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# Surface-Enhanced Raman Scattering (SERS) and Surface-Enhanced Resonance Raman Scattering (SERRS): A Review of Applications

Surface-enhanced Raman scattering (SERS) and surface-enhanced resonance Raman scattering (SERRS) can provide positive identification of an analyte or an analyte mixture with high sensitivity and selectivity. Better understanding of the theory and advances in the understanding of the practice have led to the

development of practical applications in which the unique advantages of SERS/SERRS have been used to provide effective solutions to difficult analytical problems. This review presents a basic theory and illustrates the way in which SERS/SERRS has been developed for practical use.

Index Headings: **Surface-enhanced Raman scattering; SERS; Surface-enhanced resonance Raman scattering; SERRS; Spectroscopy; Resonance; Signal enhancement; Applications.**

## INTRODUCTION

Surface-enhanced Raman scattering (SERS)<sup>1–3</sup> and surface-enhanced resonance Raman scattering (SERRS)<sup>4</sup> have very attractive advantages for the development of selective and sensitive analytical procedures. Both techniques combine the advantages of positive identification of

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Received 27 May 2011; accepted 27 May 2011.

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DOI: 10.1366/11-06365

a molecule in situ, standoff detection, well-established instrumentation, and little or no sample preparation with very high levels of sensitivity. The analyte needs to be adsorbed on the SERS/SERRS active substrate and this can be difficult to achieve; however, it also confers the advantage that fluorescence is quenched from adsorbed molecules. Despite the benefits of the technique, development of practical applications has proven challenging, although there are now commercial devices available. There have been concerns over the fundamental understanding of the technique and practical difficulties in obtaining reliable, repeatable, and robust measurements. Although there is more to be done, these difficulties have largely been overcome and SERS/SERRS is ready to deliver its potential in real applications. As with all new techniques, any new applications must deliver improved performance in the analytical methodology over anything currently available or must meet a currently unmet need; otherwise there is no driver to support the new development. Thus, the analytical targets are likely to be in areas where increased sensitivity, molecularly specific detection, and simplicity coupled with speed of analysis will confer important advantages. This article sets out the basic principles for practical developments and indicates areas where the technique is currently in use or showing promise for future applications.

### THEORY

In many papers, theory is dealt with by describing two mechanisms of SERS enhancement, electromagnetic enhancement<sup>5</sup> and charge-transfer or chemical enhancement.<sup>6,7</sup> Historically, these two mechanisms were thought to be quite different. In both, the analyte must be adsorbed on a SERS active substrate and the substrate irradiated by monochromatic radiation usually from a laser, and the resultant scattering analyzed using a Raman spectrometer. Electromagnetic enhancement arises when the exciting radiation interacts with the surface electrons to form a plasmon (essentially an oscillating electron wave on the surface). Plasmon energy on a smooth surface is bound to the surface, and for

scattering to occur the surface needs to be roughened to give a perpendicular component to the plasmon. Thus, on a suitably roughened surface, the plasmon is created. The plasmon energy causes the Raman process to occur in the analyte molecule, the energy is transferred back into the plasmon, and the scattered radiation, shifted in frequency by the energy transferred to the nuclei in the Raman process, is detected by the spectrometer. In charge-transfer enhancement, the molecule is bound to the metal surface to form a charge-transfer complex. The exciting radiation interacts with the metal to form an electron-hole pair and energy is transferred to the analyte through the new metal to the bonds of the molecule. The Raman process then occurs on the analyte, and the energy is transferred back into the metal for scattering. It is generally acknowledged that the electromagnetic effect is larger than the charge-transfer effect. The authors propose that almost all, if not all, the enhancement arises from one mechanism that is essentially the one described in the electromagnetic approach but with some elements of the charge-transfer approach.

The main steps are as follows:

- (1) An analyte is adsorbed on a surface patterned or roughened so that the chosen excitation frequency will excite a plasmon and create scattering.
- (2) Energy from the plasmon is transferred to the adsorbed molecules and the Raman process occurs on the molecule.
- (3) Energy is transferred back to the plasmon less the amount transferred to the nuclei and scattered from the surface as wavelength shifted light.

These simple steps are the main steps proposed in electromagnetic theory but they contain some fundamentally important aspects of SERS that require more explanation.

**The Surface Species.** The way in which an analyte is adsorbed on a surface is dependent on the nature of the surface and the nature of the analyte. Most substrates are made from gold or silver and these surfaces have quite different chemistries. The surface of

gold is likely to consist of Au<sup>0</sup> atoms coated with a layer of physisorbed inorganic and/or organic ions from the media. However, silver may have a surface layer of oxide or a related layer of chloride or other reagents including organic complexing agents such as citrate as well as physisorbed ions. As a result, the silver on the surface will be mainly present as Ag<sup>+</sup> ions and the chemistry of these two surfaces are different.

In the case of gold, many analytes will physisorb to the surface but analytes containing a soft binding group such as a thiol will form a bond with the Au<sup>0</sup> atoms on the surface. The nature of a metal surface is such that the bond created will affect the local structure of the metal. Under these circumstances, the analyte is now a new species with “charge transfer” bonding into the metal. One way of looking at such a species is that it is a ligand attached to a cluster of metal ions. This will create large polarizable orbitals in the cluster and significantly increase the intensity of scattering from the analyte. This “charge transfer” bonding is one basic postulate of the chemical enhancement mechanism but described in this way it is not necessary to postulate a different method of light adsorption and it is compatible with steps 1 to 3 above.

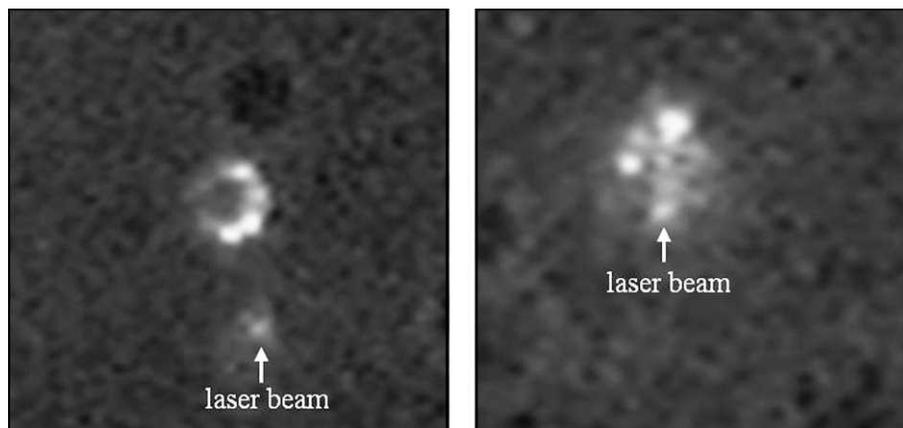
Thus, there is no need for two mechanisms, simply one mechanism with the actual magnitude of the enhancement dependent on the cross-section of the surface species present, whether it is a physisorbed molecule or a chemisorbed molecule that has created a ligand metal cluster. Of course, the clean distinction between physisorption and chemisorption will in practice be more blurred, with a range of bonding types possible, particularly on clean metal surfaces in high vacuum on which much of the early chemical enhancement experiments were carried out.<sup>8</sup>

For silver, the situation is somewhat different. Again, molecules can physisorb but for chemisorption, the likely chemistry is different. In aqueous solution, the electrochemical potential of silver is such that if oxygen is present, the surface will oxidize and Ag<sup>+</sup> ions will be created. Ag<sup>+</sup> ions bond with a wider range of donor groups, including

carboxylate groups as well as thiol groups, and will form molecular  $\text{Ag}^+$  complexes on the surface. In high vacuum, the likely surface is  $\text{Ag}^0$ . As an illustration of how different this will make the bonding, consider what will happen with pyridine. The nitrogen lone pair will bond to  $\text{Ag}^+$  to form a coordinate bond but with  $\text{Ag}^0$  this will not occur because the outer shell of a  $\text{Ag}^0$  atom cannot accept the lone pair electrons, leading to quite different surface bonding. This change in the surface of silver can be observed in the low-frequency region of a SERS spectrum, where bands due to silver oxide or, if formed, silver chloride can be observed. This tendency to form oxide layers can cause problems in surface stability and if the oxide layer is thick enough will affect the surface plasmon appreciably. However, as for gold, the intensity again depends on the SERS cross-section of the surface species.

**Scale.** Plasmons involve the cooperative movement of many electrons across the surface and are inherently not a single-molecule property. Much is now known about the properties of the plasmon required to create effective enhancement, with many good modeling calculations indicating the shape and separation of particles required for good enhancement. In addition, it is known that strong field gradients across the molecule are essential. Other studies indicate that cooperative effects between surface roughness features lead to hot spots of intense activity as discussed below. However, the Raman process occurs at the single-molecule level and much more work is required to understand the exact nature of the enhancing site at the molecular level.

**Orientation.** The orientation of the analyte on the surface is important. Scattering comes from the component of the plasmon perpendicular to the surface and this in turn relates to the molecular polarization vertical to the surface. Thus, at low concentrations, pyridine on silver tends to lie with the ring plane parallel to the surface but as monolayer coverage of the surface is approached, the rings tend to stack with the plane perpendicular to the surface.<sup>9</sup> This means that in-plane vibrations involving displacements of the carbon



**Fig. 1.** SERRS from 4  $\mu\text{m}$  sized particles coated with a rough layer of silver with a dye adsorbed indicating the hot spots on the surface and the effect of laser position. Scattering collected using 532 nm excitation and observed through a filter to remove Tyndall and Raleigh scattering. Reproduced with permission of *Optics Letters*.<sup>11</sup>

atoms will increase in intensity at the higher concentrations because they will have larger polarizability vertical to the surface.<sup>9</sup>

**Selection Rules.** Scattering from molecules with higher degrees of symmetry is dependent on symmetry-related selection rules.<sup>9</sup> The most obvious is in the case of a centrosymmetric molecule. Once adsorbed on the surface, the surface may break the symmetry and enable bands to be enhanced that would otherwise be forbidden.<sup>9</sup>

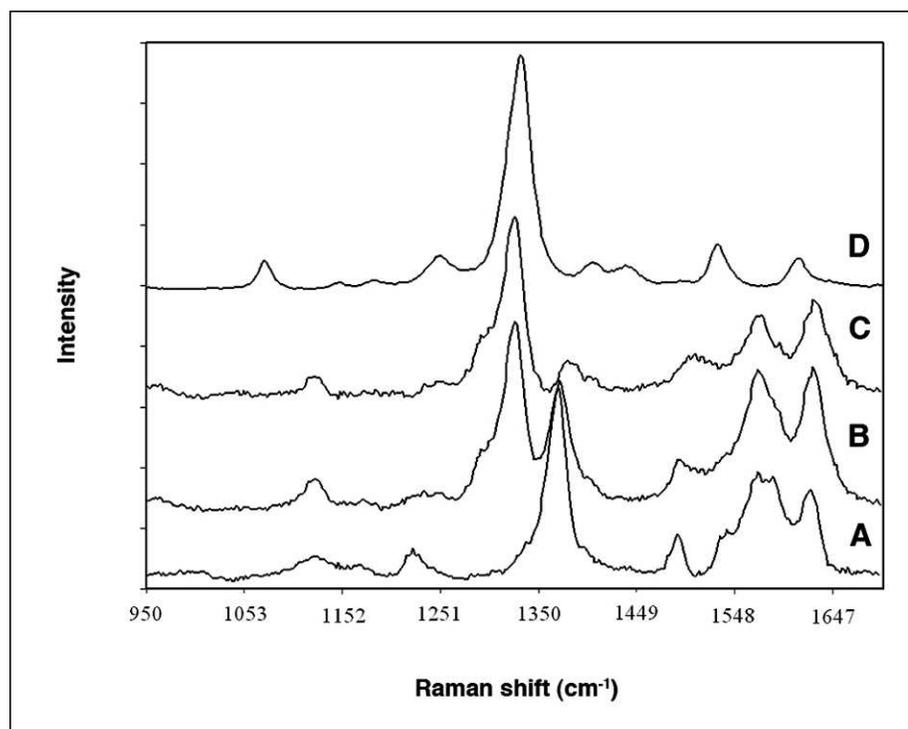
In practice, it is usual to determine the cross-section of an analyte experimentally so it is possible to use the method without an in-depth understanding of the surface provided that the ligand attaches well to the surface. However, the surface chemistry, coupled with the selection rules, does make ligand design more difficult and shows why SERS is better employed where the chemistry is controlled rather than as a broad-range screening technique like mass spectroscopy.

## SINGLE PARTICLES AND HOT SPOTS

Good SERS can be obtained from single isolated nanoparticles but the degree of enhancement of individual particles is dependent on shape and size and can vary widely. Recently, particu-

larly for gold, this problem is being overcome. Larger enhancements are obtained in areas of high field gradients such as the interaction between two particles. Fractal clusters give larger enhancements and in certain areas of the clusters more intense spectra are obtained.<sup>5</sup> These studies have led to work optimizing the spacing required between particles for optimum enhancement. This area is reviewed elsewhere.<sup>10</sup> This additional enhancement is of value in increasing sensitivity but can also make reliable reproducible measurements more difficult. Figure 1 shows an experiment taken while using micro-particles coated with a nanoscale rough layer of silver and a dye.<sup>11</sup> The effect of bringing the laser close to the surface is to induce SERRS but the hotspots on the surface are clear and vary with laser position relative to the particle.

A large number of silver particles coated with a dye to give monolayer surface coverage were isolated as single particles and small clusters on a surface suitable for transmission electron microscopy.<sup>12</sup> The single particles were too small to efficiently excite the surface plasmon with the 514 nm excitation used. It was found that SERRS could be detected only in about 2% of dimers. However, as the cluster size rose, the percentage of clusters that could be detected increased linearly until all



**Fig. 2.** SERRS spectra of the enzyme P450 taken with 457.9 nm excitation. (A) The spectrum of P450; (B) the spectrum of P450 treated with NO showing the bands from the nitrotyrosine formed; (C) the difference spectrum between A and B; and (D) resonance Raman spectrum of nitrotyrosine. Reproduced with permission from *Biochim. Biophys. Acta*.<sup>17</sup>

clusters with more than 15 particles could be detected. However, the magnitude of the enhancement varied between clusters.

To obtain reproducibility in a practical measurement, unless single-particle properties are well controlled and there is no aggregation, or in the case of clusters or extended layers of particles the surface is designed to control hot spots, any measurement should cover a sufficient area or volume to average a number of active sites. This makes microscale rather than nanoscale sensors easier to construct.

### SERS/SERRS

In many articles, SERS and SERRS are not discriminated but in practical applications the two can have very different properties. SERRS arises when the analyte has a chromophore close in energy to the frequency of the excitation used to excite the plasmon and create SERS. Thus, both enhancement from the

plasmon resonance (SERS) and molecular resonance from the analyte (R) contribute to give very intense scattering.<sup>4</sup> Exactly matching the plasmon resonance frequency to the frequency of the electronic transition in the analyte in SERRS is quite difficult and restricting, but in practice it has been found that SERRS works quite well even with about a 250 nm separation between the maximum resonance frequencies of the two.<sup>13</sup> Using unaggregated colloid as a substrate and where the two resonances were well separated, it was found that the maximum scattering intensity was obtained close to the frequency of the electronic transition maximum rather than the plasmon maximum, emphasizing the difference between SERS and SERRS.

One major difference between SERS and SERRS is the huge additional enhancement found in SERRS.<sup>14,15</sup> For example the SERS enhancement for pyridine is calculated at  $10^6$  whereas

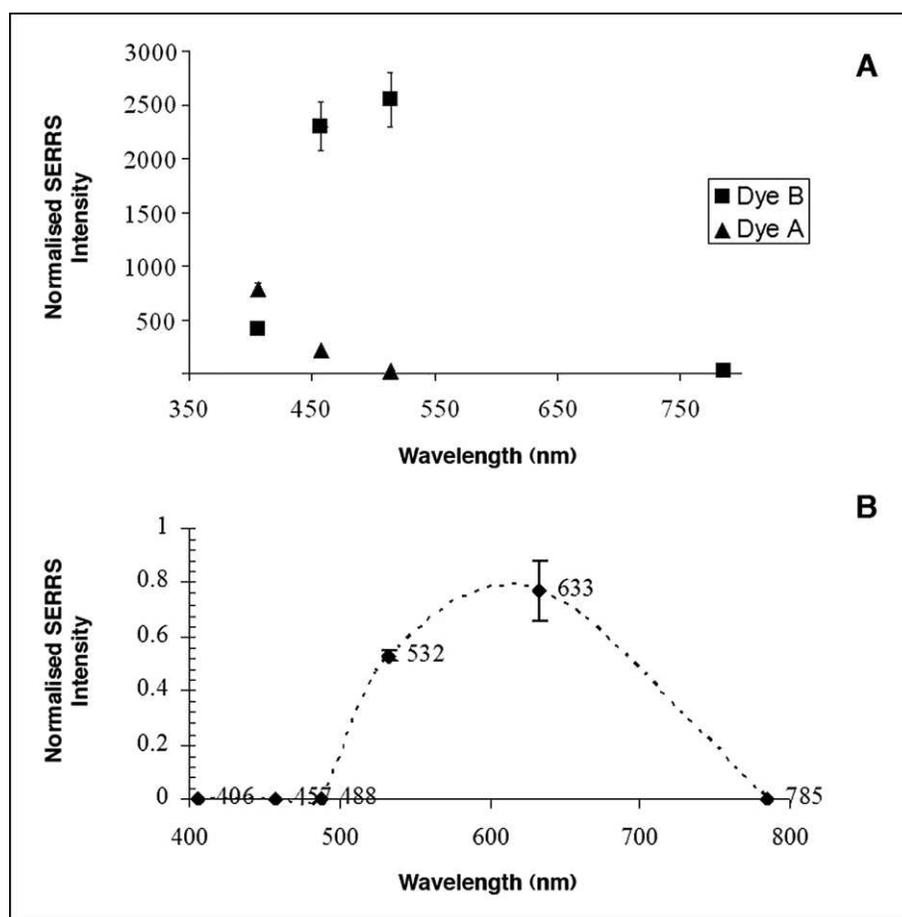
the SERRS enhancement factor for rhodamine is estimated at between  $10^{13}$  and  $10^{15}$ , much higher than most theories would predict for a combination of SERS and molecular resonance. From a practical point of view, the result is that bands associated with the chromophore dominate in SERR spectra and scattering from the rest of the molecule is usually so weak by comparison that it is not observed in most experiments. A good example of this is the spectrum obtained from the enzyme cytochrome C. Using excitation that coincides with the electronic transitions of the heme group in the protein, only vibrations attributable to the heme are observed despite the fact that the heme is not directly attached to the surface and the number of non-heme bonds is far greater than the number of heme bonds.<sup>16</sup> Another example demonstrating the advantages of this selectivity is in the study of the interaction of NO gas (muscle relaxant factor in biology) with the liver heme enzyme P450 in the presence of oxygen<sup>17</sup> (Fig. 2). The NO reacts with tyrosines in the active site close to the heme to nitrate a tyrosine, and because nitro tyrosine is pre-resonant with 457.9 nm excitation, new bands appear in the spectrum. One disadvantage of SERRS for these studies is that the protein must be adsorbed onto the SERRS active surface and this can cause conformation changes in the protein, but the selectivity of SERRS gives unique advantages in that this reaction can be observed in aqueous buffer and the spectra are sufficiently sensitive to the environment to discriminate between tyrosines in different environments. The heme pocket is buried in the P450 structure so that other parts of the enzyme are in direct contact with the active surface. However, the SERRS enhancement is so large that the reaction can be observed clearly.

The second major difference is the wavelength dependence of the intensity obtained from SERRS. This is illustrated in Fig. 3 for two different cases. In case 1 (Fig. 3A), two dyes were chosen that attached to the active surface in similar ways and only differed in that one dye had an amine group pointing away from the surface and the other had a hydroxyl

group. Silver colloid was used as substrate and kept in suspension. The dye with the amine group (dye B) reduced the negative charge on the colloid and caused clustering, thus creating aggregates of particles with high fields between the particles and hot spots. The dye with the hydroxyl group is negative at the pH of the colloid and thus maintained the negative charge on the colloid and no aggregation was observed. Thus, spectra were obtained from single particles rather than aggregates. The plasmon resonance of the unaggregated colloid was at a maximum at 406 nm and the dyes had chromophores at 442 and 413 nm, respectively. Excitation was at 406 nm and longer wavelengths. As might be expected, the maximum enhancement was obtained with the excitation closest to the dye absorption and plasmon resonance frequencies. Aggregation of the colloid produces a range of cluster sizes, each with a different resonance maximum. The result is that there is appreciable SERRS across the visible range but with any one frequency of excitation only a subset of particles will be SERS active. The effect of clustering is shown in that the SERS intensity is at a maximum away from the molecular resonance maximum and the maximum of the unaggregated plasmon resonance frequency. This is due to the greater enhancement obtained from aggregates even if fewer particles are involved. A similar result would be expected for SERS.

However, in contrast to what would be expected for SERS, when a chromophore was chosen with frequency in the red region and the plasmon resonance frequency was as before in the blue region, the greatest enhancement was found with excitation close to the molecular resonance frequency (Fig. 3B). This shows the major effect the molecular resonance process has on the enhancement. In this experiment, the huge enhancement obtained by SERRS was used to keep the concentrations of analyte down to well below that required to aggregate the colloid.

A final advantage to SERRS in practical applications is that fluorescence is quenched on the active surface. This is also true for SERS and can be

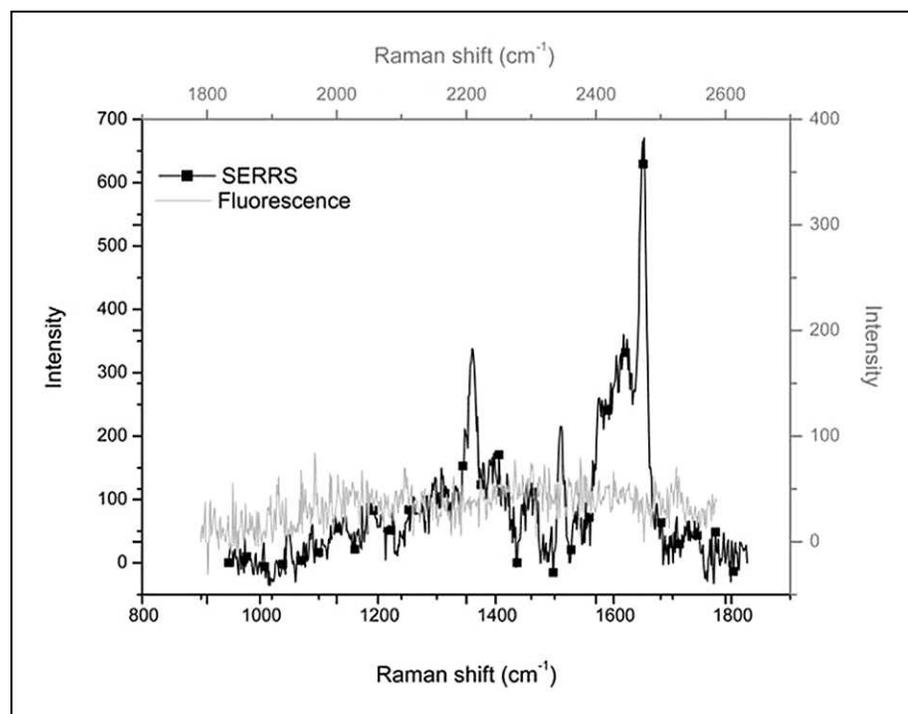


**Fig. 3. (A) Intensities of SERRS/SERS from two dyes using excitation frequencies close to or at longer wavelength than the absorption maxima of the dye chromophores and the plasmon resonance. The points from dye A are from single particles and those from dye B are from aggregates. (B) Intensities of spectra from single particles with an adsorbed dye with a chromophore with a maximum absorbance at a longer wavelength (580–650 nm) than the plasmon resonance, indicating maximum intensity at the molecular absorption frequency.**

used to reduce fluorescence interference, but for SERRS it means that excellent Raman scattering can be obtained from fluorescent molecules, allowing a very wide range of dyes, both fluorescent and non-fluorescent, to be used as labels. It should be noted that this quenching is only effective for surface-bound species and non-bound species will still fluoresce, causing interference.

Thus, the advantages of SERS for analysis are that it directly detects the actual molecule under study in a way that is molecularly specific, allowing in situ identification without the need for separation. However, it is prone to

interference from other species present in the reaction mixture and to changes in the spectra due to orientation changes of the analyte on the surface. In particular, if surface coverage is above about 1/10 monolayer or if parameters such as pH are changed then changes in the spectra are observed. The advantages of SERRS are the extremely high sensitivity, which makes it a good labeling technique and reduces interference. Further, because fluorescence is quenched by the metal surface, both fluorophores and non-fluorophores are effective analytes, giving a wide range of labeling chemistries. Although there is still considerable



**Fig. 4. SERRS versus fluorescence signals for a dye-labeled antibody close to the detection limit using a Raman spectrometer to record both SERRS and fluorescence (514 nm excitation). Reprinted with permission from *Anal. Chem.* 80, 2351 (2008). Copyright 2008 American Chemical Society.<sup>21</sup>**

potential for development, some specific SERRS labeling chemistry has been developed.<sup>18,19</sup> Although changes in orientation of the analyte on the surface can be observed, they are generally less pronounced than for SERS.

A number of experiments have been carried out comparing SERRS and fluorescence sensitivities. However, when comparing two techniques using detection by different instruments, it can be difficult to obtain a good comparison. Nevertheless, in experiments where state-of-the-art instruments are compared<sup>20</sup> or where the same spectrometer is used to detect both SERRS and fluorescence from analytes that are effective fluorophores,<sup>21</sup> SERRS is shown to have lower detection limits.<sup>20</sup> Detection limits were calculated using analytical methodology, where the detection limit is defined as three times the standard deviation of the blanks divided by the slope of the calibration graph rather than simply the concentration at which the signal is three times the noise level.

This indicates an element of sensitivity for SERRS that is as good as, or better than, that for fluorescence. Quantitative analysis of SERRS data is still at the development stage but, practically, one reason the detection limits are so low is that the sharp Raman scattering peaks can be discriminated from the baseline noise more effectively than broad fluorescence peaks<sup>21</sup> (Fig. 4).

### EFFECTIVE SUBSTRATES

For practical applications, a substrate needs to provide reasonable enhancement, be reasonably robust, provide even enhancement across the whole substrate, and be able to be made reproducibly. Many reports of substrates with high enhancement factors do not make clear whether the above criteria are met. Finding an area on a surface that produces a high enhancement is much easier than being able to make a substrate that will reproduce the same enhancement on each successive measurement. It is also important that a

substrate be reproducible such that another operator can make it provide the same enhancement factor.

To make an effective substrate the metal chosen must be suitable to make a roughened surface that will have a reasonable product lifetime. A number of metals have been reported as giving SERS with the visible and near-infrared lasers normally used for SERS/SERRS (see references cited below). These include silver,<sup>22,23</sup> gold,<sup>24,25</sup> copper,<sup>26,27</sup> lithium, palladium, cadmium, and nickel.<sup>5</sup> However, the usual choice is to use gold or silver, which combine good enhancement with reasonable chemical stability. Gold is the more inert and is the choice for most solid-state substrates. However, when light interacts with the metal both scattering and absorption are possible. Gold absorbs quite strongly at around 500 nm, unlike silver, and consequently silver is far more effective with 514 and 532 nm excitation. Where these wavelengths are used silver is usually the preferred choice. However, due to the greater chemical reactivity of silver there are fewer reproducible substrates with a good lifetime. Therefore, when silver is desired, substrates are usually made from colloidal particles. These are usually prepared in aqueous solution in the presence of oxygen and, if the colloid is stable for a long period, it is likely that the surface does not change appreciably because there is a protective layer on the surface produced during the preparation process.<sup>28</sup> In another approach, silver substrates were prepared in situ at the time of measurement by shining ultraviolet (UV) light on a titanium dioxide layer containing silver ions, thus ensuring a newly prepared substrate for every measurement.<sup>29</sup>

The highly sensitive substrates produced for SERS/SERRS can be good detectors of trace material both in the gas phase and in solution. As a result, careless handling or exposure to the atmosphere can lead to the adsorption of interferents with strong SERS signals. Surfaces therefore must be protected from the atmosphere during storage.

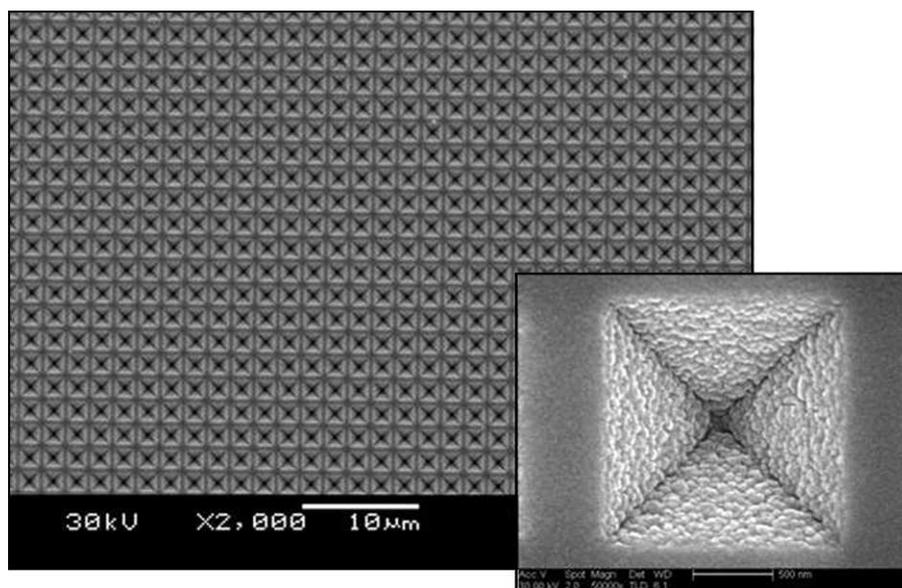
There are many different kinds of substrate that are effective, including vapor-deposited films,<sup>30</sup> shaped particles

and spheres,<sup>31,32</sup> deposited polystyrene spheres with metal deposited to produce arrays of structured metal,<sup>33,34</sup> designed metal surfaces,<sup>35,36</sup> and colloidal suspensions.<sup>37,38</sup> Most of these substrates have to be made by the user but colloid can be purchased, as can a designed solid surface<sup>35,36</sup> (Klarite®) and a surface consisting of deposited nanoparticles (QSERS).

The plasmon properties essential for SERS/SERRS can be controlled by surface design to give effective scattering. As an example, the commercially available substrate Klarite is prepared using semiconductor manufacturing methods to make a silicon surface with square pyramidal pits designed to localize the plasmon<sup>35,36</sup> (Fig. 5). The dimensions of the pits are calculated to produce localized plasmons with ideal properties for SERS. To give the most effective scattering, this surface is coated with a rough gold layer. Many similar designs are possible but the standard Klarite surface is set to give robust long-lived surfaces that can be made reproducibly and can be used with a range of exciting wavelengths.

A range of substrates can be made starting with a surface consisting of a monolayer of polystyrene beads of a chosen size.<sup>33,34</sup> Deposition of silver or gold to the correct depth onto the surface creates a roughened surface that forms an even surface of coated holes. This has proved to be an effective substrate but other substrates can also be developed from it. Dissolution of the polystyrene beads leaves a surface consisting of evenly distributed triangular-shaped deposits that give good enhancement.<sup>39</sup> Nanofabrication can also be used to create an even layer of holes in a gold or silver surface.<sup>40</sup> The size and separation of these holes can be controlled to give the best enhancement.

Many other surfaces are possible and the choice depends on the application. For qualitative applications where the ultimate in sensitivity is not required and reproducibility may be less of a concern, simply prepared substrates such as cold-deposited island films may be sufficient. However, one problem with SERS/SERRS is the inability of other laboratories to reproduce reported results and one reason for this, although not the



**Fig. 5.** SEM of the Klarite surface showing the regular pit structure and the gold coating.

only one, is the difficulty of reproducing the same substrate each time. If the technique is to be used practically, it is important that development of assays be based on substrates that are reproducible.

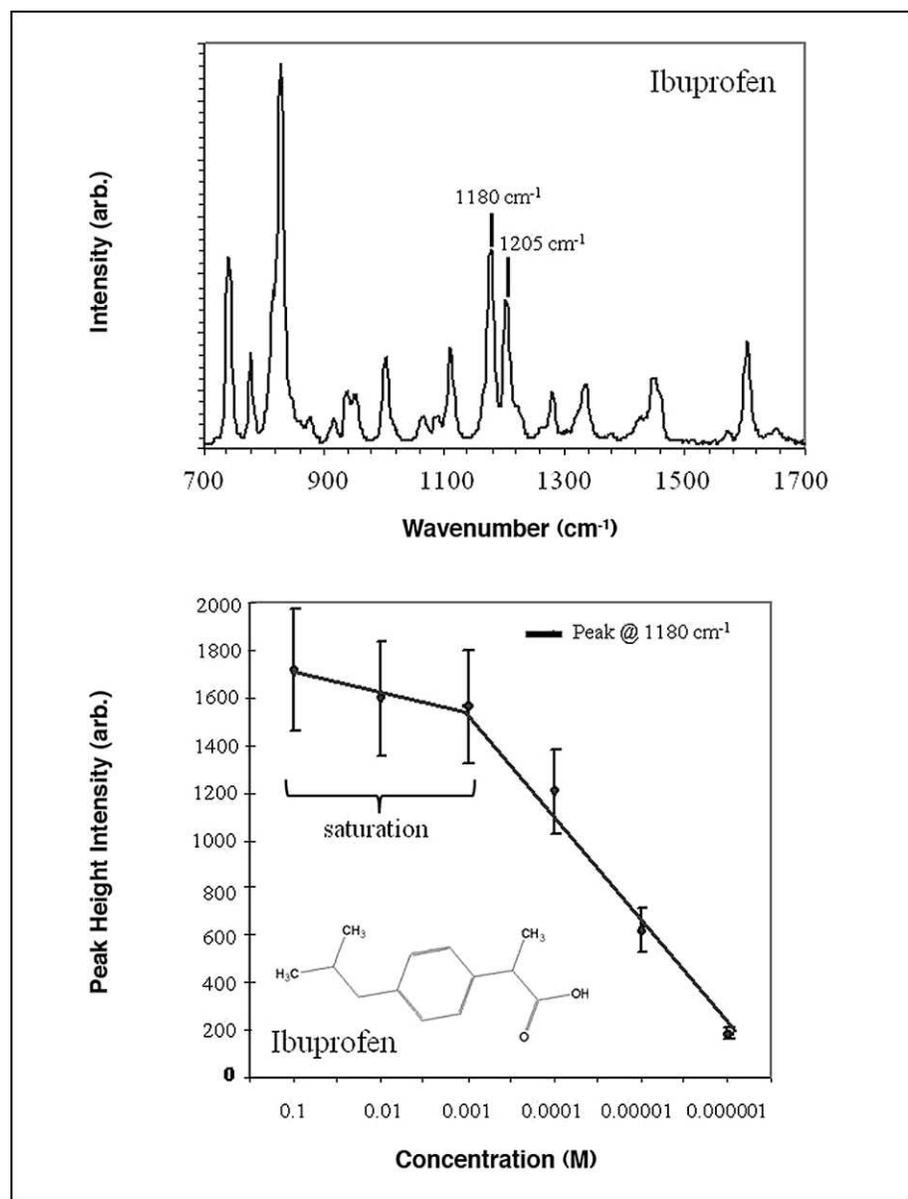
Good SERS/SERRS can also be obtained from particles. Hollow spheres give good enhancement<sup>31</sup> and the plasmon properties can be controlled by choice of sphere and thickness. Shell-isolated particles have been made and form an effective substrate. These consist of gold nanoparticles with a very thin film of silica on the surface, used as “smart dust,” which gives an effective enhancing layer that isolates the metal from the surface to be interrogated and takes up the configuration of the surface.<sup>41</sup> Nanorods are effective enhancers<sup>32</sup> and these can now be controlled in size and shape, making the use of size- and shape-selected nanorods an effective approach. Other shapes such as triangles also have potential.

One advantage of colloidal suspensions is that the suspensions can be handled like liquids and are more compatible with some analytical procedures.<sup>37,38</sup> The particle size can be chosen to give a plasmon resonance that can be excited with the chosen laser

frequency. However, many assays using this approach use aggregating agents to produce aggregates of particles that provide a rougher surface with high fields at the points of contact between the particles and hence greater enhancements.<sup>5,42,43</sup> Sol gels containing colloidal particles are also used as substrates.<sup>44</sup>

## QUANTITATIVE APPLICATIONS

For analytes that adsorb effectively on an active surface and have a reasonable cross-section, SERS is a very effective qualitative technique. It is sensitive, sample preparation is simple, and detection uses standard equipment. Very often, the requirement is to positively identify an analyte in situ with reasonable sensitivity. For this type of qualitative analysis the requirements are that the molecule adsorbs onto a substrate with reasonable enhancement factor and suitable reproducibility and shelf life. SERS/SERRS can be very effective for this type of analysis. In particular when the analyte has a high Raman cross-section compared to other species in a sample matrix the analyte can be identified directly due to its molecularly specific



**Fig. 6.** *Ibuprofen spectrum recorded on a Klarite substrate and the resultant concentration graph monitoring the peak at 1180 cm<sup>-1</sup>.*

spectrum, enabling sample identification with no separation steps.<sup>45</sup> Because most SERS enhancement comes from the first layer on the metal surface, the amount of sample added should be such as to allow sample adsorption without steric crowding or multilayer adsorption. This can be counterintuitive. It can often be better to reduce the amount of sample to improve the signal-to-noise ratio rather than to increase it. In

samples with a significant content of other species, such as for drug analysis in blood plasma, even if the proteins present in the plasma have low SERS cross-sections, they are usually present in larger concentrations than the drug and may adsorb to the surface, preventing analyte adsorption.

Greater-than-monolayer coverage of the surface may mean that more complex selection rules may apply for

multilayers and the material located at distance from the surface may cause appreciable self-absorption of the scattered radiation and affect scattering efficiency from the analyte in the monolayer. In the extreme, for solid-state substrates in particular, uneven drying of a sample can lead to thick deposits on parts of the surface, which, with a Raman microscope, can give Raman scattering rather than SERS. In addition, coverage close to monolayer may cause changes due to stacking, causing re-orientation of the molecule.<sup>9</sup> For qualitative analysis this may not be a bad thing because the signal may be enhanced, but it needs to be recognized and factored into the analysis of the results. However, in many circumstances, the creation of complete layers does give spectra with little, if any, change in relative intensity. By ensuring even surface coverage, it is still possible to obtain quantitative results from films above monolayer coverage using SERS (Fig. 6). The graph shown is linear within the limits set by the error bars, as is Fig. 8. Some studies use log plots. The reason for linearity here is that there is essentially no interference in collecting the scattered signal. This is also the case in the later example because the colloid suspension used is dilute and self-absorption and scattering of the light is minimal.

The high sensitivity of SERS means that requirements for less-than-monolayer coverage are often not difficult to achieve in practice but the possible complications of using too high a concentration of analyte need to be borne in mind when planning the analytical methodology.

If quantitation is required, the substrate used must be able to be made reproducibly both within a batch and between batches and, in the case of a solid-state substrate, enhancement across the surface must be even. When the SERS effect has been investigated in detail, large variations in activity have been observed between single active sites in nanoparticle clusters.<sup>46</sup> This wide variation is an extreme case and designed surfaces are better, but variability between individual active sites is still likely to be present with most substrates. Thus, for quantitative

measurement, careful choice of substrate, strong adherence of the analyte to the chosen surface, and surface coverage within a suitable range are required. However, because of the likely inherent differences in enhancement, some form of averaging over many individual SERS/SERRS sites is usually a requirement for effective quantitation. Usually the averaging comes from two sources. First, quantitative measurements are taken with excitation and collection optics set to measure from an area or volume containing a number of SERS/SERRS sites. Secondly, the Raman process occurs in less than  $10^{-13}$  of a second but most SERS measurements are taken over a period of more than a second to ensure there is time for multiple scattering events to occur. The following examples show that this is an effective procedure.

## EXAMPLES OF PRACTICAL APPLICATIONS

In essence, SERS provides a label-free molecularly specific detection technique whereas SERRS uses labels, whether naturally occurring or added. Thus, SERS is less sensitive and more prone to interference but can detect a wide range of analytes. Because of the need for surface adsorption and because of the wide variations in SERS cross-section between different analytes, SERS is less effective as a broad-range screening technique than techniques such as mass spectroscopy. It is much more effective for specific applications where analytes that give both strong surface adsorption and large cross-section aid selectivity and permit positive *in situ* analyte identification. SERRS sensitivity and molecular specificity make it useful for very selective analysis where the label can be identified, largely interference free, in a matrix. The use of nanoparticles with SERS/SERRS active molecules on the surface as labels in such fields as security marking and *in situ* bio-labeling is also proving effective.

**SERS Applications.** There are a very wide range of possible SERS applications including its use to identify many analytes such as DNA bases,<sup>47</sup> explosives,<sup>48</sup> therapeutic agents,<sup>49</sup> drugs of

abuse,<sup>50</sup> food additives,<sup>51</sup> and cells and spores.<sup>52</sup> As examples, three applications illustrating the potential of the technique are highlighted.

Glucose sensing *in vivo* is of great medical value but the SERS spectrum of glucose is generally of relatively low intensity. To overcome this, Van Duyne and co-workers created a modified surface designed to adsorb glucose effectively on a solid-state substrate made by depositing silver on a layer of polystyrene beads and then removing the beads to reveal a SERS active surface of closely spaced essentially triangular deposits of silver.<sup>53</sup> A carefully chosen layer consisting of two lipid-like thiols was prepared on the surface to create an adsorption site for glucose. This provided very efficient glucose adsorption and excellent SERS. With a reproducible surface and strong signals it has been possible to create a monitor for glucose detection. This is a good illustration of the advantages surface treatment can have for SERS analysis. Other examples include the use of self-assembled monolayers to aid water solubility<sup>54</sup> and the use of thin layers of imprinted polymers.<sup>55</sup>

Also, SERS has been proposed for detection of a range of explosives and other trace materials. Here there are two main problems. One is the sampling itself, because the explosive is often dispersed over a wide area or present in the open air or contained in a large volume. Some of the key explosives such as TNT, and especially components of plastic explosives such as RDX and PETN, have very low vapor pressures so the detection limits of any analytical method are required to be low. TNT, the most widely researched, does not give very strong signals in most circumstances but again will respond to the correct surface treatment. A device is reported that is effective if a gold substrate is treated with sodium hydroxide<sup>48</sup> and chemical derivatization of TNT produces a molecule that adheres strongly to silver surfaces, again giving good detection limits.<sup>56</sup>

Entities such as mammalian cells and spores give broader, more complex spectra. The origin of these signals is likely to be mainly from the part of the cell or spore closest to the enhancing

surface and various bands that are indicative of proteins can be identified. Remarkably, the spectra can be used to discriminate different cell types effectively. Given the complex nature of the spectra, a reliable substrate and correct deposition techniques are again required. The data is usually analyzed by methods such as partial least squares (PLS) and principal component analysis (PCA) and can discriminate between genetically different species of intact bacillus spores.<sup>52</sup> As an illustration of the differences between species, Fig. 7 shows the spectra of three distinct cell types and their classification using a standard PCA method.

In a related approach, chemometric methods for SERS are used for the rapid analysis of microbiological systems.<sup>57</sup>

**SERRS Applications.** Most effective SERRS applications either use naturally occurring chromophores to investigate *in situ* changes to proteins and enzymes<sup>16,17</sup> or use specific labeling chemistry to tackle high-value problems such as detection of DNA fragments<sup>38</sup> or antibodies.<sup>58</sup> In forensics, the technique can be effectively applied to determine the dyes in inks on paper<sup>59</sup> and in fibers.<sup>60</sup>

Proteins with heme chromophores form good candidates for SERRS. The heme group in proteins such as cytochrome C and P450 dominate the SERRS spectrum with little to no contribution from the many other groups present. The SERRS data can be used to probe the oxidation state, spin state of the iron, and changes in protein configuration.<sup>16,17</sup> This can also be done by resonance Raman, although the sensitivity and ability to quench fluorescence of SERRS can be an advantage. However, one of the big advantages is the ability to study protein conformation on a surface. For example, layers of proteins on electrodes can be used to study redox cycles and SERRS is one of the few techniques that can give evidence of protein conformation *in situ* in an electrolyte on a surface at monolayer coverage or less.

Analysis of DNA is widely used for many purposes including determining a disease state or identifying a specific bacteria or virus in a blood sample. To achieve sensitivity and specificity, fluo-

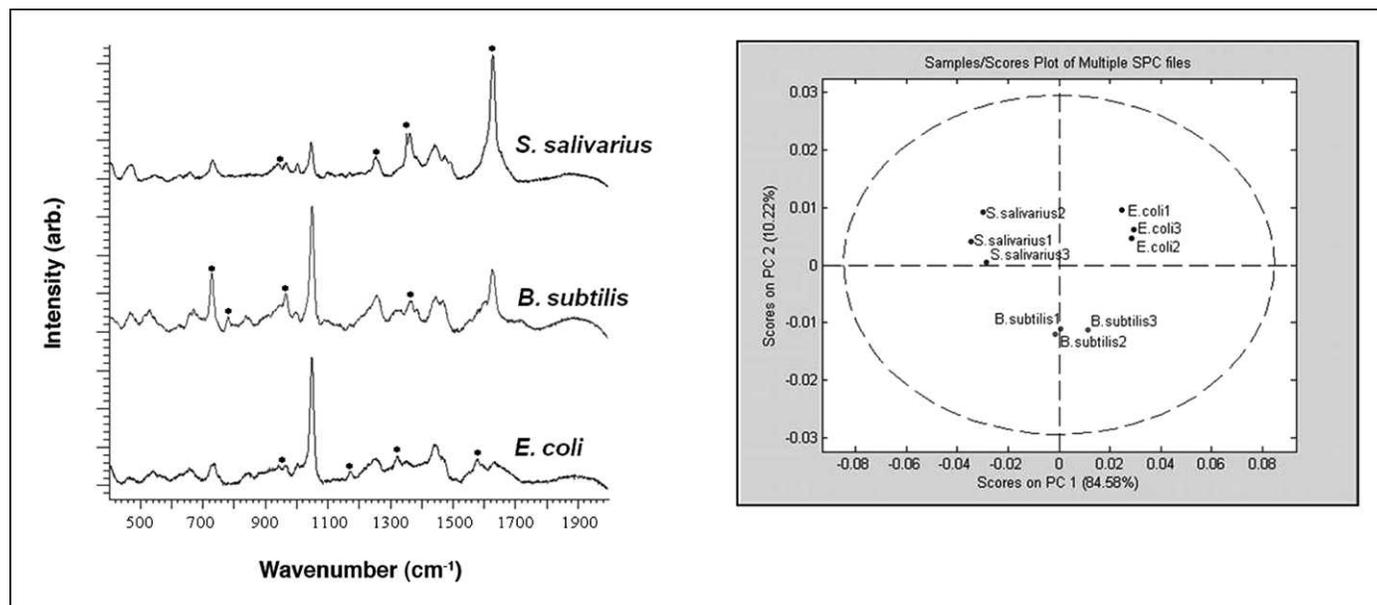


Fig. 7. SERS of different cells on a Klarite surface with 785 nm excitation and the difference analyzed by PCA.

rescence is often used as a detection technique. However, SERRS is very sensitive and provides molecularly specific spectra that can be identified in mixtures of products. Thus, as a technique it has key advantages over fluorescence in terms of the ability to detect multiple species without separation steps. One key factor of the success of SERRS in this field has been the very effective surface quenching of fluorescence. As a result, fluorophores currently used in the industry can be used with SERRS, greatly reducing the barrier to commercialization. Using silver colloid, quantitative behavior with long, approximately linear ranges have been reported with detection limits down to  $10^{-13}$  molar (Fig. 8).<sup>61</sup> Determination of any one SERRS label in a mixture of eight has been reported along with a multiplexed detection of six differently labeled DNA sequences simultaneously.<sup>61</sup> Sequences characteristic of cystic fibrosis were identified originally<sup>62</sup> and a commercial product offering detection of ten analytes in one microtitreplate well has been announced.

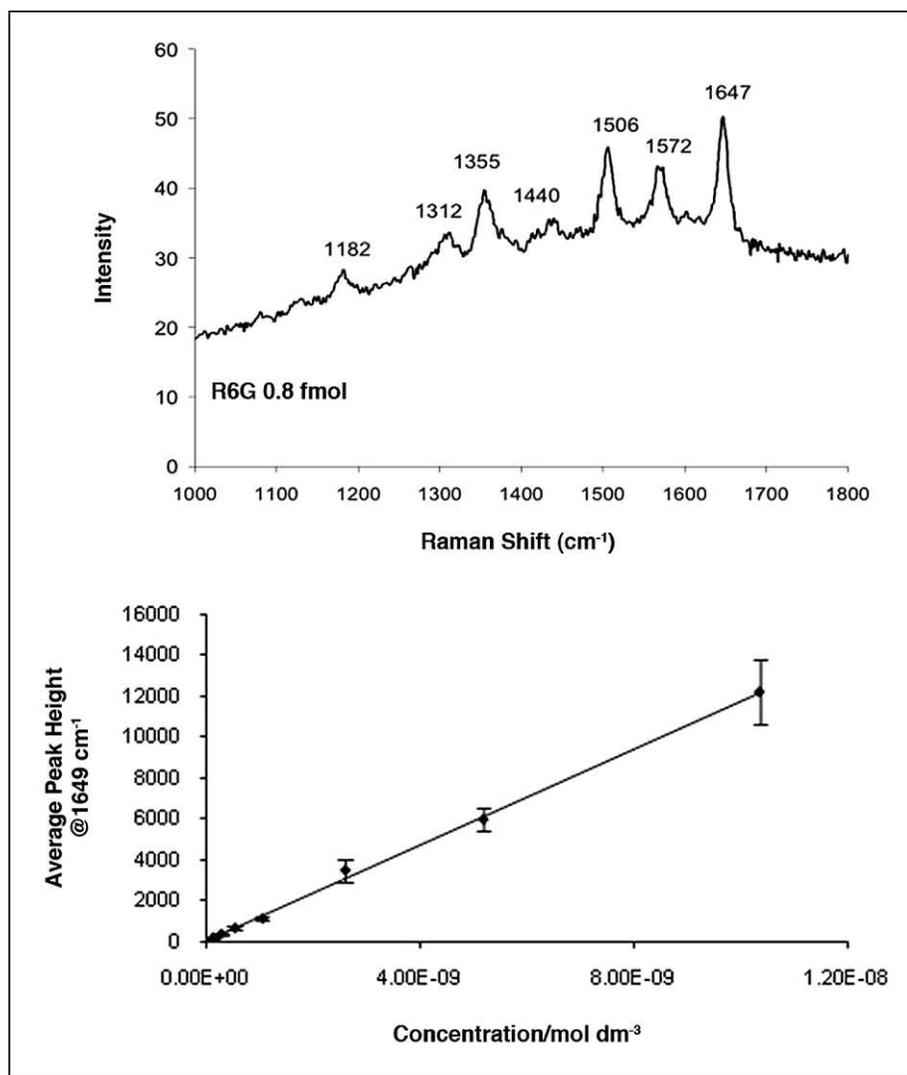
SERS has similar advantages for methods of detection using antibodies but it is important that the label used is

close to the enhancing surface and this affects the design of the assays used. Some of the most interesting approaches use labeled nanoparticles and are dealt with below. However, displacement assays in which a blocking peptide modified to have a SERRS label attached have been patented and assays in which a labeled antibody is displaced from a sandwich assay have been reported.<sup>63</sup>

**Labeled Nanoparticles.** Nanoparticles of gold and silver provide very effective labels. By coating nanoparticles with a layer of SERS/SERRS active material, a very specific label or series of labels can be made that can be detected in situ at very low concentration. As such, these are good candidates for adoption to increase security of documents, for example, by incorporating the particles in ink and for use for in situ analysis in cells and mammals. The main problem with this approach is to ensure stability of the particle, including the coating, within the analysis environment. This can be achieved in a number of ways. Silica coating of the labeled particle is one effective approach, and nanotags with high sensitivity and selectivity<sup>64</sup> can be bought commercially. As in the exam-

ple for DNA, this approach highlights the multiplexing potential for SERS/SERRS in that the specific signals for each reporter molecule on the surface means that mixtures can be created and each label identified in the mixture. This approach can be used in biology as well by attaching specific labels to the silica surface. However, an alternative approach is also being developed in which an organic layer is added to the surface.<sup>65</sup> These types of tags have been used in in vivo applications.<sup>65,66</sup> This indicates the potential for in vivo applications of functionalized nanoparticles and SERS/SERRS. Intra-cell labeling is also effective.<sup>67</sup> There are other approaches. Polymer-coated beads can be made that incorporate clusters of labeled particles,<sup>68</sup> and polymer dyes designed to bond strongly to nanoparticle surfaces have also been reported.<sup>69</sup>

Labeled nanoparticles have been used to create effective antibody assays. In one form of these assays, a gold substrate is treated to bind a capture antibody<sup>58</sup> and gold nanoparticles are treated to have a surface with a number of SERS/SERRS labels and to have copies of an antibody to recognize the capture antibody/antigen complex.



**Fig. 8.** Spectrum of a labeled oligonucleotide at low concentration (0.8 fM) showing peaks only from the SERS active rhodamine label and a plot of intensity of the 1647 cm<sup>-1</sup> peak against concentration indicating the quantitative response. The spectrum was taken with 514 nm excitation, using silver colloid as the substrate.

When the antigen is added, it is captured by the antibody on the surface. The antibody on the labeled particle then binds to the antibody/antigen complex, fixing the labeled particle to the surface. After washing away unbound nanoparticles, the SERS/SERRS spectrum is recorded from the surface.

**Other Applications.** The technique of tip-enhanced Raman spectroscopy (TERS) has now developed to the extent that it requires a separate review.

However, as an example of its use, in one approach, a fiber is drawn down to give a sharp point, which is coated with a rough layer of silver or gold. This is mounted in a suitable device such as an atomic force microscope (AFM) head to control the position and distance from the surface of the tip. When the tip is brought down on the surface in the focused laser of a Raman spectrometer, SERS enhancement is obtained but only from the area where the

tip is touching. This area can vary depending on the experiment but is usually more than an order of magnitude smaller than the diffraction limit. Using this method, very high resolution images can be obtained and very precise measurements of the distance dependence of the tip from the surface can be made.<sup>70</sup>

Other applications include the use of SERS/SERRS in art,<sup>71</sup> for in situ detection in microfluidic devices,<sup>72</sup> in optical tweezers,<sup>73</sup> and its use for identification at a distance where a SERS spectrum has been obtained from a preformed target of dye-labeled silver colloid particles dispersed in a varnish at 20 meters.<sup>73</sup>

## CONCLUSIONS

Many more applications than can be described here have been reported and, if more information is required, there are some very good books of reviews.<sup>74,75</sup> The reasons applications have taken time to develop are clear from the number of points discussed above. However, recent advances and the appearance of commercially viable products mean that much of this is now well understood, making the development of the technique much easier and releasing its potential for practical application. The requirements for successful development include the following:

- (1) A good substrate is essential. It must be roughened to give good enhancement and be reproducible and robust with a good lifetime and even enhancement.
- (2) The analyte must adsorb on the surface effectively and within the correct concentration limits to ensure that sufficient surface is available. Ideally it should have a higher SERS cross-section than any likely interferences.
- (3) Excitation intensity must be controlled to ensure no surface photochemistry.
- (4) For quantitative measurements it is best that many events are averaged by controlling the number of active sites in the interrogation volume and the interrogation time.
- (5) Quantitative SERS/SERRS mea-

surements are best with a standard to monitor any changes due to substrate changes.

If these conditions are met, the advantages of SERS/SERRS spectroscopy in terms of simplicity of sample manipulation, speed, in situ analyte identification, and multiple in situ analyte identification provide powerful drivers to develop new analytical procedures with unprecedented performance.

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