Quantitative SERRS for DNA sequence analysis

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SERRS is an extremely sensitive and selective technique which when applied to the detection of labelled DNA sequences allows detection limits to be obtained which rival, and in most cases are better than, fluorescence. In this *tutorial review* the conditions are explored which enable the successful detection of DNA using SERRS. The enhancing surface which is used is crucial and in this case suspensions of nanoparticles were the focus as they allow quantitative behaviour to be achieved in systems analogous to current fluorescence based approaches. The aggregation conditions required to obtain SERRS of DNA affect the sensitivity and the reproducibility and we describe the use of spermine as an effective aggregating agent to achieve excellent reproducibility and sensitivity. The nature of the label which is used, be it fluorescent or non-fluorescent, positively or negatively charged, also affects the SERRS response and these conditions are again discussed. Finally, we show how to detect a specific target DNA sequence in a meaningful diagnostic assay using SERRS and how the approaches described previously in the review are vital to the success of such approaches.

Background

Detection of specific DNA sequences is central to modern molecular diagnostics.[1] There are a number of different reasons for detecting specific DNA sequences such as identification of disease states,[2] analysis of samples relating to the identification of criminal suspects in forensic applications,[3] the identification of genetically modified organisms,[4] and the tracing of the evolutionary history of species.[5] Many methods exist for these purposes and the molecular biology involved in the different assay formats has been dictated and driven by the end point detection technique employed. In this review the approach of using surface enhanced resonance Raman scattering (SERRS) for the detection of specific DNA sequences is explored and the steps which are necessary when considering using SERRS as a meaningful detection method for DNA are discussed.

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As a detection technique for DNA analysis, SERRS has many advantages but also some limitations. The main attractiveness of SERRS is the sensitivity in terms of absolute detection limits, and also the ability to resolve multiple signals in mixtures without any separation procedures. The main limitation is that it is a surface based technique and as such requires the use of an appropriate surface to provide the desired enhancement. The dominating techniques used in molecular diagnostics are currently centred around closed tube homogeneous fluorescence assays which are able to exploit the polymerase chain reaction (PCR) to amplify specific sequences of DNA and then detect them as they are being amplified in the reaction vessel in real time.[6–9] These approaches have excellent sensitivity due to the use of PCR and also the ability to multiplex in terms of the deconvolution of the fluorescence emission profiles. However, the multiplexing capability of the real time systems is limited due to the emission profiles of the fluorophores, which can be broad and overlapping, and also the sensitivity is dependent on efficient PCR cycling of the target sample.

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As reported elsewhere in this special issue, SERRS is capable of exquisite sensitivity with single molecules detected in a number of different studies.[10,11] It also has the capability to provide higher degrees of multiplexing than fluorescence due to the sharper vibrational bands provided from the labels which are used routinely in bioanalytical studies. Previously DNA sequences have been detected using SERRS and labelled DNA fragments. For SERRS to be optimal there need to be some surface attachment properties added to the DNA to allow it to adsorb onto a metal surface and also a chromophore which will give a resonance contribution with the exciting laser wavelength, which we find gives a large increase in sensitivity.[12] In order to do this a visible chromophore needs to be added to the DNA probe sequence of interest which can be achieved using either conventional fluorescence labels, due to the quenching of fluorescence on the metal surface,[13] or through targeted designer dyes specifically used in SERRS studies. Thus through careful design of the probes and a consideration of surface adsorption, sensitive and specific DNA detection can be achieved.

Design and synthesis of SERRS active DNA probes

The basic concept in obtaining SERRS that relates to the identification of a specific DNA sequence is to couple the detection technique with a specific molecular biological assay. The final section of this review will deal with the molecular biology employed for different SERRS approaches, however, before we get to that stage there needs to be consideration given to the design of the oligonucleotide probe sequence which is used to report on the specific DNA sequence. In other words, how can we generate an oligonucleotide fragment that gives a specific SERRS signal? The basic two requirements to obtain efficient SERRS are that there must be a visible chromophore which is in resonance with the excitation frequency used, and also that the fragment of DNA with the label must adsorb onto a roughened metal surface to provide the surface enhancement of the scattered light (Fig. 1).

In terms of surface adsorption, the nature of the metal surface used for the enhancement needs to be considered. If it is a metal surface where simple evaporation of the sample will leave the label in close proximity to the metal, such as a vapour deposited metal, then this will yield surface enhancement from an appropriate surface. More often than not a hybridisation event is required in order to provide specific DNA sequence information and, as such, immobilisation of a probe on a surface is then required. In this case, the surface adsorption can be achieved through hybridisation to the probe already immobilised on the surface and the hybridisation yields the signal information required for identification of the target sequence.[14]

In order to be more analogous to the conventional closed tube solution based approaches, nanoparticles are often used for DNA detection by SERRS.[15] Metallic nanoparticles of gold or silver can be easily prepared by reduction of the corresponding metal salt and these metallic nanoparticles will have a surface layer on them as a result of the reduction process. The gold and silver nanoparticle suspensions which are commonly used have either citrate[16,17] or an EDTA[18]

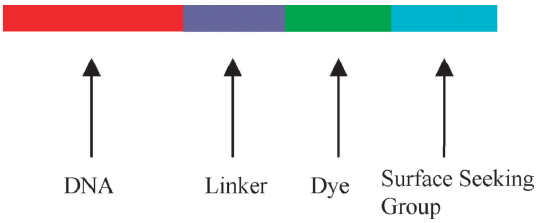

Fig. 1 Illustration of the composition of a SERRS active DNA probe. The four main components are shown and it should be noted that the dye and surface seeking group can be the same species and do not have to be separate.

surface layer. Although not complete, these layers can confer an overall negative charge on the surface of the nanoparticles and, as such, strategies need to be developed to allow adsorption of the negatively charged oligonucleotide probes onto the surface of the negatively charged nanoparticles (Fig. 2).

The simplest approach is to use electrostatic layering.[19] This involves using a layer of positive charge to attract the negatively charged oligonucleotide probe onto the negatively charged nanoparticle surface. A range of positively charged species have been investigated including metal ions, poly(L-lysine) and spermine. We have found that spermine gives the maximum surface adsorption of oligonucleotides (Fig. 3). In addition to this, the spermine can be used to aggregate the nanoparticles to give higher enhancement of the Raman scattering due to the electromagnetic enhancement experienced through the coupling of the plasmons when the nanoparticles interact.[20] Therefore spermine serves a dual purpose in these types of studies, namely it promotes surface adsorption of the negatively charged oligonucleotide probe and also aggregates the nanoparticles to provide the higher surface enhancement from the aggregated nanoparticles as opposed to the individual nanoparticles.[21] Although the spermine acts as a bridge between the two negatively charged species, the surface of the nanoparticles never becomes positive when the spermine is added alone although it does reduce the amount of negative charge. This means the introduction of regions of positive charge such as modified bases or labels can enhance adsorption.

Having neutralised the negative charge of the phosphates, it is then necessary to add the visible chromophore to be in resonance with the excitation source used to provide the SERRS. We can split the types of labels used for DNA detection into two categories, fluorophores and non-fluorophores.

1. Fluorophores

The easiest way to obtain SERRS active probes is to use commercially available fluorophores for labelling DNA sequences. Due to the quenching mechanism of the nanoparticles as a consequence of the SERRS procedure, and the availability of these types of labels and the simple chemistry involved, this is also the most commercially viable way to obtain SERRS probes. Due to the massive amount of research and development work on fluorescently labelled oligonucleotides the availability and number of labels far outweigh those of any of the other types of labels.

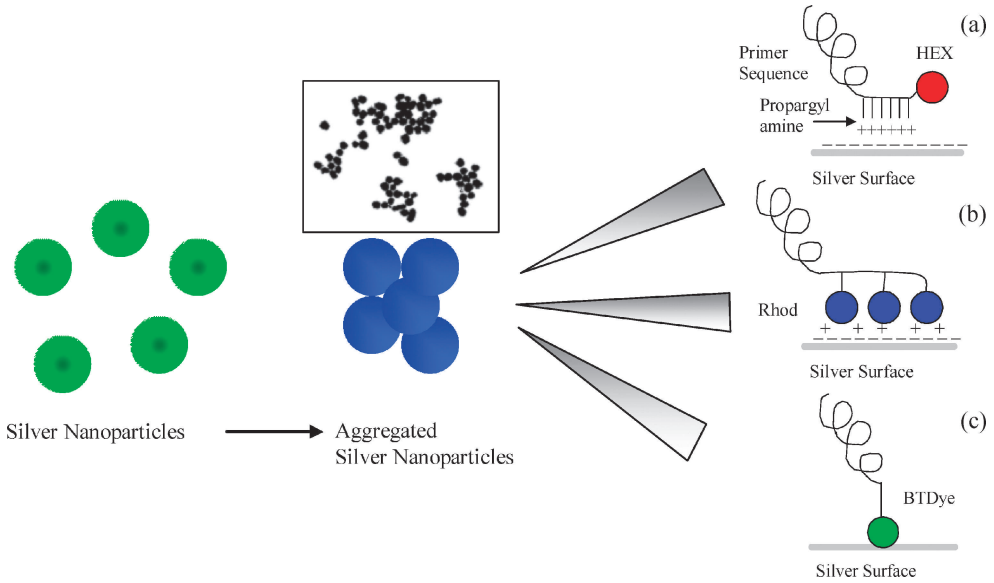

Fig. 2 Schematic representation of the aggregation process required to achieve detection of labelled DNA by SERRS: (a) shows the negatively charged label HEX, which requires propargylamine modification to confer a positive charge on to the molecule and allow surface adsorption to occur; (b) represents a R6G label already positively charged, therefore no surface modification is required, and (c) represents a specially synthesised dye containing the benzotriazole group which attaches strongly and essentially irreversibly to silver surfaces. *Chem. Commun.*, 2006, 4363—Reproduced by permission of The Royal Society of Chemistry (RSC).

There have been a number of studies carried out where commercially available labels have been attached to oligonucleotide probes and then detected using SERRS.[20,22–27] We have studied a large number of commercially available fluorescent labels and all can be made to give a quantitative response when attached to DNA. These are summarised in Table 1 where the charge of the dye is given as well as the structure to indicate the electrostatic interaction of the dye on the surface. If the dye is a negatively charged dye then we have found that we can improve the signal-to-noise ratio and obtain lower detection limits if we introduce a region of positive charge into the oligonucleotide probe sequence which is close to the position of the label. This is achieved by using modified deoxyuridine nucleosides which contain a propargylamino residue at the 5′ position of the pyrimidine ring system. At physiological pH this primary amine group is protonated and, combined with the neutralizing effect of spermine, confers an overall positive charge on the oligonucleotide probe. Originally we had used 6 propargylamino residues in a 12 base spacer at the 5′ end of oligonucleotide probes which was adjacent to the 5′-label used to provide the SERRS signal.[20]

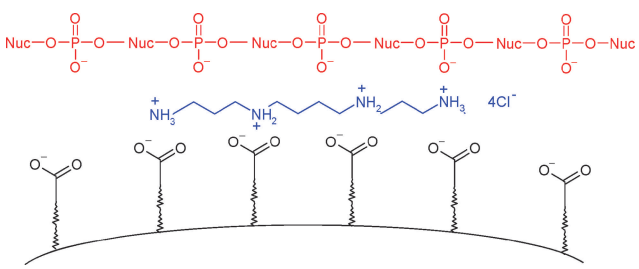

Fig. 3 Representation of the use of spermine to promote adsorption of oligonucleotides onto nanoparticles surfaces. The surface of the nanoparticle is coated in citrate and is negatively charged. The positively charged spermine interacts with the carboxylates and the phosphate groups of the oligonucleotide. There appears to be excess citrate groups on the surface hence the observation that regions of positive charge on the oligonucleotides enhance adsorption.

In a recent study we have optimised the number of modified bases required and shown that a minimum of 2 propargylamino modified residues can be used to provide the same detection limits as those obtained when 6 modified bases are used with negatively charged dyes.[28] If the dye is neutral or positively charged then the propargylamino groups will still increase the surface adsorption and hence the efficiency of the SERRS but not to the same extent as with the negatively charged dyes. As such we do not see modification of oligonucleotide probe sequences containing positive and neutral dyes to be necessary. It should be pointed out that the propargylamino modification cannot be used with every dye due to the requirements of the solid phase synthesis of the dye labelled oligonucleotides and specialist advice is recommended when looking to create new probe conjugates.

2. Non-fluorescent labels

In the non-fluorescently labelled category we can break this down into dyes designed to label the DNA and dyes designed to complex the oligonucleotide probes more effectively onto the nanoparticle surface. Labels can be used which simply adsorb onto the surface of the nanoparticle such as DABCYL, phthalocyanines and black hole quenchers (BHQs) (Table 2). Alternatively dyes can be designed for complexing directly to the metal surface through formation of bonds between the metal and a complexing group which is part of the dye, as opposed to simple electrostatic interactions. These strategies have been based on the use of simple azo dyes which contain the benzotriazole motif which has been shown to be very effective in complexing onto silver nanoparticles. Two main

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Table 1 Structures and charges of the commercially available dye labels attached to oligonucleotides and used in SERRS

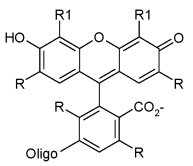
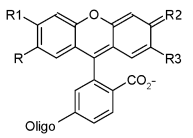
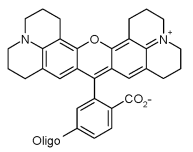
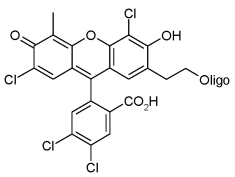
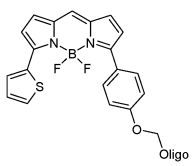
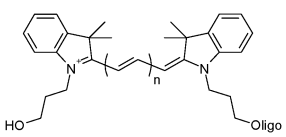
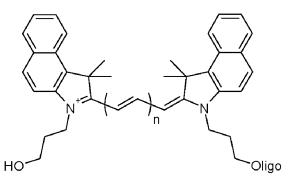
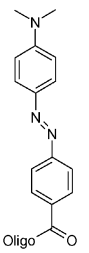
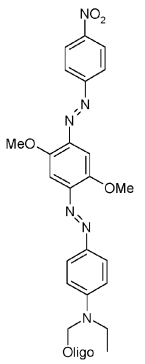
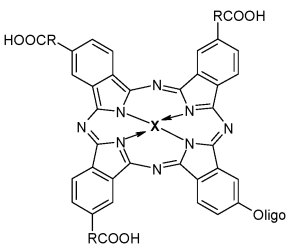
Dye label	Structure	Charge
R, R1 = H = FAM R = Cl, R1 = H = TET R, R1 = Cl = HEX		Negative
R, R3 = H, R1 = $(CH_3)_2N$, R2 = $N^+(CH_3)_2$ = TAMRA R, R3 = CH_3, R1 = CH_3CH_2NH, R2 = $N^+HCH_2CH_3$ = R6G		Positive
ROX		Positive
Yakima Yellow		Negative
BODIPY TR-X		Negative
n = 1 = Cy3 *n* = 2 = Cy5 *n* = 3 = Cy7		Positive
n = 1 = Cy3.5 *n* = 2 = Cy5.5		Positive

Table 2 Non-fluorescent dyes used to provide SERRS from oligonucleotides through adsorption onto the silver nanoparticle surface

Dye label	Structure	Charge
DABCYL		Positive
BHQ2		Positive
X = Co = PtcCo X = Al = PtcAl X = Zn = PtcZn		Positive

approaches exist for the addition of benzotriazole azo dyes to oligonucleotides (Fig. 4). These are the synthesis of a dye phosphoramidite (**1**) which can be used during solid phase synthesis of the oligonucleotide probes to incorporate the dye at the 5′-terminus of the probe[29] or to use a post-synthetic coupling strategy whereby a reactive benzotriazole azo dye (**2**, **5**) is reacted with a functional group on an oligonucleotide probe such as an amine or thiol.[30,31] Alternatively the maleimide dye (**2**) can be coupled with a diene modified oligonucleotide *via* a Diels–Alder cycloaddition.[32] These strategies then produce 5′-labelled oligonucleotides (**3**, **4**, **6**) capable of complexing directly to the metal surface and displacing the surface citrate layer, which can be then measured as it is removed.[30]

Thus, there is a combination of different dye approaches which allow exploitation of the different chemistries for surface adsorption in terms of the molecular biology and detection of the specific DNA sequence. It should also be noted that almost all of the labels to date have been used to label the 5′-position of the oligonucleotides, however 3′-labelling has been reported in some studies.[33,34] Since it is the label which is being detected, and not the DNA sequence directly, the position of the label appears to be dictated by the requirements of the molecular biology and in some cases there may be an advantage to placing the label at different positions on the oligonucleotide probe.

Sensitivity

When oligonucleotide probes are designed as described above, and used with nanoparticles, a quantitative response can be obtained where the concentration of the labelled oligonucleotide is below monolayer coverage of the nanoparticle surface (Fig. 5 and 6).[24,35] The quantitative behaviour allows the

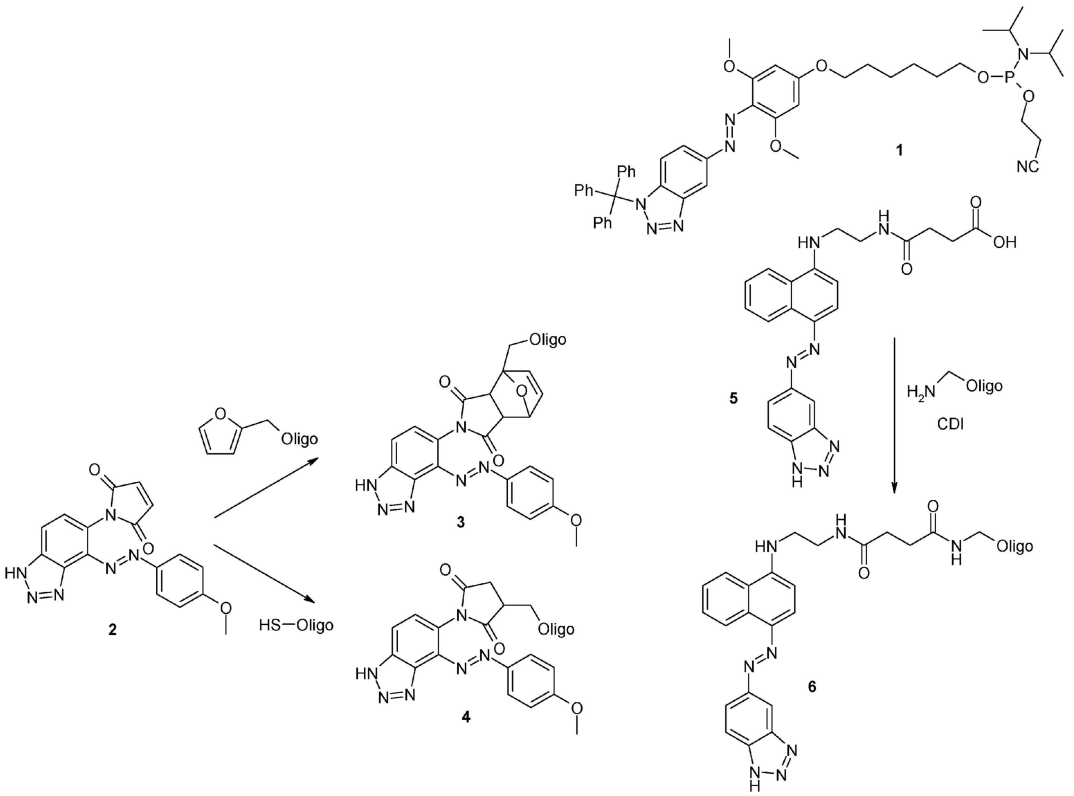


Fig. 4 The different approaches to attach benzotriazole and benzotriazole azo dyes to oligonucleotides.

SERRS detection limits to be obtained. Since some of these dyes used for the SERRS probes are also fluorescently active the same probes can be used to calculate limits of detection by fluorescence as well as for SERRS, thus allowing a direct comparison of the two techniques.[35] Table 3 shows the limits of detection (LoD) for a range of different labels, excitation frequencies and gold and silver nanoparticles. The fluorescence detection limits were calculated using routinely available fluorimeters and the detection limits for the SERRS probes were calculated using a range of different spectrometers, including Raman microscopes and fibre optic based systems.[27]

The detection limits were approximately the same for the different Raman systems, regardless of cost or configuration. This indicated that, although there is a compromise in terms of resolution as the specification of the spectrometer is lowered, the absolute sensitivity in terms of limits of detection does not appear to be greatly compromised. Obviously this depends on the laser excitation frequency and the power used. The fluorescence detection limits were obtained on fluorimeters which included quantitative PCR instrumentation, thus giving a direct comparison to instruments currently found in a functioning molecular diagnostic laboratory.

The main conclusions which can be drawn from Table 3 are that SERRS is significantly more sensitive than fluorescence for straightforward detection of labelled DNA sequences and

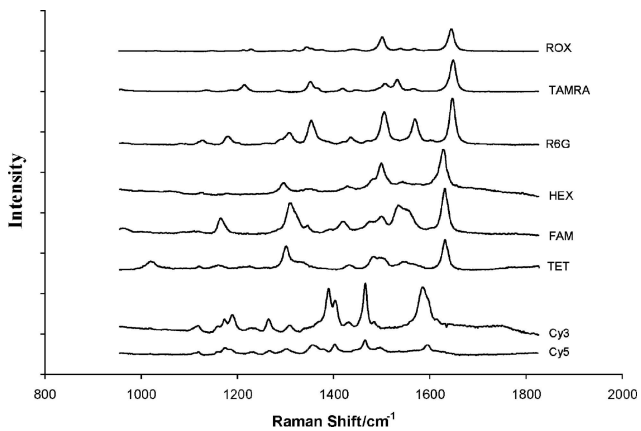


Fig. 5 SERRS Spectra of 8 dye-labeled oligonucleotides, obtained using 514.5 nm laser excitation at $\sim 1 \times 10^{-8}$ mol dm^{-3}. *Anal. Chem.*, 2004, **76**(2), 412. Reproduced by permission of The American Chemical Society (ACS).

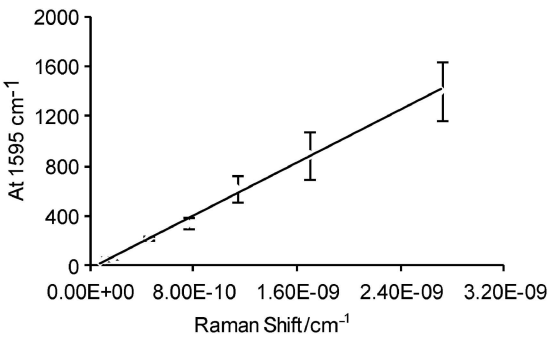


Fig. 6 Calibration graph for rhodamine 6G labeled oligonucleotide using the main peak at 1595 cm^{-1} and 514.5 nm excitation. *Anal. Chem.*, 2004, **76**(2), 412. Reproduced by permission of The American Chemical Society (ACS).

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Table 3 SERRS limits of detection for a range of dye-labelled oligonucleotides (calculated from the mean of the blank (x_{BL}) plus 3× standard deviation of the blank ($3\sigma_{BL}$) divided by the slope of the calibration graph (m), $x_{BL} + 3\sigma_{BL}/m$). No value indicates the experiment was performed but the limit of detection is higher than the base concentration of each dye ($\sim 1 \times 10^{-8}$ M). The fluorescence detection limits were not measured (N.M.) for all of the dyes hence the not measured entries

Dye label	λ_{max}/nm	λ_{ex}: 514.5 nm	λ_{ex}: 632.8 nm		λ_{ex}: 785 nm	Fluorescence LoD/mol dm^{-3}
		Silver/mol dm^{-3}	*Silver*/mol dm^{-3}	*Gold*/mol dm^{-3}	*Silver*/mol dm^{-3}	
FAM	492	2.7×10^{-12}	2.0×10^{-9}	—	—	6.5×10^{-8}
TET	521	1.6×10^{-11}	2.0×10^{-9}	—	—	2.6×10^{-8}
R6G	524	1.2×10^{-12}	1.1×10^{-10}	—	—	3.5×10^{-8}
Yakima Yellow	526	1.7×10^{-11}	—	—	—	N.M.
HEX	535	7.8×10^{-12}	1.2×10^{-9}	—	—	1.1×10^{-8}
Cy3	552	2.6×10^{-10}	1.5×10^{-10}	—	—	4.6×10^{-9}
TAMRA	565	3.5×10^{-12}	1.8×10^{-10}	—	—	1.1×10^{-8}
Cy3.5	581	2.5×10^{-11}	7.5×10^{-13}	2.5×10^{-10}	—	N.M.
ROX	585	8.1×10^{-11}	3.3×10^{-11}	1.1×10^{-9}	—	2.3×10^{-8}
BODIPY TR-X	588	1.3×10^{-10}	7.9×10^{-12}	4.9×10^{-10}	—	N.M.
PtcCo	625	—	3.2×10^{-11}	N.M.	—	Not fluorescent
PtcAl	640	—	2.8×10^{-11}	N.M.	—	Not fluorescent
Cy5	643	—	8.3×10^{-11}	1.7×10^{-9}	—	3.1×10^{-9}
PtcZn	680	1.4×10^{-10}	3.2×10^{-11}	N.M.	—	Not fluorescent
Cy5.5	683	—	5.2×10^{-12}	7.3×10^{-11}	—	N.M.
Cy7	748	—	—	—	5.8×10^{-11}	N.M.

that, in terms of SERRS, the lowest detection limits were obtained using 632.8 nm excitation with silver nanoparticles and a Cy3.5 label. When the excitation frequency is increased the detection limits increase as the availability of chromophores in resonance with that excitation frequency diminishes, however, new chromophores that are in resonance at the higher wavelengths are currently being synthesised.[36] The other interesting feature to arise from this study is that silver nanoparticles consistently gave lower limits of detection than gold nanoparticles. There is also a clear correlation between the λ_{max} of the dye and the excitation frequency used. This informs us that the resonance contribution of the label is important to the sensitivity of the system and that for maximum sensitivity, a resonance contribution with the molecular label as well as a resonance contribution with the surface plasmon is necessary for maximum enhancement.

Choice of surface

As stated above there are two commonly used types of surface used in the SERRS detection of DNA. These are colloidal suspensions of nanoparticles and metal coated surfaces. In terms of nanoparticle suspensions, gold and silver are the most obvious choices. This is due to the ease of their synthesis. Suspensions of nanoparticles are typically prepared by reducing the metal salt using citrate, borohydride, hydroxylamine or EDTA, with citrate being the most commonly used. It is also known that not all nanoparticles or aggregates of nanoparticles will be SERRS active or active to equal extents. However, by using a suspension of aggregated particles a large number of particles are interrogated by the laser beam at any one time so that the signal detected is an average over a number of particles and is stable. Various batches of nanoparticles can be blended to give a large stock of nanoparticles with a consistent enhancing comparability and aid reproducibility. Brownian motion also mitigates against deactivation by photodecomposition, sample drying and sample heating which can be an issue with metal surfaces. This control over the SERRS signal is achieved by the averaging out of the enhancements through the aggregation procedure to allow quantitation. As seen above silver nanoparticles give lower detection limits than gold nanoparticles in these types of studies and the theoretical enhancement from silver should outweigh that of gold.[27] This means that the majority of studies for maximum sensitivity use silver nanoparticles, however, due to the difficulties in reproducible synthesis of silver nanoparticles a number of studies have used gold nanoparticles. However, silver nanoparticles with reasonable degrees of monodispersity can also be synthesized in a controlled manner which is necessary when looking for a genuinely reproducible technique (Fig. 7).[37]

The alternative to using nanoparticles is to use a roughened metal surface. This can, for example, take the form of a roughened metal electrode, a thin vapour deposited metal film or a specially designed structured surface. Allain and Vo-Dinh

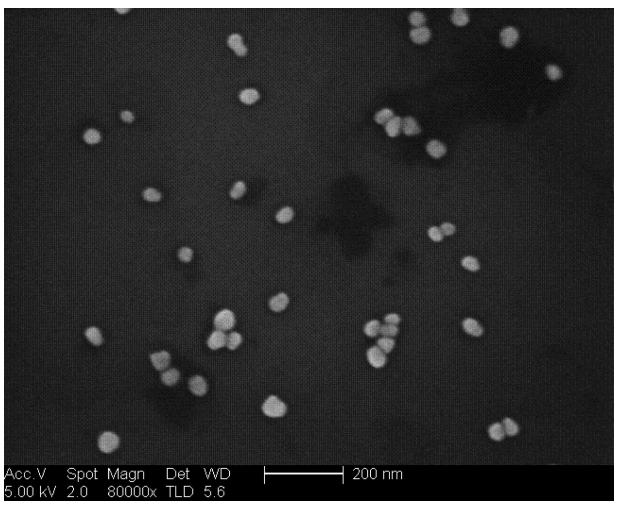

Fig. 7 SEM of silver nanoparticles prepared by reduction of silver onto core silver nanoparticles to give improved monodispersity (average diameter 52 nm, relative standard deviation = 12%).

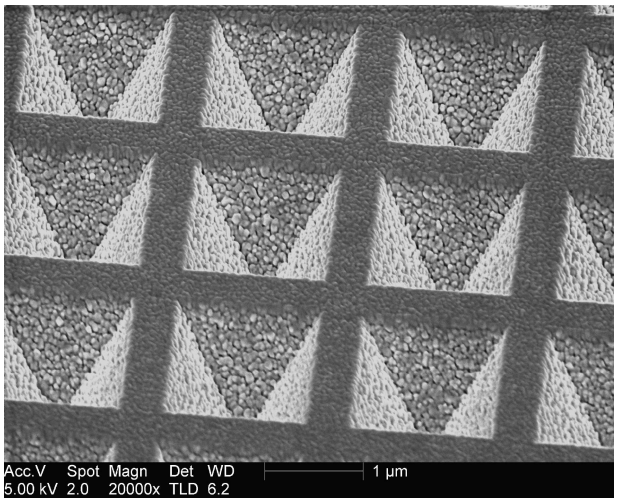

Fig. 8 SEM of the Klarite SERRS surface consisting of inverted square based pyramids coated in gold.

have utilised a 9 nm thick silver island film to detect the breast cancer gene BRC*A1* by labelling an oligonucleotide probe with the dye rhodamine B and using a λ_{ex} of 632.8 nm.[38] These surfaces have been used in a number of different detection systems including that of a gene chip,[14] SERRS molecular beacons,[33] and molecular sentinels,[34] to yield highly sensitive detection of specific DNA sequences. An alternative to these PVA silver nanoparticles surfaces or the vapour deposited films which are often used in SERRS studies is that of Klarite (Fig. 8).[39]

Klarite is a structured metal surface that has been designed to have a particular plasmon frequency and is fabricated as inverted square based pyramids with a gold coating.[39] This surface has recently been used as a substrate for the detection of DNA through hybridisation onto an immobilised probe on the surface.[40] Although not a genuine biological assay, this format is of interest due to the enhancement of the label even although it is still a considerable distance from the surface. In addition, it was shown that by getting the surface chemistry right for the immobilisation of the capture probe the relative standard deviations (RSDs) of the detection by SERRS came down to lower than 10% as opposed to much higher values when a non-specific capture methodology was used.

Multiplexing

By multiplexing we mean the ability to detect multiple labels attached to different DNA sequences at the same time, but without using any separation procedures. Obviously if an array based format is being used then spatial separation can be employed to increase the multiplexing capability of the system. This review is focusing on the suspension/solution phase multiplexing capability of SERRS and as such is not going to address the array format for this particular type of analysis. The simplest multiplex detection to achieve is obviously a 2-plex which was achieved when the multiplexed genotyping of the mutational status of the cystic fibrosis gene was obtained. During this study it was shown that by varying the ratio of the oligonucleotide probes present the change in the ratio of the SERRS signals indicated the amounts of the different probes present.[25] In that particular study a rhodamine labelled oligonucleotide was compared and mixed with a HEX labelled oligonucleotide. An advancement of the 2-plex is obviously a 3-plex which was used to indicate the presence of three mixed oligonucleotide probe sequences which corresponded to gene sequences from *E. coli* O157:H7.[41,42] In addition to this 3-plex a different 3-plex was performed using phthalocyanine labelled oligonucleotides where the central metal ion was changed in the phthalocyanine to give different SERRS signatures.[43] Cobalt, aluminium and zinc were all used to provide different labels which could then be quantified in terms of the SERRS response and when mixed in a multiplex in different ratios easily identified through their signature fingerprints.

In a recent study a 5-plex was analysed using a mixture of 5 dye labelled oligonucleotides and 2 excitation frequencies.[44] In this case the information obtained in Table 3 was used to pick 5 dyes, three of which would give excellent limits of detection using 514.5 nm excitation and two of which would give excellent limits of detection using 632.8 nm excitation. It was ascertained that the ROX labelled oligonucleotide gave signals at both 514.5 and 632.8 nm excitation. When the oligonucleotide probes were mixed together and excited using 514.5 nm, signals that could be clearly identified as coming from three of the probes were easily recognised, namely FAM, R6G and ROX (Fig. 9). When the mixture was excited using 632.8 nm excitation, three signals could again be easily identified as coming from ROX, BODIPY and Cy5.5. The limits of detection for the individual dyes, as well as the dyes in the

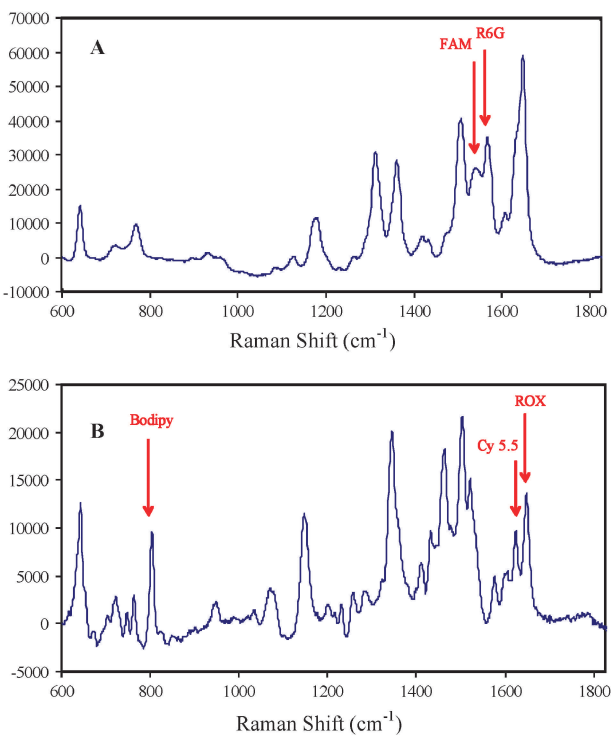

Fig. 9 Multiplexed SERRS spectra of the 5-plex at 1.82×10^{-9} mol dm^{-3} using A, 514.5 nm and B, 632.8 nm excitation. *Angew. Chem., Int. Ed.*, 2007, **46**, 1829. Reproduced by permission of Wiley.

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multiplex, were determined and compared. An interesting point here was that the limit of detection for the labelled oligonucleotides in the multiplex was almost identical to that of the labelled oligonucleotides on their own, indicating that the multiplexed format did not compromise the sensitivity and hence limit of detection of this system. This is an important point as it is the surface coverage which determines the limit of detection and it appears that the choice of these labels and the design of the probes meant there was no fierce competition for available metal surface area on the nanoparticles.

In order to go beyond a 5-plex, chemometrics need to be employed and a recent study where a 6-plex of labelled oligonucleotides was investigated has shown that use of statistical analysis can clearly identify the presence or absence of each labelled oligonucleotide in the mixture of the other similarly labelled probes and also identify the components of a complex mixture with high certainty.[45]

Assays

All of the above information can be used to provide a solid foundation from which to generate meaningful detection that relates to a specific DNA sequence of interest in a genuine biological sample. In order to do this some molecular biology to bridge the gap from taking a sample to obtaining an answer needs to be employed. When detecting DNA targets then the most obvious way to do it is to use a PCR based approach. As stated previously, there are studies in the literature relating to array based formats for specific DNA detection by SERRS, however, this review is focusing on nanoparticle and solution phase based detection systems and as such the assays reported here relate only to this type of format. An early study for the detection of the mutational status of the cystic fibrosis transmembrane conductance regulator gene using an amplification refractory mutation system (ARMS) approach was successful using SERRS.[46,47] The assay, although primitive by today's standards, was an excellent example of using the selectivity of SERRS to provide a 2-plex identification of different gene sequences from human patient examples. There are three different possibilities for the genetic status of this particular gene and these are either the wild type, where both alleles are normal, the heterozygote, where one allele is mutated and one is normal, and the fully homozygote mutant, where both alleles have the mutated DNA sequence. In this case the main mutation was the absence of the 3 bases which corresponded to the tryptophan residue that this particular sequence codes for in the protein.

The design of specific primers to amplify the region relating to this mutation were designed and used in a multiplexed PCR assay and the PCR amplicons identified by SERRS after removal of unincorporated primers. This allowed the successful elucidation of the mutational status of these particular samples without the additional separation steps commonly performed in the fluorescence assays.

An advancement of that particular assay was to miniaturise the handling conditions and to transpose the assay into a microfluidics device. To achieve this, a different strategy to the molecular biology was adopted. This involved using primers which were unmodified and the presence of a biotin tag and a

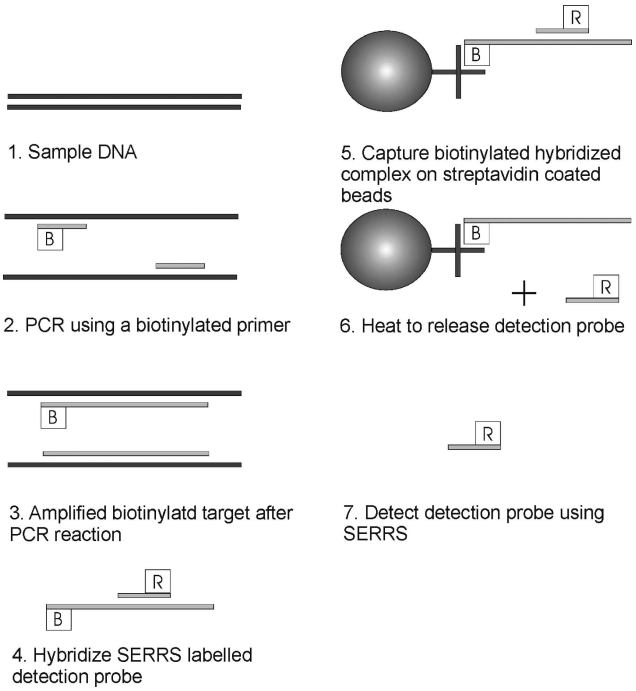

Fig. 10 Outline of the steps involved during the performance of the bead-based assay for *Chlamydia trachomatis*. *Anal. Chem*., 2007, **79**(7), 2844. Reproduced by permission of The American Chemical Society (ACS).

probe which was SERRS active and which would hybridise to part of the newly amplified PCR product.[48] The assay is outlined in Fig. 10 and involves the immobilisation of the amplified product followed by hybridisation of the SERRS active probe, immobilisation of this complex onto streptavadin coated beads or a surface which allows the excess probe to be washed away and then denaturation of the probe target duplex allows washing of the beads to generate the SERRS active probe which was then detected to indicate the presence or absence of that particular sequence of DNA. In a microfluidics format there were posts engineered into the channels to capture beads coated with streptavadin which were used for the immobilisation of the complex which was then chemically shocked to release the oligonucleotide SERRS active probe following the washing away of any of the excess reagents (Fig. 11).

In a different approach, a molecular beacon assay was performed whereby a dual labelled probe was synthesised to contain a dye at the 5′ terminus and a benzotriazole dye at the 3′ terminus[33] (Fig. 12). In this example a combination of a benzotriazole azo dye was used with a commercially available fluorophore. The beacon sequence was chosen such that it was held in a stem loop and the labels were close to each other in proximity when the beacon was closed. In this state there were SERRS signals which correspond to both the benzotriazole as an azo dye and also the fluorophore. In addition, the fluorescence was quenched as the fluorophore was held onto the metal surface due to the close proximity of the fluorophore to the anchoring group of the benzotriazole due to the stem loop formation (Fig. 13). When the beacon sequence hybridised to its complement the SERRS beacon then opened up and

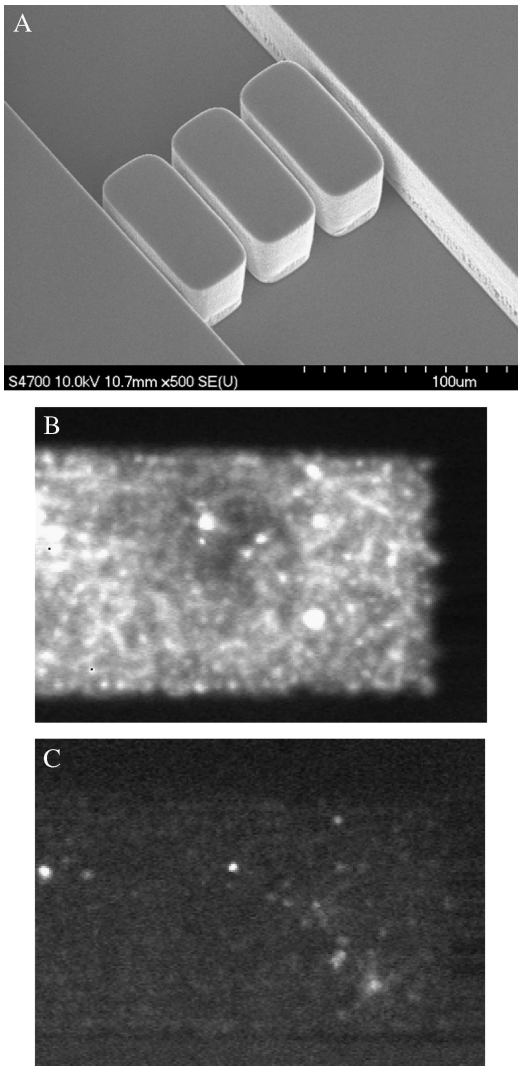

Fig. 11 (A) SEM of the channel containing the posts. Photomicrographs of the beads trapped within the microfluidic device: (B) before release of detection probe and (C) after heating and release of the detection probe. *Anal. Chem.*, 2007, **79**(7), 2844. Reproduced by permission of The American Chemical Society (ACS).

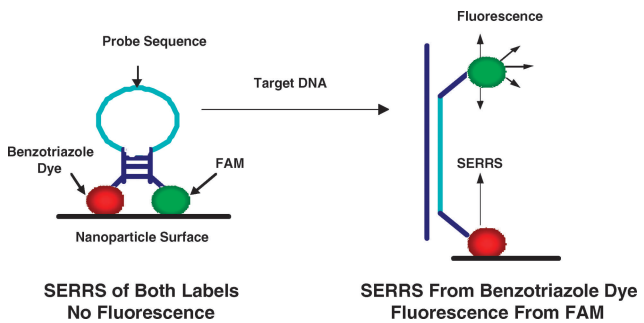

Fig. 12 The concept of the SERRS beacon. The beacon was produced with a 3′-FAM fluorophore and a 5′-benzotriazole azo dye. The azo dye has been designed to complex to silver metal surfaces and produce SERRS. *Faraday Discuss.*, 2006, **132**, 261.—Reproduced by permission of The Royal Society of Chemistry (RSC).

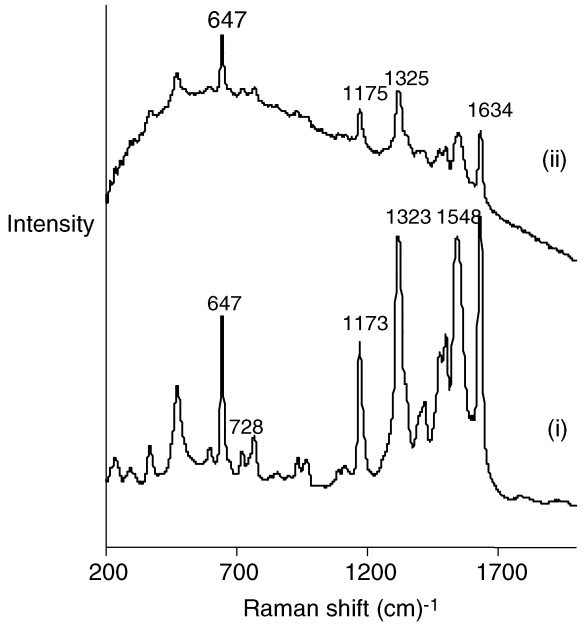

Fig. 13 SERRS spectra of (i) the closed SERRS beacon and (ii) the hybridised product at 3.4×10^{-8} mol dm^{-3}. *Faraday Discuss.*, 2006, **132**, 261.—Reproduced by permission of The Royal Society of Chemistry (RSC).

effectively formed a linear piece of DNA which held the fluorophore away from the metal surface and changed the SERRS signals as well as removing the quenching mechanism of the nanoparticle. In this particular study a FAM benzotriazole azo dye beacon was synthesised and used to detect a synthetic DNA sequence. Although this format has not been used in a genuine biological assay as yet, it indicates the ability of SERRS to deconvolute combinations of labels and offers the opportunity for further development work in this field.

Conclusion

This review describes how oligonucleotide probes can be synthesised and designed to be SERRS active and to obtain low levels of detection in a quantitative manner. This can be achieved through careful choice of the molecular label and also the nanoparticles used for the enhancement of the scattering. In addition, the choice of the excitation source does affect the limits of detection and also use of multiple sources of excitation can lead to increased multiplexing due to the vibrational nature and excitation profiles of the different dye combinations possible. The use of SERRS detection in genuine molecular diagnostic assays is still in its infancy but shows excellence promise and a number of different avenues are currently under investigation by many groups to exploit the detection technique of SERRS as a high information quantitative ultra-sensitive bioanalytical technique.

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References

1 S. Yang and R. E. Rothman, *Lancet Infect. Dis.*, 2004, **4**, 337.
2 L. Bissonnette and M. G. Bergeron, *Expert Rev. Mol. Diagn.*, 2006, **6**, 433.
3 A. M. T. Linacre and D. Graham, *Expert Rev. Mol. Diagn.*, 2002, **2**, 346.
4 V. Garcia-Canas, A. Cifuentes and R. Gonzalez, *Crit. Rev. Food Sci. Nutr.*, 2004, **44**, 425.
5 D. C. Wallace, *Annu. Rev. Biochem.*, 2007, **76**, 781.
6 P. M. Holland, R. D. Abramson, R. Watson and D. H. Gelfand, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 7276.
7 S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303.
8 D. Whitcombe, J. Theaker, S. P. Guy, T. Brown and S. Little, *Nat. Biotechnol.*, 1999, **17**, 804.
9 C. T. Wittwer, M. G. Herrmann, A. A. Moss and R. P. Rasmussen, *Biotechniques*, 1997, **22**, 130.
10 K. Kneipp, H. Kneipp, G. Deinum, I. Itzkan, R. R. Dasari and M. S. Feld, *Appl. Spectrosc.*, 1998, **52**, 175.
11 S. M. Nie and S. R. Emery, *Science*, 1997, **275**, 1102.
12 D. Cunningham, R. E. Littleford, W. E. Smith, P. J. Lundahl, I. Khan, D. W. McComb, D. Graham and N. Laforest, *Faraday Discuss.*, 2006, **132**, 135.
13 A. Ruperez, R. Montes and J. J. Laserna, *Vib. Spectrosc.*, 1991, **2**, 145.
14 T. Vodinh, K. Houck and D. L. Stokes, *Anal. Chem.*, 1994, **66**, 3379.
15 K. Faulds, W. E. Smith and D. Graham, *Analyst*, 2005, **130**, 1125.
16 G. Frens, *Nature*, 1973, **241**, 20.
17 P. C. Lee and D. Meisel, *J. Phys. Chem.*, 1982, **86**, 3391.
18 N. S. Lee, *Bull. Korean Chem. Soc.*, 1991, **12**, 601.
19 L. P. Zhou, J. Yang, C. Estavillo, J. D. Stuart, J. B. Schenkman and J. F. Rusling, *J. Am. Chem. Soc.*, 2003, **125**, 1431.
20 D. Graham, W. E. Smith, A. M. T. Linacre, C. H. Munro, N. D. Watson and P. C. White, *Anal. Chem.*, 1997, **69**, 4703.
21 K. Faulds, R. E. Littleford, D. Graham, G. Dent and W. E. Smith, *Anal. Chem.*, 2004, **76**, 592.
22 Y. W. C. Cao, R. C. Jin and C. A. Mirkin, *Science*, 2002, **297**, 1536.
23 K. Faulds, W. E. Smith and D. Graham, *Anal. Chem.*, 2004, **76**, 412.
24 K. Faulds, L. Stewart, W. E. Smith and D. Graham, *Talanta*, 2005, **67**, 667.
25 D. Graham, B. J. Mallinder and W. E. Smith, *Angew. Chem., Int. Ed.*, 2000, **39**, 1061.
26 N. R. Isola, D. L. Stokes and T. VoDinh, *Anal. Chem.*, 1998, **70**, 1352.
27 R. J. Stokes, A. Macaskill, P. J. Lundahl, W. E. Smith, K. Faulds and D. Graham, *Small*, 2007, **3**, 1593.
28 K. Faulds, F. McKenzie and D. Graham, *Analyst*, 2007, **132**, 1100.
29 R. Brown, W. E. Smith and D. Graham, *Tetrahedron Lett.*, 2003, **44**, 1339.
30 D. Graham, R. Brown and W. E. Smith, *Chem. Commun.*, 2001, 1002.
31 D. Graham, K. Faulds and W. E. Smith, *Chem. Commun.*, 2006, 4363.
32 L. Fruk, A. Grondin, W. E. Smith and D. Graham, *Chem. Commun.*, 2002, 2100.
33 K. Faulds, L. Fruk, D. C. Robson, D. G. Thompson, A. Enright, W. E. Smith and D. Graham, *Faraday Discuss.*, 2006, **132**, 261.
34 M. B. Wabuyele and T. Vo-Dinh, *Anal. Chem.*, 2005, **77**, 7810.
35 K. Faulds, R. P. Barbagallo, J. T. Keer, W. E. Smith and D. Graham, *Analyst*, 2004, **129**, 567.
36 R. J. Stokes, A. Ingram, J. Gallagher, D. R. Armstrong, W. E. Smith and D. Graham, *Chem. Commun.*, 2008, 567.
37 P. J. Lundahl, R. J. Stokes, R. M. Martin, W. E. Smith and D. Graham, *Micro Nano Letters*, 2008, submitted.
38 L. R. Allain and T. Vo-Dinh, *Anal. Chim. Acta*, 2002, **469**, 149.
39 N. M. B. Perney, J. J. Baumberg, M. E. Zoorob, M. D. B. Charlton, S. Mahnkopf and C. M. Netti, *Opt. Express*, 2006, **14**, 847.
40 R. J. Stokes, A. Macaskill, J. A. Dougan, P. G. Hargreaves, H. M. Stanford, W. E. Smith, K. Faulds and D. Graham, *Chem. Commun.*, 2007, 2811.
41 F. T. Docherty, M. Clark, G. McNay, D. Graham and W. E. Smith, *Faraday Discuss.*, 2004, **126**, 281.
42 F. T. Docherty, P. B. Monaghan, R. Keir, D. Graham, W. E. Smith and J. M. Cooper, *Chem. Commun.*, 2004, 118.
43 A. Macaskill, A. A. Chernonosov, V. V. Koval, E. A. Lukyanets, O. S. Fedorova, W. E. Smith, K. Faulds and D. Graham, *Nucleic Acids Res.*, 2007, **35**, e42.
44 K. Faulds, F. McKenzie, W. E. Smith and D. Graham, *Angew. Chem., Int. Ed.*, 2007, **46**, 1829.
45 K. Faulds, R. Jarvis, W. E. Smith, D. Graham and R. Goodacre, *Analyst*, 2008, submitted.
46 D. Graham, B. J. Mallinder, D. Whitcombe and W. E. Smith, *ChemPhysChem*, 2001, **2**, 746.
47 D. Graham, B. J. Mallinder, D. Whitcombe, N. D. Watson and W. E. Smith, *Anal. Chem.*, 2002, **74**, 1069.
48 P. B. Monaghan, K. M. McCarney, A. Ricketts, R. E. Littleford, F. Docherty, W. E. Smith, D. Graham and J. M. Cooper, *Anal. Chem.*, 2007, **79**, 2844.