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Recent developments and future directions in SERS for bioanalysis

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The ability to develop new and sensitive methods of biomolecule detection is crucial to the advancement of pre-clinical disease diagnosis and effective patient specific treatment. Surface enhanced Raman scattering (SERS) is an optical spectroscopy amenable to this goal, as it is capable of extremely sensitive biomolecule detection and multiplexed analysis. This perspective highlights where SERS has been successfully used to detect target biomolecules, specifically DNA and proteins, and where *in vivo* analysis has been successfully utilised. The future of SERS development is discussed and emphasis is placed on the steps required to transport this novel technique from the research laboratory to a clinical setting for medical diagnostics.

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Introduction

The rapid and sensitive detection of certain disease biomarkers is of vital importance to the progression of the medical diagnostic field. It is now becoming essential to be able to detect multiple biomarkers from complex biological matrices, within the fastest possible time frame, in order to enable efficient patient diagnosis and treatment. Surface enhanced Raman spectroscopy (SERS) has a multitude of potential applications that could improve upon conventional medicinal detection methods, and is a viable analytical technique for the future of disease diagnosis.

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In 1974 Fleischmann *et al.* first reported on the SERS phenomenon when he observed that pyridine gave an enhanced Raman scattering signal at roughened silver electrodes.[1] This was found to be the result of an electromagnetic enhancement arising from interaction of the exciting radiation with the surface electrons on the metal to create a plasmon.[2–4] The large enhancement factor observed for pyridine was in the order of 10^6 in comparison to conventional Raman scattering measurements and it was soon discovered that SERS could be obtained from a variety of molecules, by adsorbing analytes onto a roughened metal surface.[2,3] Surface enhanced resonance Raman scattering (SERRS) arises when the analyte has a chromophore close in energy to the exciting radiation.[5] Enhancement is the result of surface plasmon resonance and molecular resonance, allowing for greater enhancement factors to be observed enabling single molecule SERS.[6,7] Most SERS

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substrates consist of the coinage metals, predominantly gold and silver.[8,9] The surface plasmons for these metals lie in the visible region of the electromagnetic spectrum, and hence coincide with common Raman excitation wavelengths.[8] Colloidal suspensions of gold and silver are the most common, as they are easily and reproducibly prepared, and remain stable over long periods of time.[10,11] Additionally, fluorescence interference can be quenched by gold and silver nanoparticles resulting in Raman scattering being observed from fluorescent molecules allowing a wide range of dyes to be used as labels for biomolecule detection.[12] Other SERS substrates exist in the form of structured metal arrays,[13–15] shaped nanoparticles[16,17] and spheres[18,19] and specially designed solid surfaces,[20,21] such as Klarite.[22] However, this perspective will focus mainly on the use of gold and silver colloid for the detection of specific biomolecules using SE(R)RS.

The advantage of SER(R)S for the analysis of biomolecules such as proteins and DNA lies in its distinct advantage over conventional fluorescence or chemiluminescent based techniques. Generally detection methods for biomolecules use labelling techniques involving molecular fluorophores. These measurements give rise to spectra with broad emission bands, which provide limited characteristic structural information, and suffer from large spectral overlap when multiple molecules are present. Raman spectroscopy, however, provides sharp fingerprint spectra, that are molecularly specific, and can therefore allow for multiplexed analysis of different analytes of interest.[23] SERRS has already been shown to be 3 orders of magnitude lower in sensitivity than conventional fluorescence for the detection of dye-labelled oligonucleotides.[24] Therefore, the sensitivity obtained from SERRS analysis combined with the vast opportunity for multiplexed analysis has resulted in a number of potential diagnostic applications being developed.

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This perspective aims to highlight the recent developments in SER(R)S for the detection of biomolecules such as proteins and DNA, with a view to highlighting where this optical spectroscopy has the potential for advantages over conventional diagnostic testing methods. Additionally, the use of SER(R)S as a new tool for intracellular and *in vivo* analysis will be discussed, and how future developments in this area may finally lead to implementation of this technique in a clinical setting.

DNA detection

The sensitive and selective detection of DNA sequences is central to modern bio-analysis, therefore research is continually being progressed to develop more effective and competitive molecular diagnostic approaches. Conventional DNA analysis methods have employed fluorescence emission spectroscopy as a detection technique. Fluorescence is a well-established and robust detection technique, with low limits of detection routinely obtained,[25] and single molecule detection achievable.[26,27] Spatial multiplex analysis of DNA has been shown through use of array based fluorescence,[28] however, multiple analyte detection from one sample or spot is limited by the broad overlapping spectra generated. Previous studies have shown SE(R)RS to be more sensitive, with the potential of surpassing fluorescence detection by three orders of magnitude,[24] and multiple analyte detection can be easily obtained through narrow spectral SE(R)RS peaks.[29] As a result, a substantial amount of research has been undertaken into using SE(R)RS as a DNA detection tool.

Label-free DNA detection. SE(R)RS has been shown to be a highly sensitive analysis method with the detection of individual DNA nucleotides possible, this has allowed approaches to label-free detection of DNA to be developed.[30,31] The SERS spectra generated by single constituent bases, nucleosides, nucleotides and oligonucleotide chains can vary greatly depending on structure and experimental conditions, allowing structures to be clearly discriminated.[32–34] Bell *et al.* have shown the orientation of unlabelled DNA can be utilised to determine the presence of target DNA.[35] Thiolated poly A DNA sequences bound to gold nanoparticles will give preferential enhancement of the adenine breathing SERS band depending on the orientation of the sequence to the surface. Sequences which are perpendicular to the metal surface have an increased signal intensity at 736 cm^{-1}, whereas sequences which lie flat to the surface show weaker bands as seen in Fig. 1. This principle was utilised within a molecular beacon assay to detect the closed or open beacon conformation through hybridisation of a target sequence which will change the oligonucleotide orientation.

Recently detection of single base mismatches within unmodified DNA has been achieved through distinctive SERS responses.[36] Detection was achieved through the non-specific adsorption of oligonucleotides onto silver nanoparticles *via* the nucleotide side chains. The spectra obtained from unmodified sequences generated bands from all four DNA bases and single base mismatches were calculated by digitally subtracting spectra, resulting in positive and negative features corresponding to the

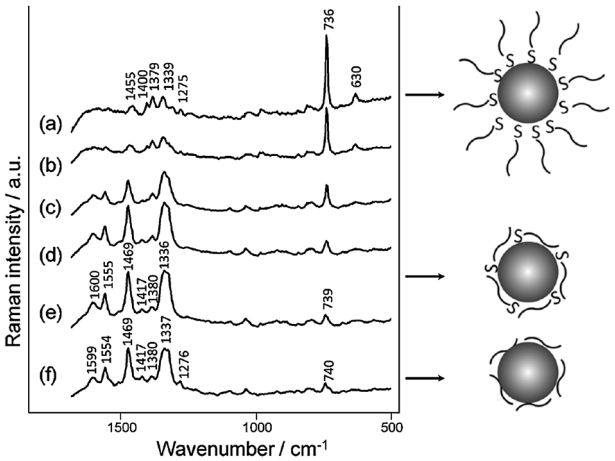

Fig. 1 SERS spectra of thermally treated 24-mer thiolated poly A at concentrations (a)–(e) 10^{4}–10^{8} M and (f) 10^{5} M unthiolated poly A on AuNP aggregated with $MgSO_4$.[35]

exchanged nucleotide. This approach has shown successful detection of single nucleotide changes, however, elucidation of results can be time consuming and non-sequence specific signals are obtained since the order of the bases could not be elucidated by this approach. Therefore, commonly used methods which can produce sequence specific detection generally rely on attachment of a fluorophore to the nucleic acid sequence, therefore the presence of a particular fluorophore is indicative of the presence of a particular DNA sequence.

Quantitative labelled DNA detection. Fluorophores can be covalently attached to specific DNA sequences and detected by fluorescence emission. Many different commercially available fluorophores have been attached to oligonucleotides and quantitative SERRS responses achieved.[37] Stokes *et al.* demonstrated the versatility of SERRS through detection of numerous fluorophores with different absorbance maxima using three different excitation wavelengths using both silver and gold nanoparticles.[38] This demonstrated that by careful selection of the fluorophore label, and the excitation wavelength, it is possible to achieve very sensitive quantitative linear SERRS responses from DNA concentrations below monolayer coverage on the nanoparticle surface.

The simultaneous detection of multiple labelled probes has also been achieved by exploiting the sharp molecularly specific spectra that are generated. Faulds *et al.* were able to simultaneously detect 5 labelled oligonucleotide probes within one sample without the need for chemometric analysis.[39] Two excitation wavelengths were used to generate strong distinctive SERRS responses from individual labels. Two labels (FAM and R6G) were detected due to their intense resonance contribution at 514.5 nm and three labels (BODIPY-TRX, ROX and Cy 5.5) were detected using 632.8 nm laser excitation to generate the spectra. The sensitivity of the probes was not reduced within the 5-plex compared to SERRS detection of the isolated labelled DNA probes. This multiplex was then advanced to detect six different DNA sequences using one excitation wavelength through aid of multivariate analysis.[29] In this approach, the whole SERRS spectrum is considered and partial least squares analysis was used to detect every possible combination of the six labelled sequences. Successful discrimination of the probes was achieved with high accuracy and sensitivity. Bayesian methods have also been used to discriminately resolve a mixture of 6 dye labelled oligonucleotides.[40]

MacAskill *et al.* adopted a different approach for the SERRS detection of multiple oligonucleotides using probes labelled with specially synthesised phthalocyanine labels.[41] Phthalocyanines have porphyrin ring structures with metal centres and can give rise to distinct SERRS spectra by altering the incorporated metal. This transformation varies the optical properties of the phthalocyanine allowing fluorescent and non-fluorescent states to be generated. The limits of detection of three different phthalocyanine labelled sequences were determined and found to be comparable to the fluorescently labelled probes described in previous studies. It was also shown using SERRS that a mixture of two phthalocyanine labelled oligonucleotides containing Co and Zn metal centres, could easily be distinguished. The use of SE(R)RS to detect short oligonucleotide labelled probes has therefore been shown to be versatile, sensitive and reliable and as a result diagnostic assays have been developed based on this detection strategy.

DNA detection assays. The first use of SERS active labelled primers within a PCR assay was reported in 1998 for the specific detection of the human immunodeficiency virus (HIV) *gag* gene sequence.[42] The SERS gene probe was detected through DNA hybridisation of the dye labelled primer to the target template giving inherent molecular specificity *via* selective hybridisation. The polymerase chain reaction (PCR) amplified the labelled DNA generating high levels of labelled oligonucleotide enabling sensitive levels of detection. The probe was then immobilised onto a solid support through selective hybridisation to a capture probe and detected by deposition of a silver layer over the samples. The coupling of SERS analysis and PCR has allowed the qualitative detection of the *gag* gene region of HIV1 to be successfully achieved. However, to drive SERS as a clinical diagnostic technique, rapidly acquired accurate and simple results using low sample volumes are required. The development of SERS based microfluidic devices have the potential to eliminate a number of manual handling steps whilst providing highly accurate results. Monaghan *et al.* have developed a DNA diagnostic assay within a lab-on-a-chip format for the detection of *Chlamydia trachomatis*.[43] Off-chip PCR was initially carried out to amplify a biotinylated primer, before addition of a complementary dye labelled probe to the PCR product. A solid-phase capture of the biotin probe could be performed through binding to streptavidin beads. The functionalised microspheres were then trapped within the microfluidics channel using an integrated microfilter. Finally, the specifically bound labelled detection probe was thermally released and detected by SERS through mixing with a nanoparticle stream. The potential application of SERS as a diagnostic method was shown and it has initiated the use of SERS detection within standard fluorescence based DNA assays.

The TaqMan assay is a widely utilised detection method which conventionally uses fluorescence analysis.[44] The assay

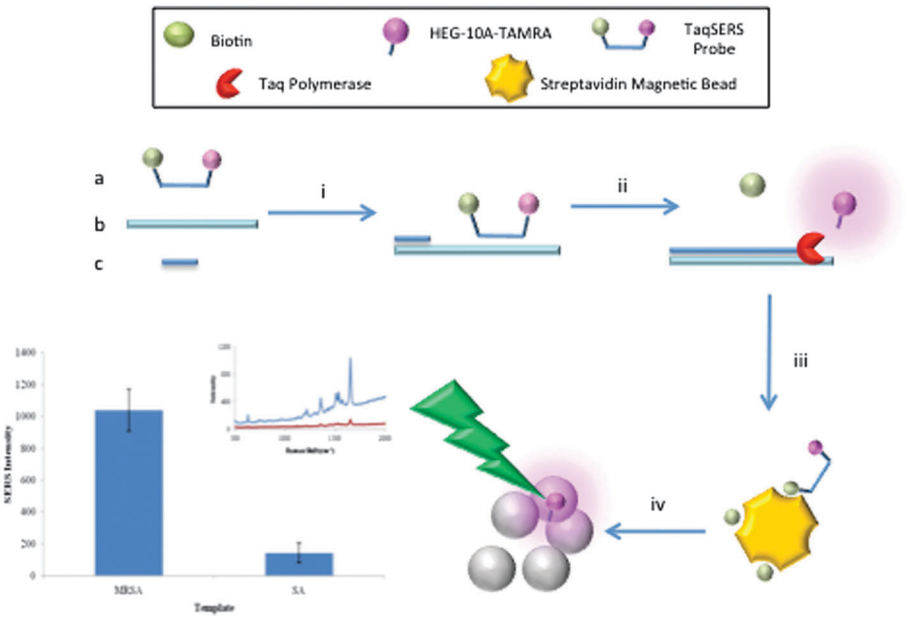

Fig. 2 TaqSERS assay; (i) hybridisation of TaqSERS probe a, target sequence b and primers c, (ii) Taq polymerase enzyme simultaneously elongates primers and digests probe, (iii) streptavidin coated magnetic beads are introduced to remove any undigested biotinylated probe and free biotin, (iv) dilute citrate-reduced silver nanoparticles with spermine hydrochloride (0.1 mol dm^{-3}) were added to detect TAMRA labelled DNA in supernatant. Insert shows SERS spectra of genomic MRSA and SA control.[45]

employs PCR to enable signal to be generated through enzymatic probe cleavage, which is subsequently detected and quantified. Harper *et al.* developed a modified TaqMan assay by designing a specific SERS probe which could be used within a TaqMan assay to obtain enhanced sensitivity and specificity (Fig. 2).[45]

The TaqSERS probe was modified to incorporate a 5′ biotin residue and a 3′ TAMRA dye. The assay utilised the 5′ to 3′ exonuclease activity of the *Taq* enzyme to simultaneously amplify the target and digest any probe which had hybridised to the template within PCR. Any undigested probe was then removed from the reaction through a wash step that exploited a biotin–streptavidin interaction. The remaining TAMRA dye could then be quantified using silver nanoparticles and SERS. Selective detection of clinically relevant samples of methicillin-resistant *Staphylococcus aureus* (MRSA) was achieved and the limits of detection obtained, 7 fM, were over a magnitude lower than conventional fluorescence detection.

Conventional fluorescence detection has also been employed within a molecular beacon assay. This approach uses competitive oligonucleotide hybridisation to generate fluorescent signal in the presence of a specific target DNA sequence. Studies have incorporated SE(R)RS to obtain sensitive detection with the possibility for multiple analyte analysis.[46] Standard molecular beacon probes are held in a stable hairpin-loop structure through a specifically designed self complementary stem region. This particular beacon incorporated a 3′-FAM (6-carboxyflorescein) fluorophore and a 5′-benzotriazole azo dye, that strongly complexes to the silver metal surface. Within the closed loop beacon, any fluorescence emitted by the fluorophore, FAM, is quenched by the azo dye and the metal surface and a SERS signal is observed from both the fluorophore and the azo dye. However, in the presence of complementary target DNA, the beacon is opened through selective hybridisation. This separates FAM from the azo dye and the surface, removing the quenching action, and increasing the fluorescence emission from the FAM dye and resulting in a SERS signal only being obtained from the azo dye. This specific recognition assay shows the presence of unlabelled single stranded DNA. Further work has shown that sensitive detection of specific DNA hybridisation using a molecular beacon has also been achieved by SERS within a PDMS microfluidic channel.[47] Jung *et al.* utilised a molecular beacon probe containing a donor dye, TET and acceptor dye, TAMRA which was used to detect the azoospermia target gene, DYS 209. In the microfluidic device, silver nanoparticles and the beacon were first mixed, generating strong SERS signals as a result of the close proximity of the Raman reporters to the surface. Addition of the target DNA, reduced the SERS response obtained since the dyes became separated in the open conformation, resulting in them being moved further away from the surface.

In 2007, Vo-Dinh *et al.* reported a plasmonics-based nanoprobe approach using molecular sentinels.[48] The sentinels also adopted a hairpin loop conformation, with one end of the stem functionalised with a thiol group, allowing attachment to a silver nanoparticle and the other end functionalised with R6G (carboxyrhodamine 6G) dye. In the closed loop conformation, an intense SERS signal is obtained due to the close proximity of the dye and the silver nanoparticle. However, in the presence of complementary PCR target a reduced SERS signal was observed due to the dye being held distal to the metal surface upon target hybridisation. Subsequent to this study, multiplex detection of molecular sentinels was demonstrated.[49] Two genes associated

with *erbB-2* and *ki-67* breast cancer biomarkers were used to design two sentinels labelled with either TAMRA or Cy3 dyes, allowing each sentinel nanoprobe to be distinctly detected.

The varying affinities which single stranded DNA and double stranded DNA have for the surface of metal nanoparticles has been exploited to determine the presence of target DNA. Different analysis techniques have been used including colourmetric[50] and SE(R)RS detection.[51] A homogeneous assay was developed that discriminately detected multiple target sequences through correlation to specific DNA hybridisation events.[52] The assay exploited the principle that single stranded DNA has a stronger affinity to a metal nanoparticle surface than double stranded DNA, consequently SERRS spectra with significantly different intensities can be generated in the presence of target. In the absence of complementary target, no hybridisation to the single stranded dye labelled probe will occur, allowing it to strongly adsorb onto the silver nanoparticle surface. This electrostatic binding event generated highly intense SERRS signals. Whereas, in the presence of target sequence, hybridisation occurs forming double stranded helix which has a lower affinity for the metal surface. Thus, the SERRS signal generated is significantly lowered. Three clinically relevant sequences of MRSA were discriminately detected within a closed tube assay without the need for separation. This specific SE(R)RS detection method has shown vastly reduced analysis times compared to standard culturing methods, however, this assay has a distinct disadvantage in the fact that it is a negative assay and a decrease in signal intensity is generated in the presence of target DNA.

A recent study has developed a positive homogeneous assay which generates enhanced SERS signals in the presence of target DNA.[53] The assay utilises the different propensities of DNA for silver nanoparticles surfaces using specifically designed SERS primers (Fig. 3). The primers contain a SERS active dye and are self-complementary, thus they form a region of double stranded DNA in the absence of target. As shown previously,[51] this reduces the affinity to the metal particle surface and a weaker SERS response is obtained. The addition of genomic DNA from *Staphylococcus epidermis*, opened the primer through preferential binding, resulting in a region of dye labelled single stranded DNA remaining free to adsorb to the nanoparticle. Highly intense SERS signals were obtained when compared to no target being present demonstrating a novel positive SERS assay with the possibility for the detection of multiple targets in clinical samples.

The detection of multiple analytes within a sample mixture without the need for separation is one major benefit which SE(R)RS can offer due to the molecularly specific narrow spectral bands that are produced. In 2002, Graham *et al.* reported the first SERRS multiplex for DNA genotyping.[54] The detection of cystic fibrosis transmembrane conductance regulator (CFTCR) gene was achieved through use of SERRS active primers in an amplification refractory mutation system (ARMS) assay. Three possible variants of the gene can be present, wild type, heterozygote and mutant homozygote. Specially designed primers containing either HEX or rhodamine dyes were used to differentially detect these gene sequences. The primers were incorporated into PCR product and any remaining dye labelled primer was removed through a biotin–streptavidin wash step. SERRS was used to successfully detect each variant *in situ* without sample separation.

The ability to detect single nucleotide polymorphisms (SNPs) within a diagnostic assay is vital. Batt *et al.* have developed a triplex genotype detection system capable of identifying SNPs.[55] A ligase detection reaction was combined with SERS detection to accurately discriminate multiple alleles. The reaction binds a fluorophore labelled probe and probe bound to a silver nanoparticle through a mutual template, bringing the fluorophore in close contact with the silver nanoparticle, enhancing the SERS response obtained. Three diagnostic primers containing distinctive dye labels, TAMRA, 6-FAM and fluorescein were used to discriminate template sequences which varied by one nucleotide base.

Many highly sensitive DNA diagnostic assays have been developed which can discriminate single base sequence mutations. Often PCR is utilised to gain the lowest levels of sensitivity through exponential target amplification. PCR has been shown to be an effective technique for the real-time detection of low copy numbers of DNA, however the method can suffer from contamination and non-specific amplification within the reaction, requiring strict laboratory procedures and highly trained personnel. Consequently, there is a distinct move towards

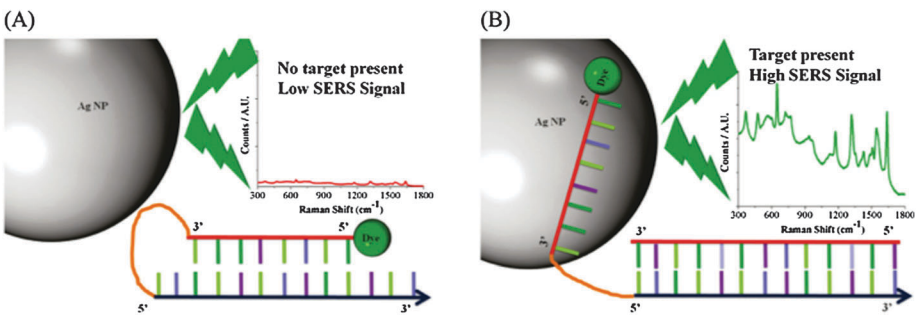

Fig. 3 SERS probe: when target DNA is absent (A) the primer is closed and predominantly double stranded DNA which results in a low SERS response. In the case of a positive sample (B), the complementary target DNA displaces the partly self-complementary region of the SERS primer, which consists of a dye labelled single stranded DNA region. This is then free to adsorb onto the negatively charged nanoparticle surface resulting in a high SERS response.[53]

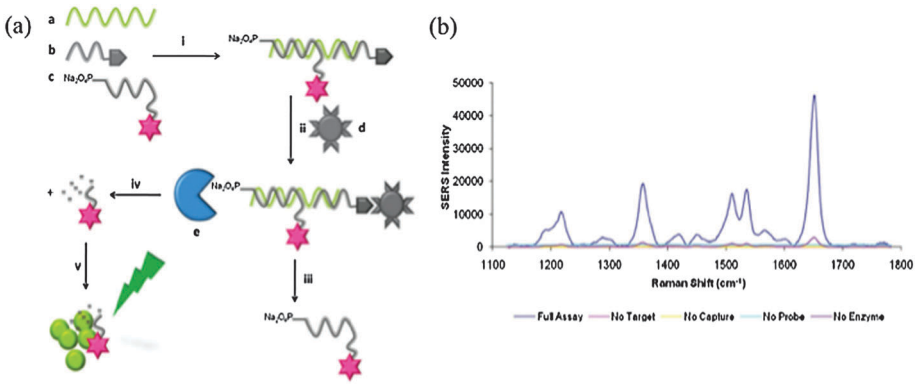

Fig. 4 (a) Schematic representation of split probe *exo*-SERS assay of this assay. (i) Sandwich hybridisation between an unlabelled target, a, biotinylated ''capture'' probe, b, and 50-phosphorylated dye-labelled ''reporter'' probe, c. (ii) The duplex is captured on streptavidin coated magnetic beads, d, before undergoing stringent washing to remove excess probe (iii). (iv) Lambda exonuclease, e, is introduced to the beads suspended in buffer and incubated at 37 °C for 1 h. (v) The supernatant is removed from the beads and added to diluted citrate-reduced silver nanoparticles with the addition of spermine hydrochloride (0.1 M). Within 5 min the SERS spectra were recorded using a Renishaw Probe system with an excitation wavelength of 532 nm. (b) SERRS enhancement when all components of the assay are present.[58]

developing alternative detection assays that use signal amplification approaches rather than having to rely on the problematic amplification of DNA by PCR.

Towards amplification free detection. The increase in signal from DNA with time, as opposed to increasing the target copy number, has previously been successfully shown to be reliable and sensitive when using fluorescence detection.[56,57] More recently a SERS based signal amplification approach has been developed, achieving high levels of sensitivity, specificity and potential for multiplexing.[58] An *exo*-SERS split probe assay for the detection of the sexually transmitted infection *Chlamydia trachomatis*, was developed through use of the enzyme lambda exonuclease to cycle a specific DNA sequence generating amplified SERS signals (Fig. 4). Lambda exonuclease was used to digest the phosphorylated reporter probe only when it had specifically hybridised to the unlabelled target sequence. A biotinylated probe was also hybridised to the capture duplex, before washing steps were carried out to remove excess probe. In theory, this approach allows free target to be continually rehybridised with undigested probe, cycling the process and building up the SERS dye signal. Although in this initial assay PCR was used to generate target, optimisation of signal amplification strategies coupled with self aggregation of DNA–nanoparticle conjugates to detect DNA could eventually eliminate the need for PCR.

DNA–nanoparticle conjugates. Since Mirkin[59] and Alivisatos[60] demonstrated the ability to detect DNA using localised surface plasmon resonance (LSPR) using thiol modified oligonucleotides and a target sequence, much research has focussed on DNA conjugated nanoparticles for the detection of target DNA. Thus, an alternative approach to the detection of target oligonucleotides by SERS makes use of DNA functionalised nanoparticles that use a sequence specific hybridisation event to promote aggregation and enhance signal output.

Cao *et al.*[61] achieved multiplexed SERS detection of oligonucleotide targets using gold nanoparticles labelled with thiolated DNA and Raman-active dyes. Using a split probe system and a microarray format, six different DNA targets with six different Raman reporter molecules were distinguished with a detection limit of 20 femtomolar. Although gold nanoparticles were used in this assay, the gold nanoparticle–DNA conjugate was subsequently coated with silver metal to enhance the signal generated from the Raman label. An alternative method of increasing visibility of the label through silver enhancement is to use silver nanoparticles. Thompson *et al.*[62] first reported the use of oligonucleotide–silver nanoparticle conjugates for the detection of target DNA sequences and Graham *et al.*[63] demonstrated how the surface enhanced resonance Raman scattering effect could be turned on using dye-labelled, DNA functionalised, silver nanoparticles, as a result of a natural biological recognition process (Fig. 5).

Two batches of silver nanoparticles were functionalised with different non-complementary probe sequences. In the presence of a target sequence complementary to both probes, the hybridisation event resulted in aggregation of the silver nanoparticles into large assemblies, which provide a large electromagnetic enhancement. Subsequently, Qian *et al.* reported on the use of SERS beacons and long range plasmonic coupling for the detection of DNA.[64] However, due to the better SERS enhancement seen in comparison to gold,[62] nanoparticle assemblies from silver particles result in much lower and more sensitive detection levels using SERS.

Additional work has been done on improving the surface chemistry of the DNA–nanoparticle conjugates in order to achieve the best SERS response possible when targeting oligonucleotides. Different anchors for the DNA strands have been used with thioctic acid proving to be more effective at stabilising oligonucleotides on nanoparticles than conventional thiol groups.[65,66] Additionally, McKenzie *et al.*[67] demonstrated the success of using mixed metal nanoparticle assemblies, which combined the surface enhancement of silver, with the more favourable surface chemistry of gold nanoparticles.

Due to the disadvantages associated with PCR, the ultimate aim of developing SERS based DNA diagnostic methods, is to exploit the advantages of SERS to develop an amplification-free method of analysis. Thompson *et al.* in particular noted that

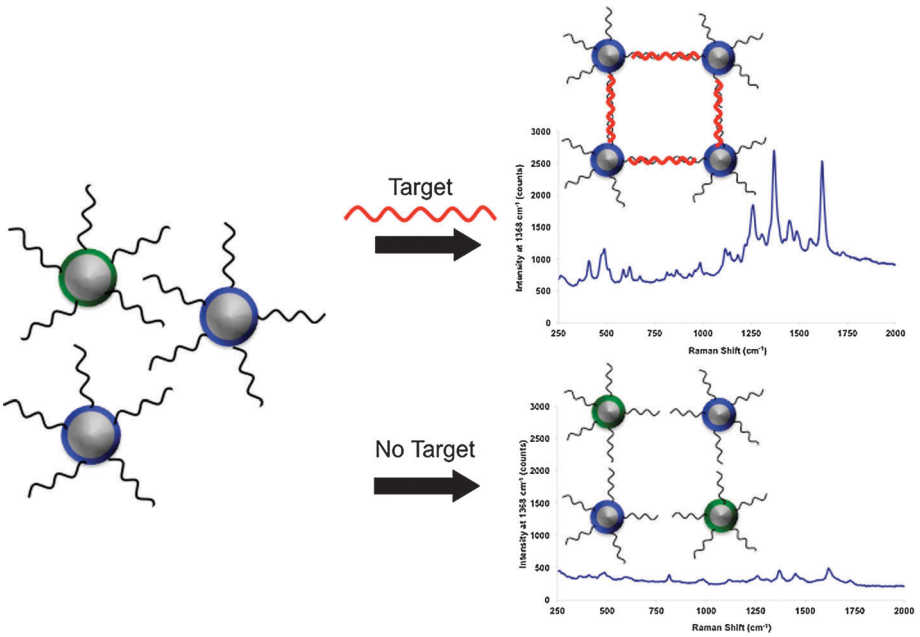

Fig. 5 Oligonucleotide–silver nanoparticle conjugates for DNA detection. The top image shows the enhanced SERRS signal obtained as a result of nanoparticle assembly into large aggregates when a target is present. The bottom image shows that in the absence of target dispersed single nanoparticles lead to a decrease in signal.[62,63]

even though multiple sequence analysis could be obtained using oligonucleotide–silver conjugates at low limits of detection, the assay was not yet capable of providing PCR-less detection of genomic material.[62] However, exploiting the optical properties of metallic nanoparticles and inducing aggregation through a significant biological recognition event is definitely a step in the right direction towards amplification-free analysis.

Other methods of signal amplification as an alternative to target amplification have been developed using oligonucleotide conjugates. Xu *et al.* used nicking endonuclease assisted nanoparticle amplification (NEANA) for the sensitive and colorimetric detection of DNA.[68] A 3-component sandwich assay was used that included two sets of gold nanoparticle oligonucleotide conjugates, a target strand and a nicking endonuclease enzyme that cleaves only one of the two strands in hybridised DNA. When the target sequence is present, it will hybridise to the linker probe in the system, which will be digested into two strands by the enzyme leaving the target to recycle. The digested parts of the linker are complementary to the nanoparticle probes and will hybridise preventing aggregation of the nanoparticles. Thus, aggregation will only be apparent in the absence of a target, and a red to blue colour change will be observed. Using this method a colorimetric detection limit of 0.5 femtomolar was obtained and the ability to detect single base mismatches demonstrated. While this assay does not include the use of Raman analysis, it shows the development of conjugate based methods of detection. Signal amplification and SERS have been discussed previously using dye labelled oligonucleotides, however the use of SERS for analysing signal amplification based conjugate protocols for DNA detection is a relatively novel technique. As research groups focus on methods of combining signal amplification with oligonucleotide–nanoparticle conjugates for SERS analysis, it is hoped that detection of genomic concentrations of DNA will be achieved, which will be a considerable step towards implementation of SERS into the medical diagnostic community as a replacement for PCR.

Protein detection

The informative detection of proteins and their interactions is central in advancing many aspects of modern biology, especially for the understanding of biological pathways and for the detection of disease states. Consequently, selective and sensitive information at a molecular level must be generated from the vast number of proteins that may have concentrations at or below the sensitivity limit of many detection techniques. Current protein detection methodologies commonly employ immunoassays and protein arrays coupled with fluorescence as a readout tool, however, SERS has been shown to be highly adaptable for the detection of proteins.[69,70]

Direct detection of proteins. Proteomic research often involves isolation and investigation of single proteins at very low concentrations, however target amplification strategies, such as PCR for DNA molecules, do not exist for proteins. Therefore, single molecule detection of proteins is critical to obtain accurate and meaningful results. In 2003, single molecule detection of green fluorescent protein by SERRS was reported through adsorption onto silver citrate nanoparticles.[71] McGuinness *et al.* then demonstrated the enhanced limits of detection of the fluorescent protein allophycocyanin (APC) by SERRS.[72] SERRS was utilised to obtain a detection limit of 1×10^{-13} mol dm^{-3}, which was over a magnitude lower than comparative fluorescent measurements.

To enable the detection of non-fluorescent proteins, labelling techniques have been developed allowing analysis to be extended to a greater range of proteins. One such method involves the specific attachment of SERS active dyes to proteins. This labelling enables maximum SERS sensitivity to be obtained when analysing an increased number of proteins. Douglas *et al.* attached a benzotriazole dye to a heme protein *via* a Michael addition.[73] The protein cytochrome C and the dye compound, *p*-anisidine maleimide (PAM) were selectively coupled through the thiol of the only available cysteine residue on the protein, providing a single dye attachment per protein. The PAM compound contains a benzotriazole dye that has dual functionality, as it is strongly adsorbed onto the silver nanoparticle surface providing strong SERRS signals through the azo group. Two wavelengths were used, 406 and 514 nm, which coincided with the frequency of an adsorption band of either the protein or maleimide azo dye and distinctive SERRS spectra could be obtained. A duplex with discernible vibrational bands from both components was also generated after coupling of the compounds *via* Michael addition and selection of wavelength allowed selective enhancement of modes from either the protein or dye. This study has illustrated that through this biodetection labelling approach a larger range of proteins can be sensitively and specifically detected by SERRS analysis.

Indirect detection of proteins – immunoassay detection. Immunoassays are routinely used for the measurement of proteins in clinical testing, disease progression and diagnostic treatments. Within a typical sandwich immunoassay, a monoclonal capture antibody which is specific for a particular antigenic site is absorbed onto a solid surface. When the sample containing the target antigen is added, the capture antibody binds to the target antigen, retaining it on the solid support. A polyconal detection antibody is then added, binding to the target antigen through a different epitope. This specific biorecognition ''sandwich'' can subsequently be detected. Moreover, proteomic studies have used protein labelling and SERRS to improve the development and performance of protein detection techniques.[74]

SERRS immunoassays for the specific detection of a target antigen through selective antibody–antigen binding have been rapidly progressed. A simple biorecognition assay has been developed which utilises a SERRS labelled secondary detection antibody to directly detect the presence of a target antigen. The intracellular signalling protein P38 was quantitatively detected by SERRS, through a sandwich immunoassay on a magnetic micro-bead surface.[75] In the presence of P38 protein a detection antibody labelled with a rhodamine dye was captured. A SERRS response was then obtained through addition of silver nanoparticles and an aggregating agent, poly-L-lysine. This caused a nanoparticle cluster formation around the labelled antibody, which was subsequently dissociated from the immunocomplex. This method has demonstrated quantitative detection of a single target protein within an immunoassay by SERRS, obtaining a limit of detection of 6 ng mL^{-1}. However, lower detection limits have been achieved in assays that directly incorporate nanoparticles within the assay sandwich.[76]

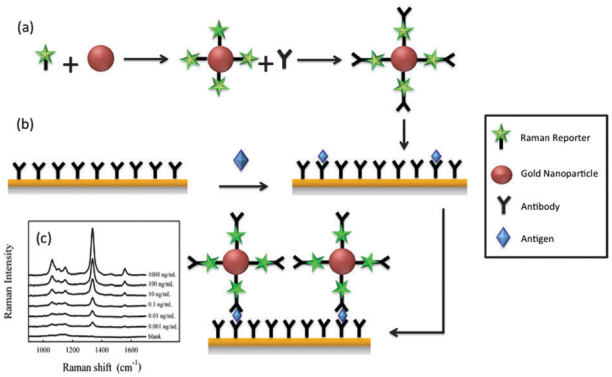

Fig. 6 Schematic representation of (a) preparation of Raman reporter and antibody functionalised Au nanoparticles, (b) capture of functionalised Au nanoparticles within a specific protein recognition on a gold coated glass slide, (c) SERS spectrum produced from captured Raman reporter functionalised nanoparticle.[77]

Grubisha *et al.* developed a SERS protein assay, through incorporation of target antibodies and Raman reporter molecules onto a gold nanoparticle.[77] A monolayer of bioselective detection antibodies are attached to the nanoparticle surface by coupling through a strong Raman reporter molecule, 5,5′-dithiobis(succinimidyl-2-nitrobenzoate) (DSNB). This design ensures that an increased number of Raman reporters were attached to each particle, thus, maximising the SERS signal obtained. In this experimental setup, the functionalised nanoparticles were captured onto a gold metal coated glass slide through a selective target antigen immunoassay, (Fig. 6) and strong SERS signals obtained from the Raman reporter molecules. An increased SERS enhancement is obtained as a result of the surface coupled dye being in resonance with the localised surface plasmon on the gold particle surface. Subsequently, enhanced detection limits were achieved by incorporation of nanoparticle conjugates into a SERS protein recognition assay, with femtomolar detection of prostate-specific antigen (PSA) achieved.

Reports suggest that SERS detection assays using silver and gold nanoparticles can increase sensitivity over that of rival fluorescence detection.[7,78] Labelling and detection strategies are being continually advanced, leading to enhanced SERS signals. This work has shown a developed method for the synthesis of nanoparticle conjugates, through formation of a monolayer of bifunctional Raman reporter dyes coupled to bioselective antibodies. This generates the strongest SERS responses as a greater number of Raman molecules can be attached to the nanoparticle surface.[79] The continued progression in conjugate design will enable increasingly lower limits of protein detection to be obtained in the future.

The low detection concentrations and sharp molecularly specific peaks achieved using SE(R)RS for the detection of proteins, were exploited to positively identify a number of analytes *in situ* without separation.[80] A protein immunoassay has been developed that utilises SERS detection to simultaneously detect a set of four target antigens. Wang *et al.* has described an approach for the detection of multiple proteins

using self-assembled mixed monolayer gold nanoparticles through a sandwich type immunoassay.[81] Nanoparticles were functionalised with antigen specific antibodies and a unique Raman reporter resulting in specific SERS spectra from each functionalised nanoparticle. Capture antibodies relating to each target antigen were immobilised on a substrate surface and through biorecognition protein binding, distinctive SERS responses were generated. This immunoassay has shown it is possible to simultaneously distinguish multiple immunoglobulin G proteins *via* SERS.

Recently, SERS has been shown to outperform conventional assays for the detection of the mucin protein MUC4, expressed in pancreatic adenocarcinoma cell lines and tissues.[82] The level of MUC4 is undetectable in normal pancreas and chronic pancreatitis, thus MUC4 has the potential to function as a detection marker for pancreatic cancer. However, the measurement of MUC4 in human sera using conventional ELISA and radioimmunoassay (RIA) techniques has been shown to be unsuccessful due to unacceptably low sensitivity and specificity levels.[83] Wang *et al.* have developed a novel SERS-based sandwich immunosorbent assay for the detection of MUC4. Quantitative MUC4 measurements where obtained through detection of gold nanoparticles that had been functionalised with the Raman reporter molecule, 4-nitrobenzenethiol. The detection antibody, 8G7, was immobilised onto a gold substrate and used to capture MUC4 marker. The functionalised gold nanoparticles could then bind to the target through a different epitope and a strong SERS response generated. Owing to the application of SERS, MUC4 has been successfully detected for the first time and limits of detection have been lowered by 1000× and sample volumes required reduced by 10×, over standard immunoassays.

Indirect detection of protein – enzyme-linked immunosorbent assay detection. The enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used analytical techniques available in clinical immunology. It is a highly sensitive and specific method used to detect and quantify the presence of either an antigen or antibody in serum. However, the technique is subject to high fluorescent background signals from the biological media and the inability to detect interactions over distances greater than 10 nm. Thus, SERRS based ELISA methods have been developed, which can overcome these issues.[84–86]

Rohr *et al.* were the first to pioneer the use of SERS for proteomic investigation within an ELISA.[87] The sandwich immunoassay was carried out on a silver surface generating a strong SERS signal through immobilisation of a thyroid stimulating hormone antibody with an attached resonant dye label, *p*-dimethylaminoazobenzene. This assay incorporated directly labelled antibodies, however, subsequent work by Dou *et al.* utilised specific enzymes to convert substrates to SERS active dyes within an HRP-conjugate sandwich ELISA.[84] A secondary antibody labelled with peroxidase was captured through a sandwich immunoassay which in the presence of *o*-phenylenediamine, converts it to the coloured product, azoaniline, which is SERS active (Fig. 7). The concentration of antigen (mouse IgG) was then quantified by observing the SERS signals of

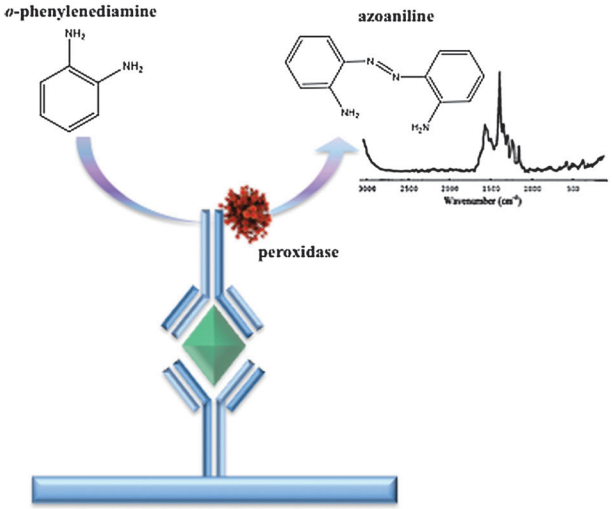

Fig. 7 SERS enzyme immunoassay system with a SERS spectrum of the resulting enzymatically produced dye.

azoaniline absorbed onto silver colloidal suspension and the limit of detection was determined to be 1.8×10^{-15} mol ml^{-1}.

Another substrate/enzyme combination that was exploited for SERS was the commonly used substrate bromochloroindolylphosphate (BCIP) which, in the presence of alkaline phosphatase, generates a blue dimeric species with an absorption maximum at 615 nm, this transformation to the blue product can be followed using SERRS.[88] Detection of the generated chromophore was obtained through resonance enhancement using a 633 nm laser excitation source producing a SERRS spectrum with a distinct Raman shift at 600 cm^{-1}. Campbell *et al.* employed this method within an immunoassay format to quantitatively detect C-reactive protein (CRP).[85] A BCIP-SERRS-ELISA was developed that could detect the target protein at concentrations as low as 0.3 ng mL^{-1} through correlating the intensity of the Raman shift at 600 cm^{-1} to the concentration of CRP in standard samples following successful recognition.

Stevenson *et al.* were the first to use SERS as a readout technique in an HRP ELISA using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) for the detection of PSA within human serum.[86] Upon reaction with hydrogen peroxide, ABTS forms a green oxidised product with an absorbance at 420 nm, which could be detected by SERS using a commercially available PSA ELISA kit in conjunction with gold nanoparticles. This work has shown that the intensity of the Raman peaks were directly related to the concentration of enzyme reacted, allowing the detection and quantification of the target antigen. The limit of detection obtained was reduced to within the picomolar range, significantly below the accepted decisive limit of PSA levels required for further investigation of prostate cancer.

The work discussed in this section has so far shown modified ELISAs coupled with SERS detection. However, Wu *et al.* have developed a different approach by carrying out the SERS assay directly on the surface of a gold nanoparticle as a method of quantitatively measuring glucose.[89] Gold nanoparticles were

functionalised with glucose oxidase (GOD) and horseradish peroxidase (HRP) and mixed with *o*-phenylenediamine and a target glucose solution. During the reaction the glucose is oxidised by GOD, producing hydrogen peroxide that when combined with *o*-phenylenediamine is converted to azoaniline by HRP. The SERS of the resulting azoaniline product was used to measure the enzyme activity and correlate this to the detection of glucose. The resultant detection range of glucose was determined to be 0.50–32 mM, a detection range six times wider than previous glucose sensors.[90]

These studies have shown the use of SERS analysis for the indirect detection of target proteins. However, this method is restricted as a result of the reduced number of substrate/enzyme combinations that can yield a SERS active dye. Consequently, applications have been developed which directly measure enzyme activity by generating a specific SE(R)RS active dye.

SERS of enzymatically unmasked dyes. Masked dyes have been specifically designed to be directly detected by SERS when in the presence of a selective enzyme. The experimental detection process uses a modified dye containing a surface seeking group and a chemical 'mask'. The dye is rendered SERS inactive while masked since the dye is prevented from binding to the metallic nanoparticle surface. The facilitation of surface adsorption is essential to achieve effective SE(R)RS, as increasing the distance of the analyte from the surface reduces the enhancement that is obtained from the surface plasmon resonance and rapidly diminishes the signal. However, the mask is synthesised to be specifically enzyme cleavable, revealing the surface seeking group upon action of the enzyme which drives surface adsorption of the target dye molecule onto the nanoparticle. This adsorption generates a highly intense SERS spectrum, easily identifiable above other constituents within the sample solution. This approach was first developed by Moore *et al.*, detecting ultra low concentrations of hydrolases using masked benzotriazole azo dyes.[91] Studies have shown that benzotriazole based dyes are ideally designed to adsorb onto a silver nanoparticle surface by displacing the citrate surface layer and generating distinct SERS spectrum through the azo group.[92–94] Thus, to release the benzotriazole azo dye and generate the SERS signal, an enzyme-cleavable linker and an enzyme recognition site were attached to the dye. Addition of a suitable enzyme will selectively cleave the linker, removing the mask and allowing the SERS signal to be turned over. This investigation established the development of a biocatalytic assay that used the lipase, *Pseudomonas cepacia*, to enzymatically hydrolyse esters of 3-phenylbutyric acid. The collapse of this linkage exposed the nitrogens of the benzotriazole dye, providing the binding site for complexation to the silver nanoparticle surface. SERS analysis of the masked parent substrate gave negligible signals, whereas, the free dye produced strong and distinct spectral peaks.

The sensitivity of this method was further improved in 2007 by Ingram *et al.*, through synthesis of masked 8-hydroxyquinolinyl azo dyes.[95] These novel SERS active dyes improved the sensitivity up to 100-fold when compared to the previously used benzotriazole dye, achieving a lower detection limit of 0.2 ng mL^{-1}. 8-Hydroxyquinoline is known to strongly chelate to the silver nanoparticle surface and the aryl azo derivatives fulfil the SERS dye requirements. The dye mask was generated through *o*-acylation of the 8-hydroxy moiety, preventing surface complexation in advance of linker hydrolysis by enzyme action. Preparation of the 8-hydroxyquinoline substrate dyes was simpler and more straightforward as there are a reduced number of synthesis steps and the initial hydrolysis rate with lipase was found to be greater. As a result, an enhanced detection method with decreased limits of detection was developed when compared with the masked benzotriazole dyes. Studies investigating SERS-based techniques for proteins detection through lipase hydrolysis of the surface masking moiety on the dye have been studied.[91] To extend the applicability of this system, Ingram *et al.* have reported application of the method for the detection of proteases.[96] The technique was adapted to synthesise masked protease substrates, by attaching phenylalanine to a dye labelled benzotriazole SERS active species *via* a chloromethyl ester linkage. At concentrations of 10^{-7} M, no SERS spectra were obtained from the masked species. When exposed to *Subtilisin carlsberg*, the SERS response was significantly enhanced, attributing this to the enzymatic cleavage of the mask linker.

The concept of direct detection of enzyme activity by SERS has been shown to be highly sensitive and selective and in 2009 the potential to analyse multiple enzymes simultaneously without sample separation was achieved when simultaneous detection of alkaline phosphatase and β-galactosidase activity was carried out.[97] Phosphorylated and galactosylated analogues of 8-hydroquinolinylazo dyes were prepared, which in the presence of alkaline phosphatase and β-galactosidase respectively cleaved the masks from the dye substrates enabling SERS responses to be obtained. The SERS spectra of the different dyes provide Raman bands that are easily differentiated, allowing each substrate to be identified within a sample mixture. The sharp molecular specific peaks obtained from SERS have been exploited to identify two components within a mixture and suggests great promise for the development of higher multiplexing.

The progressive development of protein detection through SE(R)RS analysis has shown enhanced sensitivity, specificity and the detection of analytes which are unable to be detected by conventional methods. Current research is being aimed at developing a clear understanding of protein–ligand interactions using SERS.

Functionalised nanoparticles for protein detection. The understanding of protein–ligand interactions is essential for the continuing development of biomolecular diagnostic assays. A number of studies have been carried out to monitor the assembly of functionalised nanoparticles *via* protein–ligand interactions using optical absorption spectroscopy[98,99] and transmission electron microscopy.[100] However, these techniques lack the sensitivity which SERS can offer. Robson *et al.* have recently developed a nanosensing approach to investigate a specific protein–peptide interaction.[101] The interaction between the tumour suppressor protein, p53 and a bivalent mouse double minute two (MDM2) protein was followed by SERS.

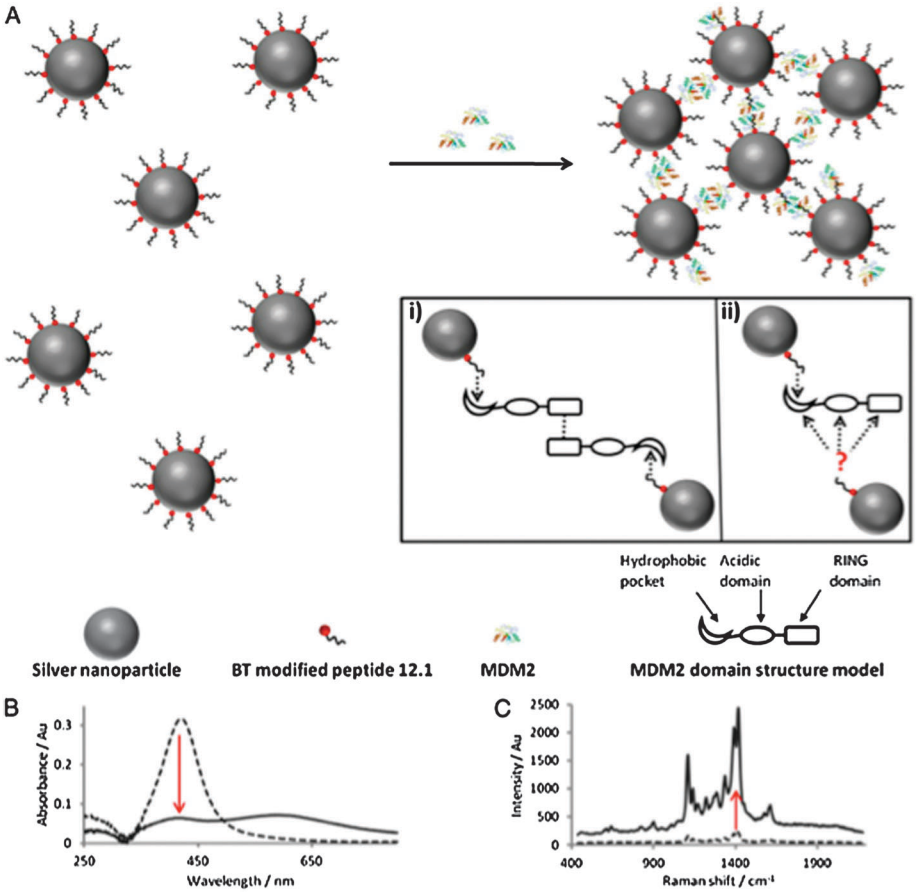

Fig. 8 Schematic of the proposed assembly of silver nanoparticles through specific interactions between MDM2 and peptide 12.1.[101]

P53 is either mutated or absent in >50% of cancers and is negatively regulated by MDM2 protein, therefore the understanding of this interaction is vital for the progression of chemotherapeutics as a result of the critical role of MDM2. SERS active nanoparticles were functionalised with a p53 peptide mimic labelled with a SERS active benzotriazole azo dye, which through the specific interaction with MDM2, resulted in controlled nanoparticle-assembly (Fig. 8). This aggregation event causes a significant enhancement in SERS signal, resulting from a large increase in the electromagnetic field strength between particles. Using this approach the potential that SERS has to provide insights into the selective allosteric binding of proteins was illustrated. More recently SERS has also shown specific and sensitive monitoring of protein–carbohydrate interactions.[102] Craig *et al.* demonstrated the first example of SERS template nanoparticle assembly *via* the interactions of carbohydrates and the lectin, ConA. This study has demonstrated picomolar detection, currently unachievable by conventional techniques and has achieved high specificity between two carbohydrate moieties.

SERS monitoring has also enabled specific enzyme detection using peptides and gold nanoparticles. Stevens *et al.* have demonstrated a method which allows low concentration of disease-specific enzymes to be detected for medical diagnostic applications.[103] This was achieved through the self-assembly of peptide functionalised gold nanoparticles and the extreme sensitivity of SERS. The peptide functionalised gold particles were formed and aggregated through π–π interactions between the FMOC groups of the peptide chain, generating a strong SERS band at 1090 cm^{-1}. The aggregates will then be dissociated upon addition of a protease enzyme, which cleaves the peptide sequence and increases electrostatic repulsion. This process decreases the SERS signal over time as a result of a reduced number of hot spots and greater nanoparticle separation. Potential detection limits of the enzyme were calculated to be 0.003 ng mL^{-1}, which significantly exceeds current medical standard methods that are in the order of 10 ng mL^{-1}. These studies show the importance of studying protein interactions and highlight the benefits of SERS for such studies.

SERS analysis for intracellular applications

A number of different methods for the direct detection of biomolecules using Surface-enhanced Raman spectroscopy have emerged in recent years. The success of SERS in the analysis of target molecules such as DNA and proteins, along with the ability to detect single molecules and single nanoparticles,[7,104] means that it is fast becoming one of the premier techniques in providing sensitive and selective methods of analysis. Therefore, in an effort to move towards implementation of SERS in a clinical setting, research has recently focussed

on the ability of surfaced enhanced Raman to probe intra- and extra-cellular activity, and moving towards *in vivo* analysis.

As with many of the aforementioned assays, the field of cellular analysis has previously been dominated by fluorescence based techniques. Recently, the use of quantum dots have proven to be advantageous in biological labelling, as these semi-conductor nanoparticles improve upon basic molecular fluorophores by providing a more intense emission spectrum and narrower bandwidths.[105,106] Additionally the biocompatibility problems associated with quantum dots due to their toxicity have been largely overcome by encapsulating the semiconductor in more biocompatible materials such as mercaptoacetic acid.[106] More recently, Nanotags have been developed for *in vivo* SERS analysis.[107,108] These tags are based on nanoparticles with Raman reporter molecules on their surface and have become an exciting new avenue for the investigation of cellular activity. It has been reported that Raman tags are more than 200 times brighter than quantum dots[108] and have the added advantage of the ability of SERS to provide sharp molecular signatures which allow for multiplexed analysis. This is of great importance for *in vivo* detection where it is becoming essential for different biomarkers to be targeted simultaneously in order to provide faster and more accurate patient diagnosis.[23]

SERS nanotags. Kneipp *et al.* investigated the behaviour of gold nanoparticles in living cells and their ability to serve as SERS nanoprobes in varying subcellular environments.[109,110] The SERS signal from native constituents of the cell was obtained and allowed for the detection of biological molecules such as DNA and proteins.[109] Additionally, the SERS spectra from endosomes in living individual epithelial and macrophage cells were investigated using gold nanoparticles in cell culture media that were taken up by endocytosis.[110] Based on the SERS enhancement factors seen, it was reported that after uptake, the gold nanoparticles formed aggregates which changed in size and morphology depending on their cellular environment. As such it was concluded that intracellular SERS investigations enable controlled molecular probing, and that gold nanoparticles are particularly useful due to their small size, meaning they can be introduced to a cell and distributed within the cellular environment with little damage to its structural integrity.[109–111] Similarly, Tang *et al.* used gold nanoparticles to probe single cancer cells, and emphasised the importance of aggregation control in obtaining a SERS spectra for detailed characterisation of cellular components.[112]

These studies on colloidal gold focused on obtaining the SERS spectra of cellular biomolecules surrounding the nanoparticles, and limitations exist due to the complex matrix of molecules found within the cell that will produce a SERS signal, and which will lead to an extremely complicated spectrum which can be difficult to analyse.[113] Therefore, it is of great benefit to functionalise the nanoparticle surface with a Raman reporter molecule in order to obtain a specific and known SERS spectrum from the cell. Kneipp *et al.* reported on SERS studies of silver and gold nanoparticles labelled with indocyanine green. The group demonstrated the application and biocompatibility of this probe in living cells and concluded that the indocyanine spectra can be used for both the determination of the nanotags position in the cell, and also to deliver information on the local molecular structure.[114] Additionally, silver nanoparticles have been used by Talley *et al.* to probe intracellular pH by functionalising the nanoparticle surface with 4-mercaptobenzoic acid.[115] SERS measurements showed that these functionalised nanoparticles responded to a change in pH and can be incorporated into living cells with almost no change in signal intensity. While both gold and silver nanoparticles have proven successful for biological SERS measurements, limitations still exist when it comes to controlling uptake and distribution. In particular, due to the electromagnetic enhancement gained from nanoparticle ''hot spots'' it is necessary to control the aggregation of sub-cellular nanoparticles in order to gain the maximum SERS signal.[112,116] As such, research has focussed on methods of physically shielding the nanoparticles and reporter molecules in order to prevent aggregation resulting from environmental conditions.

Encapsulated SERS nanotags. Several research groups have investigated various methods of encapsulation of SERS active nanoparticles to improve biocompatibility and stability in native biological environments.[117–119] Coating the core material not only aids cellular uptake and aggregation control but also allows for a variety of attachment protocols to attach targeting biomolecules onto a surface where the chemistry has been well studied.[117] Thus intracellular SERS probing can be seen as having two distinct functions.[111] It can be used for sensing purposes, whereby un-functionalised nanoparticles are used to examine the spectra of pure analytes such as proteins, which sit at hot spots between aggregates. It can also be used to target particular biomarkers by functionalising the surface of the encapsulated SERS nanotags with certain biomolecules. For example, the surface can be functionalised with a DNA sequence complementary to a sequence of interest or with a particular antibody in order to detect certain antigens present in a cell. This will provide SERS spectra of a particular Raman reporter molecule in a sub-cellular compartment when the targeted biomolecule of interest is present.

A popular method of encapsulation is that of silica. These Raman tags were first reported by Mulvaney[117] and Doering[118,119] and consist of a SERS-active metal nanoparticle, a submonolayer of reporter molecule and a silica coating. Both publications reported on the advantages of this design, whereby multiple tags can be employed for different targets to give multiplex spectra with no peak overlapping, all tags can be examined with one excitation source, and photobleaching is not a factor. Also the silica encapsulation provides a barrier between the metallic core and external environment, providing enhanced stability. Doering *et al.* additionally noted that a reactive isothiocyanate group could be used as a ''molecular anchor'' as it is compatible with silica encapsulation and thus can be used for embedding organic dyes onto core shell particles. Using these nanotags an enhancement factor of 10^{13}–10^{15} was observed which could allow for single particle or single molecule spectroscopy. While initial publications focussed on gold core nanotags, the successful synthesis of

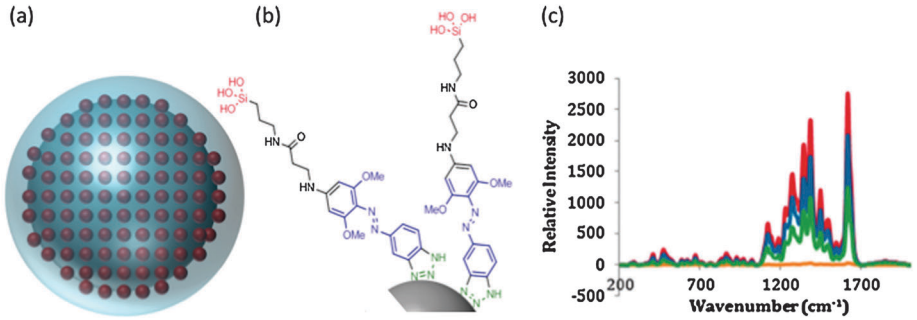

Fig. 9 (a) Schematic of a SERS active silica coated silver nanoparticle. (b) Surface modifications incorporating a Raman active molecule and silica shell. (c) SERRS spectra obtained at 514 nm from silver nanoparticles (yellow), nanotags with a full monolayer (red) and mixed monolayer of dye (green and blue).[120]

silica coated silver nanoparticles has since been published by Rocks *et al.*[120] Four tri-functional molecules were synthesised that stabilised the silver nanoparticle core, acted as a Raman reporter and provided a precursor for silica encapsulation (Fig. 9).

These silver core SERS nanotags have all the advantages of gold core nanotags while also demonstrating the advantageous optical properties of silver nanoparticles. Küstner *et al.*[121] demonstrated silica encapsulation of a self-assembled monolayer (SAM) on gold/silver nanoshells. The advantages of SAM based nanotags is discussed in terms of spectral multiplexing and signal strength, and gold/silver nanoshells are compared to traditional gold nanoparticles. It was noted that the two parameters resulted in an increase in overall brightness compared to existing SERS labels on gold nanoparticles with submonolayer reporter molecule coverage. These tags were then used for tissue imaging using prostate-specific antigen (PSA) as the target protein.

Graham *et al.*[122] utilised a functional polymer to create encapsulated aggregates of silver nanoparticles, in an effort to take advantage of the sensitivity gained from an increase in SERS activity through intense local fields. Four specifically designed SERS dyes were used based on benzotriazole and 8-hydroxyquiniline groups, and three were combined in multiplexed analysis. While the polymer core was used to hold aggregates together an outer polymer shell was also incorporated which allowed for the attachment of oligonucleotide probes. These SERS labelled beads can therefore be used for the detection of specific DNA targets.

***In vivo* SERS analysis.** The first *in vivo* application of SERS was used to obtain quantitative glucose measurements from an animal model.[123] Stuart *et al.* used silver film over nanosphere surfaces functionalised with a two-component self-assembled monolayer composed of decanethiol (DT) and mercaptohexanol (MH). The SAM helped to improve upon the signal from the analyte by partitioning glucose and localizing it within a few nanometres of the SERS-active surface, which is optimum for analysis. These surfaces were implanted subcutaneously within a rat and the glucose concentration of the interstitial fluid measured using SERS through an optical window.

Recent work by Qian *et al.* demonstrated the success of Raman nanoparticle tags for *in vivo* tumour targeting in live mice.[108] The group designed a class of biocompatible and nontoxic nanoparticles based on 60 nm colloidal gold functionalised with a Raman reporter molecule and stabilised by a layer of thiolated polyethylene glycol (PEG). The thiol-PEG-coated particles were extremely stable under very harsh conditions and the PEG coating allowed for the conjugation of tumour-targeting ligands. In particular an ScFv antibody was conjugated to the surface to specifically recognise the epidermal growth factor receptor (EGFR) that is overexpressed in malignant tumours. These SERS nanoparticles were successful in the active targeting of both cancer cells and tumours in animals models.

Zavaleta *et al.*[107] demonstrated the multiplexing capabilities of SERS nanotags in live mice. Initially, 10 different tags, each consisting of a unique Raman active molecule adsorbed onto a 60 nm gold core and coated with silica, were injected subcutaneously and the corresponding SERS intensities were identified. *In vivo* multiplexing was proven by intra-venous injection of 5 different SERS batches, whereby non-specific bioaccumulation of the tags in the liver allowed for correct identification by SERS analysis. The software used in experimentation was also able to linearly correlate Raman signal and the concentration of SERS nanoparticles, using a multiplex of four different SERS tags, to a depth penetration of 5 mm. In comparison to the previously mentioned study, this particular work did not target any specific biomolecules, however it is important in demonstrating the ability of SERS nanotags to provide multiplexed analysis *in vivo*.

However, a limiting factor of *in vivo* SERS analysis is the depth of penetration available to the Raman systems. There are multiple scattering and absorption events that occur on the surface of the skin and throughout the deeper layers, which lead to a loss in signal intensity. Zavaleta *et al.* noted that their images were not fully quantitated due to the inability of deep tissue mapping.[107]

Surface enhanced spatially offset Raman spectroscopy. Spatially offset Raman spectroscopy (SORS) is a variant of Raman spectroscopy whereby Raman scattered light is collected from regions offset from the point of laser excitation, and is used to provide better depth resolution in sample analysis. A very recent combination of SERS and SORS has lead to a new and powerful bioanalytical method of spectroscopy in the form of SESORS. This was first reported by Stone *et al.*[124]

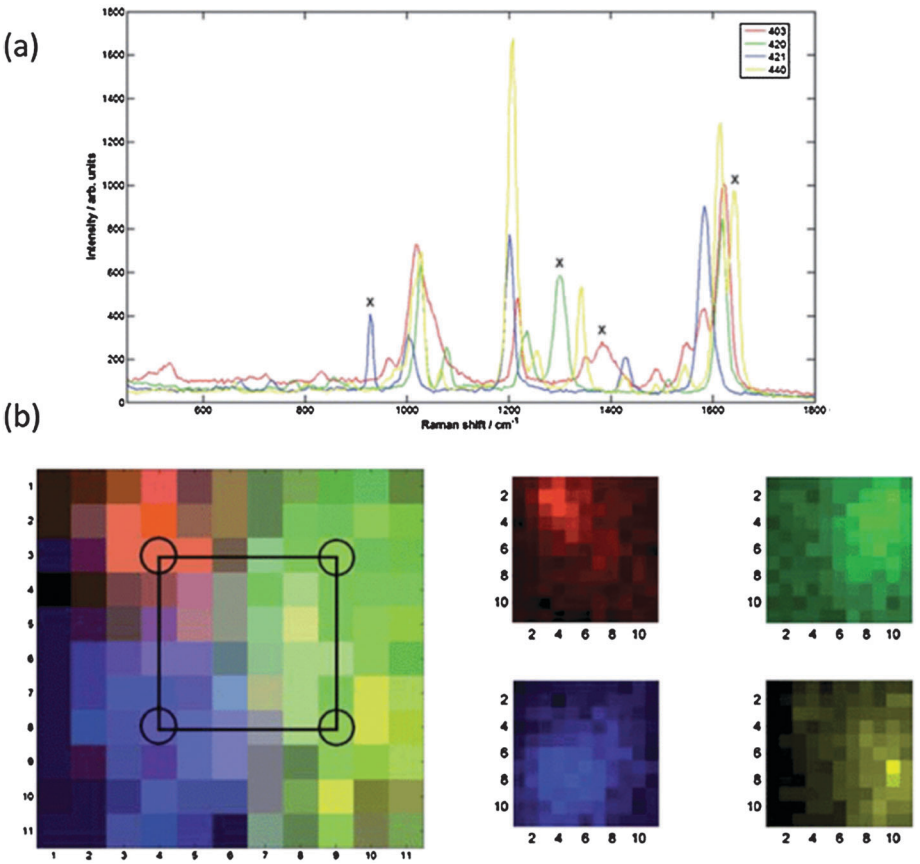

Fig. 10 (a) SESORS spectra at 830 nm of 4 different SERS nanotags. (b) False colour images of the obtained SERS nanoparticle signals.[124,126]

where transmission Raman spectroscopy, a special form of SORS where laser delivery and collection is on either side of the sample, was used to probe mammalian tissue. Dye-tagged silver nanoparticles were injected into porcine tissue allowing for the detection of a 1.1×10^9 nanoparticles at a depth of 25 mm, showing significant improvement over traditional Raman signals by 2 orders of magnitude.

Van Duyne's group used SESORS to improve upon their previous research into monitoring the glucose concentration of interstitial fluid in a rat model.[125] They used silver film over nanospheres as the substrate functionalised with a mixed self-assembled monolayer of DT and MH as with their previous SERS experiments.[123] This was the first reported case of *in vivo* SESORS and allowed for the acquisition of transcutaneous spectra giving a direct and quantitative method of glucose detection.

SESORS has also been used by Stone *et al.* for multiplexed SERS imaging.[126] Encapsulated Nanotags were used to detect 4 different SERS reporters from a sample of porcine tissue at a depth of 20 mm and reconstructed to produce a false colour image (Fig. 10). Additionally, without the multiplexing aspect, Raman signals from SERS nanoparticles could be recovered from depths of 50 mm. The authors noted that there are significant prospects for SESORS to be implemented in a clinical setting, as it has the potential to be used in numerous applications as an efficient medical tool.

Conclusion

SERS spectroscopy provides many advantages over alternative spectroscopies, including high sensitivity, selectivity, molecularly specific identification and high levels of multiplexing through the generation of narrow peaks which when combined provide a uniquely distinct detection technique that has the potential for extensive diagnostic applications. This highly versatile spectroscopic method has been rapidly progressed since it was first experimentally demonstrated in 1974.[1–3] Since its discovery SERS has been used to detect a vast array of relevant biological molecules such as DNA, proteins, peptides, enzyme activity and glucose. This perspective has highlighted recent advances in the use of SERS spectroscopy as an analytical tool, with sensitive and specific detection of target molecules achieved through use of intrinsic SERS signal of the biomolecule or by direct and indirectly labelling. The detection of multiple analytes *in situ* has shown to be one of the main advantages offered by SERS and detection of meaningful clinical samples has been shown. More recently work has focused on the successful application of SERS for targeted analysis *in vivo*, which will concentrate considerable future investigation. For example recent work by Gambhir *et al.* on using a multimodal approach to brain tumour imaging using a combination of magnetic resonance imaging, photoacoustic imaging and Raman to allow for delineation of a noninvasive

tumour through the skull of live mice is highly exciting and shows where the future advantages of SERS lies.[127]

Although significant advances have been achieved in the use of SERS detection in the last 35 years, the technique has not been fully exploited and work so far has only scratched the surface of the potential which SERS holds. However, with the more recent advances in the use of SERS in the field of bioanalysis it seems certain that this is the area where SERS will come into its own and have the potential to become a disruptive technology for the market leader, fluorescence, due to its distinct advantages in terms of multiplexing and sensitivity. Subsequent work will aim to fully unearth the promise of this method and establish SERS as one of the primary bottom-up nanotechnology techniques for diagnostic and clinical applications.

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