reporter dye molecule and then stabilised with thiolated polyethylene glycols (PEGs). Targeted SERS nanoparticles were prepared by having a mixed monolayer of thiolated PEG and a heterofunctional thiolated PEG with a carboxylic acid terminal group. The carboxylic acid group allowed the covalent addition of the ScFv antibody, which binds to the EGFR receptor of cancer cells, using EDC coupling. Using these functional nanoparticles it was possible to target the cancer cells *in vivo* by locating the nanoparticles using SERS. However this was not carried out to detect multiple targets.

Using a different nanoparticle approach, Gambhir *et al.* used commercially available 'nanotags' consisting of gold nanoparticles functionalised with non-resonant Raman reporters stabilised with a silica coating.[60] Two differently labelled SERS nanotags were injected at three different injection sites and it was possible to detect all three using their SERS signature. Multiplexing was also used to study the fate of two different SERS nanotags, one functionalised with PEG and one without, where it was found that both the PEGylated and non-PEGyated nanotags accumulated in the liver to the same extent.

In a further study by the same group, 10 different SERS nanotags were injected sub-cutaneously and their corresponding SERS intensities mapped. However, multiplexing was shown *in vivo* by intra-venous injection of 5 different SERS nanotags.[61] Non-specific bioaccumulation of all the tags in the liver allowed the ability of deep-tisssue multiplex SERS imaging to be evaluated. Furthermore, four tags of differing concentrations were

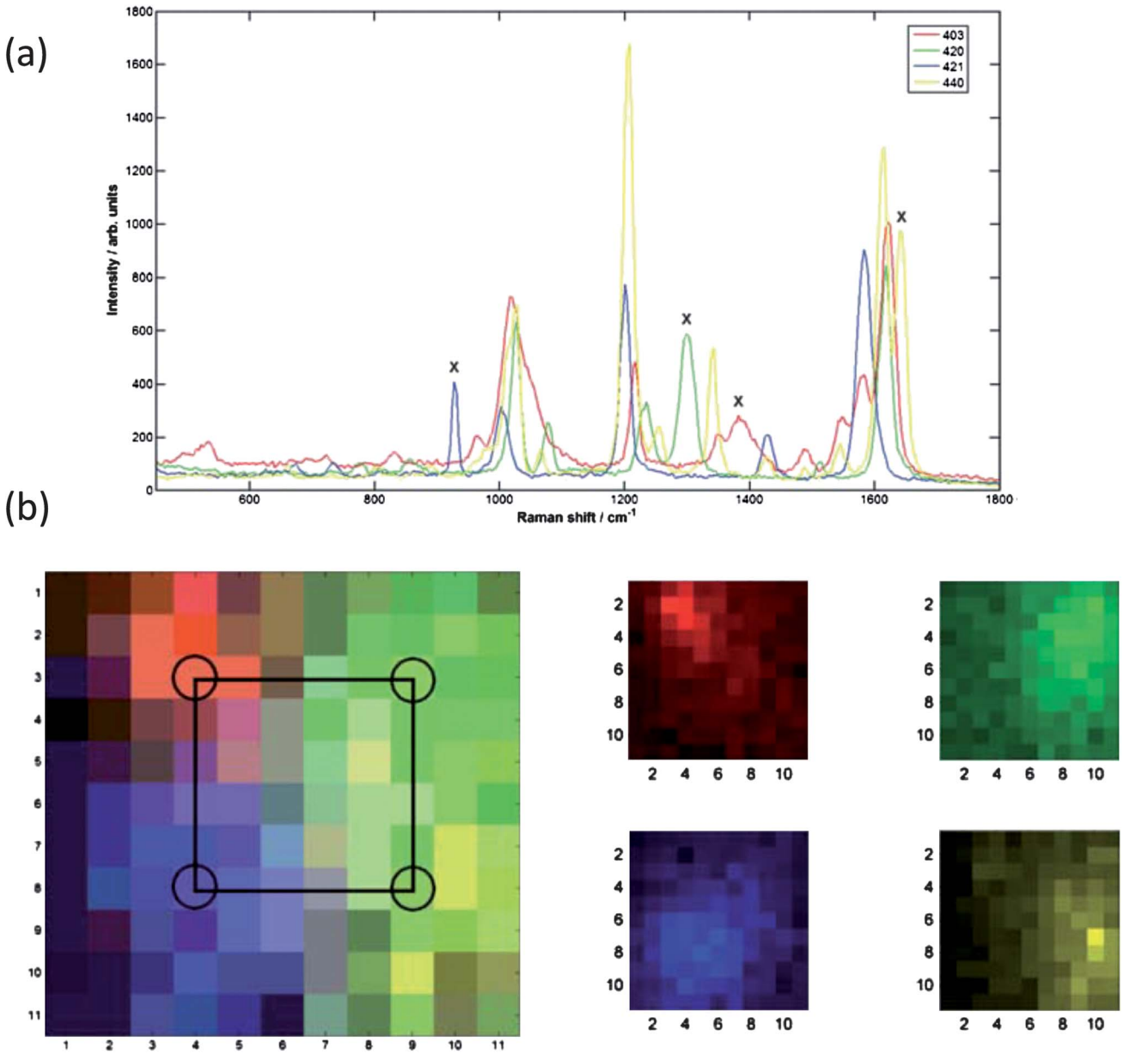

Fig. 9 (a) SESORS spectra at 830 nm of 4 SERS nanotags. (b) False colour images of the SERS nanoparticle signals. In each case red, green, blue and yellow represent differently labelled nanotags. The Xs signify the identity of the peaks used to measure the individual nanotag signals in the SESORS spectrum.[62]

then injected and allowed to bioaccumulate in the liver and a linear concentration dependent response was observed. Although this study did not target any biomarkers, or indeed, undergo a bio-recognition event it was signficant in showing the applicability of SERS multiplexing in an *in vivo* imaging scenario.

A particular difficulty in the analysis of tissue samples is the issue of depth penetration. A combination of spatially offset Raman spectroscopy (SORS) and SERS known as SESORS has been achieved by Stone *et al.*[62] The combination of the two techniques has allowed the multiplexing of 4 different SERS reporters from within a sample of porcine muscle tissue.[63] The spectral discrimination of all four samples could clearly be seen. The multiplex spectra were obtained from sample depths of 20 mm, Fig. 9. Indeed, extending the methodology further allowed SERS spectra to be recorded (albeit not multiplexed) from a 50 mm depth. This advancement of the SERS methodology will have significant impact upon the use of SERS in a clinical setting.

Conclusions and future developments

This review has focussed on the multiplexing capabilities of SERS for bioanalysis. It has been demonstrated here that, by careful choice of Raman reporter, significant multiplexing can be achieved. This can be carried out by either using the intrinsic SERS signal of a biomolecule or by direct labelling of species or by co-functionalising a nanoparticle with a recognition molecule and a reporter molecule, allowing flexibility in the approach used

to suit the requirements of the assay system. This holds significant advantages over other techniques where separation of components is required before analysis or where fluorescence is used which can be limited by the spectral overlap of fluorophores and requires multiple excitation wavelengths. In theory, the multiplexing capability of SERS is limited only by finding suitable reporter molecules with non-overlaping spectral signatures.

As previously stated, multiplexing is one of the main advantages of using SERS as an analysis technique. SERS was first reported over 30 years ago however it is only in the last few years that the multiplexing capabilities of the technique have been successfully demonstrated on meaningful samples. Future research in the area will further exploit these capabilities to allow the advancement of the technique and allow it to compete successfully with more established techniques such as fluorescence. Future areas will focus on expanding the multiplex capabilities to allow screening of real biological samples for a large number of biomolecules. The application of multiplex SERS to carrying out targeted analysis *in vivo* is a very exciting recent application of SERS which will be the focus of a significant amount of future research.

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