Biosensing using silver nanoparticles and surface enhanced resonance Raman scattering

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Silver nanoparticles can be used to provide excellent surface enhanced resonance Raman scattering. Control of the surface chemistry and the use of appropriate protocols enables effective sensing of biomolecules.

Introduction

Interest in the detection of various biomolecules such as DNA, proteins (including enzymes, antibodies and receptors) and small molecular species that are bioactive is at an all time high. The ability to fulfil the desire to understand how molecular interactions shape the function and nature of biological systems is dependent on the detection of the molecules listed above. As such this has led to a massive increase in activity in this particular field of detection science. Over the last couple of decades there have been many techniques adopted for the measurement and investigation of the structure and functional relationships of these types of bioactive molecules and there are many elegant new approaches currently under investigation which will impact on this field in due course and allow faster acquisition of more detailed data. Here we describe how we have combined the technique of surface enhanced resonance Raman scattering (SERRS) with silver nanoparticles to produce nanosensors for various biological targets. Most of this work has been conducted *in vitro* but has been designed to ultimately move towards the *in vivo* environment. The main challenge for the future of biosensing is to create chemistry and spectroscopy that can work in living systems and our interests lie in using SERRS for this approach. This article will describe the technique of SERRS and the parameters that it imposes on our chemistry and detection science and also how we have exploited this technique in combination with novel chemistry to produce nanosensors.

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SERRS

SERRS is a form of Raman spectroscopy in which a combination of surface enhancement provided by immobilising an analyte onto a suitable surface and of molecular resonance from a chromophore in the analyte gives a vibrational spectrum at very high sensitivity.[1,2] The surface enhancement of Raman scattering from pyridine adsorbed onto silver electrodes was first reported in 1974 by Fleischmann *et al*.[3] Since then the field of SERRS has rapidly moved to a situation where the technique, although not fully understood at a fundamental level,[4–8] can now be used for quantitative analysis of meaningful targets.[9–18] The crux of the technique is that the analyte or molecule of interest must be adsorbed onto the enhancing surface. A number of different metals such as silver, gold, copper and aluminium can be used to provide the surface,[1,2] however, silver tends to give the largest enhancement factors when using routinely available

Duncan Graham obtained his PhD in Bioorganic Chemistry in 1996 with Prof. Tom Brown at the University of Edinburgh then moved to the University of Strathclyde as a postdoctoral fellow with Prof. Ewen Smith. In 1997 he was awarded a BBSRC David Phillips fellowship, in 2002 he won the RSC's Analytical Grand Prix Fellowship, in 2004 he was awarded the SAC Silver medal and in 2005 he was presented with the Nexxus Young Life Scientist of the Year award. He was appointed a lecturer in 2002 and professor in 2004. In 2005 he cofounded the Strathclyde Centre for Molecular Nanometrology which operates jointly between the Departments of Chemistry and Physics and exploits the natural synergy in molecular manipulation and optical spectroscopy for the study of biological systems on a nanoscale.

Karen Faulds was appointed as a proleptic lecturer in March 2005 and started her full time position in April 2006. She completed her PhD with Prof. Ewen Smith and two postdoctoral positions at the University of Strathclyde in the area of surface enhanced resonance Raman Scattering (SERRS). She was the first to demonstrate the quantitative detection of DNA and show SERRS was more sensitive than fluorescence. In addition she has published the first quantitative detection of amphetamine by SERS. Her interests are in using the power of SERRS to create new analytical methods that can be used reliably.

Ewen Smith completed his PhD at ICL in 1965 then moved to Oak Ridge National Laboratory for two years before returning to UCL to take up a postdoctoral position with Ronald Nyholm. He joined the University of Strathclyde as a lecturer in 1969 and was appointed a professor in 1987. In 1983 he was awarded a DSc from the University of Glasgow and is a Fellow of the Royal Society of Edinburgh. He has served as Head of Department and in many other senior roles within the UK chemical community. He has published over 300 papers and is a world recognised expert in the area of surface enhanced resonance Raman Scattering (SERRS). He has been responsible for the dramatic increase in the profile and interest in the use of SERRS in practical applications both within the UK and worldwide.

visible excitation wavelengths. If a coloured molecule *e.g.* a dye is used as the target analyte then a resonance contribution to the enhancement is obtained from the electronic transition, the frequency of which must be close to or coincide with the laser excitation frequency used to provide the Raman scattering.[19–22] This combination of surface and molecular resonance contributions to the enhancement provides SERRS which has been reported to give enhancement factors of up to 10^{14} over normal Raman scattering.[23] Single molecule detection has been reported by a number of groups[23–25] indicating the extreme sensitivity of the technique. Further, due to the sharp vibrational nature of the spectrum, identification of specific components of mixtures *in situ* without separation is also increased relative to other spectroscopic techniques such as fluorescence. An additional benefit of SERRS is that only the species on the surface will give rise to signals. Therefore by designing strong surface seeking species, background signals can be kept to a minimum.

SERRS has been used to provide unique information from a number of biological species. The first well defined studies were of cytochromes and demonstrated the ability to selectively analyse the resonant heme chromophore in the presence of the full protein.[26] Recently SERRS has been shown to be useful for the detection of DNA, antibodies, antigens and enzyme activity. It offers significant advantages over fluorescence or other ultra sensitive spectroscopies. The power required to obtain scattering is very low and the molecules do not photofade, the sensitivity is at the single molecule level and, since water is a poor Raman scatterer, SERRS is also compatible with aqueous environments. It provides information from complex mixtures and is selective for the chromophore adsorbed onto the surface. In addition, modern Raman spectrometers have decreased considerably in price and suitable and portable equipment for the detection of Raman scattering can be acquired for less than £10 000. This makes the technique more widely available.

However, there are a number of limitations which must be considered before choosing to use SERRS as a detection technique. The first is that the molecule of interest must adsorb effectively onto the metal surface. The format of this surface will be discussed in the next section but it can be either a solid surface or a suspension of particles compatible with solution phase analysis procedures. The other major limitation is that not all molecules have a large Raman cross section and as a result, some molecules provide better SERRS signals than others. In some cases such as the case of heme proteins this can be an advantage but for most analyses it makes it difficult to construct analytical methods which can be employed to determine a wide range of analytes. To overcome this, we have adopted the same approach used for fluorescence detection whereby we attach a strong SERRS label to a molecule of interest using the biochemistry of the molecule to provide the analysis procedure.[27,28] An additional benefit of SERRS is that any fluorescence produced is quenched by the metallic surface used for the enhancement (Fig. 1). This means that a wide range of dye chemistries are available and that fluorescent molecules which are routinely available can also be used as long as they can be made to adhere to the metal surface.

There are various formats which can be used in SERRS experiments and these depend on the different types of metal surface used. Electrodes have been used previously[3,29,30] as have vapour deposited films[31,32] and a whole variety of different roughened surfaces.[33,34] For many of these surfaces, there is little control of the nanoscale roughness features to ensure that enhancement factors across the surface are uniform and reproducible. As such this led to large variations in the enhancement of SERRS when these types of surfaces were used for quantitative analytical work. More recently, engineered surfaces with controlled regularity have been introduced. These can give very good and much more reproducible surfaces.[35] These are very effective for qualitative analysis but for quantitative analysis care must be taken to use delivery methods which result in even coverage of the surface by the analyte. Simple methods such as drying out a spot of analyte on the surface results in variable surface layers and hence non-reproducible SERRS.

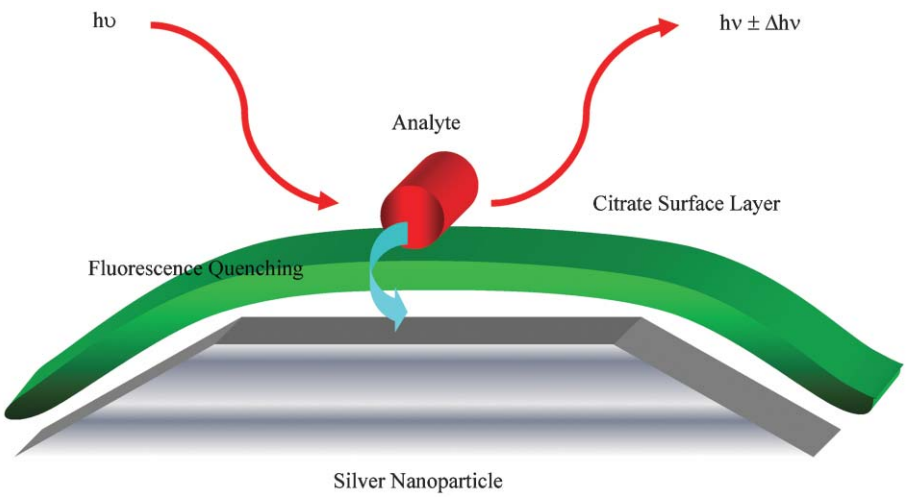


Fig. 1 The SERRS process involving adsorption of the coloured analyte onto the silver nanoparticle which is covered in a citrate surface layer. The excitation source is in resonance with the molecule and surface enhancement arises from the interaction with the plasmon band of the silver. Any fluorescence produced is quenched very efficiently by the metal.

One way to overcome some of the variability is to average the surface enhancement by looking at a number of sites at any one time. This can be done, for example, by focusing on a large area or spinning the sample with the point of focus off set from the central point so that the beam tracks a circle of material.[36] The use of colloidal suspensions of metallic nanoparticles stabilised by a surface coating to ensure that they do not aggregate and precipitate has proved a particularly effective method of obtaining quantitative results. Most commonly silver and gold nanoparticles are used to provide SERRS since they can be routinely produced and are relatively stable. Individual nanoparticles will give rise to surface enhancement under the appropriate conditions.[19] However, maximum signal intensity can be obtained by aggregating the particles in a controlled manner to turn on a larger surface enhancement due to the increased electric field in the interstices between the nanoparticles.[19] This appears to average the enhancement from these aggregates and allows quantitative measurements to be obtained from colloidal suspensions. Of these two different types of metal nanoparticles we have found that silver nanoparticles give greater enhancement factors with visible excitation and as such we have developed an extensive surface chemistry to work with silver nanoparticles which is discussed in the next section.

Surface chemistry

As stated above, the most essential requirement for SERRS is to ensure that the molecule of interest is effectively adsorbed onto the metal surface. Silver nanoparticles are most commonly produced by the reduction of a silver salt (normally silver nitrate) by a variety of different reducing agents such as sodium citrate,[37] EDTA, or sodium borohydride.[38] The nanoparticles produced from citrate or EDTA reduction tend to be more stable over time and as a result we favour these colloids.[39] This is because the nanoparticles produced have an organic surface layer, which in the case of citrate colloid is largely citrate, giving an overall zeta charge of around −40 mV. This relatively high surface charge creates a suspension which is stable over a long period of time. The typical size of a silver nanoparticle produced by the citrate method is 36 nm in diameter.[39]

We have taken two approaches to ensure effective adsorption of molecules onto the surface to produce SERRS (Fig. 2) We can either rely on electrostatic interaction where the negative surface of the particles is used to attract a positively charged dye which can either be attached to the biomolecule of interest or be a positively charged species of interest in its own right. For example to produce SERRS from DNA sequences, a coloured molecule has to be attached to the DNA to produce the resonant chromophore necessary and also some form of positive charge has to be attached to the overall negatively charged DNA to form the electrostatic interaction. We have examined a number of different methods for achieving this and have found the best way to obtain SERRS from oligonucleotides is to use a conventional fluorescent label and if that fluorescent label is positively charged then the phosphate backbone can be neutralised by spermine.[40] The labelled oligonucleotide adheres to the surface and produces substantial SERRS signals in a quantitative manner.[10,12] If the dye is

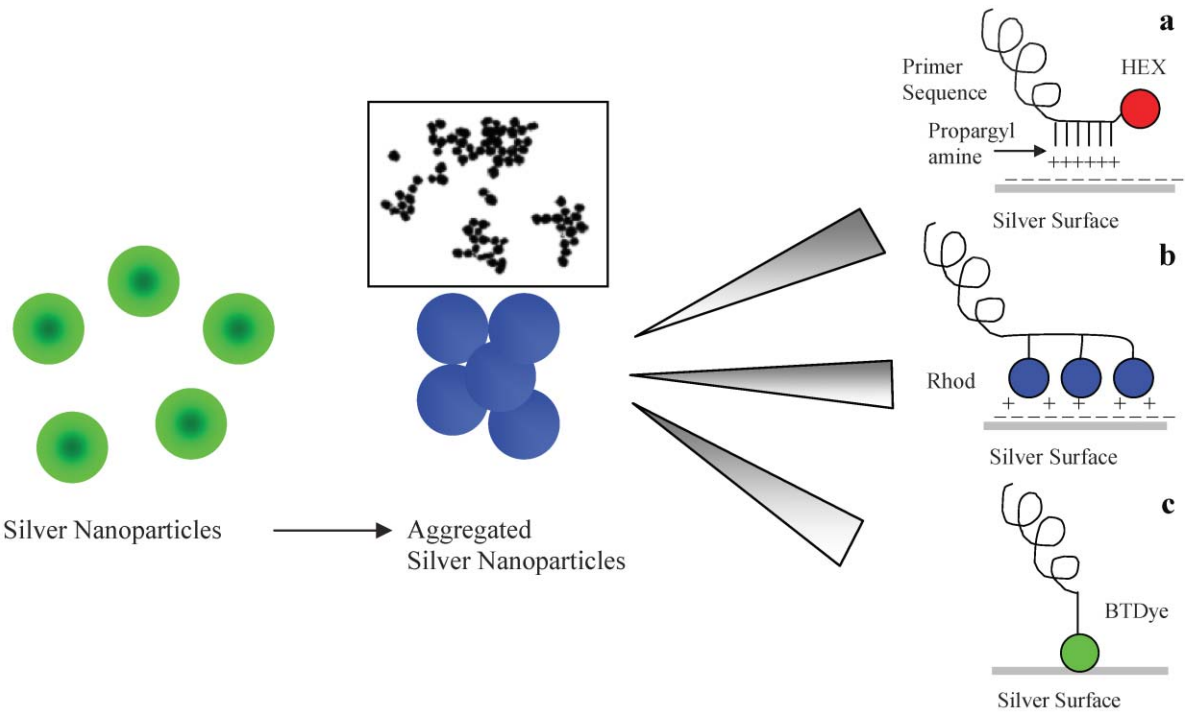
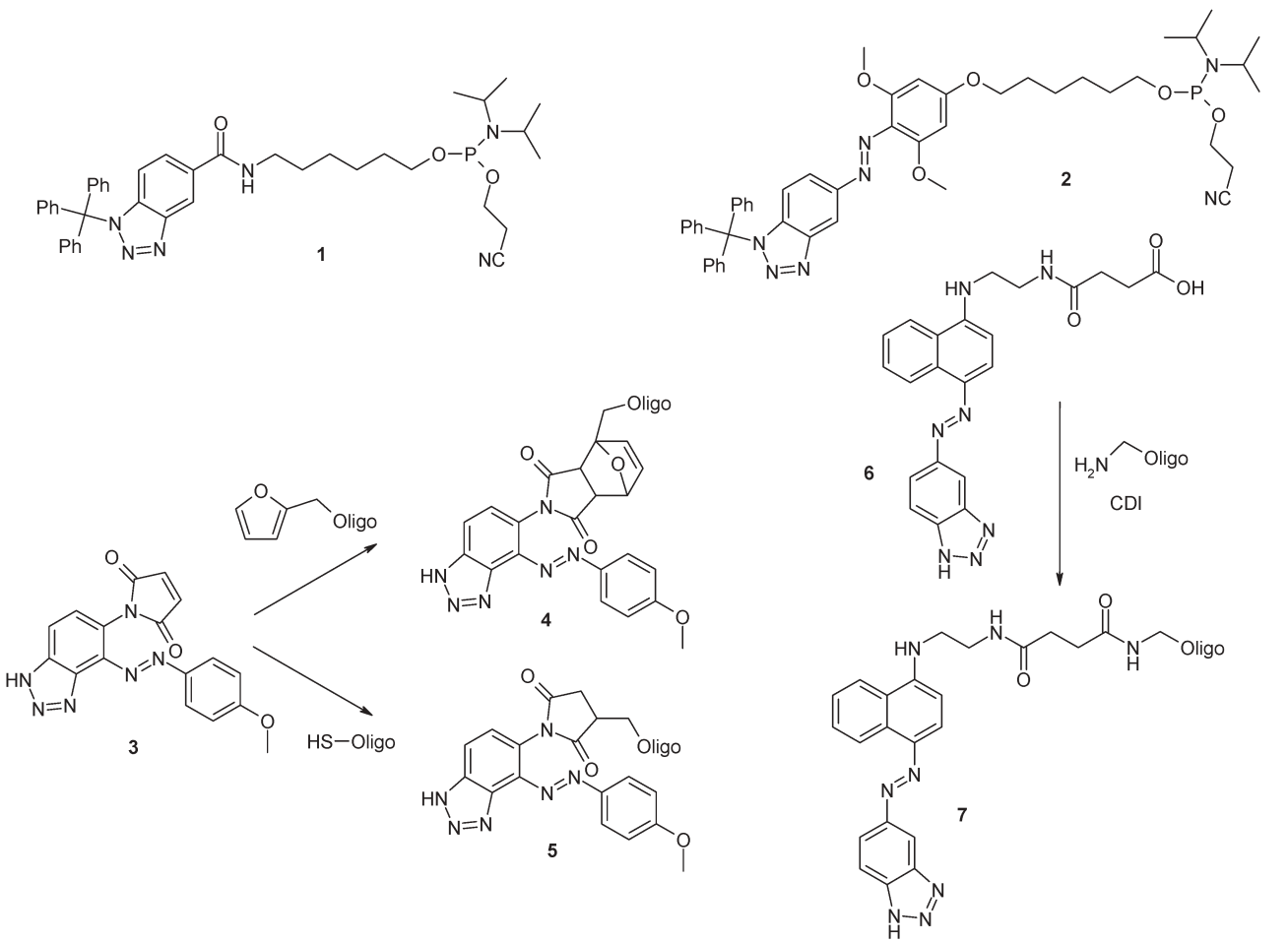


Fig. 2 Schematic representation of the aggregation process required to achieve detection of labelled DNA by SERRS: (a) shows the negatively charged label HEX, which requires propargylamine modification to confer a positive charge on to the molecule and allow surface adsorption to occur, (b) represents 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.

negatively charged then some positive charge can be added to the backbone of the DNA through modification of deoxyuridine bases by propargyl amine and again use of spermine allows this overall positively charged molecule to now adhere to the silver citrate surface.[41] The spermine serves two roles in this particular scenario. The first is to neutralise the phosphate backbone and prevent repulsion between the phosphates and the citrate surface layer but it also reduces the overall surface charge of the silver nanoparticles. This allows them to aggregate to turn on the enhancement effect to a greater extent and to produce more intense SERRS from the system. A number of different aggregating reagents have been used for DNA. However, we have found that spermine gives the optimal results. An alternative to an electrostatic attraction is to use a group which will complex directly to the silver metal and displace the citrate.

Benzotriazole

Benzotriazole is an aromatic heterocycle which has a strong affinity for a number of different metals including copper and silver.[42,43] Benzotriazole is known to complex very strongly with these metals and as such we have attached it to DNA in a number of different ways in order to promote adsorption onto silver nanoparticles and also obtain SERRS (Scheme 1). In the simplest approach, we took benzotriazole and synthesised a phosphoramidite [**1**] which could then be used in routine solid phase synthesis of oligonucleotides, at the 5′-end, to provide a method of surface attachment to silver nanoparticles.[44] Although simple in chemical terms, benzotriazole does not contain a coloured group and as such does not provide a resonance contribution and lacks the same sensitivity of a chromophoric system. Therefore the next step was to make a benzotriazole azo dye phosphoramadite [**2**]. We have a range of benzotriazole azo dyes which are excellent SERRS dyes in their own right.[45–48] However, in order to make them compatible with bioanalysis they have to be functionalised in such a way that they can be attached to biomolecules. In the case of DNA, a phosphoramidite is desirable as it can be incorporated using solid phase synthesis into any oligonucleotide probe used in, for instance, a PCR based assay. The benzotriazole dye phosphoramidite was synthesised but it required several steps and ultimately gave a low yield despite the fact it coupled well during solid phase synthesis.[49] This approach was effective for in-house use but to make our chemistry more widely available we developed alternative methodologies for labelling. Also, we wanted to make the benzotriazole labels more generic in their approach and compatible with other biomolecules such as proteins. To this end a benzotriazole azo dye maleimide was designed and



Scheme 1 The different approaches to attach benzotriazole and benzotriazole azo dyes to oligonucleotides.

synthesised [**3**]. This dye can be considered to be trifunctional in its nature. The benzotriazole group is used to adhere onto the silver surface, the azo group provides the resonance chromophore and the maleimide provides a point of attachment to other molecules of interest. In the first example of attachment of a benzotriazole azo maleimide very simple Diels Alder cycloaddition to a furan tagged oligonucleotide was used.[50,51] This can be considered as a post synthetic modification of the oligonucleotide and occurred rapidly in aqueous solution. This is a type of click chemistry[52] for labelling of biomolecules to make them SERRS active since the biomolecule containing the diene is SERRS inactive until the maleimide spontaneously reacts to make the biomolecule a SERRS active species. In addition, a difference between the SERRS of the maleimide and the cycloadduct clearly indicates that the reaction has taken place (Fig. 3).

Maleimides are useful for Michael additions and we have also shown that a benzotriazole azo dye maleimide can be attached *via* a thiol group, which is either artificially introduced into an oligonucleotide or is exposed on a protein or antibody. This reaction takes place very rapidly and again in high yield.

Purification of the labelled molecules obtained from either the Diels Alder cycloaddition [**4**] or the Michael addition reactions [**5**] requires only a few minutes using size exclusion chromatography. This makes it a very attractive approach for labelling. One aspect of this approach that has to be kept in mind is that the coupling reaction must be carried out in an aqueous buffer. The maleimide group is susceptible to hydrolysis[53] under different buffer conditions and is also dependent on the nature of the dye used. Therefore a slight excess of maleimide dye tends to be used to overcome the competing hydrolysis.

The final approach adopted was to synthesise a carboxylic acid derivative of the benzotriazole azo dye [**6**] and conjugate that to amine linked oligonucleotides and also peptide nucleic acids (PNA). The choice of coupling agent was crucial and by use of carbonyl diimidazole (CDI) we were able to selectively couple the primary amine group and the carboxylic acid without any side reaction from the benzotriazole group.[54]

Therefore we have developed a number of approaches that can be used for labelling biomolecules which themselves are SERRS inactive to make them active and thus provide ultrasensitive and quantitative information. The next step is to demonstrate how we have exploited this surface chemistry to provide information from a biological system.

Exploiting surface chemistry

We have shown that by using labelled oligonucleotides and suspensions of silver colloid, quantitative detection over wide concentration ranges can be achieved[10] (Fig. 4). Further, the labels used in this experiment were commercially available fluorophores enabling comparisons between detection limits obtained using fluorescence and SERRS with the same labels. With routinely available instrumentation, lower limits of detection by three orders of magnitude or more were obtained for seven out of eight dyes using SERRS[55] (Table 1). Partly this is due to the efficiency of Raman detection equipment but additionally the sharp Raman signals make discrimination of the background simple and in general it appears to give superior performance to fluorescence.

Previously we have shown how we can use these labelled oligonucleotides as primers in a genotyping assay for cystic fibrosis.[56,57] This allowed us to multiplex the genotyping experiments and determine the identity of both homozygotes

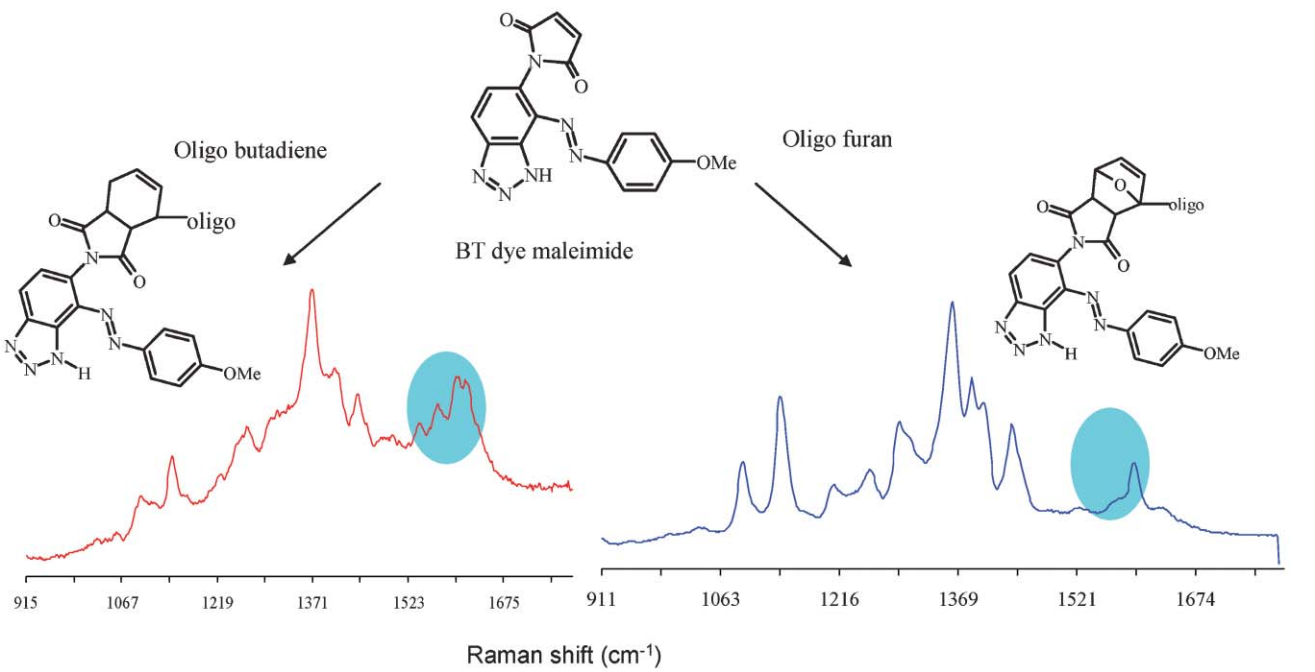


Fig. 3 SERRS from an oligonucleotide labelled with the same dye *via* Diels Alder cycloaddition but showing different signals relating to the diene attached to the oligonucleotide.

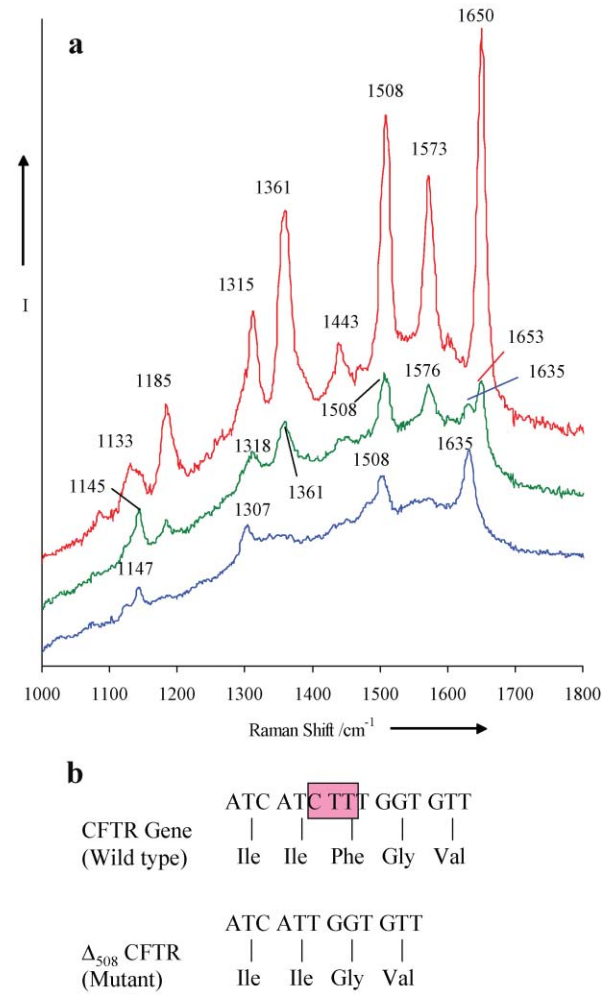


Fig. 4 Multiplexed genotyping of the mutational status of the cystic fibrosis transmembrane conductance regulator gene (CFTR) using SERRS. (a) SERRS spectra from an amplification refractory mutation system assay. Red—rhodamine signals only = homozygote mutant, blue—HEX signals only = homozygote wild type, green—HEX and rhodamine = heterozygote. (b) The gene sequence of the wild type and mutant.

and the heterozygote for the status of the main mutation found in the majority of cystic fibrosis sufferers *i.e.* the cystic fibrosis trans conductance membrane gene. One probe was produced

Table 1 Detection limits obtained using SERRS and two fluorescence instruments

Dye label	Fluorescence detection limits/mol dm^{-3} [a]	Fluorescence detection limits/mol dm^{-3} [b]	SERRS detection limits/mol dm^{-3}
HEX	8.2×10^{-8}	1.0×10^{-8}	7.8×10^{-12}
TAMRA	6.0×10^{-8}	1.1×10^{-8}	3.5×10^{-12}
Cy3	—	4.3×10^{-9}	2.6×10^{-10}
ROX	1.7×10^{-8}	2.1×10^{-8}	8.1×10^{-11}
Cy5	3.1×10^{-9}	4.2×10^{-7}	8.3×10^{-11}
R6G	—	4.9×10^{-8}	1.2×10^{-12}
FAM	3.0×10^{-8}	5.8×10^{-8}	2.7×10^{-12}
TET	—	2.2×10^{-8}	1.6×10^{-11}

[a] Carried out using the Stratagene MX4000 in plate read mode [b] Carried out using the ABI Prism 7700

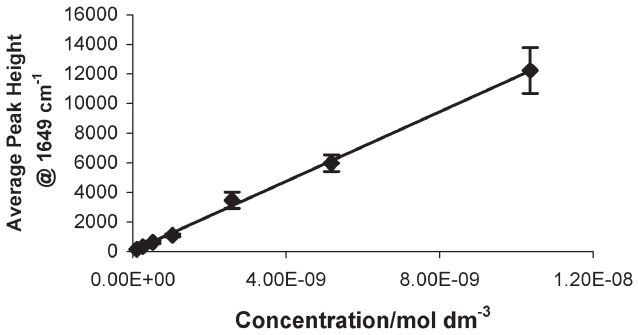


Fig. 5 Example of the quantitative response obtained for an oligonucleotide labelled with rhodamine 6G.

that corresponded to the normal DNA sequence and was labelled with HEX and a corresponding probe which hybridised to the mutated sequence labelled with rhodamine (Fig. 5). These probes were then used in an amplification refractory mutation system (ARMS) to determine the mutational status of the gene.[58]

In the case of the homozygote wild type we only observed HEX signals, in the case of the homozygote mutant we only observed rhodamine signals and in the case of the heterozygote we observed both a HEX and a rhodamine signal. This was the first multiplexed SERRS genotyping to be conducted. Since then we have investigated a number of different methodologies for exploiting the surface chemistry and one particular example of this is SERRS beacons.

In SERRS beacons one end of the beacon is functionalised to contain a benzotriazole azo dye and the other end a fluorophore which does not show great affinity for the metal surface (Fig. 6). The beacon in its closed state shows a SERRS signal corresponding to both of the chromophores attached to the DNA probe and the fluorescence is quenched. The fluorescence is quenched due to the presence of the azo dye, which is a good molecular quencher and to the presence of the silver surface. The signal from the fluorophore is also the dominant signal due to its higher SERRS cross section. When the beacon hybridised to its corresponding complementary sequence it opens up and removes the fluorophore from the metal surface. This then reduces the fluorescence quenching and fluorescence emission as observed but also the SERRS signals change to indicate the different arrangement of dyes on the surface[59] (Fig. 7). We have shown that single base mismatches can be detected using this methodology and are currently working on new molecular diagnostic approaches based on SERRS and its ability to multiplex to a greater extent than corresponding techniques with higher degrees of sensitivity and these assays will be reported in due course.

The final example of how we have exploited the surface chemistry to provide biosensing using nanoparticles and SERRS is in enzyme analysis. As has been stated many times in this feature article the key to obtaining SERRS is adsorption onto the surface. The benzotriazole azo dyes were specifically designed to complex directly to the metal surface by displacing the citrate.[54] We can alter the nature of these benzotriazole dyes and prevent them sticking to the silver surface by functionalising the benzotriazole complexing group.

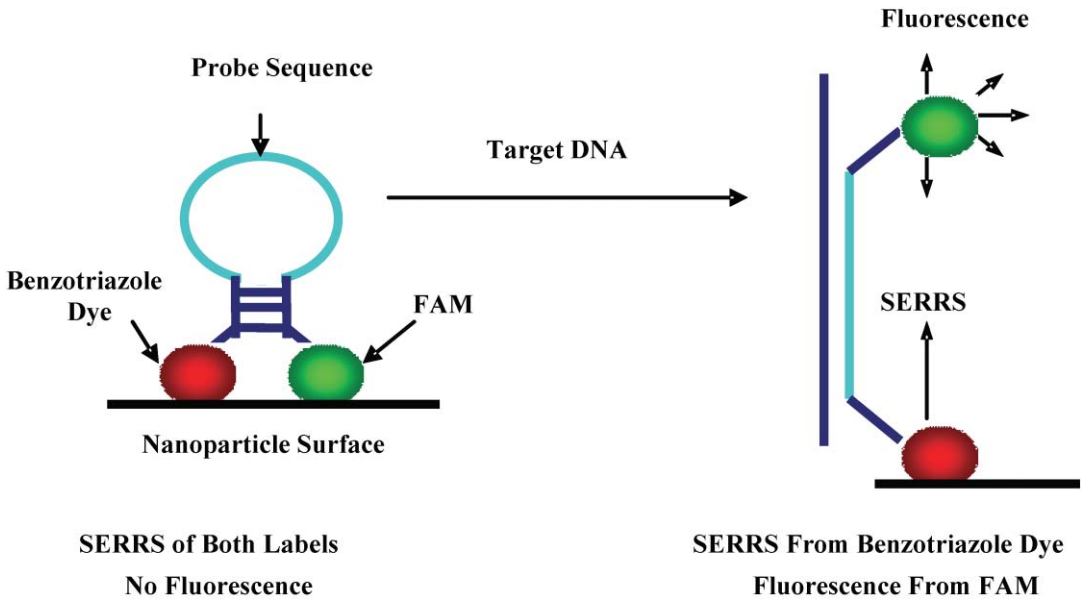


Fig. 6 The principle behind the SERRS beacon approach. A single stranded DNA stem loop is attached to a silver nanoparticle *via* a benzotriazole azo dye at one end and has a fluorophore at the other. In the closed state the fluorescence is quenched by the nanoparticle and SERRS is observed from both dyes. When the loop sequence hybridises to its target sequence it opens the loop and separates the fluorophore from the silver nanoparticle and reduces the quenching effect. In addition the SERRS signals change to reflect this new conformation.

If we functionalise the benzotriazole group in such a way that the molecule which is preventing adsorption or complexation is a substrate for an enzyme then we can potentially use an enzyme to unmask the benzotriazole dye and allow it to stick down to the metal surface (Fig. 8).

This indeed turns out to be the case and we have synthesised a number of different enzyme substrates attached to benzotriazole azo dyes. The initial example was based around a set of lipases which recognised an ester linkage and hydrolysed to release the azo benzotriazole dye to complex to the metal surface. By using a substrate that contained a chiral centre, we were able to demonstrate that the enantioselectivity could be determined using SERRS at enzyme levels far lower than any other conventional technique[60](Fig. 9). The data acquired was quantitative and could be used with a number of different enzymes. Since then we have synthesised a number of different linkages including amides for proteases, phosphates for phosphatases, ethers for P450s and glycosides for glycosidases. These different substrates are currently under investigation for their ability to act as enzyme substrates to produce SERRS signals. One of the advantages of this approach is that a combination of masked dye substrates can be added to an enzymatic system to allow multiplex analysis of enzyme affinities for different stereoisomers without individual experiments being conducted in parallel. This offers great advantages to current enzyme based detection methodologies.

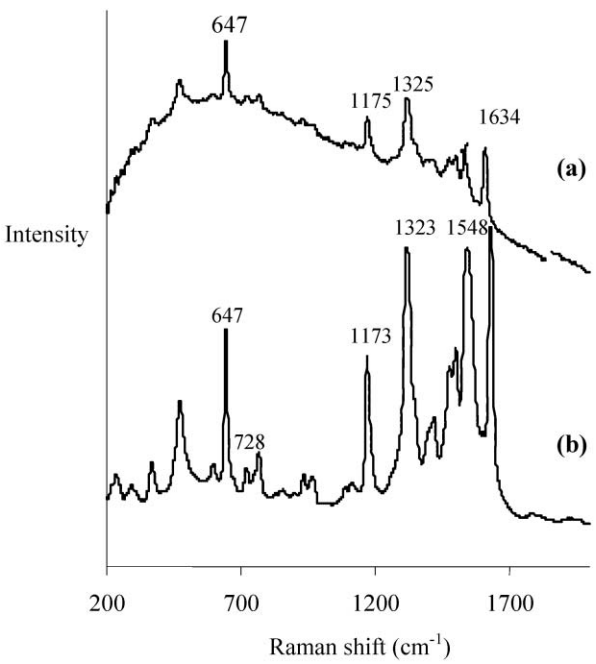


Fig. 7 SERRS spectra of (a) the closed SERRS Beacon and (b) the hybridised product at 3.4 × 10^{-8} mol dm^{-3}.

Conclusion

This *Feature Article* has demonstrated the advantages of using SERRS as an analytical technique and has also highlighted the need for robust surface chemistry appropriate for the surface used for the enhancement. We have illustrated how different surface chemistries can create SERRS active biomolecular probes and also how we have exploited these biomolecular probes in bioanalytical applications. The chemistry is not overly difficult and most of the reagents are commercially available as are the spectrometers which have decreased dramatically in price in recent years. There is currently great interest in the use of SERRS for a number of different bioanalytical or biosensing applications and the number of applications looks set to increase. However, the success of all

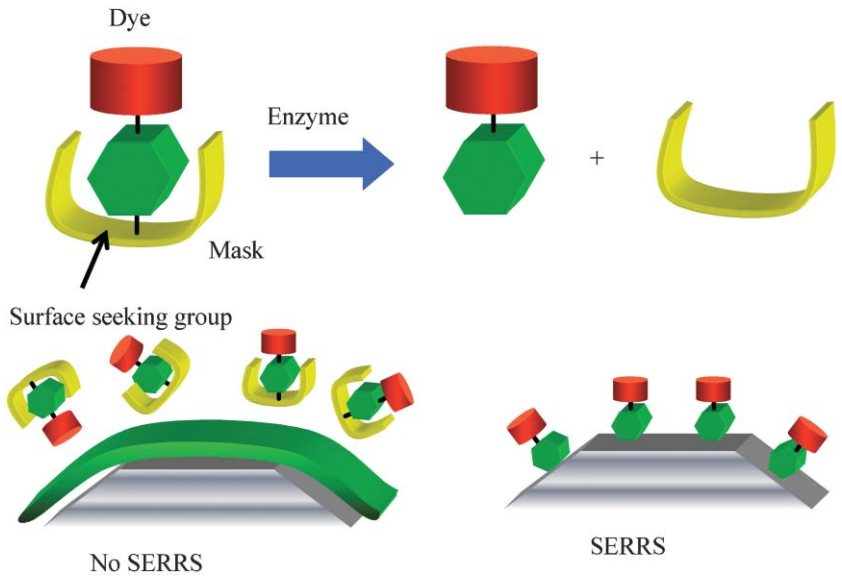


Fig. 8 The principle of obtaining SERRS through the unmasking of a benzotriazole azo dye that is incapable of surface adsorption due to the presence of an enzyme substrate. Enzymatic hydrolysis of the bond linking the substrate to the dye releases the dye which then gives SERRS.

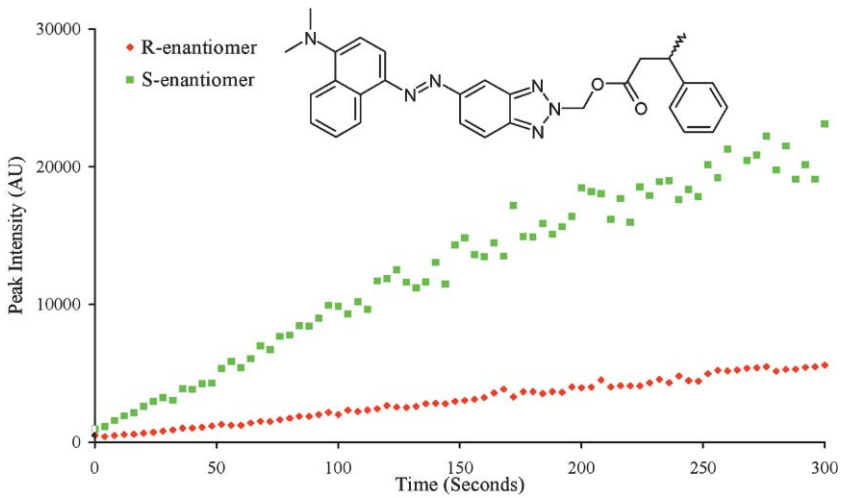


Fig. 9 SERRS Intensity of the major band from the unmasked dye for each enantiomer.

of these applications depends on understanding and controlling the surface chemistry as demonstrated in the examples above.

Acknowledgements

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