Multiplex in-vitro Detection using SERS

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ABSTRACT

The ability to detect multiple disease-related targets from a single biological sample in a quick and reliable manner is of high importance in diagnosing and monitoring disease. The technique known as surface enhanced Raman scattering (SERS) has been developed for the simultaneous detection of multiple targets present in biological samples. Advances in the SERS method have allowed for the sensitive and specific detection of biologically relevant targets, such as DNA and proteins, which could be useful for the detection and control of disease. This review focuses on the strengths of SERS for the detection of target molecules from complex mixtures and the clinical relevance of recent work combining SERS with multiplexed detection of biological targets.

INTRODUCTION

The sensitive and specific analysis of biomolecules from complex mixtures is essential in the field of clinical diagnostics. The presence and progression of disease generally involves a multitude of different biomolecules; thus the detection of multiple events in tandem can reduce time and cost, as well as allowing significantly more information to be obtained from a small clinical sample.

Raman scattering is an inelastic process involving the gain or loss of energy between an incident photon and the vibrational and rotational motions of a specific target molecule.[1] The sharp, molecularly specific spectra that are obtained make it possible to specifically identify individual components from a mixture, therefore making it an ideal technique for the detection of multiple analytes. Raman scattering is a relatively weak

process with approximately only 1 in 10^6 photons being inelastically scattered.[1] However, it was discovered by Fleischmann *et al*.[2] and developed by Jeanmaire and Van Duyne,[3] that the Raman signal could be significantly enhanced by adsorbing the target molecule onto a roughened metal surface.[4] This method, known as surface enhanced Raman scattering (SERS), produced enhancement factors of 10^4-10^8 in comparison to normal Raman scattering.[5, 6] In order to achieve this enhancement, Au and Ag are the predominantly utilised metals as their surface plasmons lie in the visible region of the electromagnetic spectrum, which coincides with the laser excitation wavelengths commonly employed for Raman. The metals are often used in the form of colloidal suspensions as these are relatively easily prepared and are compatible with solution-based analysis.[7] Furthermore, Au and Ag nanoparticles can quench fluorescence, which allows the use of fluorescent dyes as Raman labels. The use of Raman labels is beneficial in obtaining further enhancement in the form of surface enhanced resonance Raman scattering (SERRS). This occurs when the analyte contains a chromophore close in energy to the exciting radiation. However, a dye label can also be used to achieve this enhancement if the chromophore is not present in the analyte itself. This combination of resonance with surface enhancement has resulted in reported enhancements of up to 10^{14} in comparison to normal Raman scattering.[8]

Although the molecule of interest does not need to be directly adsorbed onto the metallic surface for SERS to be observed, the degree of enhancement is distance dependent. This was demonstrated by Van Duyne *et al*. who found that when the distance between the target molecule and a silver substrate was greater than 2.8 nm, the SERS intensity decreased by a factor of ten.[9] Therefore, it has been shown that to observe the most intense SERS signal, the target molecule must be within a few nanometers distance from the metal surface. To enable target molecules to be within the required range, target molecules modified with thiol and amine groups have been used to enable the molecule of interest be adsorbed onto the metallic surface.[10-13] Furthermore, dyes have been specifically designed for use in SERS analysis with a chromophore, or reporter molecule, and a surface-seeking group to facilitate attachment to the enhancing metal surface.[14-17] The suitability of these dyes in a multiplex detection system has also been demonstrated.[16]

The use of SERS for the detection of biomolecules has been widely investigated and successfully applied for the direct detection of DNA,[18, 19] proteins[20] and cellular components,[21] due to their unique Raman spectra. Alternatively, as already mentioned, the target biomolecule can be labelled with a dye and detected, in this case the observation of the dye Raman spectra is indicative of the presence of the target DNA, proteins or of specific biological interactions.[22-24] SERS is an ideal method of choice for clinical target detection, owing to its high levels of sensitivity and specificity due to the characteristic fingerprint spectra obtained. SE(R)RS is a highly sensitive technique which has been shown to offer an improvement in detection limits of three orders of magnitude, in comparison to fluorescence, for the detection of dye-labelled DNA.[25] However, the main advantage SERS has compared to fluorescence spectroscopy is the ability to detect multiple components simultaneously within the same sample. The peaks obtained in Raman spectra have narrow spectral widths, allowing for ease of spectral separation between components. This is clearly advantageous over the broad fluorescence emission bands, which possess large spectral overlaps, making multiplexing more difficult and also providing limited structural information. This is also the case for colorimetric detection where SERS offers greater sensitivity and an increased potential for multiplexed analysis.[26] Culture-based methods are commonly employed in molecular diagnostics; however, these are time consuming and can be limited in sensitivity.[27] Electrochemical sensing offers quick, simple and sensitive detection but has issues with interference, long-term stability and non-specific adsorption.[28, 29] Microarray technology has also been widely investigated for diagnostic applications due to its high-throughput and multiplexing capabilities.[30-32] Microarrays can be combined with each of the detection techniques mentioned, along with many others, to achieve the desired sensitivity along with the rapid detection of multiple biological targets.[33-36] Compared to other methods available, advantages to using SERS include minimal sample preparation and the ability to detect target molecules in aqueous samples, as water exhibits very weak Raman scattering due to its small Raman cross-section.[37, 38] This allows the analysis of biological samples in a multiplex format which is ideal for clinical applications. Additionally, instrumental advances are allowing the development of handheld, portable instruments with comparatively low cost so that multiplexed analysis could potentially be performed at a bedside or in a clinician's office in a timely and accurate manner.[39, 40]

The multiplexing capabilities of SERS have been demonstrated in the field of molecular diagnostics over the last decade. A popular method of detection involves targeting specific DNA sequences that code for various diseases. These methods are mainly based on the Watson and Crick base pairing where a probe sequence complementary to the target DNA sequence of interest is labelled with a fluorescent dye.[41] There have been numerous clinical targets of interest detected, for example, different strains of the *E. coli* bacterium,[42] three forms of the cystic fibrosis trans-membrane regulator (CFTCR) gene[43] and three genes associated with methicillin-resistant *S. Aureus* (MRSA).[44] Nanoparticles have also been functionalised with DNA sequences to allow for target detection. Graham and co-workers functionalised metallic nanoparticles with DNA and used the base pairing methodology to observe an increase in SERS signal due to controlled nanoparticle aggregation induced by DNA hybridisation.[45] Following this, Vo-Dinh *et al*. developed "molecular sentinels", which consist of molecular beacons attached to the nanoparticle surface via a thiol group, for the multiplex detection of two breast cancer biomarkers.[46]

SERS multiplexing has also made an impact on the detection of proteins where SERS analysis methods have been developed for the detection of specific antigens[47] and protein interactions,[48] to monitor specific biorecognition events[49] and for the detection of cellular proteins.[50] Successful SERS analysis of enzymes has also been applied, where the action of the enzyme results in the production of SERS-active dyes.[51-54] This concept was extended to the multiplex detection of two enzymes; alkaline phosphatase and β-galactosidase.[55]

Multiplex SERS has also been extended to cellular detection. Detection of proteins *in vivo* has been possible on a multiplex level using SERS for the detection of two cell surface proteins, β_2-adrenergic receptor and caveolin-3[56] and for the detection of two co-cultured cell lines using functionalised nanoparticles that targeted epidermal growth factor receptors (EGFR) and the HER2 biomarker expressed in breast cancer cells.[57] SERS has also been used to successfully detect multiple bacterial pathogens simultaneously.[39, 58] Successful multiplex SERS analysis of tissue samples has also been achieved. For example, SERS has been applied for the detection of prostate cancer biomarkers from prostate tissue samples.[59, 60]

Therefore, SERS has been shown to be an ideal method of choice for multiplex detection and the technique meets the requirements for selective and sensitive detection in molecular diagnostics. This review will focus on the various approaches, which have been developed for the use of SERS for the analysis of biomolecules, as well as the recent advancements of SERS in multiplex detection.

DETECTION OF DNA BY SERS FOR CLINICAL APPLICATIONS

The sensitive and specific detection of DNA sequences coding for particular diseases is extremely important when trying to understand disease progression and in developing novel detection methods. Current methods of DNA detection, such as PCR and fluorescence, are limited in multiplexing capabilities and also pose issues such as contamination. The molecule-specific Raman spectra with distinct narrow bands make the technique suitable for multiplexed analysis. Furthermore, the high sensitivity of the technique means that strong Raman signals can be obtained from low sample concentrations, so that amplification is not always necessary, thus overcoming potential contaminations issues.

Label-free DNA detection using SERS

For successful SERS analysis, the analyte must be adsorbed onto or in close proximity to the enhancing metal surface. Adsorption of DNA nucleotides directly onto a metal surface can allow the direct detection of a SERS signal from the constituent bases.[18, 61-63] Barhoumi and Halas investigated the SERS of thiolated single stranded and double stranded DNA oligomers bound to Au nanoshells.[61] They found that thermal pre-treatment of the DNA prior to adsorption onto the Au nanoshell changed the conformation of the DNA molecules, resulting in improved reproducibility of the SERS spectra. They developed a spectral correlation function (SCF) to quantify differences in SERS spectra due to the chemical modification of adsorbed DNA and applied their method to monitor changes in the DNA spectrum resulting from conformational changes which occurs upon interaction with the chemotherapeutic agent cisplatin. They were able to observe greater spectral changes on interaction with cisplatin than with its analogue transplatin, which possesses a lower affinity for DNA, therefore it was

postulated that the method could be used for studying the kinetics and interaction of DNA with various molecules.[61] However, regardless of the DNA composition or base sequence, the spectra obtained from the DNA were similar and dominated by adenine due to its greater SERS cross section over the other bases. In a further study, they used this concept to show that adenine can be used as a marker in a label-free SERS assay for the detection of DNA hybridisation.[62] By substituting adenine with 2-aminopurine (2-AP) in the probe sequence, the hybridisation characteristics of the probe were maintained while significant changes in the SERS spectra were observed. This allowed the 2-aminopurine-substituted probe to be used for the detection of DNA hybridisation where hybridisation with the adenine-containing target sequence resulted in the presence of the adenine peak at 736 cm^{-1}, which was not observed in the spectrum of 2-aminopurine-substitued DNA or in the presence of the non-complementary control sequence. The ratio of this adenine peak to the 2-AP peak at 807 cm^{-1} could be used to determine hybridisation efficiency, as the 2-AP peak was constant. In both of these studies, the DNA sequences were thiolated to facilitate adsorption onto the metal surface; however, although label-free, this method of adsorption still requires modification of the DNA.[61, 62] Additionally, non-specific adsorption of the DNA bases may occur which will result in different orientations on the metal surface compared to specifically oriented, covalently attached thiol modified DNA sequences. Papadopoulou and Bell have shown that differences in orientation of thiolated DNA sequences on a metal surface can result in variations in the spectra obtained.[64] They also investigated the variation in SERS spectra of the DNA base adenine, deoxyadenosine and deoxyadenosine-5'-monophosphate (5'-dAMP) with changes in experimental conditions such as pH.[65-67] Recent work from this group has shown that SERS detection of unlabelled single and double stranded DNA is possible without the need for labelling, thiolation or use of linkers.[19, 68] By allowing spontaneous adsorption of the DNA onto Au and Ag nanoparticles via the nucleotide side chains, spectra were recorded with high signal to noise and excellent reproducibility. Changes in Raman bands were observed corresponding to each of the bases, which could be attributed to the difference in scattering cross sections between the different bases. This method has been applied to the detection of single base mismatches, in this case spectra were collected for different DNA sequences before being digitally subtracted to give a difference spectrum with positive and negative features corresponding to the changes in base sequence.[19] An

extension of this work enabled the detection of both single and double stranded DNA at 10^{-9} M, as well as the separation of five DNA sequences corresponding to five different strains of *E. coli* bacteria without any need for multivariate analysis.[68] Figure 1 shows the spectra obtained for the five different *E. coli* DNA sequences as well as the number of each of the four bases present in each strand. It can be observed that visible differences are present in the spectra, relating to the different compositions of the DNA sequences. For example, the adenosine ring breathing mode at 737 cm^{-1} and the large adenosine band at 1329 cm^{-1} are more intense in the spectrum of strains 4 and 5 where there are more adenosine nucleotides present. This shows that the simple method can be applied for the detection of biologically relevant samples without any need for modification or labelling. They also observed small changes in spectra when the order of the bases was changed, indicating that this method could also potentially be used to obtain information on the order of bases in the DNA sequence.

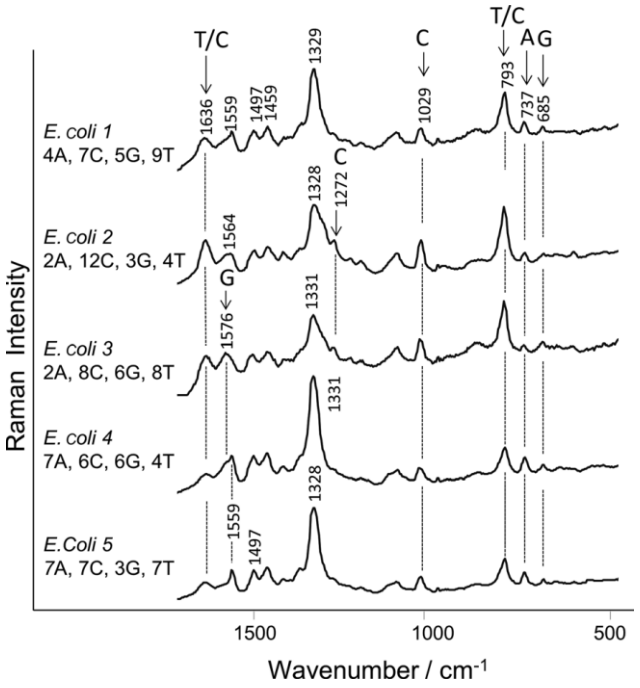

Figure 1. *SERS spectra of five single stranded DNA sequences corresponding to five different strains of E. coli, recorded on hydroxylamine hydrochloride reduced silver nanoparticles at 10^{-6} M DNA. The number of each of the four nucleotide bases are indicated for each sequence.[68] Reprinted by permission of John Wiley and Sons. Copyright © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.*

Guerrini *et al*. have recently applied the use of spermine-stabilised silver nanoparticles for the direct analysis of DNA duplexes.[69] The positively charged silver nanoparticles, originally prepared by van Lierop *et al*.,[70] were able to undergo controlled aggregation, which provided the hotspots required for SERS but without over aggregation of the sample. This means that no aggregating agent is required as the DNA itself induces the aggregation into small clusters via electrostatic interactions. As well as simplifying the analysis, this increases stability which permits analysis over a greater period of time. Using these nanoparticles, significant information could be obtained without a need for labelling or DNA amplification. Furthermore, single base mismatches and base methylations could be detected from DNA duplexes for the first time using SERS. The sensitive assay was highly reproducible between batches, providing a useful and efficient method of analysis.[69]

SERS detection of dye-labelled DNA

Although label-free detection has its advantages, it is limited in its capabilities for diagnostic applications as it is very challenging to accurately determine the specific order of the bases in the DNA sequence from the SERS spectrum. The use of dye-labelled DNA allows the monitoring of signal variations arising from the presence or absence of target sequences related to disease. In these applications the target DNA will not be labelled, therefore a sequence complementary to the target is generally utilised and labelled so that when hybridisation to the target sequence occurs, a change in the signal arising from the dye label can be observed, allowing detection of a specific target sequence. Hybridisation events give the required selectivity as these are based on Watson and Crick base pairing,[71, 72] while the narrow bands present in the SE(R)RS spectra of dyes introduce the potential for multiplexed analysis. Various approaches for the SERS detection of dye-labelled DNA sequences have been investigated, with sensitivity approaching the level of single molecule detection.[14, 24, 41, 73-77] Many of these assays use spermine hydrochloride to facilitate the adsorption of the DNA to the metal surface for successful SERS analysis. The spermine forms electrostatic layers, allowing the negative DNA to adsorb onto the negatively charged nanoparticle surface. Furthermore, this induces aggregation forming the hotspots required to obtain intense SERS signals.[73]

When dye labels are used, careful consideration of the properties of the dye must be made in order to achieve optimal SERS signals and the largest possible discrimination between target and controls. It is important to consider how the dye may interact with other assay components as this could affect overall performance. In a recent study, Gracie *et al*. compared two bases, spermine and triethylamine (TEA), and optimised the experimental conditions for FAM- and TAMRA-labelled DNA.[78] They found that dye-spermine and dye-DNA interactions cause changes in the observed fluorescence intensity, which is also dependant on experimental conditions such as the pH and concentration. It was also observed that these fluctuations in intensity are dependent on the particular dye label used and therefore careful consideration must be made when considering the experimental design for SERS-based DNA detection using dye-labelled DNA. This is of particular importance for multiplex assays where multiple dyes are used, since changes in signal could be mistaken for varying amounts of target.

When DNA is double stranded and therefore in its coiled conformation, the negative charge of the backbone results in repulsion from the negatively charged nanoparticle surface. In contrast, single stranded DNA is uncoiled with the bases exposed, which have an affinity towards the negative metal surface.[68] Therefore, adsorption of single stranded DNA to a colloidal metal surface is more efficient than that of double stranded DNA, which is clearly a significant advantage when using SERS for the detection of DNA. This concept has therefore been extensively studied and exploited in DNA detection assays.[44, 79, 80] Following on from a study by MacAskill *et al*.,[44] Harper and co-workers studied the varying affinity of dye-labelled DNA to the surface of silver nanoparticles and the effect these differences have on the overall SERS response.[81] A difference in the intensity between dye-labelled single stranded and double stranded DNA was observed, with the single stranded DNA giving the largest response. It was also found that no SERS signal was obtained for the dye alone under the conditions used, indicating that without the DNA the dye doesn't get close enough to the nanoparticle surface, thus proving that the DNA rather than the dye drives the adsorption. Importantly, it was shown that by optimising experimental conditions such as pH, volume of colloid and salt concentration of buffers, the largest possible discrimination between single and double stranded DNA could be achieved so that the system can be successfully applied to assays that use the difference in SERS response of single and double stranded DNA for the detection of target DNA. Therefore when designing an assay it is ideal that single

stranded DNA is detected rather than double stranded DNA, however, commonly the case is the opposite where the DNA becomes double stranded upon hybridisation with the target DNA sequence. This would result in a negative assay where the SERS signal is "on to off" rather than "off to on".[44] Although a change in signal is still observed, negative assays are unfavourable as it can be difficult to distinguish between a reduction in signal due to the presence of target or because of other reasons such as poor assay performance. Furthermore, it can be very challenging to multiplex using this assay type. In order to overcome the disadvantages of negative assays, van Lierop *et al*. developed a SERS primer assay.[79] In this work, the target sequence was the femA gene of *Staphylococcus epidermidis*, which can be used for bacterial identification of *Staphylococcus epidermidis* and *Staphylococcus aureus*. The SERS primer contains a dye-labelled region that was rendered single stranded when DNA hybridisation occurs with a specific target sequence. When these SERS primers are closed, they are predominantly double stranded DNA and therefore do not adsorb strongly onto the nanoparticle surface (Figure 2). However, when target is present, it will hybridise to the SERS primer, which then opens the partly self-complementary region of the primer, that contains a single stranded region with a dye label attached, allowing for adsorption onto the nanoparticle surface resulting in an increased SERS response. This separation-free, positive assay was successfully applied for the detection of femA bacterial DNA, as well as PCR product from the amplification of 1 ng genomic DNA from *Staphylococcus epidermidis*, showing the applicability of the SERS method for the detection of biologically relevant samples.

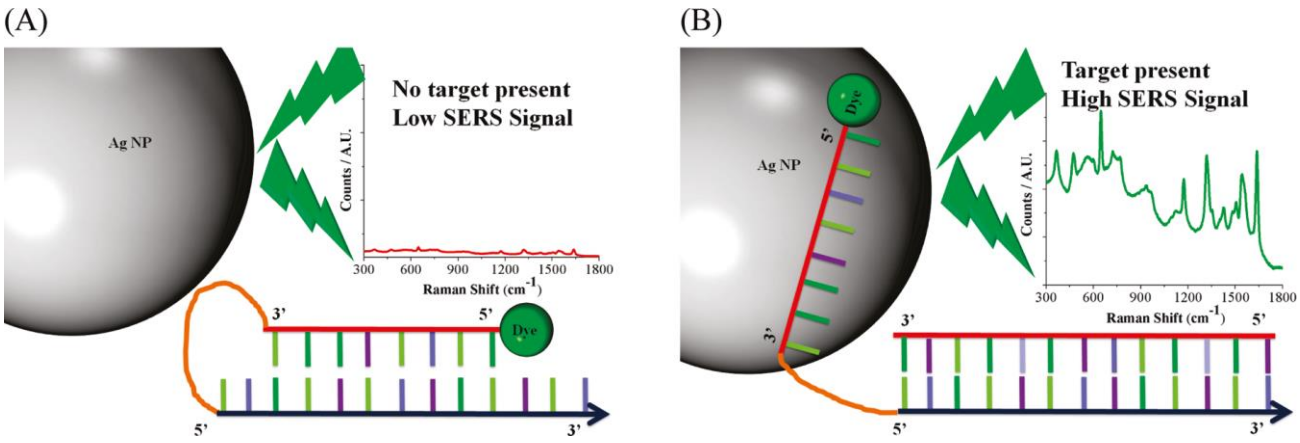

Figure 2. *When no target is present (A), the SERS probe is closed and the DNA is double stranded and therefore unable to adsorb onto the nanoparticle surface. When complementary target is present (B) this displaces the partly self-complementary region of the primer, destabilised by mismatches, leaving the dye-labelled single stranded DNA able to adsorb onto the nanoparticle surface to give an increased SERS response.*[79] *Reprinted with permission from D. van Lierop, K. Faulds and D. Graham,* Anal. Chem.*, 2011, 83, 5817-5821. Copyright © 2011 American Chemical Society.*

The optimisation of the SERS primers and demonstration of their capabilities in different assay types was further investigated to determine how the SERS primers performed in model assay systems.[82] The synthetic model systems were used to optimise parameters such as the specific design of the primers, type of nanoparticles and the nanoparticle and analyte concentration. The results of these experiments led to the development of a new assay which involved the SERS primers being incorporated into PCR product and combining this with the 5'-3' exonuclease activity of *Taq* DNA polymerase.[82]

Harper *et al*. developed a new detection assay based upon the commonly used TaqMan assay, where SERS detection was used to improve sensitivity over the commonly employed fluorescence detection.[83] TaqMan assays involve enzymatic probe cleavage where the TaqMan probe contains both a fluorophore and a quencher, connected by a DNA sequence, allowing quenching of the fluorescence due to the close proximity of the fluorophore and the quencher.[84, 85] The TaqMan probe hybridises to the target sequence at a site adjacent to the primer binding site and, during PCR, the *Thermus aquaticus* (Taq) polymerase enzyme will both amplify the target DNA and, in the process, digest the TaqMan probe. Upon digestion, the distance between the fluorophore and the quencher will be increased resulting in increased fluorescence signal, which is proportional to the amplification of the target sequence. The TaqSERS

assay developed in this work required the design of a DNA probe containing a SERS-active dye compatible with the enhancing metal surface. The optimum probe design included a 5' biotin followed by a 29 base sequence complementary to the target gene sequence, which was a sequence within the MRSA bacterium. The 3' modification consisted of a HEG-spacer, to terminate enzyme progression, followed by 10 adenine bases and, finally, a 3' TAMRA dye. The 10A base region attached to the TAMRA acts as a tail and facilitates adsorption of the DNA onto the colloidal surface since dye-labelled DNA gives a stronger SERS response than dye alone, this results in an increase in SERS signal.[83, 86] Following amplification of the template and digestion of the probe to separate the biotin and the TAMRA dye, any undigested probe was removed from the mixture using streptavidin-coated magnetic beads so that SERS signal was only obtained from the TAMRA from the digested probe (Figure 3). As well as the target sequence coding for the methilin resistant mecA gene from MRSA, the authors analysed a nonsense sequence, which did not contain a region complementary to the mecA probe region so that no hybridisation or digestion should occur. They found that, in comparison to the nonsense sequence, a much more intense SERS signal from the TAMRA dye was observed in the presence of the target sequence, proving that the assay was sensitive and specific to the target. The sensitivity of the assay was also studied and a comparison was carried out between SERS detection and fluorescence detection, which is normally the method of choice for this assay type. It was shown that this new TaqSERS assay could achieve detection limits an order of magnitude lower than when using the more conventional fluorescence detection method. In addition, the novel assay with SERS detection overcomes issues such as high background signal and broad overlapping peaks, which limit the use of the assays for simultaneous detection of multiple DNA sequences by fluorescence.

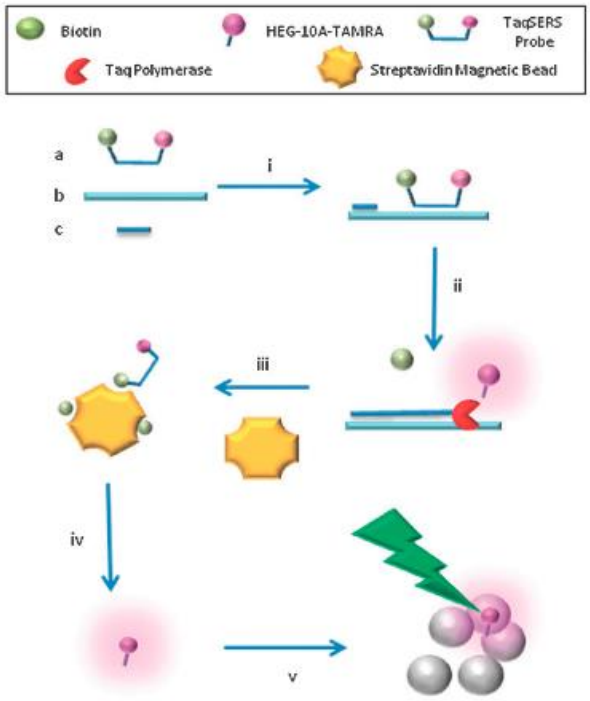

Figure 3*. TaqSERS assay; (i) hybridisation of TaqSERS probe, a, target sequence, b, and primers, c, (ii) Taq polymerase enzyme simultaneously elongates primers and digests probe, (iii) streptavidin-coated magnetic beads are introduced to remove any undigested biotinylated probe and free biotin, (iv) magnet removes beads from system leaving TAMRA labelled DNA "tail" in supernatant, (v) dilute citrate-reduced silver nanoparticles with spermine hydrochloride (0.1 mol dm^{-3}) were added. Within 5 min the SERS spectrum was recorded using 514.5 nm laser excitation.*[83] *Reproduced from Ref. 71 with permission from The Royal Society of Chemistry.*

In an alternative approach, Dougan *et al.* developed an exo-SERS signal amplification assay using the enzyme lambda exonuclease, where the aim was to amplify the signal rather than the amount of target present, for the detection of *chlamydia trachomatis*.[86] A reporter probe was designed which was 5' phosphorylated for enzyme recognition, with a 15 base detection region which was complementary to a region of the target sequence. This was followed by a HEG spacer to stop the enzyme activity then 10 adenines that act as a tail and a 3' dye (TAMRA) label. To reduce the possibility of non-specific adsorption and digestion and allow separation of unhybridised probe, a split probe system was designed where the reporter and biotinylated capture probes hybridise to the same target sequence in a sandwich format (Figure 4). The target, reporter and capture probes were hybridised before being captured on streptavidin-coated magnetic beads. After thorough wash steps to remove excess probe, lambda-exonuclease was added and DNA digestion was allowed to occur. Supernatant was

separated from magnetic beads and added to citrate-reduced silver nanoparticles followed by addition of spermine hydrochloride and SERS analysis using 532 nm laser excitation wavelength. TAMRA signal was observed in the presence of target but not when any components were missing from the assay. To determine if the assay was quantitative, the concentration of target was varied with all other components remaining constant. A linear concentration versus intensity curve was obtained, which was impressive considering the number of events taking place (hybridisation, washing and enzyme activity) and proves that the assay was working effectively. When the assay was applied to *chlamydia trachomatis* PCR product, a clear distinction was observed between target and nonsense DNA sequence and a detection limit was obtained which was comparable to molecular beacon assays.

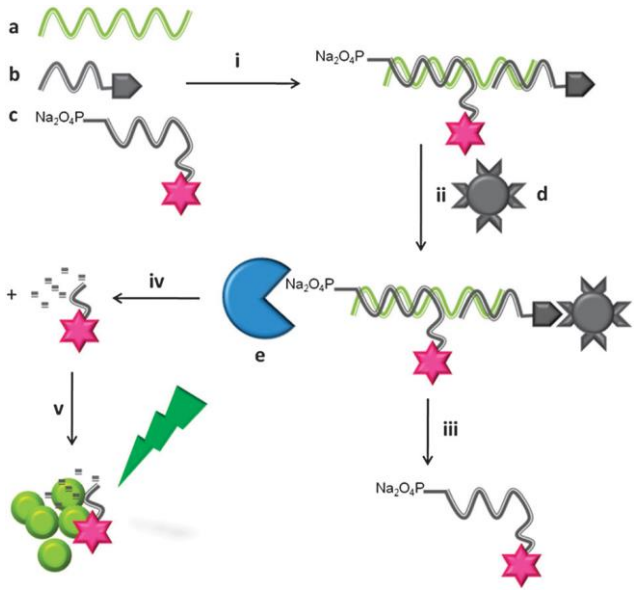

Figure 4. *Schematic representation of the exo-SERS assay. Hybridisation between the unlabelled target, a, the capture probe, b, and the reporter probe, c. (ii) the duplex is captured on streptavidin-coated magnetic beads, d, before washing to remove excess probe (iii). (iv) lambda-exonuclease, e, is incubated with the beads and the supernatant is removed and added to diluted citrate-reduced silver nanoparticles with the addition of spermine hydrochloride (v) before SERS spectra were recorded at 532 nm laser excitation wavelength.*[86] *Reproduced from Ref. 74 with permission from The Royal Society of Chemistry.*

Although these assays have been applied for the detection of clinically relevant targets and have shown potential for multiplexed analysis, the detection of multiple targets was not demonstrated in the work discussed. Since the focus of this review is multiplex

detection, the following section will discuss how these different assay formats have been applied for the detection of multiple DNA sequences using SERS.

Multiplex detection of disease-related DNA targets

The simultaneous detection of multiple disease-related DNA sequences from one sample could result in simplified, efficient and cost-effective detection and diagnosis of diseases. Due to the compatibility of SERS for the analysis of biological samples, as well as the advantages of SERS for multiplexed analysis, the technique is a suitable candidate for the detection of multiple clinical targets. Consequently, SERS has been extensively investigated for the multiplexed detection of DNA sequences and has been successfully applied for the analysis of biologically relevant targets.[43, 44, 76, 77, 87-90] For example, the multiplexed detection of DNA sequences has been carried out to identify 5 different labelled DNA sequences using two excitation wavelengths without the need for any data analysis.[76] The detection of 6 labelled DNA sequences was also carried out using one excitation wavelength with the aid of chemometrics for deconvolution of the data[77] and also using Bayesian statistics methods.[91]

Recently, Gracie *et al*. further developed the exo-SERS split probe assay discussed previously (Figure 4) for the detection of multiple gene sequences.[92] They applied the assay, based on the activity of lambda-exonuclease, for the detection of three bacterial meningitis pathogens: *Neisseria meningitidis*, *Streptococcus pneumonia* and *Haemophilus influenzae*. By using chemometrics, each of the three pathogens in the multiplex were quantified. The detection of each pathogen was carried out individually to determine the sensitivity of the assay, followed by the design of a triplex to detect the three DNA sequences simultaneously, with chemometric analysis for the quantification of each. A synthetic target pathogen sequence was hybridised to two DNA sequences, one modified with biotin and the other with a fluorescent dye and 5'-phosphate. Streptavidin-coated beads were added to attach to the fully formed duplex DNA before wash steps were carried out to remove any excess/unhybridised DNA from the mixture. Lambda-exonuclease was then added to digest the 5' phosphate modified probe (with the fluorescent dye attached) resulting in release of the dye from the DNA duplex, attached to the streptavidin-coated beads. The digestion products were then added to

citrate-reduced silver nanoparticles with addition of spermine prior to analysis by SERS. For each of the three pathogens, picomolar detection limits were obtained using FAM, TAMRA and Cy3 as labels and 532 nm laser excitation wavelength. The lower detection limit was comparable to the previous use of the assay for the detection of *chlamydia trachomatis*.[86] PCR was then carried out on plasmid DNA from each pathogen and the assay was applied for the detection and quantification of the target sequences from PCR product. Even though the spectra are similar, peak differences could be observed between each of the dyes and so multiplexing was possible. Figure 5 shows the spectrum obtained for each individual dye, corresponding to each of the three bacterial meningitis pathogen DNA sequences, as well as the spectrum for the multiplex sample. Here it can be clearly observed that there is at least one peak exclusively present in each dye spectrum and the individual peaks for each dye can also be observed in the multiplex spectrum, indicating that each of the three pathogens can be identified from the mixed sample. Further to the detection of each individual pathogen, a chemometrics model was built which was able to quantify each of the pathogens in the sample. This was the first time SERS was used for the detection and quantification of each component in the multiplex, with consistent results obtained much quicker than with the conventional culture-based method. This assay is potentially applicable to a wide variety of diseases and thus shows promise in clinical applications. This has recently been demonstrated when the assay was applied to the simultaneous detection of two bacterial pathogens in clinical samples obtained from meningitis patients.[93]

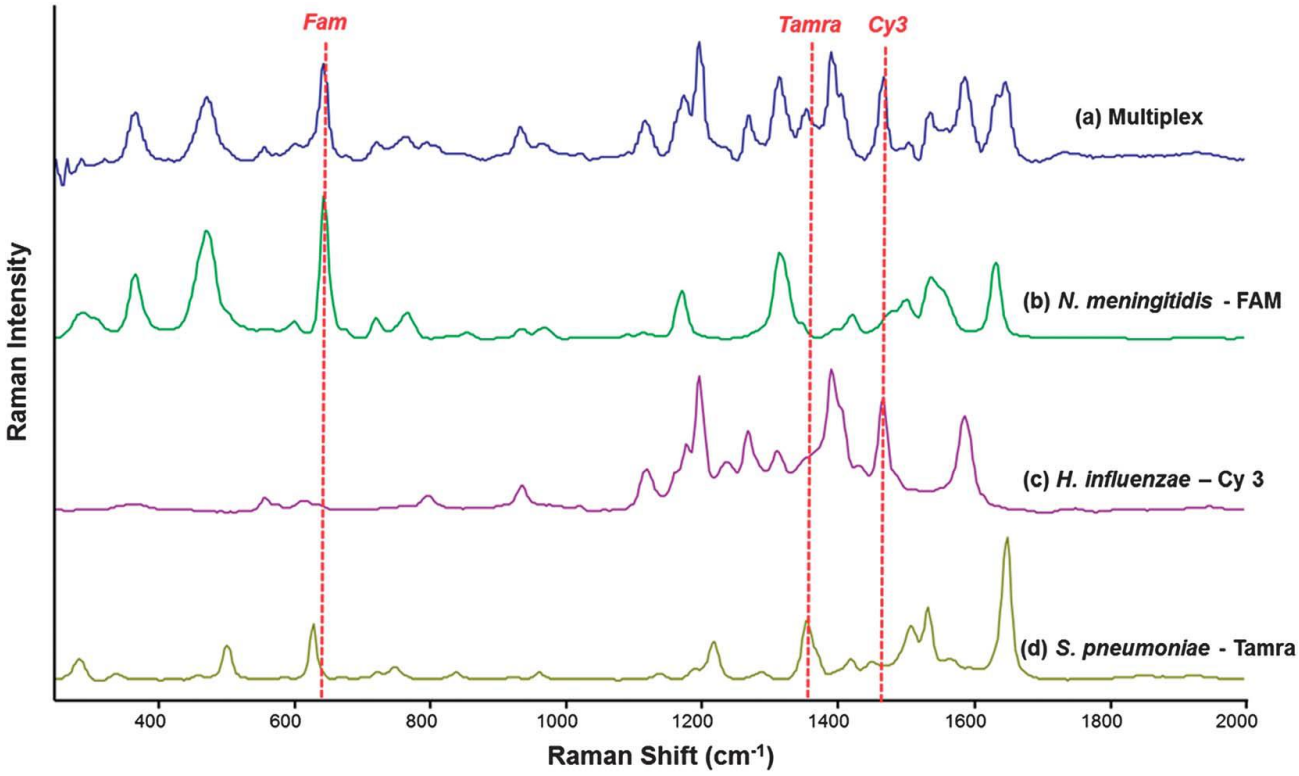

Figure 5. *SERS spectra obtained from the simultaneous detection of all three bacterial meningitis pathogens using the Exo-SERS assay (a) and the SERS spectra obtained from the PCR product of each pathogen separately; N. meningitidis (b), H. influenzae using (c) and S. pneumonia (d). Red dotted lines show peaks that are unique to each SERS spectrum and hence each pathogen.*[92] *Reproduced from Ref. 80 with permission from The Royal Society of Chemistry.*

Another approach in SERS-based detection of DNA is using nanoparticles functionalised with two non-complementary DNA sequences, which are both complementary to the target sequence. Therefore, when the target is introduced, hybridisation occurs resulting in the aggregation of the sample. This nanoparticle assembly has been exploited for the detection of DNA sequences for several years, the first reported by Graham *et al.* in 2008 where three different DNA sequences were detected using DNA-functionalised silver nanoparticles combined with SERS-active reporter molecules.[45, 94-97] This method has recently been combined with the use of magnetic manipulation for the sensitive detection of two DNA sequences, both individually and in a duplex.[98] In this work, Donnelly *et al*. used DNA-functionalised silver nanoparticles and DNA-functionalised magnetic nanoparticles, where the nanoparticles were each functionalised with a different 12-base DNA sequence that were both complementary to a section of the 24-base target DNA sequence. The silver nanoparticles were also functionalised with a SERS reporter molecule so that when the target was introduced,

hybridisation occurred between the two probes resulting in aggregation of the nanoparticles due to the reporter molecules location in hotspots between the particles. Therefore, in the presence of the target DNA, a strong SERS signal could be obtained from the Raman reporter. An external magnet was used in order to concentrate the sample and excess material was removed by washing prior to direct SERS analysis of the magnetic plug. This assay was used for the detection of specific *Candida* fungal species important in the detection of fungal infections: *C. krusei* was detected by monitoring the Raman spectrum of 4-mercaptopyridine (MP) and *C. albicans* using malachite green isothiocyanate (MG) as the reporter on the silver nanoparticle conjugates. A detection limit of 20 fmol was obtained for each of the two target sequences, giving a ten-fold improvement over using suspension-based systems. The assay was then applied for the detection of both sequences using a mixture of the two probes and a strong MP signal was obtained in the presence of the *C. krusei* target sequence and the MG signal was strongest when the *C. albicans* was present. Although small background signals were observed, the presence of each target gave clearly distinguishable results.

The "molecular sentinel" (MS) technique was developed by Vo-Dinh's group for the sensitive and specific detection of multiple DNA sequences.[46] In this approach, two MS nanoprobes were designed for the detection of separate DNA sequences. The probes consist of a DNA hairpin with a Raman probe at one end and a thiol group at the other to attach to the metal surface. Without the target DNA, the MS probe is in a hairpin loop so that the Raman reporter is close to the metal surface resulting in a high SERS signal. Hybridisation of a complementary sequence separates the probe from the metal surface thus decreasing signal. Since SERS decreases when the target hybridises to the probe, this is a negative "on to off" assay which has disadvantages as discussed previously. However, the assay has been successfully applied for the detection of multiple DNA sequences without a need for target labelling or wash steps. This work has recently been further developed into a SERS-based molecular sentinel-on-chip (MSC) assay (Figure 6).[99] This MSC assay involved the functionalisation of a nanowave chip with MS probes to detect DNA sequences for the diagnosis of viral infections. The nanowave chip used in this work was a plasmonic substrate developed by Vo-Dinh in 1984.[100] This metal film over nanosphere (MFON) is a close-packed nanosphere substrate with a thin gold film that provides a high enhancement factor with low production costs and

high reproducibility. In the current work, a bimetallic film (Ag and Au) was used, as opposed to the Au film used previously, to provide further SERS enhancement. To investigate this enhancement, the authors fabricated Au film over nanosphere (AuFON) and bimetallic film over nanosphere (BMFON) substrates and found that BMFON exhibits a 3.6 times greater SERS signal than the AuFON. This can be explained by the fact that the BMFON has greater surface roughness than the AuFON and its LSPR is red-shifted due to the greater nanoprotrusions.

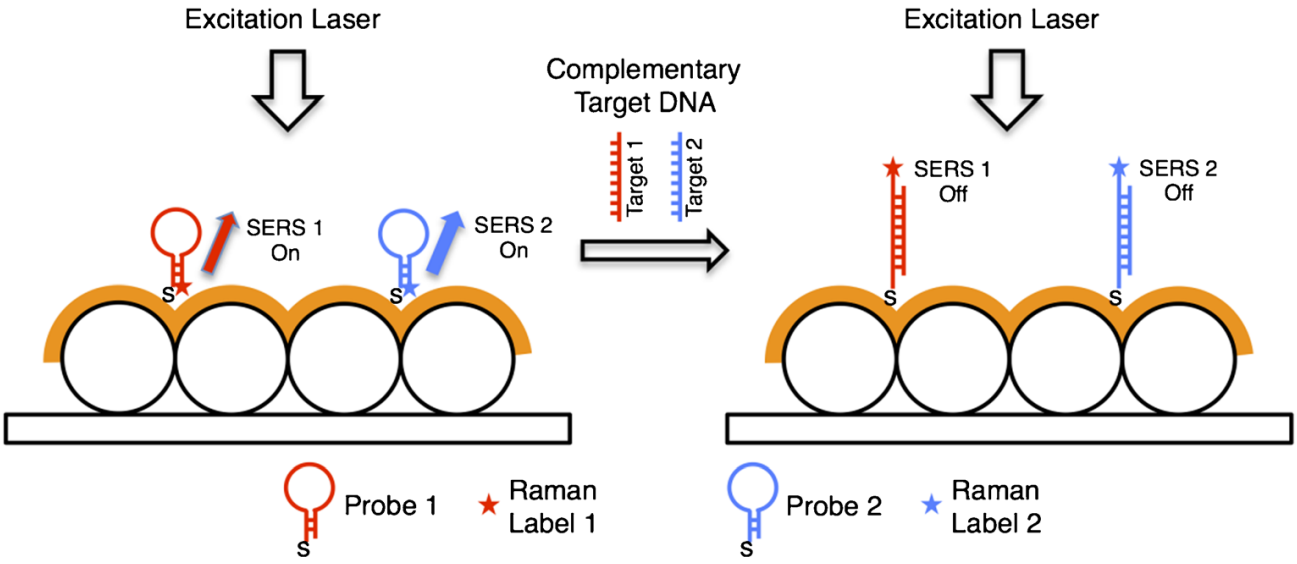

Figure 6. *Schematic representation of the MSC approach for the multiplex detection of two target DNA sequences. BMFON substrates are functionalised with MS probes. When no target is present, the hairpin loop is closed and SERS signal is observed. On hybridisation with the target DNA sequence, the probe is opened resulting in a decreased SERS signal due to separation of the reporter molecule with the metal surface.*[99] *Reprinted from Springer, Analytical and Bioanalytical Chemistry, volume 406, 2014, page 3338, H. T. Ngo, H. N. Wang, T. Burke, G. S. Ginsburg and T. Vo-Dinh, Figure 1, © Springer-Verlag Berlin Heidelberg 2014. With kind permission from Springer Science and Business Media.*

MS hairpins were functionalised at the 3' ends with two Raman labels (Cy5 and ROX) with an alkyl thiol group at the 5' end of each probe for attachment to the metallic substrate. Each hairpin was at least 35 bases (~10 nm) in length to allow for effective separation of the probe from the metal surface upon hybridisation. The BMFON substrates were functionalised with the MS probes, interferon alpha-inducible protein 27 (IFI27) and interferon-induced protein 44-like (IFI44L), both individually and as a mixture in order to demonstrate the separate detection of the targets, as well as

multiplex detection. For individual detection, a decrease in the SERS signal for the dye label (Cy5 or ROX) was observed when single stranded complementary DNA was present, compared to when no ssDNA was present or when non-complementary DNA was present. This proves that the Raman labels were separated from the nanowave surface when hybridisation occurred between the probe and single stranded complementary target DNA. When a mixture of the two nanoprobes were analysed, a reduction in signal for each dye was observed when the complementary DNA was present and for the individual probes when only complementary target was introduced. This change in signal from "on to off" when the relevant targets are present shows that the system can be used for multiplex detection; however, the authors used a parameter they developed previously[101] called relative diagnostic index (RDI), to convert the change in signal to an "off to on", since a positive assay is usually preferred. This assay was the first use of the MSC approach for the label-free multiplex detection of genetic disease biomarkers. The fact that the assay requires only a single hybridisation step and has no need for washing is advantageous, as is the lack of requirement for labelling the target molecules. The same group have recently developed the first "off to on" SERS nanobiosensor for the detection of DNA.[102] This was a proof of concept for the novel "off to on" system and so it has yet to be applied for multiplexed detection, however, a limit of detection of approximately 0.1 nM for target DNA was obtained. Since the authors used significantly small amounts of sample (2 μL), they estimated the absolute limit of detection to be 200 amol.

Kang *et al*. have developed a Au particle-on-wire system which involves the self-assembly of Au nanoparticles onto Au nanowires in the presence of target DNA, resulting in a SERS signal which is proportional to the amount of target present in the sample.[103] Au nanowires were functionalised with thiolated DNA probes and Au nanoparticles were functionalised with reporter DNA molecules containing a Cy5 label. After blocking remaining sites on the Au nanowire surface, incubation with target DNA, which contains sequences complementary to the immobilised DNA probes and the reporter DNA, results in the capture of the target DNA in a sandwich format while creating SERS hotspots between the nanowire and nanoparticle gap (Figure 7(a)). This results in a high SERS signal being obtained characteristic of the Cy5 Raman reporter (Figure 7(b)), which increased with increasing concentration of target DNA resulting in a lower detection limit of 10 pM.

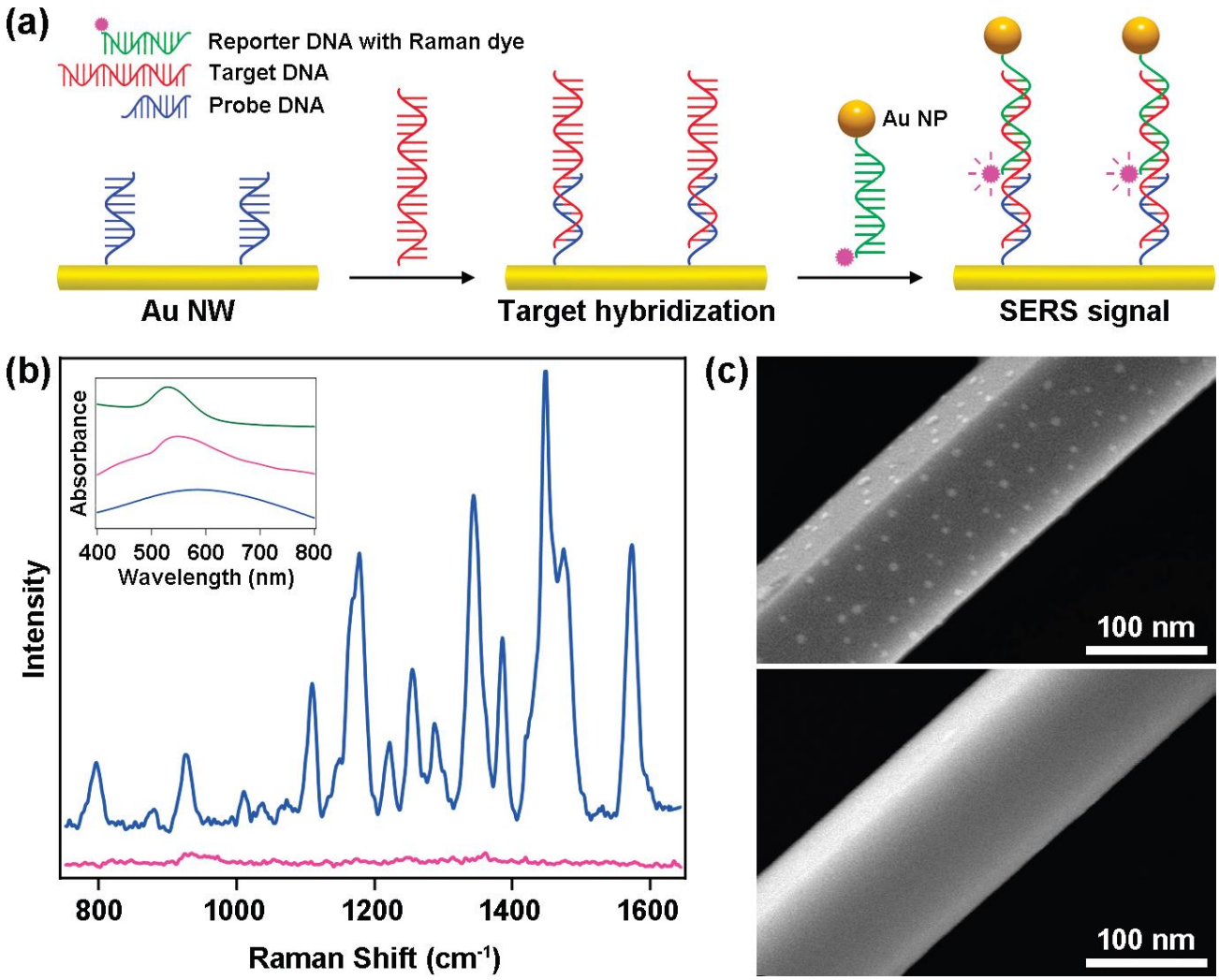

Figure 7. *(a) Schematic representation for the detection of target DNAs by Au particle-on-wire system. (b) SERS spectra collected using 633 nm laser excitation in the presence of complementary target-reporter DNA sequences (blue spectrum) and that of a single Au NW prepared by non-complementary target DNA sequences (magenta spectrum). The inset is UV-vis absorption spectra of Au NPs (green spectrum), Au NWs (magenta spectrum), and Au particle-on-wire systems (blue spectrum) illustrating why the 633 nm laser excitation was selected. (c) SEM image of a typical Au particle-on-wire structure constructed by adding complementary target DNA sequences (top) and a clean NW in the presence of non-complementary target DNA (bottom).*[103] *Reprinted with permission from T. Kang, S. M. Yoo, I. Yoon, S. Y. Lee and B. Kim,* Nano Lett.*, 2010,* ***10****, 1189-1193. Copyright © 2010 American Chemical Society.*

To test the system in a multiplex format, the Au nanowires were functionalised with two different probe sequences and incubated with a mixed solution of two target DNA sequences. Distinguishable SERS spectra were obtained for each of the two Raman reporters, Cy5 and TAMRA, with no cross-hybridisation occurring. The Au nanoparticle-on-wire system was then applied for the simultaneous detection of PCR product of four pathogenic DNA sequences: *Enterococcus faecium*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* and *Vibrio vulnifus*, to demonstrate its applicability as a diagnostic assay. Although a decrease in SERS signal was observed

when all four targets were present, each pathogen could be correctly identified from the mixture. Furthermore, the assay was applied for the detection of the four bacterial pathogens from real clinical samples, where target pathogenic DNA was extracted from cerebrospinal fluid, stool, pus and sputum, with results comparing well to those obtained from the conventional culture-based method. This system has therefore shown significant promise for application in clinical diagnostics and demonstrates the potential of SERS as a detection technique for bioanalysis of clinical samples.

Au nanoparticle-decorated silicon nanowire arrays (AuNPs@SiNWAr) have been reported to give an excellent enhancement factor when used as a SERS substrate.[104-106] Wei *et al*. have recently utilised these substrates for the multiplexed detection of DNA using a molecular beacon-based system.[107] Dye-labelled stem loop DNA sequences were immobilised onto the AuNPs@SiNWAr and in the absence of target DNA, a SERS signal is obtained from the dye label. When target DNA is present, the stem loop opens, separating the dye molecules from the Au nanoparticle surface and resulting in a decrease in signal. This was applied for the detection of synthetic DNA sequences with a 10 fM detection limit which is comparable to positive assays, although this approach still has the disadvantages of being a negative assay. Nonetheless, the assay was applied for the detection of three DNA strands, labelled with three different dyes, and distinguishable signals were obtained. Since the fabrication of these substrates is low cost and accessible, the authors envisage potential for this technique in SERS–based sensing applications.

The use of SERS for the detection of disease-related target DNA sequences and the advancements in methods for application in multiplexed analysis have been discussed herein. Parallel to the success in DNA analysis, SERS has also been applied for the detection of multiple proteins relating to disease. The following section focusses on the development of SERS-based methods for the detection of proteins for diagnostic applications.

SERS-BASED PROTEIN DETECTION FOR CLINICAL APPLICATIONS

The understanding of specific protein interactions can provide abundant information on particular biological pathways, especially in disease progression. Furthermore,

detection of specific disease-related protein biomarkers can be invaluable for the detection and diagnosis of disease. Compared to amplification methods used for DNA, such as the polymerase chain reaction (PCR), there are no such available methods for protein amplification. Therefore, it is crucial that highly sensitive detection methods are developed for the detection of the low concentrations of proteins present in biological samples. Highly sensitive methods involving electrochemistry[108-110] and fluorescence spectroscopy[111, 112] have been developed for the specific detection of protein-protein interactions. However, as discussed previously, SERS has some advantages over these methods and the technique has therefore been extensively applied for the analysis of proteins, with detection at the single molecule level being achieved.[113-115] Although there are potential limitations to a direct SERS approach, mainly the permanent damage to the native protein structure, these issues can be overcome with careful consideration of the experimental conditions and the SERS substrate used for analysis.[116-120]

Label-free protein detection by SERS

Proteins can be referred to as simple, where they consist of only amino acids, or they can be conjugated, where the protein is covalently attached to a prosthetic group. SERS studies of simple proteins was performed many years ago,[121, 122] where the focus was on the method of attachment of the proteins to the metallic surface, for example, through a carboxyl group or an amine group. Haemoproteins are the most commonly known conjugated proteins characterized by SERS. The SERS spectra of haemoproteins are easier to interpret compared to the spectra of simple proteins, as almost all of the SERS bands arise from the haem group. An example of this is the extensive SERS analysis of cytochrome *c*, where information on the molecular orientation,[117, 123] electron transfer mechanism[124, 125] and the ability to detect single molecules was achieved.[126, 127] SERS of haemoglobin has also been reported,[113] and further to this, the oxygen released from haemoglobin monitored by SERS.[128] Moreover, Feng *et al*. have also successfully characterised the native structure of myoglobin using SERS.[116]

More recently, novel methods have been developed based on a label-free detection approach. Wang *et al.* successfully monitored the progression of colorectal cancer using

the SERS spectra obtained from serum proteins and compared the spectral differences between serum proteins from cancer patients and those from healthy volunteers.[129] Principle component analysis (PCA) and partial least square regression (PLS) demonstrated the high level of accuracy of the proposed SERS method with 99.5% and 93.5% levels of accuracy obtained, respectively. Another novel SERS method for the detection of adenoviral conjunctivitis was proposed by Choi *et al*. in 2014.[130] The method, which involved drop-coated deposition surface enhanced Raman scattering (DCD-SERS), required very low sample volumes (2 μL) and was shown to give highly reproducible SERS spectra. Erythropoietin was also detected using SERS without the need for Raman labels with high levels of sensitivity (LOD 3.5×10^{-13} M).[131] The substrate used here was composed of gold nanoparticles with an ultrathin silica shell and an erythropoietin-specific antibody attached to the surface. The detection was based on the conformational changes that the antibody undergoes in the presence of erythropoietin by comparing the SERS spectra of unreacted substrate to that in the presence of the specific target protein. This method was also extended to the successful detection of caffeine.[131]

C-reactive protein (CRP) is a biological marker of infection and inflammation. Sensitive detection of the protein is therefore important for diagnostic applications.[132, 133] Kim *et al*. have recently developed a label-free SERS method for monitoring CRP-ligand specific interactions.[134] They used a substrate comprised of concentration-induced silver nanoparticle aggregates that possessed a phosphocholine-terminated self-assembled monolayer, with a high affinity for CRP. Due to the short distance between the CRP and the metallic surface (<0.4 nm), a high level of sensitivity was obtained with a reported limit of detection of approximately 100 fM. Most recently, Kahraman *et al.* designed a metallic 3D structure to detect six different proteins (bovine serum albumin (BSA), haemoglobin, thrombin, avidin, cytochrome *c* and lysozyme) using SERS without the need for Raman labels.[135] The metallic structures were produced via nanopatterning with latex nano/microparticles combined with Cr and Ag sputtering. This method generated nanovoids within the 3D structure that allowed for the generation of protein specific SERS spectra that were background free with reported protein concentrations of 0.05 μg/mL. PCA analysis was used, which demonstrated the significant differences present in the SERS spectra between the six proteins, allowing for successful label-free SERS detection (Figure 8).

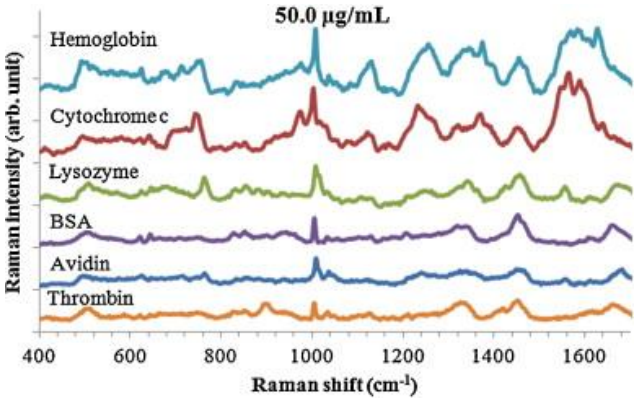

Figure 8. *SERS spectra of hemoglobin, cytochrome c, lysozyme, BSA, avidin, and thrombin dropped on the nanovoid structures. All protein concentrations are 50.0 µg mL^{-1}.*[135] *Reprinted from Analytica Chimica Acta, Vol 856, M. Kahraman and S. Wachsmann-Hogiu, Label-free and direct protein detection on 3D plasmonic nanovoid structures using surface-enhanced Raman scattering, pages 74-81, Copyright © 2015, with permission from Elsevier.*

Indirect detection of proteins using SERS

As mentioned above, SERS is an ideal method of choice for the analysis of specific protein interactions and with the addition of Raman reporters the levels of sensitivity can be improved. Similarly to the detection of DNA, labelled proteins can be adsorbed onto the enhancing metal surface with the use of an aggregating agent to create the desired hotspots between the metal particles.[136] Alternatively, aggregation can be induced using a specific nanoparticle assembly approach. In 2012, Robson *et al.* developed a SERS-based assay for the detection of the mouse double minute (MDM2) protein, which plays a critical role in the progression of many cancers.[137] The method involved using a peptide mimic of the tumour suppression protein, p53, which is negatively regulated by MDM2, chemically attached onto the surface of silver nanoparticles. In the presence of MDM2, the p53-functionalised nanoparticles underwent assembly, resulting in a significant increase in the SERS intensity of the reporter (benzotriazole dye) also present on the silver nanoparticle surface. Based on a similar methodology of nanoparticle assembly, Craig *et al.* designed a SERS detection method for specific carbohydrate-protein interactions, in particular, the interaction between the lectin protein ConA and lactose-functionalised nanoparticles (Figure 9).[138] The specific sugar used to functionalise the nanoparticles has four potential binding sites, meaning that multiple carbohydrate-lectin interactions can occur simultaneously, allowing for nanoparticle aggregation to occur and therefore increasing the overall

SERS intensity of the benzotriazole dye present. Due to the high sensitivity of the SERS method, limits of detection as low as 40 pM were achieved which was a significant improvement over alternative detection methods. This study was a proof of concept that this method could be used for the highly sensitive monitoring of interactions between carbohydrates and ligands; however, the next step was to use this method to monitor these interactions in a cellular environment.

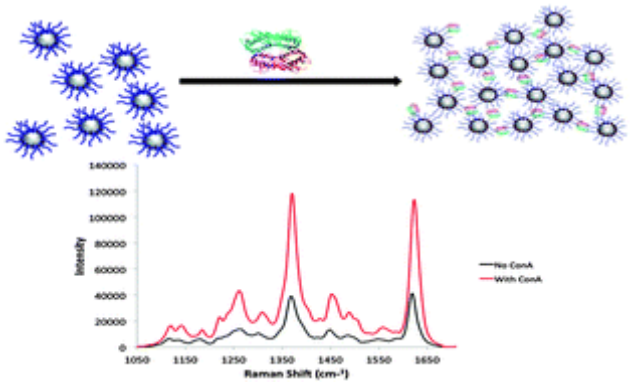

Figure 9. (top) *Aggregation resulting from the interaction between ConA and the glyconanoparticles.* ***(bottom)*** *SERS spectra with and without the addition of ConA.*[138] *Adapted from Ref. 125 with permission from The Royal Society of Chemistry.*

In 2014, Craig *et al.* used lectin-functionalised nanoparticles to monitor the expression of specific carbohydrate species at the cellular interface. By exploiting the difference in expression of glycans in cancerous and noncancerous cells, this method proved to be successful in the discrimination between healthy and cancerous prostate cells.[139]

Simpson *et al*. have recently applied the use of glyconanoparticles for the sensitive and specific detection of cholera toxin B-subunit (CTB) from synthetic freshwater, to levels as low as 56 ng/mL.[140] Particles were functionalised with a mixture of carbohydrates, sialic acid and galactose, to mimic the binding of CTB with the GM1 ligand on the surface of intestinal cells. The mixed carbohydrate coating was required as this increased the response significantly in comparison to functionalising with sialic acid or galactose alone. The selectivity of the method was shown as no aggregation, and thus a reduced SERS response, was obtained when ConA was present in place of CTB. The observed detection limit was 50 times better than that achieved using UV-Visible extinction

spectroscopy and within the recommended detection range for cholera toxin. This sensitivity also matches the limits of current WHO approved tests.

Protein Detection using SERS-based Immunoassays

The ability to detect and quantify specific protein biomarkers provides a deeper understanding of disease progression, diagnostics and is highly beneficial in the area of drug development.[141] In order to achieve this, a method of detection that is sensitive, reproducible and allows for high-throughput analysis is required. The development of enzyme-linked immunosorbent assays (ELISA) combined with Raman spectroscopy has allowed for the detection and quantification of disease biomarkers. Recently, replacing the colorimetric detection with resonance Raman scattering (RRS) in a conventional ELISA has proved successful for the detection of tumour necrosis factor α (TNF- α), an inflammatory cytokine related to inflammatory diseases.[142] Furthermore, this detection method was combined with microarray technology for the detection of prostate specific antigen (PSA).[143] Since the intensity of the resonance enhanced Raman bands was proportional to the protein concentration, protein quantification was possible with low limits of detection achieved in both examples: 90 fg/mL and 25 pg/mL for TNF- α and PSA, respectively. As well as RRS, SERS has also been used for the analysis of the coloured products generated in an ELISA, to improve the sensitivity over the generally utilised colorimetric detection.[132, 144]

Additionally, SERS-based immunoassays have been developed to combine the specificity and convenience of immunoassays with the advantages of SERS in sensitivity and the potential for multiplexing.[145] The SERS-based immunoassay platform has been used to detect protein biomarkers such as immunoglobulin G (IgG) and prostate-specific antigen (PSA) to very low limits of detection (approximately 1 pg/mL).[146, 147] In 2005, Porter *et al.* developed a SERS-based immunoassay for the detection of feline calicivirus (FCV), which causes upper respiratory infections in cats.[148] FCV could be compared to that of the human calicivirus, associated with gastroenteritis, in that it is highly contagious but difficult to study as the virus does not grow in cell culture. This was the first report of low-level detection of an intact viral pathogen in a sandwich immunoassay format using SERS as the readout method.

Following this, they used the SERS-based immunoassay format to detect the pancreatic cancer marker MUC4, a mucin protein.[149] This proved to be a simple diagnostic test for MUC4, providing rapid results from the SERS readout. This was the first report of detecting MUC4 in serum samples from patients. The results showed that serum from patients with pancreatic cancer produced significantly higher SERS response corresponding to the presence of MUC4, compared to the SERS results from the analysis of serum samples from healthy patients. This assay was shown to have significant advantages compared to the conventional immunoassay detection methods, with respect to the limits of detection, analysis time and the amount of sample volume required. Moreover, the assay has the potential to be used for the detection of other cancer biomarkers. More recently, further optimisation was performed on the aforementioned SERS-based immunoassay for the detection of MUC4 that involved the addition of a smooth mica surface to further increase the reproducibility and sensitivity (Figure 10).[150] It was reported that the presence of either a polymer or graphene monolayer as the thin protective layer further improved the sensitivity and allowed for a more stable signal from the Raman reporter (nitrobenzenethiol, NBT) to be obtained. The assay was applied for the detection of MUC4 from patient serum samples and, once again, the samples from healthy individuals could be clearly distinguished from those of patients with pancreatic cancer.

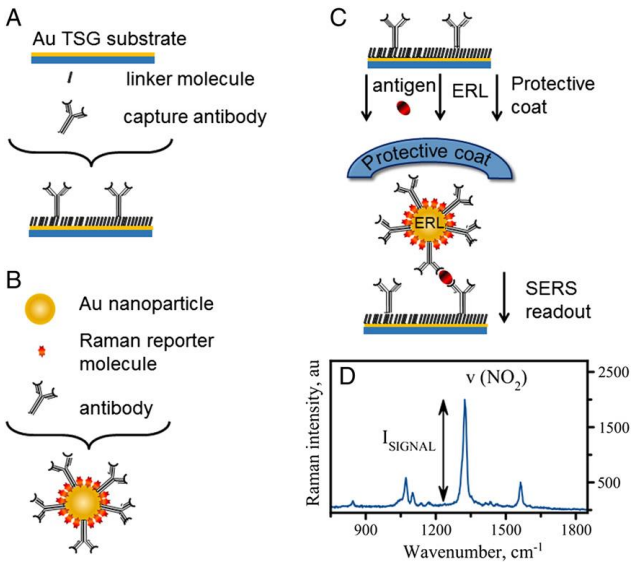

Figure 10. *Scheme showing the design of the SERS-based nano-immunoassay: (A) a gold capture substrate modified with linker molecules and antibodies; (B) gold nanoparticles functionalised with Raman reporter molecules and specific antibodies (ERLs); and (C) sandwich immunoassay where antibodies on the capture surface first bind the antigen and*

subsequently bind the ERL. (D) The sandwich assay produces a SERS readout with several characteristic bands from the Raman reporter molecule, NBT. The most prominent is ν(NO_2)— a symmetric nitro stretch at 1336 cm^{-1}.[150] Reprinted from Nanomedicine: Nanotechnology, Biology and Medicine, Vol 11, Alexey V. Krasnoslobodtsev,María P. Torres,Sukhwinder Kaur,Ivan V. Vlassiouk,Robert J. Lipert,Maneesh Jain,Surinder K. Batra,Yuri L. Lyubchenko, Nano-immunoassay with improved performance for detection of cancer biomarkers, Pages 167-173, Copyright © 2015, with permission from Elsevier.

Multiplex Detection of Proteins by SERS

Due to the rapid development of SERS-based immunoassays, they have been shown as an extremely desirable method for the simultaneous detection of multiple biomarkers. Wu *et al.* have reported success using the SERS-based immunoassay format by designing novel SERS substrates, consisting of Au@Ag core-shell nanorods, that are highly SERS-active and chemically stable.[151] The simultaneous detection of the tumour suppressor p53 and the cyclin-dependent kinase inhibitor p21 was achieved using these nanorod substrates that were functionalised with antibodies specific to the two targets and two different Raman reporters, 4-mercaptobenzoic acid (4-MBA) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).[152] High levels of specificity, reproducibility, and sensitivity (LOD 1 pg/mL) were achieved using the SERS-immunoassay format. These outcomes proved to be highly desirable in cancer diagnostics where the combined detection and quantification of p53 and p21 would be extremely informative for early cancer predictions. More recently, Wu *et al.* have developed a multiplex immunoassay using SERS and a 3D barcode chip on a microfluidic platform to allow for multiplexed high-throughput protein biomarker analysis.[153] Multiple proteins present in different samples were spatially separated using a microfluidic device that contained specific antibody patterns, allowing for the formation of a 2D hybridisation array when the target analyte is present. The novel method was used for the multiplex detection of human IgG, mouse IgG and rabbit IgG by using the unique spectral Raman bands of 4-MBA, DTNB and 2-naphthalenethiol (2-NAT), respectively.

By exploiting the robust synthesis and functionalisation methods available when designing a substrate to be used in SERS analysis, unique designs have been reported that allow for the highly sensitive detection and quantification of biomarkers in a SERS-based immunoassay format. Fluorescent dyes and Raman active small molecules have been combined to create highly sensitive, selective and multifunctional substrates known as fluorescent surface enhanced Raman spectroscopic (F-SERS) dots that are

ideal for multiplexing, as well as tracking and imaging of cellular and molecular events.[50] F-SERS dots have been used to simultaneously detect three cellular proteins: CD34, Sca-1 and SP-C, which are all expressed in bronchioalveolar stem cells (BASCs). Antibody-functionalised paramagnetic nanoparticles have also been used in conjunction with Raman reporter-functionalised gold nanoparticles for the detection of antigens specific to West Nile Virus (WNV) and Rift Valley fever virus (RVFV), with limits of detection as low as 5 fg/mL when the assay was performed in salt buffer and 25 pg/mL when the biomarkers were present in buffer spiked with fetal bovine serum.[154] SERS was combined with hollow-core photonic crystal fiber (HCPCF) to enable the ultrasensitive detection of hepatocellular carcinoma (HCC) biomarkers: alpha-fetoprotein (AFP) and alpha-1-antitrypsin (A1AT).[155] One of the main advantages highlighted when using SERS and HCPCF is the extremely low sample volume required, approximately 20 nL, which is highly desirable in clinical diagnostics where samples are likely to be small with low amounts of target protein, combined with a lack of amplification methods available for proteins. Guarrotxena *et al.* designed SERS "antitags" for the simultaneous detection of three targets: human α thrombin (THR); myoglobin (MYG) and C-reactive protein (CRP), using three different Raman reporters.[156] The "antitags" comprised of silver nanoparticles functionalised with antigen-specific antibodies and are held together by dithiolated Raman reporters. The detection method was in the form of a sandwich immunoassay where protein-specific capture antibodies were immobilised onto an epoxy-functionalised glass substrate. A solution that contained a combination of target proteins was exposed to the surface; followed by the addition of the "antitag" solution containing equimolar concentrations of all three tags. Binding of the "antitags" occurs, followed by several wash steps to remove any unbound "antitags" with subsequent SERS analysis. The sensitivity of this particular assay was tested and limits of detection around 100 pM were obtained. The level of sensitivity of the SERS-based immunoassay platform was recently further tested by Xu *et al*., where the presence of DNA aptamers induced the self-assembly of silver pyramids that enabled the multiplexed and ultrasensitive SERS detection of three disease biomarkers.[157] The three targets were: prostate specific antigen (PSA), thrombin and mucin-1, which were detected by monitoring the Raman spectra of 4-aminothiophenol (4-ATP), 4-nitrothiophenol (4-NTP) and 4-methoxy-α-toluenethiol (MATT), respectively. The limits of detection for each target were all found to be in the

attomolar range (Figure 11). This level of sensitivity is remarkable and extremely beneficial in the analysis of clinical samples.

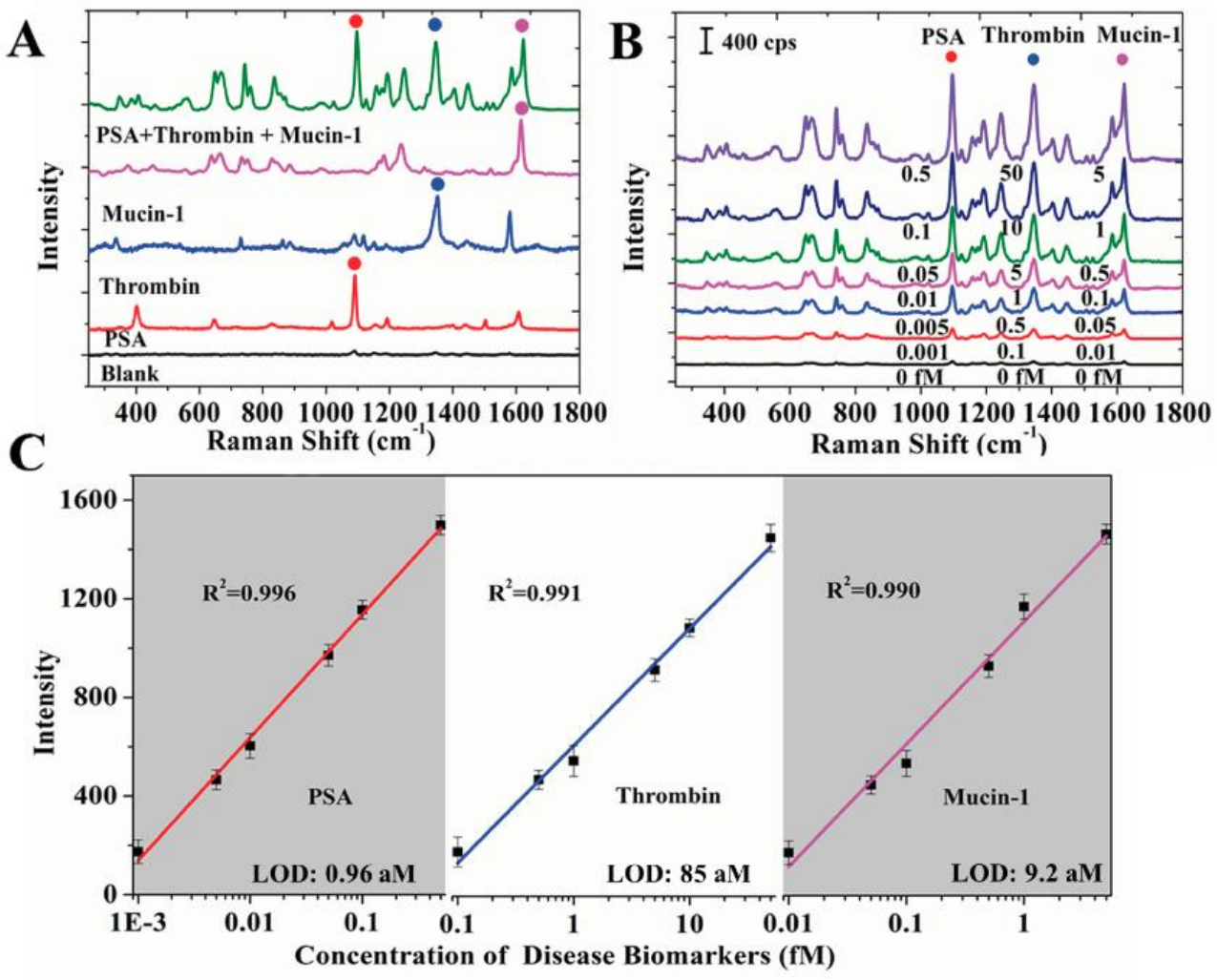

Figure 11. *A) SERS encoded pyramids for solo biomarker detection. B) SERS spectra of simultaneous multiplex biomarkers detection. C) Standard curves of SERS encoded pyramidal detection for the SERS signatures intensities versus the concentration of PSA (at 1095 cm^{-1}, left), thrombin (at 1346 cm^{-1}, middle) and mucin-1 (at 1621 cm^{-1}, right).[157] Reprinted by permission of John Wiley and Sons. Copyright © 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.*

Porter *et al*. had similar success using SERS-based immunoassays for the simultaneous detection of multiple biomarkers. One such approach was based on the self-assembly of mixed monolayers.[47] A new functionalisation method was developed for the design of extrinsic Raman labels (ERLs, Figure 10). The conventional method involves coating gold nanoparticles with both Raman reporter molecules and antigen-specific antibodies that each possess thiolates to allow for surface adsorption. The new method involves

two thiolates: one thiolate is the bifunctional compound dithiobis(succinimidyl propionate) (DSP) that contains both disulphide and succinimidyl functionalities, allowing for surface adsorption onto the gold nanoparticle surface and covalent coupling of the antibody to the gold nanoparticle; the other thiolate is a molecule that has a large Raman cross section to act as a reporter, as DSP is a relatively weak Raman active molecule. By using the newly designed ERLs, a tetraplex was developed for the detection of four different targets: mouse IgG, human IgH, rabbit IgG and rat IgG. The limits of detection were all calculated to be in the ng/mL range; however, it was noted that the level of sensitivity for each target present in the multiplex decreased compared to the targets being detected individually. This was attributed to the increase in the background in the multiplex spectra.[47] More recently, Porter *et al.* have developed a SERS-based immunoassay for the detection of two pancreatic cancer biomarkers: serum carbohydrate antigen 19-9 (CA 19-9) and matrix metalloproteinase 7 (MMP-7).[158] A comparison was made between the SERS-based method and the conventional ELISA method to demonstrate the increase in sensitivity when the chosen readout method is SERS. The limits of detection of each target when using the SERS-based immunoassay were 2.28 pg/mL and 34.5 pg/mL for MMP-7 and CA 19-9, respectively. Comparing these to the values obtained when using the standard ELISA method (MMP-7: 31.8 pg/mL, CA 19-9: 987 pg/mL), the increase in sensitivity that SERS offers as a method of detection was clearly demonstrated.

CONCLUSIONS

SERS offers many advantages over alternative methods for the detection of biologically relevant targets, particularly for multiplex detection. The technique has been successfully applied for the sensitive and specific detection of biomolecules, such as DNA and proteins, over a number of years and significant progress has been made on improving the sensitivity of detection as well as applying the method for the detection of multiple target molecules from one sample. Recent advancements have seen the application of the technique for the detection of multiple targets from real clinical samples, which is of paramount importance if the technique is to be applied for molecular diagnostics. The multiplexing capability is due to the specificity of the technique which is essential for disease detection and diagnosis. For example,

increased levels of CRP alone may be indicative of various conditions involving inflammation; however, detection of CRP along with other biomarkers, such as cardiac troponin I (cTnI) and/or B-type natriuretic peptide (BNP), could signify the presence of a cardiovascular disease. Therefore, by being able to detect multiple biomarkers simultaneously, a more accurate diagnosis can be made. One of the main advantages of SERS is the ability to directly analyse biological samples with minimal sample preparation, which is extremely useful for clinicians. The sensitivity is also of great interest to the end user although sometimes the dynamic range may be more important, depending on the clinically relevant concentration of the target. Nonetheless, sensitivity and reproducibility allow for quantitative analysis which can be imperative in distinguishing between healthy and diseased samples. Future progress in the field will likely expand each of these capabilities and we can expect to see more examples of the technique being applied to biological samples for the detection of multiple disease-related targets. Furthermore, advances in instrumentation could allow for quick and cost-effective analysis which could potentially be carried out at a bedside. This could provide an excellent tool in the field of diagnostics and will open up potential opportunities for the use of the technique in clinical applications. Potential issues such as interference, stability of SERS substrates and toxicity of nanoparticles may be of concern, although it is expected that these will be overcome with the ongoing developments in the field, particularly due to improvement in understanding of nanoparticle synthesis, surface chemistry and functionalisation.

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