Quantitative surface-enhanced Raman spectroscopy

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The purpose of this *tutorial review* is to show how surface-enhanced Raman (SERS) and resonance Raman (SERRS) spectroscopy have evolved to the stage where they can be used as a quantitative analytical technique. SER(R)S has enormous potential for a range of applications where high sensitivity needs to be combined with good discrimination between molecular targets, particularly since low cost, compact spectrometers can read the high signal levels that SER(R)S typically provides. These advantages over conventional Raman measurements come at the cost of increased complexity and this review discusses the factors that need to be controlled to generate stable and reproducible SER(R)S calibrations.

1. Background

The potential of surface enhanced Raman (SERS) and resonance Raman (SERRS) as sensitive detection techniques with high levels of molecular specificity has been recognised for many years.[1] However, it has taken considerable time for this potential to be realized and it is only recently that there has been a general acceptance that SER(R)S can, or soon will be, sufficiently reliable and low cost that it will be able to compete with established analytical techniques across a broad range of applications and sample types.

In part, the reasons for the long development time are the same as for spontaneous Raman methods where, up to the 1980s, the high equipment cost and level of expertise required to make the measurements meant that it was a niche technique, practiced by a relatively small number of specialists. However, advances in optoelectronics, particularly the development of compact lasers, CCD detectors and efficient optical filters allowed lower cost, integrated instruments to be produced commercially. This, combined with the vastly increased power of desktop computers, which enabled automated instrument control and data handling, meant that even non-specialists could easily record high quality Raman spectra. The result has been that Raman spectroscopy is no longer a technique of last resort and has been adopted as a routine method in many fields because it has been shown to be simpler and more rapid than alternative analytical approaches. SER(R)S is not yet at that stage but many specific examples which show how the potential can be translated into working methods are now published (some are discussed in detail here). In addition, the general principles of how such methods can be established are now well understood and two recent monographs have excellent treatments of the principles of SER(R)S and on quantitative SER(R)S analysis.[2,3]

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It is useful to divide SER(R)S into two broad sensitivity regimes, as discussed in ref. 2. In the low sensitivity regime (enhancements $\leq \times 10^6$), the observed signal is necessarily composed of the sum of contributions from numerous scattering molecules. The averaging effect of these numerous contributions leads to ensemble signals which are much more stable and reproducible than those obtained when substrates having very high enhancement factors (up to, or even exceeding, $\times 10^{10}$) are used to detect small numbers of scattering molecules situated at areas of high enhancement *i.e.* "hot spots". These latter signals typically show fluctuations in intensity (blinking), band positions *etc*. Here we are primarily concerned with the former since the fundamental principles underlying small number/single molecule phenomena are still being explored.

SER(R)S provides many of the advantages of spontaneous Raman measurements, such as molecule specificity but also adds high sensitivity (even in the "low" enhancement regime). However, it is more complex than spontaneous Raman spectroscopy and a number of factors need to be optimized and controlled in order to obtain reliable SER(R)S measurements. Some of these, such as the variation in signal levels which are associated with apparently small changes in the experimental conditions (even simple sample positioning) are common to all Raman measurements. Others, particularly problems with manufacturing reproducible enhancing media, are specific to SER(R)S. This tutorial review discusses all these factors, touching on the general features of quantitative Raman experiments in section 2 before dealing in more depth with reproducibility in SER(R)S media, the role of internal standards and methods to reduce interference in section 3. The discussion will be centred on the use of SER(R)S to provide direct quantitative information on the amount of a target analyte in a sample by recording the intensity of a characteristic peak or peaks in the target's SER(R)S spectrum, as shown in Fig. 1.[4] However, many of the observations made here are equally applicable to indirect measurements, where the presence of a target is signalled by the appearance of the spectral signature from a SER(R)S-active label rather than the target itself. These experiments using SER(R)S labels are an area of intense research effort in their own right and are more appropriately treated in that context, since the analytical procedure is typically bound up with selection of the best labels, the binding chemistry between target and label and so on.[5]

The number of quantitative SER(R)S studies in the literature, although small compared to the >5800 SER(R)S publications of all types that are available in a single database,[2] are too numerous to treat comprehensively in a tutorial review. However, a small number of case studies are presented at the end of this review. Examples where several different methods have been used for the same analyte have been chosen to illustrate the fact that there is often more than one way to carry out a successful analytical measurement using SER(R)S.

2.1 Instrumental factors

The instrumental requirements for SER(R)S measurements are similar to those for spontaneous Raman spectroscopy. Indeed, lower specification spectrometers whose performance has been compromised in order to reduce cost and/or increase portability may well be satisfactory for SER(R)S work.

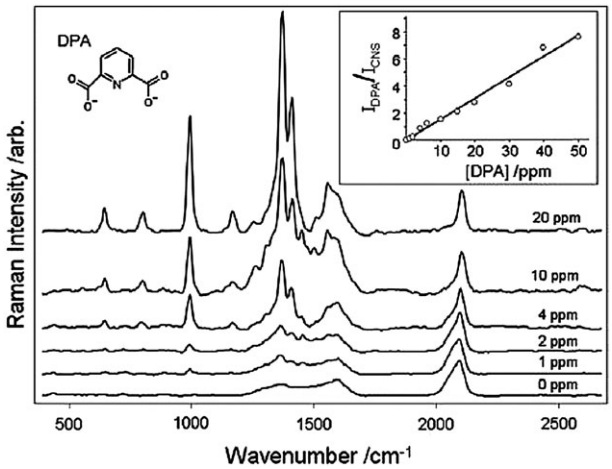

Fig. 1 A SERS assay of dipicolinoate anion (DPA). The characteristic bands of the analyte (600–1600 cm^{-1}), grow with respect to that of the internal standard (CNS^-) at 2120 cm^{-1} with increasing concentration. The insert shows a simple calibration plot of the intensity of the largest DPA band, normalised to the internal standard, against (DPA). (Adapted from ref. 4, with permission; copyright 2005 Royal Society of Chemistry.)[4]

The two main parameters that define the performance of Raman instruments are resolution and sensitivity. Having instrumentation with the highest possible sensitivity can be important in spontaneous Raman spectroscopy, since this has a significant impact on the length of time required to accumulate weak signals. However, the large signals associated with successful SER(R)S enhancement mean that lower sensitivity is needed for routine SER(R)S measurements. Accumulation times are typically just a few seconds and acceptable data can be obtained from compact or even portable Raman spectrometers.[6]

The resolution requirements of SER(R)S are also modest. Even in spontaneous Raman measurements, instrument resolutions of several cm^{-1} are adequate, both because the Raman bands of moderately-sized molecules in solution typically have natural linewidths >5 cm^{-1} and because it is not essential for bands to be fully resolved to be useful for quantitative analysis. This is particularly obvious when using the multivariate techniques described below, which were developed for near infrared (NIR) measurements where extensive band overlap is the normal situation.

In SER(R)S the possibility that molecules can adsorb to the enhancing surface at different orientations, that absorption can alter the properties of the adsorbed molecules and that there may be different types of adsorption sites present can all result in significant broadening of the observed signals. Linewidths are therefore typically at least as large as solution phase values and, as shown by the thiocyanate internal standard band in Fig. 1, can sometimes be very significantly broadened. This broadening can be included as part of the calibration so has little effect on the quantitation of the data but it does demonstrate that it is often not necessary or appropriate to record spectra at high cm^{-1} resolution.

2.2 Excitation wavelengths and resonance effects

The electromagnetic enhancement mechanism for SER(R)S requires that the incident photon excites plasmons within the enhancing medium, so there is an implicit requirement that the laser excitation wavelength needs to be chosen to match the enhancing medium. However, for many enhancing media the wavelength range over which successful enhancement is observed is quite broad (100s of nm). For example, citrate-reduced silver colloids typically display peak absorbance around 405 nm but are most often used with lasers lying in the 488–785 nm range. This is possible because the UV/visible absorption spectra of the colloid is significantly altered during aggregation, broadening to the red end of the spectrum. Similarly, in other randomly roughened surfaces the plasmon resonances would be expected to be broad. In many cases the wavelength used for a particular measurement is set by what type of laser is available. An indication of the range of sources which are suitable is given by the observation that the references in this paper include work carried out using lasers at the following wavelengths: 514 nm (Ar^+), 532 nm (Nd/YAG), 633 nm (HeNe), 647 nm (Kr^+), 785 nm (diode), 830 nm (diode) and 1064 (Nd/YAG).

There are situations in which the excitation wavelength choice is more important, most obviously when the objective is to combine resonance and surface enhancement to give the extremely high enhancement factors associated with SERRS spectroscopy. Under favourable circumstances, quantitative measurements of dye molecules down to picomolar concentrations can be carried out with SERRS, but it is necessary to find an excitation wavelength which is appropriate both for the enhancing substrate and for the absorbing target analyte. The various permutations that this can give rise to have been very clearly elucidated in ref. 7.

2.3 Internal and external standards

The signal observed in spontaneous Raman measurements is proportional to the concentration in the probed volume so, in principle, it should be possible to make a direct calibration plot of the absolute intensity of the Raman band against concentration. However, instrumental factors, including variation in the laser power and alignment of the sample with respect to the excitation laser and collection optics mean that it is difficult to reproduce the absolute intensity of signals, even from the same sample. These problems are often particularly severe with home-built systems and they are smaller with highly engineered commercial integrated spectrometers but it is still usual to make quantitative measurements by measuring relative band intensities of the compound of interest against an internal standard. In part this may be a consequence of the fact that many samples which are analysed by conventional methods also contain a second compound which is present due to the nature of the sample itself. This second component often provides a detectable signal that can be used as an internal standard. For example, in tableted pharmaceuticals the amount of active drug is often measured relative to the amount of excipient, while for solutions the intensity of the analyte bands may be normalized to those of the solvent. This approach not only eliminates the instrumental variables discussed above but can also correct for effects such as differences in focus due to non-uniform sample morphology in solids, or changes in scattering from solutions due to turbidity or inner filter effects from absorbing components. Since this method uses peaks which are being recorded in the spectra in any case it is hardly surprising that it is so widely implemented. In SER(R)S, the variation in enhancement from the substrates means that selection of internal standards is more complex. This is discussed in section 3.4.

2.4 Univariate and multivariate data analysis

Until a few years ago the normal method for quantitative analysis of Raman spectra (spontaneous or surface-enhanced) was to measure either the peak height or the area of an appropriate band of the analyte (essentially a strong band which was reasonably free from interference by other components in the sample) and then to normalize this value with respect to that of an internal standard, as discussed above. Calibration plots of relative band intensity against composition could then be used to determine the composition of test samples. One difference with SER(R)S measurements is that the curves tend to plateau at high analyte concentrations (Fig. 2).[8] This is the simple result of saturation of the surface by the analyte and can be dealt with either by confining measurements to the linear region of the saturation curve, or by linearizing the data in what is typically an empirical or semi-empirical fashion. The data in Fig. 1 show a simple linear calibration over a restricted concentration range but log/log plots can be used to give an extended linear region. In some cases, the data are fitted to Langmuir adsorption isotherms (Fig. 2) so they can be plotted in the standard linearized form of the isotherm.

The simple use of band height or intensity measurements is widely used since it is straightforward and robust. However, this is now joined by a set of much more sophisticated approaches which are based on multivariate data analysis. These multivariate methods are designed to capture the variance within an entire set of spectra (for example spectra run at different analyte concentrations) in terms of a much reduced set of latent variables. In principal component analysis (PCA) the data are reduced to a set of loadings and scores, each spectrum can then be described in terms of a set of scores for each of the loadings. Importantly, the loadings can be plotted so that their spectroscopic origin can be determined. PCA is particularly useful for distinguishing between closely related samples on the basis of their scores; for example Goodacre and co-workers have shown that it is possible to discriminate *bacillus* bacteria at the strain level using PCA of their SERS spectra.[9] For quantitative analysis the method PLS-1 (partial least squares) is often used. In this method, a set of training data with known sample concentrations is used to establish a calibration model. The most significant contributions to the variance associated with the concentration changes are found and the scores of these loadings are then related to the concentrations. The key difference from univariate methods is that this general type of analysis includes data from numerous points across the whole recorded spectral range and so is less sensitive to noise at any given point. For

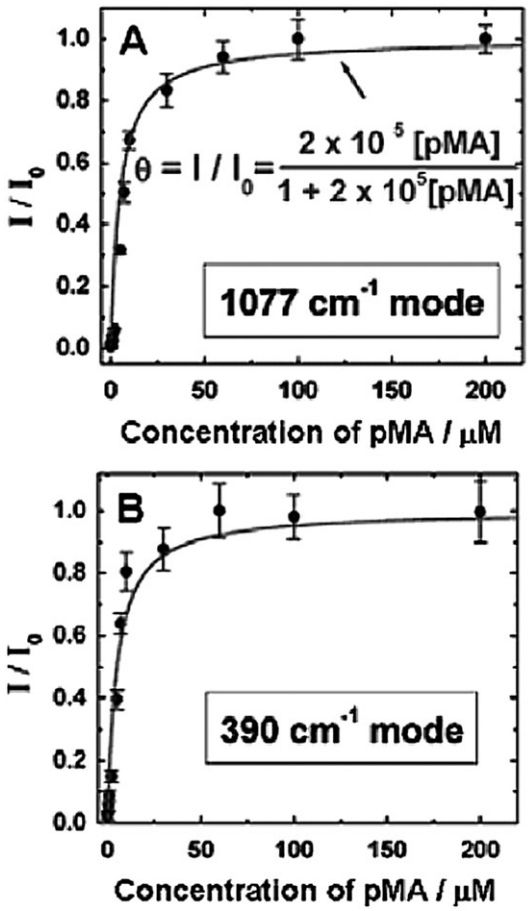

Fig. 2 Band intensity plots for *p*-mercatoaniline (pMA) deposited on nanoparticle arrays showing saturation at high analyte concentration. Intensities are shown as a ratio against I_0, the peak intensity which is obtained with a saturated pMA monolayer. The data fit to the Langmuir isotherm shown. (Reprinted from ref. 8, with permission; copyright 2005 American Chemical Society.)[8]

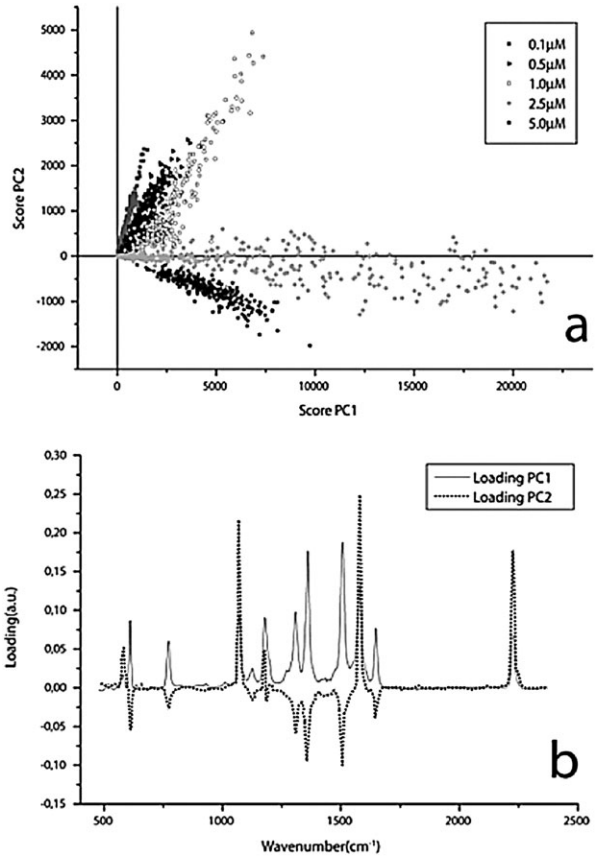

Fig. 3 Score and loading plots for the first two principal components in a PLS model for SERRS spectra of mixtures of rhodamine 6G (R6G) and 4-mercaptobenzonitrile (MBN), which is the internal standard. The first loading plot reflects the overall intensity of the spectra and contains bands from both components. The loading plot of the second principal component shows positive peaks at the positions for MBN and negative peaks for R6G. Taken together, these two components (whose scores are shown at the top of the Figure) account for the largest two sources of variance in the data. (Reprinted from ref. 10 with permission; copyright 2004 American Chemical Society.)[10]

example, Fig. 3 shows loading plots for the first two principal components in a PLS model of SERRS data for rhodamine 6G (R6G) laser dye with 4-mercaptobenzonitrile (MBN) as the internal standard.[10] Since the data were not intensity normalised the first loading reflects the overall intensity of the spectra and contains bands from both components. The loading plot of the second principal component shows positive peaks at the positions for MBN and negative peaks for R6G so these two components (whose scores are shown at the top of the Figure) account for the two largest two sources of variance in the data.

3.1 Enhancing media

Since the earliest days of SER(R)S measurements there have been two main classes of enhancing media: solid substrates with microscopically rough (randomly textured) surfaces and colloidal suspensions of coinage metal (predominantly Au or Ag) nanoparticles. A good general discussion of these is available in a recent monograph.[2] More recently, a third class of enhancing materials, generally referred to as plasmonic media, has been made possible through the widespread availability of nanoscale fabrication and characterisation tools. The primary advantage of these materials is the extraordinary degree of control which can be exercised over the physical structure of the materials. However, plasmonic substrates have by no means replaced colloidal particles and random-roughness surfaces, so all three general types of enhancing material are discussed below. This is not intended to be a comprehensive coverage of all the enhancing media which have been fabricated and tested (at the time of writing new enhancing materials are reported every month) but is intended to give some indication of the general features of each. No attempt has been made to give a detailed quantitative assessment of the enhancement factors (EFs) that each surface can provide. Although EFs might appear to be a useful figure of merit, the measurement and reporting of reliable EFs is extremely complex. Even the basic definitions are still being clarified[11] and there are no universally accepted standard test molecules. This situation is exacerbated by the tendency to test new ''general purpose'' enhancing media using compounds which are known to bind strongly to metal surfaces and to give huge SER(R)S signals, often due to resonance effects. Under these

conditions even media that are only moderately enhancing will still give very large signals, which means that there is very little opportunity for discrimination between competing substrates on the basis of such tests. Conversely, for more challenging samples the ability to obtain even moderate enhancement may be significant. For example, Ben-Amotz and co-workers were able to obtain quantitative information on the composition of small (10 μl) aliquots of 1 mM oligosaccharide solutions by drying them onto electrochemically roughened substrates even though the nominal enhancement was just $\times 100$–1000.[12]

As a very broad generalisation, it is probably true to say that the majority of the enhancing materials in the literature will give sufficient enhancement for routine analysis purposes, provided the analyte of interest can be brought to the enhancing surface. For example, Rowlen *et al.* compared 5 different SER(R)S substrates and found that despite a variation of >2 orders of magnitude in sensitivity, the limit of detection with their test molecule was still sub-picomole, even for the least sensitive of the substrates investigated.[13] In general, the choice between different enhancing media rests as much in the surface chemistry and ease of use for particular applications as in the electromagnetic properties of the enhancing substrate. In addition, the SER(R)S literature has now grown to the extent that even if the specific target molecule of interest has not been previously studied a near-analogue probably has been, so there will be data to guide the choice of an appropriate enhancing material.

Colloidal nanoparticles

Au and Ag nanoparticles have been used for SERS since 1979.[2] Their main advantages are ease of preparation, very low cost and the high enhancement factors they provide. The general procedure for preparing these colloids is that a solution of the appropriate Au or Ag salt is chemically reduced in aqueous solution to produce colloidal suspensions of particles which are typically in the nm range. The precise methodology has been the subject of numerous studies which have investigated factors as subtle as the stirring rate but for SER(R)S work the most widely used colloids are prepared by reduction of $AgNO_3$ solution by tri-sodium citrate. This generates nanocrystals in the 50–100 nm size range which exhibit a remarkably heterogeneous range of morphologies, including spheres, prisms, rods *etc*. (see Fig. 4). Smaller particles are produced by borohydride reduction. Au colloids are also most usually prepared by citrate reduction and are much more monodisperse than their Ag analogues with smaller particle sizes. Both these colloids are stabilised by a layer of citrate ions which give them a negative zeta potential.[2] More recently, a much simpler room temperature method for preparation of Ag colloids using hydroxylamine reduction has been reported.[14] The colloids prepared using this technique are broadly similar to those produced using citrate but they are covered in a layer of strongly bound Cl^- ions. One advantage of the ease of preparation is that it allows the enhancing media to be generated *in situ* which eliminates problems with degradation during storage. Numerous variations on these basic procedures which produce different particle shapes have been reported but so far none have proved to be sufficiently superior over the basic materials to have been widely adopted.

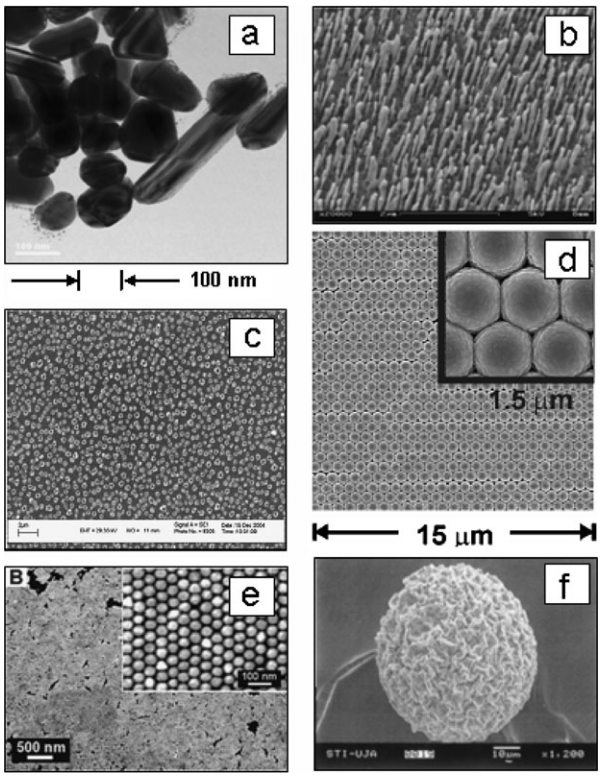

Fig. 4 A selection of SER(R)S-enhancing materials. (a) Citrate-reduced Ag colloid, (b) Ag nanorod array,[15] (c) Ag torus structures on SiO_2/Si substrate,[16] (d) silver film over nanospheres (AgFON),[6] (e) Au nanoparticle array,[8] (f) Ag layer on sephadex bead.[17] (Reprinted with permission; (b), (d) and (f) copyright American Chemical Society; (c) and (e) copyright Royal Society of Chemistry.)

The main factor which distinguishes colloidal particles from roughened or textured enhancing surfaces is the need to aggregate the particles to obtain optimum enhancement. This aggregation introduces another variable into the experimental procedure and undeniably complicates any assay. At the least it may add an additional physical mixing step. More importantly, the aggregating agent may interact directly with the enhancing surface which can lead to precipitation of the colloid rather than formation of metastable aggregates. Such precipitation can result in a marked reduction in the enhanced signals over a period of minutes, as shown in Fig. 5.[18] However, the largest enhancements have been recorded for aggregated clusters of particles and the materials lend themselves to simple "mix and record" protocols. In early SER(R)S colloid studies the aggregation was normally induced by addition of simple alkali metal halides, particularly KBr and NaCl. More recently, it has been recognised that this process alters the nature of the colloid surfaces so here the effect of various aggregating agents is discussed below in section 3.3, along with other surface modification methods.

Solid substrates

One advantage of solids over colloids is that they allow much more flexibility in the sampling. Typically, liquid samples are flowed over the enhancing surface while the monitoring beam is directed onto a single point on the surface. However, it has

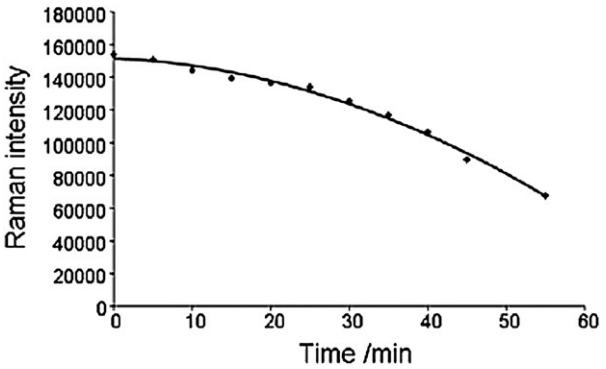

Fig. 5 Plot showing the loss in intensity of the strongest band in the SERS spectrum of nicotine following aggregation of a citrate reduced colloid with a high concentration of salt. [Nicotine] = 10 ppm, [NaCl] = 0.9 mol dm^{-3}. (Reprinted from ref. 18 with permission; copyright 2004 Royal Society of Chemistry.)[18]

been shown that the media can also be prepared on the tips of glass fibres that are inserted into the test subject or a liquid sample.[19] Solid SER(R)S substrates are also intrinsically more appropriate for array based assays.

SER(R)S was first observed for pyridine adsorbed on the surface of a rough silver electrode and the use of roughened (*i.e.* randomly-textured surfaces) still continues.[2] The traditional method of carrying out repeated oxidation/reduction cycles on Ag electrodes to build up a very rough metal layer on the surface continues to be used. However, the range of substrates is now vast. For example, Au or Ag island films, which also date back to the earliest days of SER(R)S, remain popular enhancing media. These are prepared by evaporating Au or Ag onto smooth substrates; under suitable deposition conditions the films spontaneously form microscopically rough SER(R)S-active surfaces.[2] Alternatively, uniform metal layers may be deposited onto rough or textured surfaces so that the morphology follows that of the underlying substrate.

A very successful approach has been to carry out the deposition on ordered arrays of polymer spheres. It has been found that deposition of a silver layer onto a close-packed array, followed by removal of the masking polymer spheres (nanosphere lithography) results in creation of ordered arrays of silver nanotriangles whose spacing can be controlled by altering the particle size.[20] A second variation of this technique has been to evaporate Ag or Au over a close packed layer of polymer nanospheres and to use this nanostructured AgFON directly as the enhancing medium.[20] Regularly structured SER(R)S-active materials can also be fabricated through bottom-up rather than top-down procedures, for example through the growth of Ag nanowires on planar substrates[15] or self-assembly of ordered arrays of nanoparticles on the surfaces of solid supports.[8] Hybrid approaches have included methods where patterning is achieved lithographically but Ag is deposited chemically.[16]

3.2 Stability and reproducibility

Reproducibility has been, and continues to be, a major preoccupation of researchers interested in developing practical SER(R)S assays. Of course, if infinitely stable substrates which did not degrade or become contaminated were available then reproducibility would not be a problem, since each individual system would only need to be calibrated once throughout its lifetime. Progress is being made towards this ideal situation for specific applications, in particular surface modification is now allowing reversible analyte binding to be achieved.[21] This is important for sensors that can be implanted or used remotely to provide a continuous monitoring output.[20] However, for many applications, particularly those where there is a possibility of contamination by materials which bind irreversibly to the surface, disposable substrates are useful. Indeed, with simple colloids the difficulty in removing the target analyte without destroying the colloid, combined with the very low cost of production, means that they are invariably used once and discarded. Similarly, even solid substrates are typically used for a very limited number of analyses before being discarded.

With single use media it is important that the manufacturing method is sufficiently reproducible so that there is a reliable supply of material that will conform to an established calibration model. Silver colloids, in particular, are notoriously difficult to manufacture with high reproducibility but careful work on the production of the materials can help to reduce the batch to batch variation in absolute enhancement factors of the freshly manufactured colloid to a few percent.[22] However, the enhancing ability of colloids does tend to degrade with time and the rate of this degradation also varies from batch to batch; a recent study has shown that even different storage conditions can give detectable differences.[23] This means that even if a calibration can be established for fresh batches of colloid, its accuracy will degrade over time. The timescale over which this occurs may be up to months but it will ultimately fail. An alternative approach is to find a method to preserve the colloid indefinitely and this has been achieved by use of hydrophilic, swellable, gel-forming polymers which can protect the colloids during storage but still allow ingress of the target analyte when required.[24] With high stability polymer gels a single very large batch of material can be manufactured and then provide a stock of enhancing materials which are identical to each other (measured RSD over 20 samples was 3%) since they come from the same subdivided batch (see Fig. 6). An alternative approach is to prepare the colloid *in situ*, although it should be noted that although this may eliminate storage problems it still requires the fresh colloid to be formed reproducibly every time it is required. This approach has been demonstrated with both borohydride[25] and hydroxylamine reduced Ag colloids[17] in flow cells. In the latter case the silver was formed on the surface of polymer beads and the relative standard deviation of the absolute peak intensity of a test acridine dye was an impressive *ca.* 5%.

For solid substrates the problems are slightly different because here any heterogeneity in the enhancing surface will increase the uncertainty in the absolute signal height. The simplest way to reduce this error is to take multiple points on the surface. For example, when vapour deposited films were used to detect amphetamine the between-film relative standard deviation (RSD) in the intensity of the most intense peak was reduced from 31.6% to 5.8% by taking several spectra.[26] Multipoint grid sampling is extremely easy to implement in spectrometers with motorised sample stages[18] and is very commonly used in spontaneous Raman studies of solid dosage from pharmaceuticals, for example.

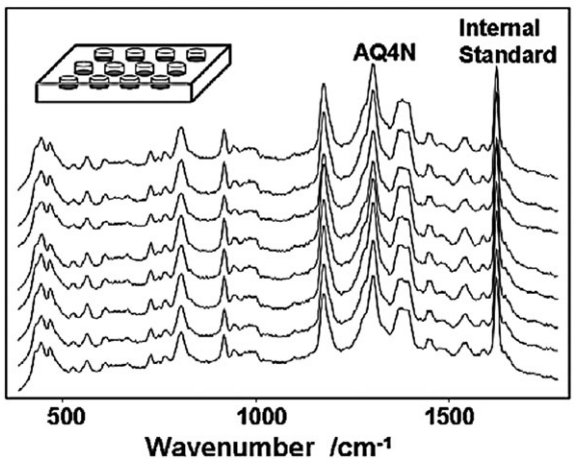

Fig. 6 Replicate SERRS spectra of an anthraquinone-derived drug (AQ4N) enhanced using Ag colloid protected within a swellable, gel-forming polymer. Spectra were recorded in a 96 well microtitre plate and normalised against a SERRS-enhanced internal standard. (Reprinted from ref. 24 with permission; copyright 2001 Royal Society of Chemistry.)[24]

It is worth stressing that the work on reproducibility discussed above is all essentially concerned with minimising the minor variations in the absolute enhancement factor which are found within nominally identical media. Although absolute reproducibility to $<\pm 1\%$ would be desirable, the fact that it has not been achieved does not mean that reproducible assays cannot be carried out, since the levels of variation in absolute enhancement shown above are still sufficiently low that they can be corrected by use of appropriate standards, as discussed in section 3.4. The most important point is that extreme irreproducibility found in earlier studies where some samples of the enhancing material were completely ineffective is now rarely encountered as the understanding of the best way to produce enhancing materials develops.

One final observation is that efforts to prepare stable and highly reproducible enhancing media need to be complemented by care during the data acquisition process. It is known that the focussed lasers used in Raman experiments can cause signal degradation either through photochemical damage to the sample or to the enhancing medium. This problem is particularly apparent with solid substrates but even with colloids the aqueous medium cannot completely protect against photodamage and it is important to ensure that the signals are acquired under conditions where the laser irradiance (power per unit area) is kept below the damage threshold. In SER(R)S experiments one particularly common indicator of sample degradation is the appearance of a strong broad doublet of bands at *ca.* 1360 and 1560 cm^{-1} which arise from formation of graphitic or amorphous carbon on the enhancing surface.[2]

3.3 Surface modification

The two fundamental processes required for surface enhancement of a target molecule is that the molecule can approach the enhancing surface and that the substrate will then enhance the signal of the adsorbed molecule. Given a reasonably enhancing medium (and such materials are easy to obtain) the main factor that will determine whether the analyte will give a large SER(R)S signal is its ability to access the critical region on or near the surface. In some cases the compounds of interest bind strongly to Ag or Au surfaces, so it is only necessary to give them opportunity to do so. Pyridine, the first molecule to have its SER(R)S spectrum recorded, falls into this category but other examples include thiols and triazoles.[3] Recently, significant advances have been made in understanding and controlling the interactions between more weakly binding analytes and metal surfaces.

One of the most important factors determining the binding of compounds is the medium's surface charge. With roughened electrodes the surface potential can be modified by an applied voltage and it is often possible to alter the potential to maximise the observed signal (see Fig. 7). However, roughened electrodes are now much less widely used than colloids for example. With colloids, the control of the surface potential is normally through chemical methods. Citrate-, hydroxylamine- and borohydride-reduced Ag colloids and citrate-reduced Au all have a negative surface potential.[2] This is presumably the reason that so many of the earlier studies using citrate colloids were of cationic compounds, particularly highly coloured dyes, which were attracted to the negative surfaces by electrostatic forces and were resonance enhanced by virtue of their strong UV/visible absorption bands. The inherent bias to obtaining strong signals from cations was reinforced by the common use of KBr or NaCl as aggregating agents which were used if the dyes did not aggregate the colloid themselves. When these salts are added to citrate-reduced colloids, the halides bind to the surface creating a strongly bound layer which gives the particles an even more negative surface potential. Although this will assist the binding of cations, anions are repelled by the negative charge on these halide aggregated colloids and even highly coloured dyes will give no signal if they carry an overall negative charge. One solution to this problem is to add a positively charged material that is capable of coordinating the surface and at least partly neutralises the excess negative charge. The most common method of doing

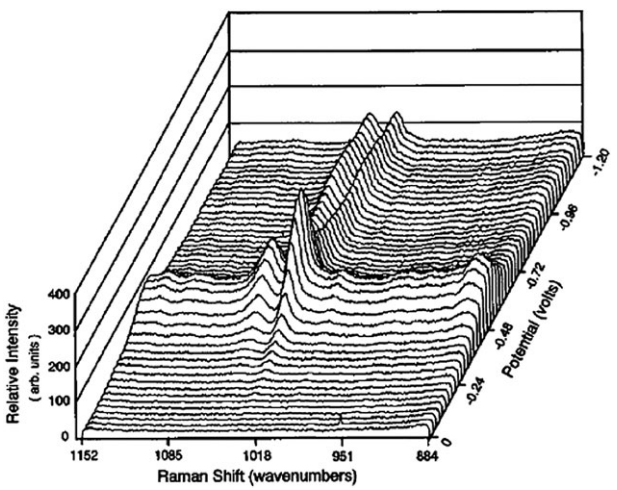

Fig. 7 Influence of applied potential on the SERS signal of 1 ppm nicotine on a Cu electrode. (Reprinted from ref. 28 with permission; copyright 1994 Society for Applied Spectroscopy.)[28]

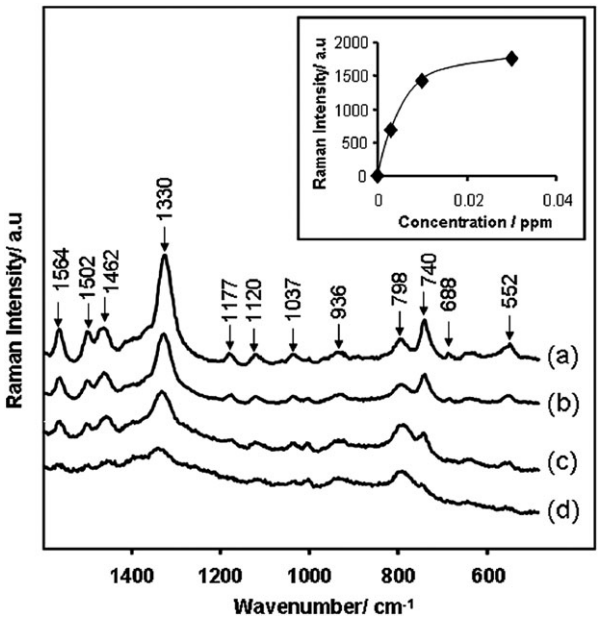

Fig. 8 SERS spectra of 2′-deoxyadenosine-5′-monophosphate (dAMP) recorded using citrate-reduced Ag colloid aggregated with $MgSO_4$ to promote binding by the anionic analyte with low spectral interference. Concentration of dAMP (a) 0.1 ppm, (b) 0.03 ppm, (c) 0.01 ppm and (d) 0.003 ppm. The insert shows the calibration plot of the dAMP (4 s accumulation times). Adapted from ref. 29.

this is to add cationic polymers such as poly-L-lysene and spermine, these poly-cationic materials both alter the charge and aggregate the colloids.[27] Of course, a consequence of adding any material that will bind to the enhancing surface is that their signals will also be enhanced. If the analytes are resonance- as well as surface-enhanced the signals may be so large that they dominate the signal and this was the case for the first studies using poly-L-lysene. However, this will not generally be the case and signals from the modifier can interfere with those of the analyte.

More recently, an alternative approach has been shown to be successful even for non-resonant SERS. In this method, citrate reduced colloids are aggregated using salts, such as $MgSO_4$, whose binding constants are so small that the aggregates still carry only the weakly bound citrate layer formed during the synthesis.[4] It was shown that when more strongly binding anions were added to this aggregated colloid they displaced the surface citrate, rather than being repelled by it, and their SERS spectra could be observed. Indeed, a series of substitution reactions using increasingly strongly binding analytes was carried out. It was found that the SERRS spectrum of each compound could be replaced in turn by the next member of the series.[4] This approach has allowed detection of DNA bases, their corresponding nucleosides and anionic mononucleotides (see Fig. 8) with good sensitivity and with low background signals, since no additional modifier was necessary.[29]

The compounds which have seen most extensive use as surface modifying agents to promote the capture of particular analytes are thiols of various types (see Fig. 9). Thiols have a particular advantage in forming a strong covalent bond to Ag

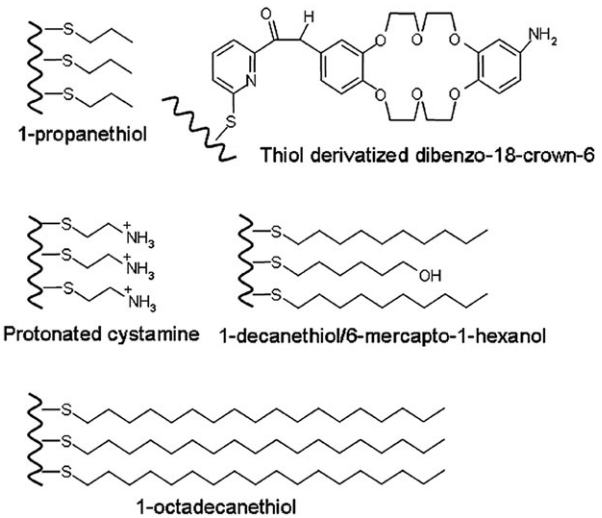

Fig. 9 Illustration of some of the thiols which have been used to modify the surfaces of SER(R)S-active substrates.

and Au surfaces, while self assembled monolayers (SAM) can be applied to surfaces by simple immersion in appropriate thiol solutions. This approach has been used to modify both colloids and roughened surfaces, for example cystamine, which carries a net positive charge when protonated in acidic solutions, has been used to provide a positive surface for binding ClO_4^-.[30] Similarly, alkane thiols have been used to create a non-specific hydrophobic surface which promotes adsorption and pre-concentration of polycyclic aromatic hydrocarbons while thio-derivatized crown ethers have detected alkali metals.[31] Thiol SAMs may also play a dual role, promoting analyte binding and protecting the surface. This has been elegantly demonstrated for AgFONs treated with either alkane-thiolate tri(ethylene glycol) or mixed 1-decanethiol/6-mercapto-1-hexanol layers designed for *in vivo* glucose monitoring where the modification of the surfaces prevents non-specific protein absorption and prolongs the lifetime of the device.[20]

Finally, the most specific surface capture agents come from molecular biology rather than organic chemistry. DNA analysis through selective capture of target strands and methods for signalling the capture event through generation of the SERRS signal from a label is already a significant research field in its own right and is complemented by studies using antibody capture of various targets signalled by SERRS labels. These labelled studies are not treated in detail here since they have been extensively treated elsewhere,[3] although it is notable that studies where binding is signalled by observation of bands of the target itself are starting to emerge.[16]

SER(R)S of mixtures

A very important feature of spontaneous Raman measurements is the ability to detect several different analytes simultaneously and to quantify each of them separately, on the basis of their distinct spectral signatures. Even if the spectra are extremely complex, with numerous overlapping bands, multivariate data analysis can be used to separate the contributions from the individual constituents. In principle, it should be

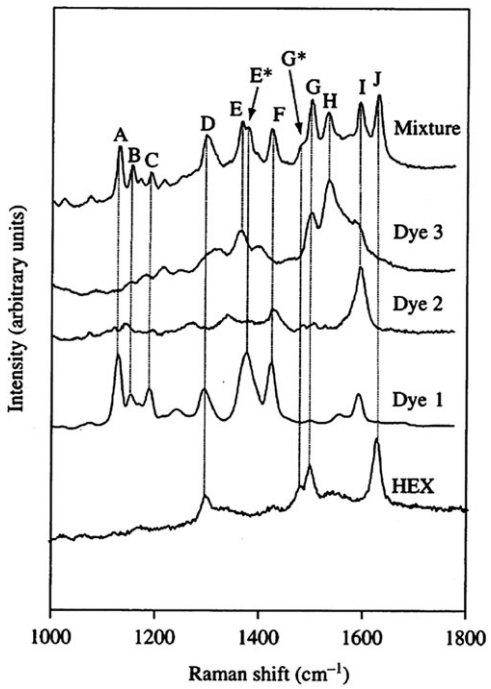

Fig. 10 SERRS spectra of a series of three dyes and a SERRS (HEX) labelled oligonucleotide illustrating the ability of SERRS to discriminate between labels in solution. (Reprinted from ref. 3 with permission; copyright 2005 Wiley.)[3]

possible to make SER(R)S measurements of even complex mixtures in the same way, but in practice it is often found that a single component from the mixture dominates. This may occur because of competition for surface sites, for example in the mixed anion solutions shown above, where the most strongly binding analytes occupied all the sites on the surface. Alternatively, if one of the constituents is resonance-enhanced, the extraordinarily high enhancement factors associated with SERRS will make its signals dominate the spectrum of the mixture. This is not a serious problem when well controlled assays in standard solvents are being developed but it is very significant when less control is possible, such as with tissue samples or environmental sampling, where a tiny amount of a strongly absorbing impurity can dominate the spectrum. The surface-modification techniques discussed above can minimize this problem since they can be used to prevent binding and detection of interfering molecules but studies where both resonance and non-resonance-enhanced compounds need to be detected in the same mixture will always be problematic.

In general, the best situation is one where the chemical properties of all the analytes of interest in the sample are similar, so that the surface populations broadly reflect the populations within the sample solution. This situation has already been realized for mixtures of closely related *bacillus* samples[9] and even for the more difficult situation of mixtures of dyes, all with strong SERRS signals, which were designed as labels for multiplexed experiments, as shown in Fig. 10.[3]

3.4 Internal standards in SER(R)S

The reasons for the almost universal adoption of internal standards for quantitative analysis using normal Raman spectra were discussed above. However, for SER(R)S measurements the situation is much less clear cut. The majority of quantitative SER(R)S experiments do not use a direct analogue of the normalisation using internal standards described above, which in SER(R)S would be use of a compound which is present in the sample along with the analyte of interest and is enhanced by the same mechanism. Reasons for this difference in approach arise from a complex combination of factors, as outlined below.

One source of variation in spontaneous Raman measurements which is often minimised in SER(R)S experiments is the reproducibility of the mechanical and optical alignment of sample and spectrometer. Quantitative SER(R)S measurements are often the result of considerable effort in optimising enhancing media and experimental conditions and this may involve standardised production of physically identical solid substrates or the use of colloids produced in a standard way. An excellent example is the use of standardised flow injection and microfluidic systems which reduce mechanical and optical variation between runs to a considerable extent and in some cases have been shown to produce quantitative intensity data directly from measurements of the absolute areas of the bands of interest.[17] However, even with flow systems it is more usual to use some form of internal standard since this can allow laser power variations to be eliminated with recourse to any external standardisation method and ensure proper correction any remaining minor optical variations. Similar arguments apply to 96 well microtitre plates, which have an advantage over flow systems that they are single-use devices so that there is no need to wash them between samples.[18]

The main difference in the use of internal standards for spontaneous Raman and SER(R)S measurements is that in SER(R)S there is often a huge disparity in the Raman scattering cross sections of the analyte and other compounds that are present in the sample. This means that often it is only the signal of the analyte that is observed. While this is excellent for selectivity (discussed in section 3.3), it means that there is no standard signal to normalise against. This effect is particularly noticeable in samples such as low dose tablets, where the predominant material is not water but a strongly scattering excipient. For example, in a recent study of illicit tablets prepared using a very potent designer ''ecstasy'' variant 2,5,-dimethoxy-4-bromoamphetamine (DOB), spectra of model tablets with 300 mg lactose excipient and up to 1 mg DOB were obtained by direct application of Ag colloid to the tablets. Even at 1 mg per tablet the SERS signal from the DOB was so strong as to completely dominate the spectra, while the bands of the unenhanced lactose excipient, although still present at the reasonably high levels associated with pure organic solids, had such a low intensity relative to the enhanced bands that they were barely detectable (see Fig. 11).[32]

A partial solution to the problem of high analyte signals is to add a non-SER(R)S-active internal standard at whatever concentration is needed to make its unenhanced signal sufficiently strong that it can be detected in the presence of the surface-enhanced analyte signal. This approach is the most widely applied of the SER(R)S internal standard methods since it is the most straightforward to implement and has the benefit that it at least corrects for variations in laser power and

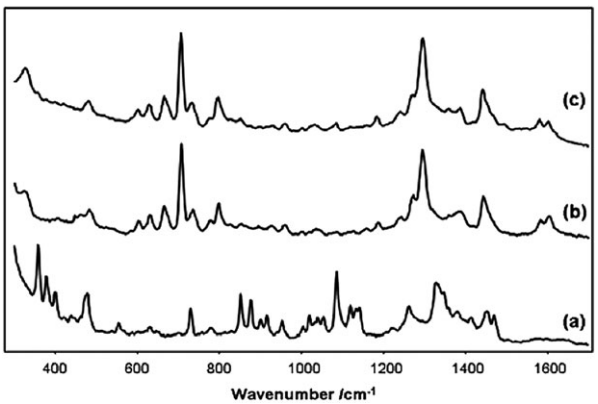

Fig. 11 Data illustrating how the signals from an enhanced drug can dominate those of an unenhanced excipient, even in low dose tablets. (a) Pure lactose excipient treated with colloid, (b) SERS spectrum of pure DOB, a highly potent "ecstasy" variant, (c) SERS spectrum of a tablet at a concentration similar to that found in seized tablets (1 mg per 300 mg tablet). Normal quantitative analysis of the tablets by measuring the drug/excipient band ratio is made difficult by the very low relative intensity of the excipient signal in (c).[32]

alignment/focussing. It has been particularly successful when combined with microfluidic sampling. For example, Choo *et al*. fabricated a microfluidic polydimethylsiloxane (PDMS) system for detection of malachite green, a dye which is widely used as a fungicide in aquaculture in some parts of the world but is banned in the US because it is carcinogenic and genotoxic.[33] In this system, acetonitrile was used as the internal standard because it gives a strong band in a non-interfering spectral region but it was essential to use a high concentration (it was mixed 1 : 3 by volume with water and then with 0.5 M NaCl as one for the feed solutions, the other being Ag colloid) to give a signal that was comparable in intensity to the ppb concentration of malachite green being recorded (see Fig. 12). With this system, the calibration graph of the intensity of the strongest dye band (I_{1615}) normalised to the acetonitrile peak at 2258 cm^{-1} was linear over the range 0–100 ppm and had a correlation coefficient $R = 0.993$.

The disadvantage of using an internal standard which is not SER(R)S active is that it cannot correct for any changes in the enhancing ability of the medium itself, which could arise for numerous reasons, including deactivation by some secondary component in the sample mixture or loss of enhancement due to aging effects. This means that it is extremely useful to have a signal that indicates whether the enhancing medium is itself acting as required. This will become increasingly important if SER(R)S sensors are to be deployed for critical detection, such as monitoring for explosives and chemical warfare agents. Enelbrektsson *et al*. have tested several thiol derivatives as potential internal standards. All these compounds formed self-assembled monolayers on the surface of the enhancing substrates, which were gold colloids dried onto glass slides.[10] The standards also contained -CN groups to give a distinct peak *ca*. 2300 cm^{-1}, a spectral region free from interference from their test analyte, R6G laser dye, which is a very well known strong SERRS target. It was found that, despite the chaotic nature of the substrates (which gave very large variation in enhancement when spectra were taken from different positions, even on the same slide), normalisation against the enhanced standard allowed an acceptable calibration over the range 0–5 μM with an RMS error of prediction of 0.5 μM.

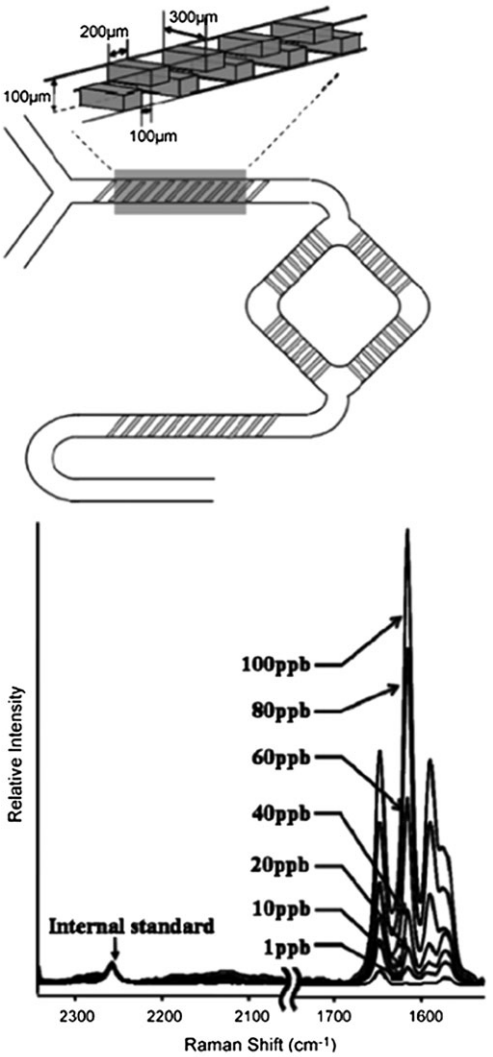

Fig. 12 A schematic drawing of a zig-zag PDMS microfluidic channel (top) designed to ensure effective mixing between Ag colloid and analyte solutions which are pumped into the two input channels shown. SERRS data recorded with this system for malachite green dye using a high concentration unenhanced acetonitrile internal standard is shown bottom. (Adapted from ref. 33 with permission; copyright 2007 Elsevier.)[33]

Finally, the best internal standard will be one which is not only SER(R)S enhanced but also chemically similar to the analyte because such a standard will be sensitive to changes in the enhancing medium which can alter its ability to enhance specific types of molecules. These changes can be very dramatic. For example, to detect anionic compounds with the cystamine modified surfaces shown in Fig. 9, the pH must be kept sufficiently acidic so that the amine is protonated to give the overall positive charge that attracts the analyte to the particles. However, if the pH is changed the analyte signal may be dramatically reduced while that of the modifying layer, which remains bound to the surface irrespective of the pH, will continue to give a strong SERS signal. The means that using

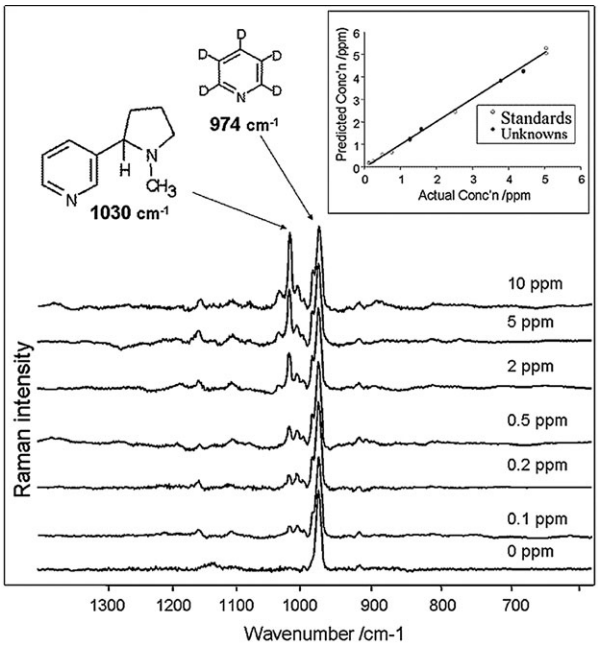

Fig. 13 Data from a SER(R)S assay for nicotine (structure shown top left) using polymer-stabilised Ag colloid (gel coll) as the enhancing medium. Spectra are normalised to the intensity of the 974 cm^{-1} band of the d5-pyridine internal standard. Linearised calibration data from the spectra is shown in the insert. (Adapted from ref. 18 with permission; copyright 2004 Royal Society of Chemistry.)[18]

the cystamine signal to confirm SERS activity is inappropriate. However, this problem can be avoided if the standard and analyte have similar chemical properties, since their response to any perturbation of the experimental conditions should be the same. For example, pyridine has been used as the internal standard in the analysis of nicotine, which is a pyridine derivative.[18] Since both compounds attach to the surface through their pyridyl ring any perturbation to the enhancing medium which affects the spectrum of one will affect the other in a similar way. The only disadvantage of using such similar compounds is that their Raman bands may lie at similar positions. In this example, the strongest bands of the nicotine and pyridine lie at *ca.* 1030 cm^{-1}. This interference was removed by the use of d_5-pyridine, which has the appropriate chemical properties but whose strongest Raman band is shifted to 974 cm^{-1}. This gave a system where a calibration plot of I_{1030}/I_{974} could be linearised to give $R^2 \sim 0.998$ over a 0–10 ppm range (Fig. 13).

The most extreme example is where the internal standard is an isotopically substituted form of the target analyte itself. In this "isotope-edited" approach the response of the analyte and internal standard to any perturbation in the experimental conditions would be expected to be effectively identical. Ben-Amotz *et al.* have demonstrated the principle using R6G and its d_4 isotopomer with very impressive results, where they showed reproducibility better than 3% could be achieved and that measurements could be carried out over very large concentration ranges.[34] Importantly, they also showed that using a standard that was SERS enhanced but chemically quite different from R6G (adenine in this case) gave much poorer reproducibility when the experimental conditions were varied by, for example, changing the total concentration of analytes to be measured in the sample. The only disadvantage of this isotope-edited approach is that isotopically substituted analogues of the analyte of interest need to be available. However, choosing standards that are chemically similar to the target can give many of the benefits of a full isotope-edited analysis without the need for synthesis of potentially very expensive isotopically substituted internal standards.

4. Examples of quantitative SER(R)S

A very large number of examples of quantitative SER(R)S studies could be used to illustrate the broad range of approaches and techniques which can be used. Here a small set of target molecules have been selected because they show how there may be several ways to carry out successful measurements of the same compound and how the level of sophistication required is set both by the nature of the target and what will ultimately be required of the assay *i.e.* field deployment, high throughput, low cost *etc.*

4.1 Nicotine

Nicotine detection and quantification has been studied for many years as a tool in smoking-related research. However, it has recently taken on much more commercial importance with the growth of a global market in nicotine replacement products (patches, gums *etc.*) which aid smoking cessation. The diversity of methods which have been applied to nicotine analysis is very striking.

In the first quantitative study by Haas and co-workers in 1994, roughened Cu and Ag electrodes were investigated as enhancing media, the electrodes were first polished, then roughened by oxidation/reduction cycles before introduction of the test solution.[28] The intensity of the signal was found to depend on the applied potential and with Cu electrodes went through a transient maximum at *ca.* −0.5 V before reaching a stable plateau at higher values (see Fig. 7). The concentration of nicotine was determined by measuring the intensity of the strongest nicotine band at 1030 cm^{-1}, the only normalisation was to scale the intensity according to the height of the broad background signal. The best results were obtained for Ag electrodes with 647 nm excitation where a linear calibration ($R = 0.99$) was obtained over a concentration range 10–900 ppb. Each measurement required approximately 5 min to carry out, which was significantly faster than chromatographic methods.

In 1999 the sensitivity of SERS for nicotine detection was exploited by Vo-Dinh and Stokes who demonstrated a SERS-based dosimeter which used Ag-coated alumina substrates and gave nicotine spectra after 4 h exposure to 265 ppb nicotine vapour.[35]

More recently, the colloids stabilised by encapsulation within gel-forming polymers (gel-colls) discussed above have been used in a study where the objective was to develop a practical analytical procedure that could be used routinely in non-specialist laboratories.[18] The basic premise was that the method would be based on the use of enhancing media which would conform to the same calibration model because they were drawn from large

stocks of identical stabilised colloids. Spectra were run at 785 nm excitation, in a motorised 96 well microtitre plate sampler using a citrate-reduced, polymer-stabilised colloid, d_5-pyridine internal standard and multivariate PLS-1 calibration. Quantitation over 0.1–10 ppm (the range appropriate for analysis of nicotine replacement products) gave RMS prediction errors of 0.1 ppm for blind trail samples. Sampling rates >50 per h were possible but the key advantage was the robustness and reproducibility inherent in the approach.

Nicotine analysis has also been carried out in a PDMS microfluidic flow system where test solutions and pre-prepared hydroxylamine reduced colloid were mixed by flowing through a zig-zag type channel of the type shown in Fig. 12. The SERS signal was measured using a 514 nm Raman microscope and quantitation was carried out from measurements of the area of the 1030 cm^{-1} nicotine band and an internal standard, which was the 1360 cm^{-1} band of the hydroxylamine reductant. A linear response ($R = 0.998$) was found over the 0.1–10 ppm concentration range, unfortunately no prediction errors were calculated.[36]

These examples show the diversity of wavelengths (514, 647, 785 nm), enhancing media (roughened electrodes, Ag-coated alumina, citrate and hydroxylamine-reduced Ag colloid) and sampling arrangements (electrochemical cell, solid substrate, 96 well microtiter plate, microfluidic channel) which can be applied to the same target molecule. In this case because nicotine has a strong affinity for Ag surface due to the Lewis basicity of its pyridyl ring, there was no requirement to modify the surfaces to promote adsorption so the as-prepared substrates were very effective in enhancing the signal.

4.2 Dipicolinolate

Dipicinolate has recently become an important target analyte because it is a useful marker compound for *Bacillus* endospores, including those of *B. anthracis* (anthrax) which are *ca.* 10% by mass calcium dipicinolate (CaDPA). The sensitivity of SERS, combined with its molecular specificity would suggest that it would be a good method for spore detection through identification of DPA. However, the affinity of DPA for Ag or Au surfaces is lower than that of pyridine so slightly different strategies need to be employed.

The first studies were carried out with Ag-doped sol–gel coated capillaries. The analyte solution was drawn up into the tubes under capillary action where it came into contact with the silver particles held in the sol–gel coating. Acceptable signals were detected from 1 ppm solutions using 785 nm excitation although no tests for reproducibility or quantitative calibrations were carried out.[37] This early work was followed by a study using citrate-reduced Ag colloids, aggregated with a non-competing SO_4^{2-} anion (aggregation with Cl^- salts completely eliminated the DPA signal) and using a negatively charged CNS^- internal standard, which was strongly SERS enhanced and used <1 ppm in the sample mixture (see Fig. 1). Quantitation was through simple plots of normalised peak height (I_{DPA}/I_{CNS}) against [DPA], which had $R^2 = 0.986$, and a detection limit <1 ppm.[4]

Finally, very sophisticated and effective AgFON substrates have been developed for DPA detection. In this system, 590 nm polystyrene spheres were chosen to generate films optimised for SERS using NIR excitation. These were then protected by atomic layer deposition of a <1 nm alumina layer which increases the affinity of the surface for DPA and stabilises the SERS activity. These substrates have a shelf life of >9 months and high sensitivity for DPA. The signals, normalised to 0.02 M NO_3^- as the internal standard (not SERS-enhanced) followed a Langmuir adsorption and fits to the linearized form were good ($R^2 = 0.99$), with an LOD of 1.9×10^{-6} M (*ca.* 0.3 ppm).[6]

Interestingly, the feasibility of using portable instruments has been investigated for both AgFON[6] using a battery powered conventional instrument and with a rugged system based on an acousto-optic tunable filter and using hydroxylamine reduced Ag colloid which gave a signal at 50 ppm.[38]

4.3 Mitoxantrone

It is useful to compare the sensitivities of the analyses above, impressive though they are, with an example of a resonance enhanced compound. Mitoxantrone is an anticancer agent with a large conjugated π system. It adsorbs readily to Ag surfaces and its UV/vis absorption spectrum has a broad band which peaks around 650 nm. Smith and co-workers have used citrate-reduced colloid in a flow cell to enhance the signal of mitoxantrone solutions.[39] Both 514 and 633 nm excitation were used and very high sensitivity was obtained; the limit of detection was 4×10^{-11} M. Moreover, the assay could be used to determine the drug level in serum as well as pure aqueous solution with a precision that was typically better than 5%.

Mitoxantrone has also been analysed in a multiplexed microfluidic system fabricated in PDMS. Here the SERRS data were recorded with 633 nm excitation. The objective of using the multiplexed system was to allow systematic parallel studies to be carried out. Fig. 14 shows the SERRS data obtained from this system using crystal violet, a strongly scattering dye, as the test compound. As is clear from the Figure, several different channels could be monitored with good reproducibility. When this system was tested with mitoxantrone good quantitative data were again obtained with a

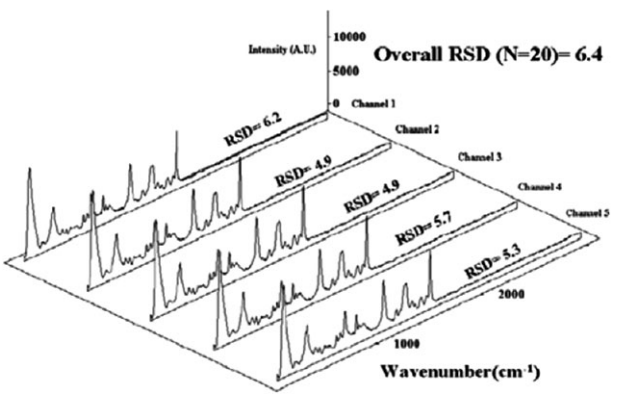

Fig. 14 SERRS spectra illustrating the signal reproducibility within and between channels in a multiplexed microfluidic system. Data were acquired from 5 channels using 1.0×10^{-6} crystal violet on Ag colloid. (Reprinted from ref. 40 with permission; copyright 2007 Society for Applied Spectroscopy.)[40]

linear response ($R = 0.982$) over the concentration range 10^{-13}–10^{-9} M.[40]

5. Conclusions

In the past, SER(R)S-based assays have been perceived as difficult and unreliable, primarily due to problems with the manufacture of enhancing materials. This situation has now changed, a broad range of strategies have been shown to be capable of providing materials which give absolute enhancement values that vary within a few percent between batches. These materials, when combined with standard techniques from analytical chemistry, particularly the use of internal standards, combined with more modern methods of multivariate data analysis and sample pre-concentration when required can make SER(R)S a reliable and highly sensitive analytical method.

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