

Bionanotechnology III: from Biomolecular Assembly to Applications

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Nanoparticle assembly for sensitive DNA detection using SERRS

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Abstract

SERRS (surface-enhanced resonance Raman scattering) is a vibrational technique, whereby a relatively weak Raman scattering effect is enhanced through the use of a visible chromophore and a roughened metal surface. The direct analysis of DNA by SERRS requires the modification of a nucleic acid sequence to incorporate a chromophore, and adsorption of the modified sequence on to a roughened metal surface. Aggregated metallic nanoparticles are commonly used in the analysis of dye-labelled DNA by SERRS, allowing for detection levels that rival those gained from standard fluorescence-based techniques. In the present paper, we report on how SERRS can be exploited for the analysis of clinically relevant DNA samples. We also report on the ability of nanoparticles to aggregate as the result of a biologically significant event, as opposed to the use of an external charge-modifying agent. The self-assembly of metallic nanoparticles is shown to be a promising new technique in the move towards extremely sensitive methods of DNA analysis by SERRS.

Introduction

The ability to detect specific sequences of DNA in a highly sensitive and selective manner is of vital importance in molecular diagnostics, an area that is continually working towards faster and more efficient techniques to enable rapid disease detection. Currently, the most successful methods for the detection of DNA involve the use of PCR coupled with fluorescence spectroscopy. However, recently, SERRS (surface-enhanced resonance Raman scattering) has been employed in a number of detection schemes as an alternative optical technique for nucleic acid sequence analysis.

Raman scattering provides a vibrational spectrum of a molecule by measuring the difference in energy between incident and scattered light. This is a relatively weak process, with only around 1 in every 10^6 photons being Raman scattered. SERS (surface-enhanced Raman scattering) was first reported by Fleischman et al. [1] in 1974. During experiments using Raman spectroscopy to detect pyridine at a silver electrode, it was noted that the Raman scattering was greatly increased when the surface of the electrode was roughened. This large enhancement in signal stimulated great interest in the technique and it was discovered that the effect was not unique to pyridine. It has subsequently been shown that it is possible to obtain SERS from a large number of molecules as long as they are adsorbed on to a roughened metal surface.

SERRS [2] can be considered to be a combination of two processes, resonance Raman and SERS. As a consequence of this, the enhancement in signal that is observed is greater than either of these two processes, and with SERRS

Key words: DNA detection, functionalized nanoparticle, nanoparticle assembly, surface-enhanced resonance Raman scattering (SERRS).

Abbreviations used: FAM, 6-carboxyfluorescein; OGN, oligonucleotide-gold nanoparticle; OSN, oligonucleotide-silver nanoparticle; SERS, surface-enhanced Raman scattering; SERRS, surface-enhanced resonance Raman scattering.

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spectroscopy an enhancement in scattering three to four orders of magnitude greater than SERS can be observed. This in turn corresponds to an enhancement of up to 10^{14} in the scattering from some molecules compared with normal Raman scattering [3]. For an analyte to exhibit SERRS, it must have a chromophore and by tuning the frequency of the laser excitation to the absorption maxima of the analyte, as well as the surface plasmon of the metal substrate, very large enhancements in signal can be observed [3,4]. This also gives a degree of selectivity, as coloured compounds with absorbance maxima close to the laser excitation will be selectively enhanced over other species that may be present. In addition, whereas conventional fluorescence detection methods provide a broad emission profile, SERRS gives unique fingerprint spectra that are indicative of the molecular structure of an analyte. As such, SERRS offers a distinct advantage over fluorescence in that it is capable of multiplexed detection and specific molecular identification [5]. This enables much higher numbers of analytes to be discriminated in the one vessel by SERS/SERRS than by fluorescence, where it is difficult to discriminate between more than three or four analytes in a mixture.

Since unmodified DNA does not contain a visible chromophore, a sequence must be altered to incorporate an optically visible label before SERRS analysis can take place. Since the metal surface will quench fluorescence, commercially available fluorescent DNA labels can be employed for analysis following conventional labelling techniques. The labelled DNA must then be adsorbed on to the surface of the metal, in this case metallic nanoparticles. Successful detection formats utilize gold and silver nanoparticles prepared via a citrate reduction method, and are aggregated via the addition of an external charge-modifying agent. Spermine is the preferable choice for DNA detection as it serves the dual purpose of aggregating the colloidal suspension to create 'hotspots' between two or more nanoparticles providing signal enhancement, while simultaneously promoting adsorption of the DNA on to the nanoparticle [6]. Since the surface is coated with negative citrate ions, and DNA is negatively charged, positively charged spermine interacts with the phosphate backbone of the DNA sequence, neutralizing the charge and thus enabling surface adhesion.

Upon addition of spermine to a solution containing nanoparticles and a labelled nucleic acid sequence, an enhanced SERRS signal will be obtained that is indicative of the label present on the strand of DNA. Using this method, quantitative analysis has been achieved and detection limits for DNA sequences labelled with a number of commercially available dye labels have been obtained [7,8], and SERRS was shown to be three orders of magnitude more sensitive than fluorescence for the analysis of dye-labelled oligonucleotides [7]. In addition, the multiplexed detection of five labelled DNA sequences in solution using two λ_{ex} has been achieved by eye [5] and six different labelled DNA sequences has been detected using one λ_{ex} combined with chemometric data analysis [9]. As such, research has moved on to focus on

detecting more clinically relevant DNA samples in an effort to move towards the implementation of SERRS in diagnostic settings.

Detecting specific gene sequences using SERRS

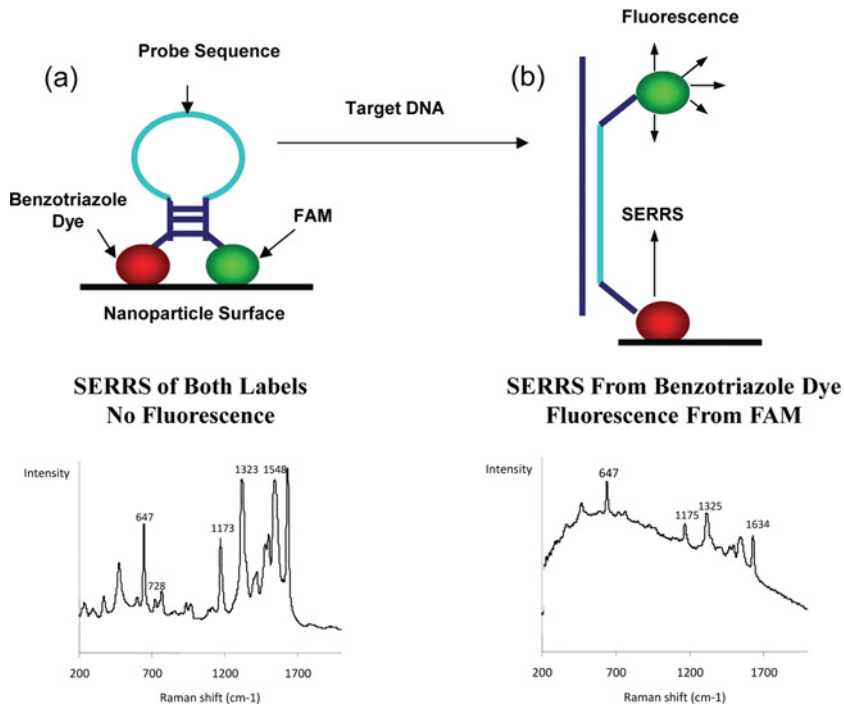
An early example of the application of SERRS for multiplex genotyping involved the detection of the mutational status of the cystic fibrosis transmembrane conductance regulator gene using an ARMS (amplification refractory mutation system) [10,11]. By designing and labelling specific primers for use in a multiplexed PCR assay, it was possible to identify the genotypes present in a sample, whether homozygotes or heterozygotes. The success of SERRS for this purpose not only lies in the ability to provide simultaneous detection of two genes, i.e. 2-plex analysis, but also in the simplicity of the process when compared with standard fluorescence-based techniques. A similar assay design was used for the detection of the clinically relevant disease target *Chlamydia trachomatis* [12]. This method involved the hybridization of a SERRS active probe with biotin-labelled amplified PCR product, followed by immobilization on to streptavidin-coated beads. After a wash step and denaturing of the probe target duplex, the appearance of a SERRS signal from the labelled probe remaining in solution provides evidence that amplification of the desired target has occurred. This assay design was incorporated into a lab-on-a-chip format, to allow for a solid-phase clean-up to remove any background contributions. Both of these assays are comparable in that they use silver nanoparticles aggregated via spermine alongside a labelled sequence of DNA gained from PCR amplification in order to obtain an enhanced Raman scattering signal.

A slightly different approach for the analysis of DNA involved the use of a specifically designed SERRS beacon [13]. This took the form of conventional molecular beacons, whereby a single strand of DNA is held in a loop by a self-complementary stem region. This particular beacon was designed with a 3'-FAM (6-carboxyfluorescein) fluorophore and a 5'-benzotriazole azo dye, which had been specifically designed to complex to silver metal surfaces. In the closed form, the beacon does not fluoresce due to the quenching action of both the azo dye and the silver nanoparticles. When the target complementary to the loop sequence hybridizes, the SERRS beacon opens up, removing the FAM fluorophore from the quenching action of the surface and the azo dye. As a result, the SERRS signal changes, which is indicative of a specific recognition event (Figure 1). Although this particular assay was not applied to biologically relevant samples, it describes a novel method of detection that involves a single sequence of unlabelled DNA.

More recently, studies have focused on the specific interaction of DNA with the surface of the nanoparticle as a basis for target sequence detection. A homogenous assay for the simultaneous detection of three DNA sequences coding for genes relating to hospital-acquired infections has been reported [14]. This assay is based on the fact that single-stranded

Figure 1 | Schematic representation of the SERRS beacon

The beacon was modified with a 3'-FAM fluorophore and a 5'-benzotriazole azo dye. The azo dye has been designed to complex to silver metal surfaces and produce SERRS. **(a)** In the 'closed' conformation, the FAM and benzotriazole dye are in close proximity to each other and the metal surface, therefore a SERRS spectrum from both is observed and the fluorescence is quenched. **(b)** Upon hybridization of the target, the beacon opens, resulting in an increase in fluorescence and a decrease in the signal from the FAM label. Reproduced from Faulds, K., Fruk, L., Robson, D.C., Thompson, D.G., Enright, A., Smith, W.E. and Graham, D. (2006) A new approach for DNA detection by SERRS. *Faraday Discuss.* **132**, 261–268 with permission from the Royal Society of Chemistry.


Figure 2 | Schematic representation of the 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 on to the negatively charged nanoparticle surface resulting in a high SERS response. Reproduced from van Lierop, D., Faulds, K. and Graham, D. (2011) Separation free DNA detection using surface enhanced Raman scattering. *Anal. Chem.* **83**, 5817–5821 with permission of the American Chemical Society.

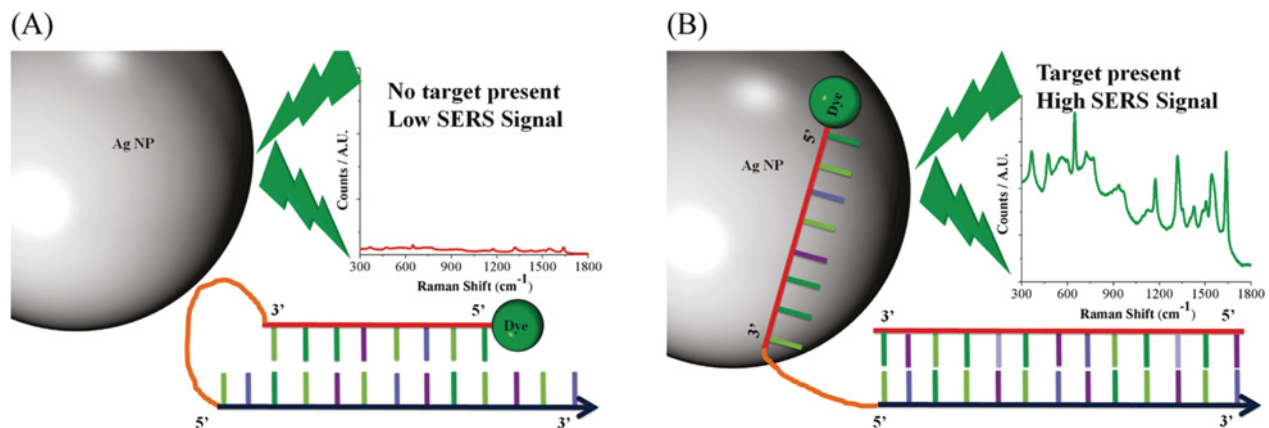
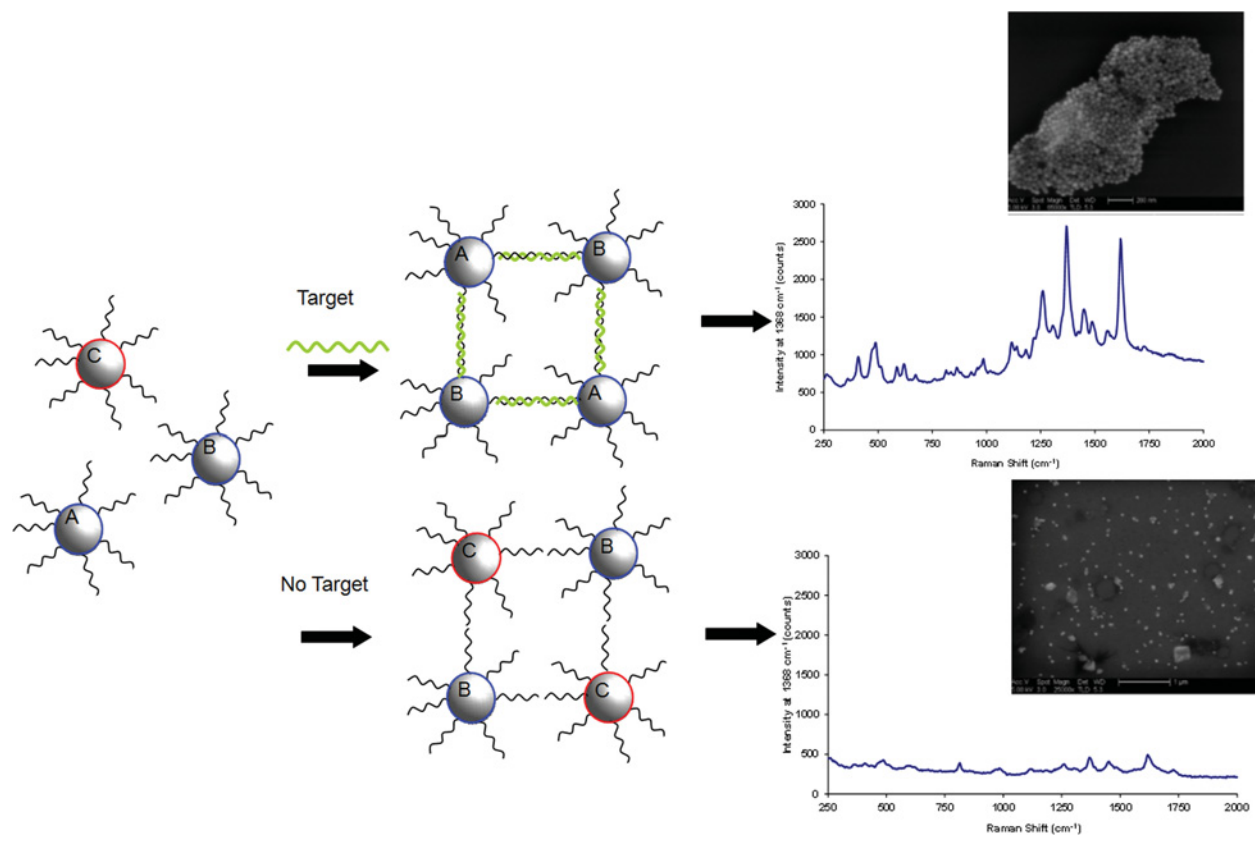


Figure 3 | Oligonucleotide–silver nanoparticle conjugates for DNA analysis by SERRS

Silver nanoparticles are functionalized with a Raman active dye and two non-complementary sequences of DNA. Upon addition of a target nucleic acid sequence complementary to both probes, the nanoparticles will aggregate. The top SEM (scanning electron microscopy) image shows the large aggregates formed by hybridization of OSN conjugates to a complementary target, and the enhanced SERRS signal obtained as a result. The sample in the bottom SEM image contains a non-complementary target and shows dispersed single nanoparticles and small agglomerates that are associated with crystals of the hybridization buffer. The accompanying spectrum shows the SERRS results when no target sequence of DNA is present. Figure based on data from [16,17].

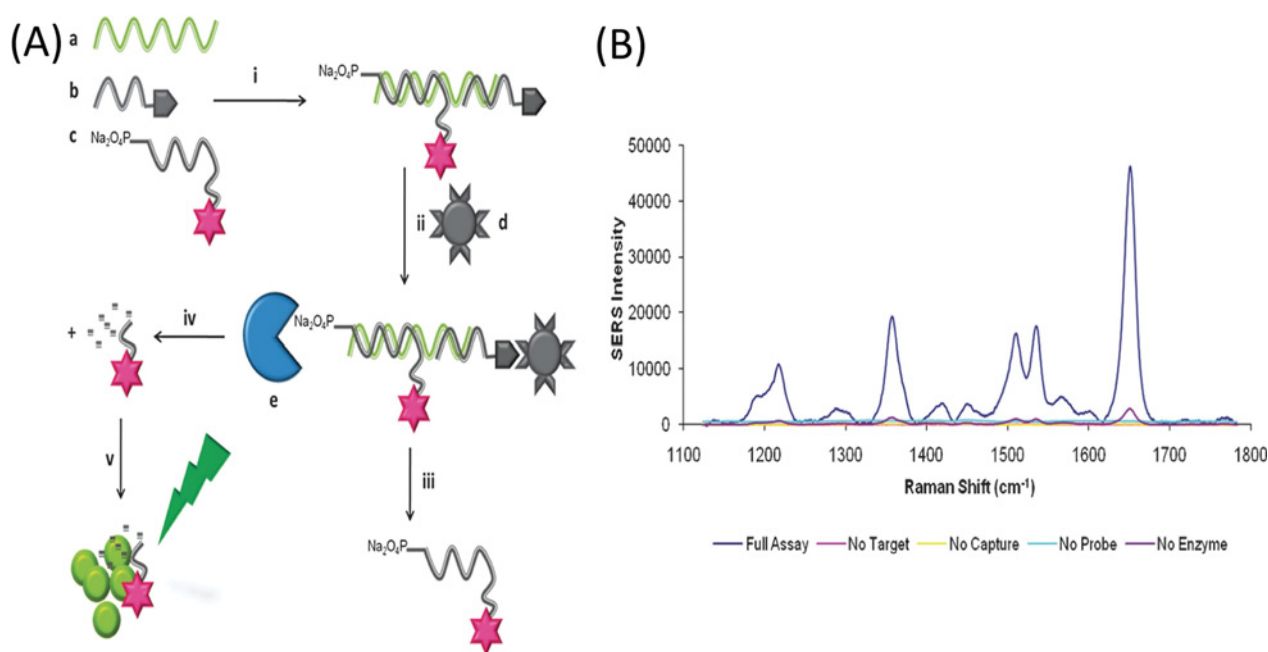


DNA has a much greater affinity for the surface of silver nanoparticles than double-stranded DNA and this phenomenon was exploited to detect a target sequence. In the absence of target, no hybridization to a single-stranded complementary probe sequence labelled with a fluorophore will occur, leaving it free to adsorb on to the surface of the silver nanoparticles via a spermine bridge and produce a strong SERRS signal. In the presence of target DNA, the signal intensity will be reduced since the target will now hybridize to the probe and form double-stranded DNA, which has a lower affinity for the metal surface. As a result, surface adsorption, and hence SERRS, is inhibited. As with the previously mentioned assays, this method was tested on PCR product to ensure the applicability of this approach to biological samples. The multiplexed detection of three possible sequences relating to MRSA (methicillin-resistant *Staphylococcus aureus*) was possible in a closed-tube system within a faster analysis time than required for conventional culturing methods. However, a drawback of

this assay is that it is a negative assay, i.e. the presence of target results in a reduction in signal rather than an increase. Therefore this assay format was further developed to design a positive homogenous assay that gives an increased SERRS response in the presence of target and is fully compatible with PCR [15]. As shown in Figure 2, the assay consists of a SERS primer that can be used to either detect DNA directly or in combination with PCR. When no target is present, the dye-labelled SERS primer is closed and therefore double stranded, due to self-complementarity, and as such does not interact with the surface of the nanoparticles. When a target sequence of DNA, in this case genomic DNA from *Staphylococcus epidermidis*, is present, the primer opens up as the complementary target displaces the self-complementary region of the primer. This leaves a section of single-stranded dye-labelled DNA free to adsorb on to the nanoparticle surface and enhance the Raman signal. This method of DNA analysis has been carried out with PCR product to ensure the suitability of this protocol for clinical samples.

Figure 4 | Split probe *exo*-SERS assay

(A) Schematic diagram of the assay. It is performed via a number of steps: (i) sandwich hybridization between an unlabelled target, a, biotinylated 'capture' probe, b, and 5'-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 λ_{ex} of 532 nm. (B) Shows the SERRS enhancement when all components of the assay are present. Reproduced from Dougan, J.A., MacRae, D., Graham, D. and Faulds, K. (2011) DNA detection using enzymatic signal production and SERS. *Chem. Commun.* **47**, 4649–4651 with permission from the Royal Society of Chemistry.



Functionalized nanoparticles for SERRS analysis

The assays described above have proven successful for the detection of labelled DNA; however, the enhancement due to nanoparticle aggregation is permanently turned on as a result of an external charge-modifying agent. Recent research has favoured the use of a biological target molecule of interest to promote nanoparticle assembly. In this case, interaction with the biomolecule of interest causes the aggregation of the nanoparticles and as a consequence turns 'on' the SERRS. The first reported use of a DNA-based assembly process for enhanced Raman scattering utilized OSN (oligonucleotide–silver nanoparticle) conjugates [16,17]. The focus of this design was to control the electromagnetic enhancement of Raman scattering through interaction of the nanoparticles. Silver nanoparticles were synthesized and coated with a Raman active dye, and 5'-thiol-functionalized oligonucleotide probe sequences were anchored to the surface. Two batches of conjugates were made in this manner with different non-complementary probe sequences, which remained as a colloidal suspension. On addition of a target complementary to both probes, hybridization takes place,

promoting nanoparticle aggregation, as shown in Figure 3, which results in an enhanced SERRS signal of the dye label. As a result, a synthetic target sequence of DNA could be successfully detected using the enhancement in the Raman signal gained from hybridization-induced nanoparticle assembly. The move to biologically relevant samples, however, has proven difficult due to the sequence-dependent stability of both OSNs and OGNs (oligonucleotide–gold nanoparticles). Research into improving the stability of conjugates has resulted in the use of multiple thiol anchor groups for oligonucleotide attachment based on thioctic acid [18]. Other stability studies have been published that aim to optimize the conjugation process [19] to implement both OSNs and OGNs in a similar assay format to that previously mentioned, and ultimately detect biologically relevant samples using nanoparticle assembly and SERRS.

Towards amplification-free detection

In addition to an irreversible SERRS response, the detection of labelled DNA by SERRS requires a target amplification step in order to obtain sensitive levels of detection. Although

PCR is a powerful technique for the detection of very low copy numbers of DNA and the success of real-time PCR is well documented, it is not without limitations. Contamination issues can complicate PCR and initiate the amplification of unwanted sequences of DNA, compromising the sensitivity of the technique, and thus it requires highly trained personnel and strict laboratory procedures. Recently, methods of signal amplification as opposed to target amplification have been developed [20,21]. Most involve fluorescence-based methods of DNA detection, whereby there is a build-up of signal as opposed to increasing the copy number of the target. Owing to the drawbacks associated with fluorescence, signal amplification has recently been implemented in an assay for SERS analysis [22]. A split probe *exo*-SERS assay has been developed whereby the enzyme lambda exonuclease is used to cycle a specific target sequence of DNA, allowing for amplification of a SERS signal (Figure 4). The unlabelled sequence of target DNA is initially hybridized to a biotinylated 'capture' probe and a 5' phosphorylated dye-labelled 'reporter' probe. This duplex is then captured on streptavidin-coated magnetic beads before undergoing a wash step to remove any excess probe. Lambda exonuclease is then introduced to the beads, and since it is a highly processive enzyme that digests 5'→3' starting at phosphorylated DNA, the hybridized portion of the 'reporter' probe is digested. The remaining dye-labelled sequence is then free to adsorb on to silver nanoparticles, giving an enhanced SERS signal. Only in the presence of target DNA will the dye-labelled sequence be present in the final step, and since the enzyme only digests one strand of a duplex, the target will be free to re-hybridize and start the process again. This has been implemented in the detection of *Chlamydia trachomatis* to show the applicability of this detection system to relevant samples. Although this required the use of PCR, optimization of such signal amplification strategies alongside target-induced nanoparticle assembly methods should eventually eliminate the need for target amplification methods in DNA analysis.

Conclusions

SERRS can be successfully utilized for the sensitive and multiplexed detection of DNA. The aggregation of colloidal suspensions of nanoparticles has been integral to the enhancement factor when compared with basic Raman scattering. As such, the ability to control this aggregation via a biologically significant event has enabled a variety of different detection mechanisms to be developed, and alongside signal amplification strategies, is paving the way towards amplification-free DNA detection.

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