Practical understanding and use of surface enhanced Raman scattering/surface enhanced resonance Raman scattering in chemical and biological analysis

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The unique ability to obtain molecular recognition of an analyte at very low concentrations *in situ* in aqueous environments using surface enhanced Raman scattering (SERS) and surface enhanced resonance Raman scattering (SERRS) detection makes these spectroscopies of considerable interest. Improved understanding of the effect coupled to improvements in practical techniques make the use of SERS/SERRS much simpler than has been the case in the past. This article is designed as a *tutorial review* targeted at aiding in the development of practical applications.

Introduction

Surface enhanced Raman scattering (SERS) and surface enhanced resonance Raman scattering (SERRS) have great potential for use for the development of methods for highly sensitive and selective nanoscale detection. SERS/SERRS has a sensitivity to rival or surpass fluorescence, a unique ability to achieve molecular recognition *in situ* in aqueous environments and, potentially, an extensive labelling chemistry. However, the potential can be difficult to realise in practical applications. Very intense signals can be obtained in ideal conditions but can be difficult to obtain reproducibly and quantitatively in media such as biological fluids. To overcome this, and to decide when detection by SERS/SERRS would be advantageous, an understanding of the effect sufficient to exert practical control is required, and the main aspects of this understanding are set out in this article.

Not all papers on SERS and SERRS discriminate between the two effects. In SERS, the analyte is adsorbed onto a suitably roughened surface, which is usually of silver or gold, and the Raman scattering is measured using a Raman spectrometer. In SERRS, the same approach is taken to achieve surface enhancement but additional enhancement is obtained through molecular resonance by using an analyte containing a chromophore with an electronic transition close in frequency to the excitation frequency of the laser used. Far higher cross sections have been reported for SERRS than for SERS and the spectrum is often immediately recognisable as closely related to the resonance Raman spectrum of the analyte. These differences mean that SERRS can give lower detection limits, reduce interference from contaminants, aid *in situ* identification and provide a different dependence of intensity on excitation frequency. However, SERRS requires that a chromophore is used as the analyte and it is scattering from the chromophore which dominates the spectrum. In some studies this distinction may not be important but in practical applications, assay development using SERS and SERRS can be quite different. For this reason the two are discriminated in this article.

A wide range of analytes give effective SERS by direct adsorption onto the correct substrate. This provides a very simple and effective method of analysis and has the advantage that the properties of the actual molecule are analysed without labels or derivatisation chemistry. With larger analytes, it must be borne in mind that the enhancement drops off rapidly away from the surface so that it can be the surface layer which dominates the spectrum and, for any analyte, distortion of the analyte due to close contact with the surface is possible. SERS has its own selection rules which have to be taken into account when comparing the spectra with Raman spectra. At a basic level, the main effect of the selection rules is that in molecules with a high degree of symmetry, attachment to the surface breaks the symmetry and can lead to the appearance of IR active bands in the spectrum. For all molecules, it is the change in polarisability perpendicular to the surface which gives rise to the scattering, so the angle of the analyte to the surface is important. The large changes caused by this are mainly in intensity with frequency positions affected only marginally if at all unless attachment at the surface creates a new species. The intensity of SERS, like Raman scattering, varies widely from analyte to analyte with some entities such as aromatic rings giving strong

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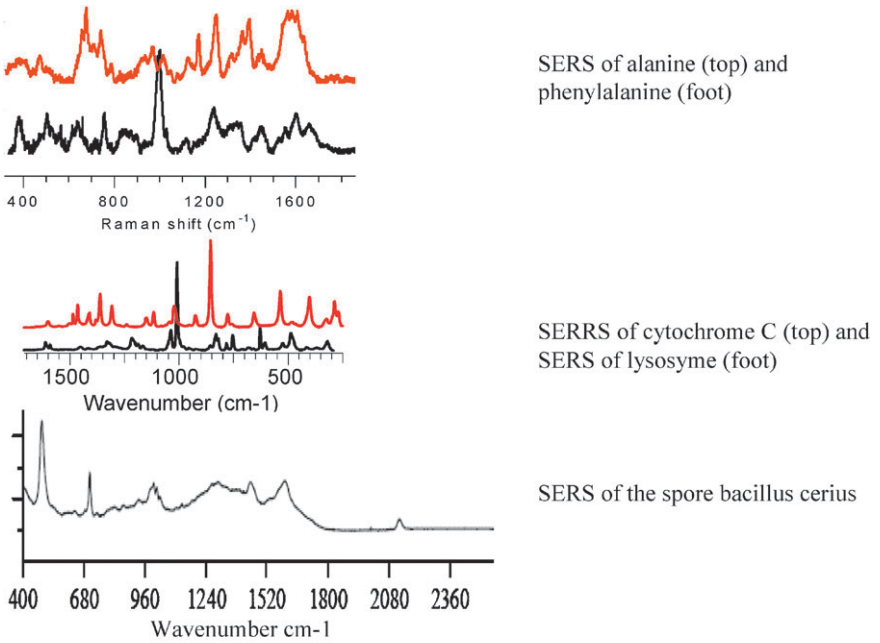

Fig. 1 SERS from a selection of analytes adsorbed on a Klarite surface (D3Technologies Ltd) with no modification or surface treatment, illustrating the specific nature of the spectrum from each analyte. Different accumulation times, instruments and power densities have been used. Spectra courtesy of C. Netti, D3Technologies Ltd. For details on SERS of *Bacillus cerius* see ref. 28.

scattering and other entities such as water molecules giving very weak scattering. A good example of this is that water is a weak scatterer so analytes in aqueous solution can be readily detected and identified *in situ* without separation at low concentrations. However, analytes ranging from small molecules such as drugs, through proteins to cells, spores and tissue samples give good results by direct adsorption on a suitable substrate and examples of spectra from different sized analytes is given in Fig. 1.

For SERRS, with the fixed excitation wavelength systems widely used, it is not always possible to find a chromophore at a frequency which exactly matches the excitation frequency of the available lasers. However, some molecular resonance enhancement may still be present even if the match is only approximate, giving sufficient additional enhancement to obtain the advantages of SERRS for practical use. SERRS often gives rise to spectra similar to those obtained in recording resonance Raman scattering under the same conditions and although there can be some intensity differences due to orientation of the chromophore to the surface, in many cases major changes in intensities relative to the resonance Raman spectrum do not occur. This makes it easy to identify the molecule on the surface and to determine properties such as the oxidation state and spin state of heme groups. In addition, as discussed above, the additional enhancement of SERRS makes it very effective for labelling. However, the selectivity of the enhancement for vibrational displacements on the chromophore means that without derivatisation, it is not applicable for the analysis of many substances.

Fig. 2 illustrates one use of SERRS in which the excitation frequency is not an exact match to the molecular resonance frequency. Cytochrome C contains a heme group which will give SERRS from the Q bands with excitation in the 500 to 600 nm region. In the example shown cytochrome C has been labelled with a dye which will give good SERRS at about 450 nm.[1] Bands due to both the heme and the dye can be seen clearly in the spectrum taken with 514.5 nm excitation. In addition, the heme group is not at the surface of the protein so that SERS might have been expected from parts of the protein directly attached to the surface. However, the strong enhancement from the two chromophores means that no peaks attributable to any other part of the enzyme are observed. SERRS provides the selectivity and sensitivity for distinctive labelling but no information about the rest of the protein is obtained. To achieve this, the weaker SERS would have to be measured and this is simpler in proteins without a SERRS active chromophore.

The development of SERS/SERRS has been truly multidisciplinary involving spectroscopy, surface science, plasmon

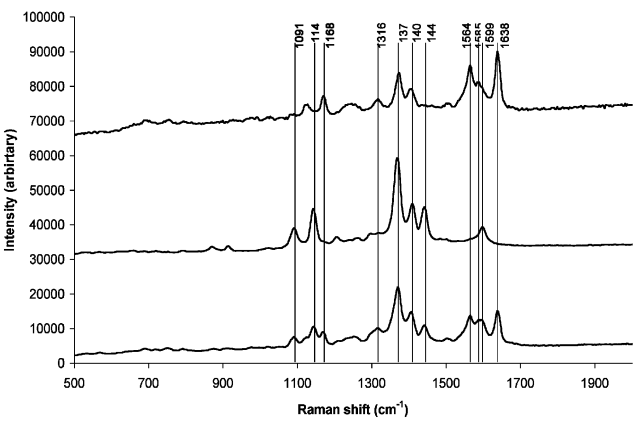

Fig. 2 SERRS spectra of cytochrome C (top), of the dye label (middle), and of the dye labelled protein (foot). Reproduced with permission from ref. 1.

physics, materials science, chemistry and bioscience and this has led to various explanations of the nature of the effect and to some confusion in the past. However, although there are intriguing problems remaining, the basic effect is well enough understood and SERS/SERRS is quite simple to apply. It requires that the analyte is adsorbed effectively on the SERS surface, that an appropriate surface is used and that the correct spectrometer parameters are applied. These points are developed below.

Chemistry of SERS/SERRS

For robust SERS/SERRS measurements the analyte requires to be adsorbed effectively on a surface which is SERS/SERRS active. This section discusses the nature of the active surface and the likely requirements to ensure that a specific analyte adsorbs strongly onto it, and remains there during the time of the measurement.

SERS/SERRS substrates

The original observation of SERS was from pyridine adsorbed on a silver electrode surface.[2] Before electrochemical treatment, no signal was observed, but following treatment, the surface became rough and good signals were observed. It was recognised later that the magnitude of the enhancement was extremely large (10^6) and that a specific surface enhancement mechanism was involved.[3,4] It is now clear that a nanoscale roughened surface is required and SERS/SERRS has been reported with various enhancement factors for many metals including gold, silver, copper, lithium, sodium, rhodium, palladium, platinum, nickel, cobalt, and iron.[5] There are three main reasons why some roughened surfaces are more effective than others. Firstly, it is generally accepted that a surface plasmon requires to be created by the excitation source which is usually in the visible or near IR region. The frequency range over which this is possible is a property of the electronic structure of the metal and the surface roughness. Secondly, to provide intense fields in the vicinity of the adsorbed analyte, it is essential that the correct surface roughness is created. Thirdly, and importantly, the surfaces must be relatively stable under the experimental conditions used and should adsorb the analyte effectively. All three criteria are most easily met in practice with silver and gold surfaces and consequently they are the most widely used.

A very wide range of roughened surfaces have been made. The original electrode surfaces are very effective but the surfaces most commonly used in current work are either surfaces for which the roughness has been engineered on a nanoscale or colloidal suspensions. The former can be made in many ways, such as by lithography[6] or by deposition of polystyrene spheres on a surface and coating them with silver.[7] Dissolution of the spheres then leaves a regularly patterned surface. There are many other methods including hollow gold nanospheres,[8] gold rods[9] and less controlled surfaces such as photochemically developed surfaces to provide a robust substrate only at the time of measurement.[10] Fig. 3a shows an electron micrograph of an engineered surface in which the plasmon properties have been shaped to give effective enhancement by creating specially designed wells, the walls of which are coated with a rough layer of gold.

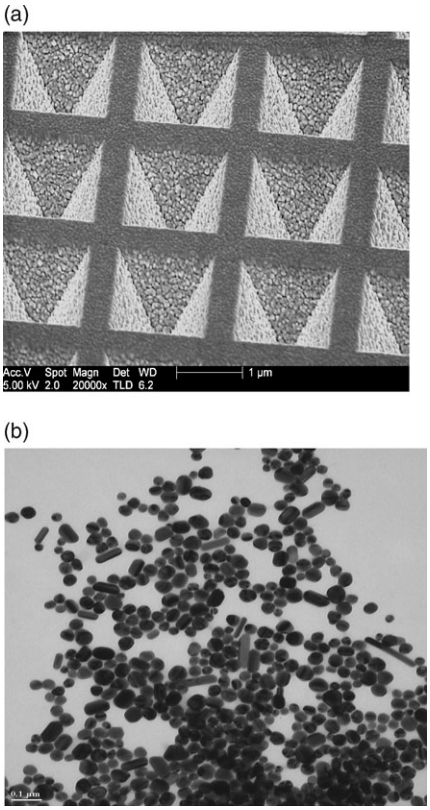

Fig. 3 Examples of (a) a substrate engineered for reproducible enhancement (Klarite) for SERS/SERRS and (b) nanoparticles from a colloidal suspension.

Colloidal suspensions are very effective, but particle size control to the degree necessary to obtain reproducible SERS/SERRS enhancements between batches of colloid is difficult. Significant improvements have been made recently but it is essential that the particle size, shape and size distribution is checked and compared for each batch. Fig. 3b shows a SEM of colloid showing a mixture of particle sizes and shapes. This colloid will give good enhancement but is very difficult to make reproducibly. Much of the variability in size and shape can be removed by careful choice of reducing agent and conditions and by attention to detail.

Nature of the surface layer on the substrate

Effective adsorption of the analyte on the chosen surface is critical for good enhancement and in some cases this has been established simply by trial and error. Although this can be effective, it limits the range of analytes which can be studied and can be misleading. For example, weakly adsorbing analytes can be observed, especially if they are present in vast excess over the surface available, since some analyte will be forced onto the surface. However, as the concentration is lowered, the analyte will desorb and the signal will drop off quickly making it easy to detect any effectively adsorbing contaminants instead. Even strongly adsorbing analytes can be a problem if they also adhere to the walls of vessels used in

the analysis procedure causing curved calibration graphs and possible interference if the vessel is reused.

The ability of SERS/SERRS to provide positive identification of an analyte at very low concentrations *in situ* creates real advantages for these methods in some analysis procedures in key areas such as analysis of low concentrations of biomolecules such as DNA[11,12] and antibodies.[13] This creates a huge opportunity for the development of a SERS/SERRS labelling chemistry to overcome the problems discussed above and to make SERS/SERRS the preferred choice of method for specific analysis procedures. For informed design of labels, an understanding of the chemistry of the surface of the roughened substrate below the Helmholtz double layer is required and this can be difficult to obtain, particularly if, in the analysis procedure, the substrate is in contact with aqueous media or a solvent.

For silver, bare roughened silver surfaces in contact with aqueous media would be expected to oxidise, unless oxygen is rigorously removed, to form an oxide layer probably of silver(I) oxide. Such a layer may not be very stable. Some silver colloid, for example, made by adding sodium borohydride to reduce a solution of silver nitrate usually lasts for a few days. However, when citrate or EDTA is added to reduce the silver nitrate, the resultant colloid can last for many years. The likely reason for this is that a layer of silver(I) citrate or silver(I) EDTA is formed on the surface by reaction with the silver(I) in the oxide layer. Provided some of the carboxylate groups remain uncoordinated, they will be negatively charged at neutral pH stabilising the colloid. SERS provides one of the best ways of confirming this and the role of citrate in particular has been studied.[14]

If citrate is added to colloid made by borohydride reduction, a SERS spectrum of citrate can be obtained. The spectrum changes with time to a very similar one to that obtained when citrate is used to reduce the colloid and the colloid lifetime increases. This suggests adsorption and subsequent reaction on the surface to form a stable citrate layer and indeed citrate colloid has a higher negative zeta potential presumably due to the presence of ionised carboxylate groups. The hypothesis that citrate is present on the surface giving the high negative charge was tested by added agents to displace citrate from the surface of the colloid. Following this treatment citrate was detected in the supernatant.[15] Thus, a general picture emerges for silver colloid of an oxide layer forming on the surface of the suspended particles which is stabilised by a layer of silver compounds of specific ligands such as citrate of EDTA. It should be noted that silver(I) citrate is easily photodegraded. Since this is what is likely to be present on the surface of the widely used citrate reduced colloid, it is not surprising that, with the high power densities sometimes used, signals due to ill defined carbon species are often seen on the surface.

Gold surfaces would be expected to be more stable and particle size control is easier. Gold colloid is stable in suspension and has a high negative zeta potential presumably caused by the layer of solvent and ions surrounding the particles. It should be noted that colloidal gold surfaces are not as inert as massive gold. It is possible over a period of a few days to oxidise and dissolve up colloidal gold with thiols and oxygen.

Although the same considerations also apply to engineered surfaces, a more important issue can be the hydrophilic or hydrophobic nature of the surface. For example, for aqueous solutions of an analyte, the solution has to wet the surface before surface adsorption of the analyte can be efficient. In addition, a common method of obtaining signals from surfaces is by adding a solution of analyte and allowing it to dry so that effective adsorption is less critical. However, if the analyte is an effective Raman scatterer, drying it on a metal backing is a very effective way of preparing a sample for normal Raman scattering and care should be taken to ensure that the scattered light collected is surface enhanced Raman and not simply Raman scattering from small crystallites or from multiple layers of analyte deposited on the surface.

Chemical design of surfaces and labels

Two alternative approaches to controlled adsorption of specific analytes are proving successful in achieving selective and efficient adsorption on the active surface. In one, the surface is modified to capture the analyte and in the other, the analyte is derivatised to create molecules which effectively attach to the active surface. The detection of glucose is a very good example of the first approach.[16,17] Glucose gives a relatively weak SERS spectrum with many surfaces. However, Van Duyne and colleagues treated a surface with a mixture of long chain molecules and created a surface which effectively binds glucose even in biological fluids. This gives a very good SERS spectrum of glucose and the ability to detect it in biological fluids. This technique is now being progressed as a biosensor *in vivo*. Another effective technique is to provide a surface coating, which incorporates the labels, and silica coatings are often used for this purpose.

An alternative approach is to derivatise the analyte with a group which will attach to the surface, while leaving enough of the molecule present to identify it by Raman scattering from specific groups. The relative ease with which many analytes can be recognised by their spectrum *in situ* during analysis also applies to many derivatives. Fig. 4, shows the spectrum of TNT derivatised to form an azo dye as analyte so that SERRS rather than SERS is obtained. An 8-hydroxyquinoline group was used to ensure that it adheres strongly to the silver surface.[18] An intense readily recognised spectrum is obtained with aggregated silver colloid as substrate.

SERRS is now becoming quite widely reported for bio labelling. In this approach, the additional sensitivity of SERRS over SERS normally ensures that the label, not the rest of the biomolecule, is observed in spectrum. This gives specific and highly sensitive detection in a manner analogous to that used with fluorescence. An additional advantage of SERRS is that fluorescence from fluorophores close to the surface is quenched so that both fluorophores and dyes which do not fluoresce can give effective SERRS. The efficiency of quenching makes it possible to use the commercially available labels used for fluorescence detection for SERRS provided almost all the label is adsorbed on the surface.[10,11] If a significant amount of label is left in solution and not adsorbed, it will fluoresce and can interfere.

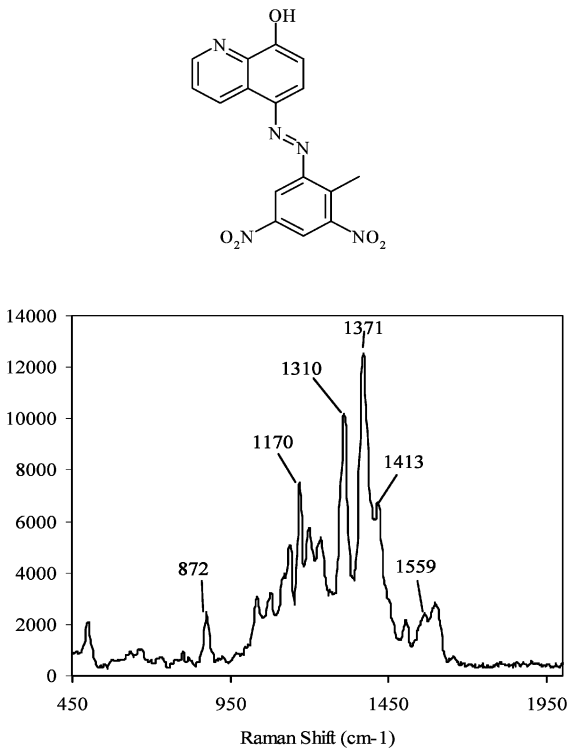

Fig. 4 SERRS spectrum of a TNT derivative and the structure of the derivative.

DNA is negatively charged on the surface and therefore does not adhere well to negatively charged colloidal surfaces or indeed to many substrates making reliable detection at low concentrations difficult. However, oligonucleotides can be modified both to include a label and to aid attachment to the surface. There are different ways of achieving this. For example, in one strategy, a dye and a number of bases which contained a positively charged side chain (propargyl amine groups) were added to an oligonucleotide. The dye provides the additional enhancement of SERRS and the positive charge aids adsorption of the oligonucleotide to the surface. The dye label is placed next to the positive charge so that it also is forced down on the surface quenching fluorescence and giving excellent SERRS. In another strategy, a dye was chosen which was positively charged so that the dye itself acted to attach the oligonucleotide to the surface.[11]

The advantages of using labels as in the example above opened up the development of labelling chemistry for SERS/SERRS. Both fluorescent and non fluorescent dyes can be used, as can other strong scatterers with no chromophore. An alternative to the development of labels described below is to provide a surface coating which incorporates the labels and silica coatings are often used for this purpose.

The most extensive family of SERRS labels currently reported uses the benzotriazole group[19,20] to adsorb to the surface (Fig. 5). Benzotriazoles are used as an anti-tarnish agent for silver due to the high affinity of these molecules for the surface. It is believed they adhere strongly by complexing through the nitrogen groups on the benzotriazole to more than one silver atom on the surface to form clusters and polymers on the surface. Over 50 of these compounds have now been made and the specificity of SERRS means that almost all can be individually recognised and discriminated. They make

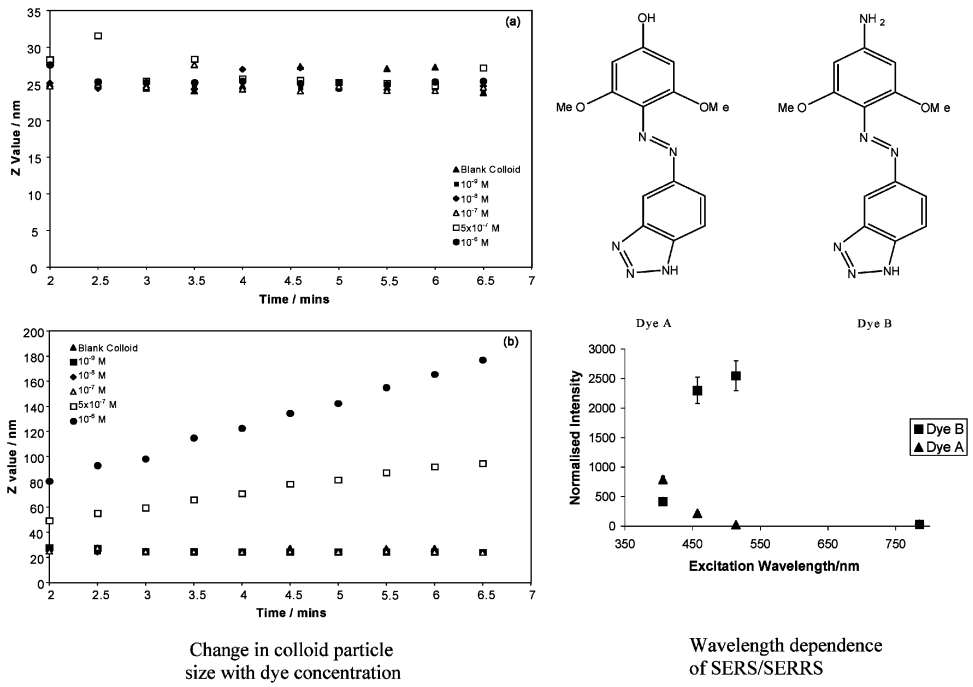

Fig. 5 Benzotriazole dyes specifically designed as SERRS labels. Dye A is negatively charged in aqueous suspension and dye B is neutral. Dye A does not cause aggregation of the colloid (top left) whereas dye B does so that the particle size changes with dye concentration (foot left). For dye concentrations close to that required for monolayer surface coverage, the magnitude of the enhancement of the major peak in the Raman spectrum at about 1370 nm follows the plasmon resonance profile for unaggregated colloid with dye A and aggregated colloid with dye B.

excellent labels for suspended silver nanoparticles with little evidence of desorption in suspension.

These ligands can control the surface properties of the substrate.[21] Fig. 5 shows the result when two different benzotriazole dyes are titrated into a suspension of silver nanoparticles in quantities which take surface coverage from below up to about monolayer coverage. Dye A has a hydroxyl group which ionises to form a negative ion at neutral pH. Even at monolayer coverage, zeta potential and particle size are not appreciably affected as the ligand is added displacing negatively charged citrate. The plasmon resonance frequency range is centred at about 406 nm and SERRS decreases as the excitation frequency shifts away from 406 nm. Dye B on the other hand is neutral at neutral pH and the zeta potential drops as the dye is added. This causes aggregation. Enhancement from aggregates is larger than for single particles and the plasmon shifts to longer wavelengths. The SERRS from dye B is larger and at a maximum at longer wavelengths.

One notable use for this type of dye has been in enzyme assays. A series of dyes have been synthesised in which the nitrogens in the benzotriazole ring are blocked from complexing to the surface by the addition of a side chain suitable for enzyme action. The addition of a specific enzyme to cut this chain and remove it frees the nitrogen groups in the dye to attach to the surface. This approach has provided a very sensitive and quantitative assay for specific enzymes which can be enantiomerically specific.[22]

Basic physics for experimental design

Having ensured effective adsorption, it is necessary to choose the correct parameters such as excitation frequency, substrate and degree of roughness. To do this in an informed manner, an understanding of the physics of the scattering event is required. The controversy surrounding the SERS mechanism is well documented and the uncertainty, although fascinating, has not helped practical development. However the prevailing view is that effective enhancement requires the interaction of the adsorbed analyte with the surface plasmon on a nanoscale roughened surface.[23] The largest SERS/SERRS enhancement requires the molecule to be adsorbed directly onto the surface. The formation of oxide, citrate or chloride surface layers on silver may make the exact nature of the surface less defined but the evidence from high vacuum experiments on clean surfaces suggest that 90% or more of the signal is achieved in the first layer. Some scattering can be detected further from the surface and SERRS has been detected up to about 20 nm from the surface.

The effect is best described in terms of a number of different steps which occur in different length and, probably, time dimensions. The main steps are,

(1) The initial interaction is between the metal surface and the incident light. In the correct frequency range a plasmon will be created. A plasmon is a wave of electrons which oscillates across the surface and the frequency range at which it occurs is a property of the metal used and the surface roughness features. Photons can also be directly adsorbed by the metal. The frequency of the incident light causing the plasmon is usually between 400 and 1064 nm and the preferred surface roughness for SERS/SERRS is about 50 nm. One effect of the roughness is to localise the plasmons on the roughness features.

(2) The Raman event is on the molecular scale and so some of the energy now in the localised plasmon must transfer for a short time to the molecule to create the change in polarisation which will give the Raman event, a process which will occur on a scale of about 1 nm or less for small analytes.

(3) For the most effective scattering the molecule must be on the surface where there will be a strong field gradient increasing the polarisability. There is considerable evidence to suggest that the intense fields present at sites such as the junction between two close colloidal particles creates intense fields and molecules trapped within such sites give particularly effective scattering. As a result fields and field gradients close to molecular dimensions require to be considered. It is also suggested that chemical bonds formed with the surface can contribute to the enhancement. It is difficult to prove that there is some specific effect but interaction between molecules and the surface is a consequence of chemisorption. Electron delocalisation could occur making for greater polarisability and larger Raman scattering.

(4) The energy transferred back into the plasmon is then scattered from the nanoscale roughness features. Since plasmons on a smooth surface oscillate parallel to the surface, they are bound to it and cannot scatter. To obtain scattering, an effective plasmon oscillation component perpendicular to the surface is required and this is created by the surface roughness. Thus, the nature of the roughness is crucial not only in creating a surface which can form a localised plasmon of the correct frequency, but in creating a perpendicular component in it to enable scattering.

As well as changes in length dimension there is also the likelihood that there is a difference in the time taken by the various processes with plasmon scattering being faster than Raman scattering due to the time taken for energy to transfer to the nucleus. Much less is known about the temporal aspect of the process.

Another area requiring more study is the effect of molecular resonance on the enhancement. In essence, molecular resonance enhancement is due to the large polarisation of the real states involved in the resonance process and the influence of the high fields at the surface on these states should provide even greater polarisability than that described in (3) above for SERS.

The above description avoids the use of the terms electromagnetic and chemical enhancement present in many articles. The electromagnetic theory[24] uses plasmons and the role of plasmons in SERS has been discussed many times with significant evidence in favour of their involvement. However, as stated above, a molecule adsorbed on a metal surface is bound to it in some way and interactions with the metal surface are likely, so the possible role of surface bonding, one key part of chemical enhancement,[25] cannot be ignored. It is the view of the author that there is one process not two with the features set out above. In any case, the above is all that is required to design practical SERS/SERRS applications.

Choice of substrate and excitation frequency

Choice of substrate

A plethora of substrates are available but the discussion below is limited to structured surfaces or suspended nanoparticles. Engineered surfaces have the advantage that they bring some control over the nature of the surface roughness and therefore the enhancement mechanism. Since different laboratories use different excitation frequencies, one criterion for a generally applicable substrate is to set surface roughness so that plasmon excitation can occur over a wide range of frequencies. The broad plasmon created makes for a more ubiquitous substrate but reduces the total enhancement that can be achieved. It is possible to tune surfaces to achieve much sharper plasmons which should improve scattering efficiency but this limits the range of excitation frequencies for which the substrate is effective.

The use of nanoparticle suspensions has the advantage that the suspensions are compatible with solution phase procedures. For example, tagged nanoparticles can be used for a wide variety of purposes such as labelling of cells, incorporation into inks, and as tracers in animal experiments.[26–29] However, it is extremely difficult to achieve the degree of control over nanoparticle assembly required to ensure that all nanoparticles are equally active with a particular frequency of excitation. In a study of over one thousand silver particles coated with about a monolayer of dye and separated on a surface, only two percent of single particles gave detectable signals. The percentage of detectable clusters rose till all clusters of 15 or more particles were detectable. In addition, clusters in general gave more intense signals and there was wide variation in signal strength for any one size of cluster.[30] In addition, clusters in general gave more intense signals but there was a wide variation in signal strength for any one size of cluster as shown in Fig. 6. This experiment indicates the variability possible between signals from nanoparticles and nanoparticle clusters and illustrates the degree of control required for quantitative sensing of individual nanotags.

The additional enhancement obtained when clusters are formed is due to the very high field strength at particle junctions and is the reason a suspension of nanoparticle aggregates gives very effective SERS/SERRS. However, this suggests that a significant proportion of the signal will come from molecules in these junctions rather than from all adsorbed molecules equally. Clustering is usually caused in colloidal suspensions by the introduction of an aggregating agent to break the colloidal suspension. If added carefully, aggregates are formed which stay in suspension for a time significantly longer than the measurement time. Essentially, this is a dynamic process in which a range of aggregates of different sizes are formed, each of which will have a different surface plasmon frequency. This gives a broad plasmon and ensures enhancement with different enhancement frequencies. It follows from this that only molecules adsorbed on that

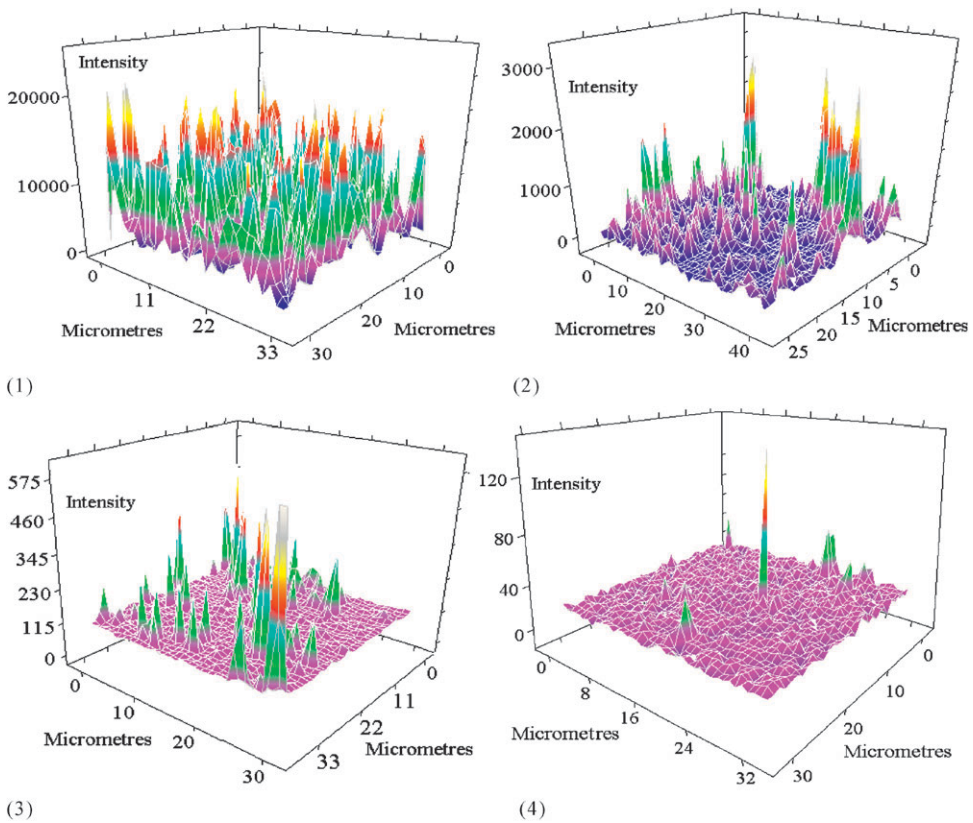

Fig. 6 Maps of the intensity against position of nanoparticles immobilised on a silicon oxide surface illustrating the variable nature of the signals. The particle coverage reduces from grid 1 to grid 4 with most particles shown on grid 4 being separated by more than the area interrogated by the beam in any one exposure. Reproduced with permission from the Strathclyde University PhD thesis of Imran Kahn.

proportion of the clusters with a plasmon in resonance with the excitation frequency chosen will give effectively enhanced scattering. Addition of more aggregating agent can increase the size of aggregates and with visible to near infrared lasers increase the amount of signal. This leads to quite large aggregates forming in suspension which can move in and out of the interrogation volume and to the precipitation of the nanoparticles during the measurement time. These practical considerations explain why the degree of enhancement achieved in practice is often less than might be regarded as theoretically possible.

Although sodium chloride is the most commonly used aggregating agent, it is not necessarily the best for all purposes. The problem is that it creates a special surface layer of sodium chloride which is not effective for the adsorption of some analytes and very effective of the adsorption of others. A more ubiquitous way of aggregating suspensions is to use the ionic strength of the solution where monovalent and divalent ions from addition of reagents such as sodium nitrate or magnesium nitrate can be very effective. Organic aggregation reagents such as poly-L-lysine are also very effective but much depends on the surface chemistry causing the chemisorption of the analyte. For example, a comparison of SERRS from a benzotriazole dye and rhodamine indicated that rhodamine was much more effective with sodium chloride than with the other aggregating agents whereas the benzotriazole dye was effective with all the aggregating agents used.

Choice of frequency

Having chosen the substrate to use with the analyte, the frequency required for the analysis can be chosen. One important consideration is whether the substrate is silver or gold. Inherently, silver provides a better ratio of scattering to adsorption from photons which interact with the surface. In addition, gold absorbs below about 500 to 550 nm. Thus, although signals can be obtained with gold using 514 nm and 532 nm excitation, it is usually a much weaker signal than can be obtained with silver. However, towards the red, where gold does not absorb effectively, the enhancement rises quickly. Using benzene thiol as analyte, SERS at different wavelengths of excitation from 514 to 850 nm was measured quantitatively for colloidal gold and colloidal silver. Gold gave a low enhancement with 514 nm excitation but the gradient for the gold line was steeper than that for the silver and this particular experiment showed gold giving a higher enhancement than silver at 785 nm excitation. The actual numbers in this figure can be changed depending on the aggregation procedures and the choice of colloid. For example, silver citrate colloid gives different enhancements from silver EDTA colloid. Thus, this experiment does not show that gold is more effective than silver at 785 nm but it shows that gold is better used with 632 nm and 785 nm excitation than with frequencies close to 514–532 nm.

For SERS, having avoided using substrates where there is strong absorption of the incident radiation and weak scattering, the major concern is that the excitation frequency is suitable to excite a plasmon on the substrate used. In the case of the most widely used substrates, roughened surfaces with broad plasmons and aggregated colloidal suspensions, this is not usually a problem in that the plasmons are broad and there may be a particular preference for a specific frequency but frequencies in the range from 514 nm and above should work with gold and frequencies right across the visible region to the infrared should work with silver.

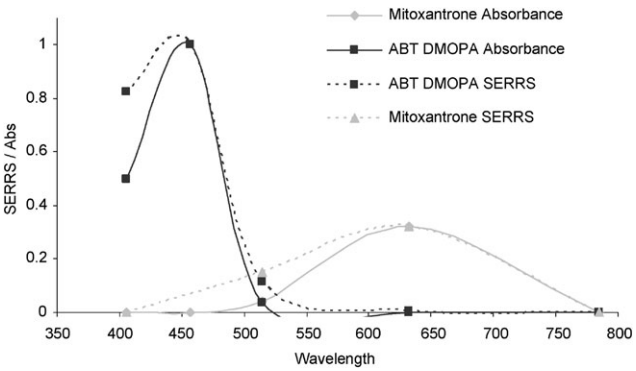

Fig. 7 Dependence of SERRS intensity on excitation frequency for unaggregated colloidal suspensions of silver for two dyes with chromophores in different parts of the visible region. Dye concentrations are less than one tenth of that required to obtain monolayer coverage and little to no aggregation occurred. Reproduced with permission from ref. 31.

SERRS gives a very much larger enhancement but the frequency response is much more complex. Fig. 7 shows wavelength dependence of the intensity of SERRS for two dyes with chromophores in different parts of the visible region, using colloidal silver as the substrate.[31] When this colloid was aggregated, a broad plasmon was created right across the visible region and good enhancement was achieved right across the visible region. However, without colloidal aggregation, and this was checked using particle size and charge measurements, the plasmon is much sharper and is located in the 400 nm to 450 nm region. In this case it was found that the maximum enhancement did not occur at the plasmon resonance frequency but close to the frequency of the electronic transition. Thus, it would appear that the molecular resonance plays a very significant part in obtaining effective SERRS. Where this additional enhancement is required, it is important to ensure that there is an activated plasmon but that the excitation is also close to the frequency required for effective resonance enhancement.

Practical applications

Advances in instrument and substrate technology have made it simple to carry out effective SERS measurements. Correct application of the sample can lead to the development of simple and sensitive analysis methods. For example, TNT can be flashed off the surface and detected readily on a target substrate. No separation procedures were required and the whole experiment is simple and relatively easy to carry out. In another example, amphetamine can be identified from a 10^{-5} to 10^{-6} M aqueous solution by addition to a suspension of aggregate nanoparticles[32] or an engineered surface. However, more complex mixtures such as drugs in blood plasma or saliva are more challenging. If material other than the analyte

adsorbs on the surface, as for example proteins tend to do, the analyte may not be effectively adsorbed and signals from adsorbed interferents may dominate the spectra. In addition, in some cases, non adsorbed material may fluoresce and this fluorescence will not be quenched. In these circumstances SERS analysis methods need a pre-separation stage. This need not always be the case. Mitoxantrone is very strong scatterer which adheres strongly to silver surfaces. When a drop of serum containing this drug at clinically relevant concentrations was added to colloid, interfering fluorescence from non adsorbed proteins was low due to the dilution, but Mitoxantrone SERRS was strong and could be detected quantitatively.[33]

In many cases, the requirement is for simple and sensitive identification of the analyte and for these cases qualitative analysis procedures are sufficient. Time stable reproducible substrates and colloidal suspensions are available to provide reliability. Quantitative analysis requires more careful consideration. The wide range of intensities evident from individual nanoparticles and nanoparticle clusters and the probability that all molecules are not equally enhanced mean that averaging of many single Raman events is required. Further the ease with which surfaces can be photodegraded limits the laser power and accumulation time which can be used. With colloidal suspensions, the use of a Raman microscope has the advantages of creating a high power density in a small volume with a relatively small laser and because of Brownian motion, particles readily move in and out of the interrogation volume during the measurement, reducing problems with photodegradation. Further, the small sample volume enables interrogation close to the surface reducing self absorption of scattered radiation and fluorescence interference. Since the Raman process occurs very quickly, there is time in even a one second accumulation for averaging and in practice this can be sufficient, although if the measurement is taken too close a liquid surface, distortion of the meniscus will cause problems. However, the interrogation volume will contain only a few particles at any one time and since the effect is variable at the individual particle level, a larger interrogation volume is probably better, giving more effective averaging. In practice this works well and enables measurements from cuvettes thus avoiding problems with meniscus distortion. However, it is essential to use a standard with every set of experiments and this is of particular importance if more than one batch of nanoparticles is used.

With substrates, if the analyte adsorbs effectively on the surface, dipping the substrate into a solution of the analyte and thoroughly washing the surface before analysis can be effective. This procedure requires a substrate which gives even surface enhancement and which can be made reproducibly. The variable individual events discussed above are on the nanoscale and even with a high powered optical microscope, the areas usually interrogated are much bigger and the accumulation times much longer than a single Raman event so there is a natural averaging. Since in simple systems the sample area is interrogated continuously, careful control of power density is required. Alternatively more complex systems involving scanning or spinning the sample can be used to reduce the time for which any one area is interrogated. Quantitative methods may require surface modification to ensure effective adsorption for some analytes. The development of a glucose sensor is a very good example of this and of SERS in practice.

The additional sensitivity of SERRS can be an advantage where the electronic structure of the analyte is suitable. Examples include heme proteins, drugs such as Mitoxantrone and pigments. However, the number of analytes is much more limited. On the other hand, for analytical methods where labelling or derivatisation is required to obtain the sensitivity and selectivity required, SERRS can be very effective and this opens up significant opportunities especially in biological labelling and analysis procedures.

As an analysis technique, SERS/SERRS can have a very bright future. The simplicity of use, the specific signals that give immediate identification, the small amounts of materials that can be detected using a microscope and the low concentrations that can be obtained from solution all give SERS/SERRS huge advantages. The potential is only now beginning to be realised because the experiments are required to be carefully designed, the technique must be understood and interdisciplinary skills from separation science and to plasmon physics are required. However, the increased understanding and simpler equipment mean that those with skills and experience in the applied areas of separation science, biochemistry and chemistry can now exploit SERS/SERRS effectively.

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