Detection of DNA and P-450s on silver colloidal nanoparticles by surface-enhanced resonance Raman scattering (SERRS)

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ABSTRACT

Surface-enhanced resonance Raman scattering (SERRS) is a very sensitive and selective detection method that can be used for the analysis of both DNA and P-450s. A number of factors have limited the broader application of the technique. These limitations are described and addressed. An approach to reduce the problems associated with variation of the silver colloids used to provide surface enhancement and chemical methodologies that ensure surface adsorption are presented. A practical approach was used to investigate the nature of the effect. This approach has highlighted the importance of resonance enhancement for ultimate sensitivity. Two approaches to achieve successful detection of DNA using SERRS are described, and, using these two approaches, the possibility of multiplexing is also demonstrated. The analysis of proteins by SERRS is discussed and P-450 is presented as a specific example of the information that may be gained from SERRS of proteins.

Keywords: surface-enhanced Raman scattering (SERS), surface-enhanced resonance Raman scattering (SERRS), silver colloid, DNA, P-450

1. INTRODUCTION

Surface-enhanced resonance Raman scattering (SERRS) is a very sensitive and selective detection method. The method requires that an analyte is chosen which has an absorption band close to the frequency of the laser to be used for Raman excitation and that the analyte is adsorbed onto a roughened metal surface, typically silver or gold. Excitation of the sample with the laser provides enhanced Raman scattering both from molecular resonance with the analyte and from surface enhancement, resulting in selective enhancement of the signal of the chosen analyte. Despite the very great potential of the method, its use has been inhibited by difficulties with regard to reproducibility and quantitation. The work reported here uses aggregated colloidal suspensions as the roughened metal surface for the analysis of biological molecules and indicates solutions to the main problems limiting the wider application of the technique. The main factors limiting the use of the technique for quantitative analysis are: the effect of particle shape and size, and state of aggregation of the colloid; uncertainties with regard to the nature of the surface adsorption mechanism; and uncertainties with regard to the actual mechanism of enhancement. These three main problems have been addressed to improve the technique.

2. SILVER COLLOIDAL NANOPARTICLES

2.1 Colloid variation

We have eliminated some of these difficulties associated with reproducibility of the SERRS signal from analytes adsorbed on silver colloids by careful preparation of the colloid in a standard manner. The initial decision as to whether or not a particular batch of colloid is acceptable is taken on the basis of the UV-visible extinction spectrum. This spectrum is related

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in a complex manner to the particle size and shape, and state of aggregation of the colloid. Whilst the spectrum alone is not a sufficient test to define these characteristics, by combining such results with the standard method of colloid production, the effect of variability on the substrate is largely eliminated.

2.2 Surface adsorption

The second major practical difficulty is in ensuring effective surface adsorption. Early experiments with rhodamine give curved concentration dependent profiles suggesting adsorption of the rhodamine onto the walls of the vessel as well as onto the particles.[1,2] New ligands—azo-dyes which contain the benzotriazole (BT) group—were designed to provide effective surface adsorption. BT is an anti-corrosion agent for copper and an anti-tarnish agent for silver. It is believed to function by forming a polymeric array by complexation with silver ions on the surface of the metal,[3] thus preventing desorption. Aggregation studies using these ligands have shown that a number of inorganic and organic aggregating agents can be used. The concentration of the aggregation agent can be set so that the aggregated colloid remains stable and in suspension. Over-aggregation provides bigger signals but reduces reproducibility, as the colloid becomes unstable in the aqueous suspension. Aggregation with reagents such as sodium chloride takes a few minutes to develop. Following that, stability is obtained for over 20 minutes. In Figure 1, a BT-dye (GM19) is illustrated along with the spectrum obtained in an experiment using sodium chloride as aggregating agent. The time stability is also shown.

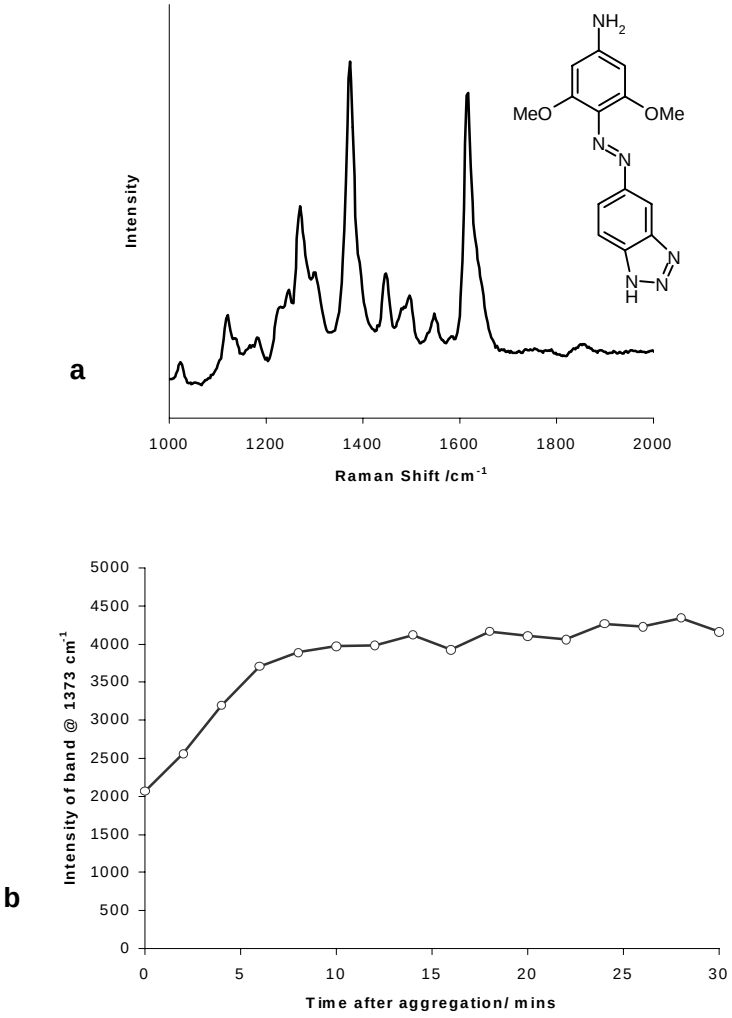

Figure 1. (a) SERRS spectrum and chemical structure of a BT-azo-dye (GM19) on aggregated silver colloid, and (b) a plot of the time dependence of the intensity of the largest peak after aggregation with sodium chloride.

To determine the reproducibility of the colloidal suspension, 6 batches were prepared, and with this dye, at a concentration of 10^{-8} M, the SERRS measured gave an inter-batch variability of ±25 %. This result indicates that semi-quantitative analysis is possible using SERRS and this may well be sufficient for many practical purposes. Other experiments in this laboratory have indicated that much better RSDs can be obtained using flowcell technology.[4]

2.3 The nature of the effect

The third major difficulty with the more general application of the technique is the lack of understanding of the effect. In recent experiments, we have addressed the problem from a practical point of view. Using the specially synthesised dye (GM19) described above, concentration dependent studies were carried out using 457.9 nm excitation, which is close in energy to the absoption maximum of the dye (which is at 450 nm), 514.5 nm excitation, which is pre-resonant with the dye, and 632.8 nm excitiation, which is further from the absorption maximum of the dye. Addition of GM19 to silver colloid produces aggregation so there is no need for an additional aggregating agent that would increase the complexity of the experiment. It was found in log-log plots of the normalised intensity against concentration—Figure 2—that the signal decreases at concentrations above ~10^{-6} M. It is thought that this concentration corresponds to monolayer coverage. Signals from molecules not on the first monolayer are much weaker and the high concentrations of dye may lead to over-aggregation of the colloid. However, significantly, at concentrations below this coverage, it was found that with the different excitation frequencies there were very considerable differences in slope of the calibration graph. With excitation at 457.9 nm the gradient is approximately 1.1 and is close to linear although the concentration profile is probably best defined by to two slopes within the concentration range studied. One gradient is found where the dye concentration is greater than 10^{-8} M and one where the dye is at a concentration of 10^{-8} M or less. With 514.5 nm excitation no signal was detected below 10^{-8} M and a much steeper gradient is obtained above this concentration. For excitation at 632.8 nm, the limit at which a signal is observed is ~10^{-7} M, and a profile steeper than that observed at either 457.9 nm or 514.5 nm is observed. At 10^{-6} M, the normalised signal with 457.9 nm excitation is approximately four times that obtained with either 514.5 nm or 632.8 nm excitation. The difference between resonance and non-resonant conditions at 10^{-6} M is remarkably small since SERRS is regularly quoted as being 10^3 times more sensitive than surface-enhanced Raman scattering (SERS) without resonant enhancement. These experiments clearly show this to be concentration dependent, with the biggest differences being obtained where the molecule is in molecular resonance and is at low concentration.

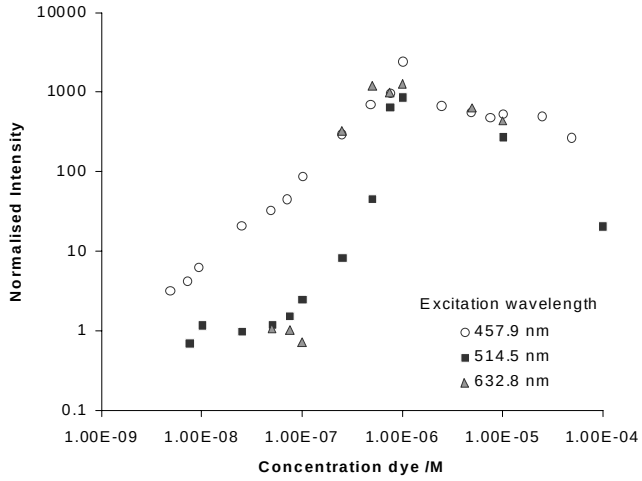

Figure 2. Plot of the concentration dependence of the intensity of the SERRS of GM19 at three different excitation wavelengths.

Two Raman maps of the same area, but with excitation at 514.5 nm and 632.8 nm respectively, are shown in Figure 3(a) and (b). These maps represent the intensity of the GM19 signal obtained from colloid adsorbed onto an amine-derivatised glass surface. It can be seen that there is significant variation in Raman signal across the surface with excitation at both 514.5 nm and 632.8 nm. The areas of high intensity (lighter shades) are thought to result from large enhancements at large colloidal aggregates that are randomly spatially distributed. An additional important observation is the fact that a significant Raman signal is obtained across the whole area examined with 514.5 nm excitation but not 632.8 nm excitation. There appears to be significant scattering from the areas of lower density of immobilised colloid, thought to consist of single particles and small aggregates when the exciting frequency is close to resonance. The explanation for this result appears to be that there is a significant enhancement closer to resonance. This supports the observation of much steeper concentration profiles and higher limits of sensitivity further from resonance. It is clear that this effect is only obtained on resonance. The slope obtained with 457.9 nm excitation is consistent with the fact that there is a single particle enhancement mechanism in SERRS but not in SERS as the data collected with 632.8 nm excitation show.

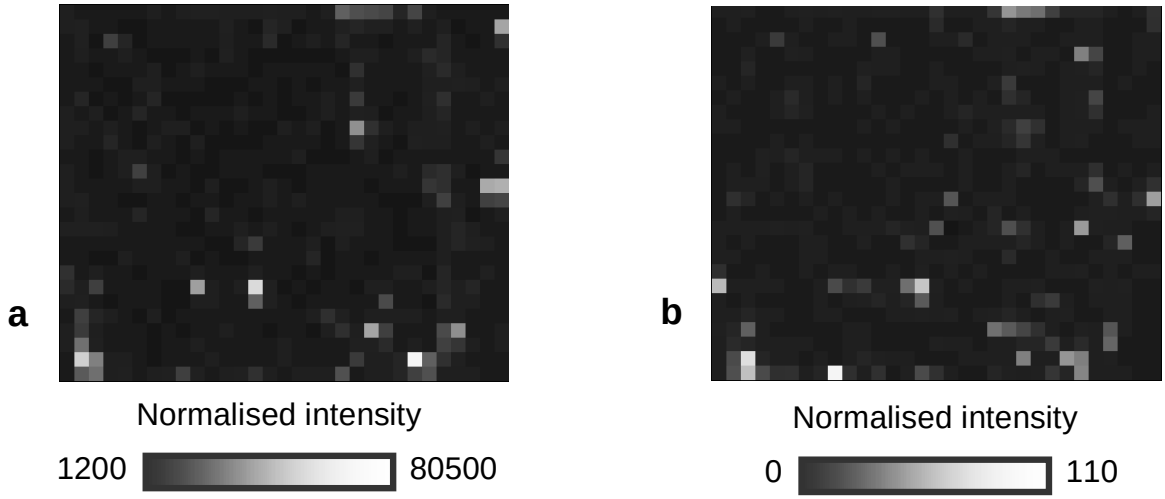

Figure 3. Raman maps of the SERRS intensity of the signal from GM19 taken from the same 62×56 µm area of immobilsed silver colloid collected with excitation at (a) 514.5 nm and (b) 632.8 nm.

3. DETECTION OF DNA AND PROTEINS

3.1 DNA detection

An area in which sensitive and selective detection of molecules would provide meaningful results is in DNA detection. DNA detection at low concentration is often carried out by the use of fluorescent tags. Since SERRS is reported to have single molecule detection limits, it should be possible to use SERRS active tags in place of fluorescent tags.[5,6] The advantage is that a vibrational spectrum is obtained from the tag giving much better molecular specificity, and the greater ability to discriminate between tags enables multiplex analysis. A further major advantage in SERRS is that there is a fluorescence quenching mechanism at the surface. This means that both non-fluorophores and fluorophores can be used as tags. This greatly extends the range of taggants that can be used. However, initial experiments using fluorophores as potential SERRS ligands were unsuccessful. The reason for this is that both the DNA, through its phosphate backbone, and the silver colloidal particles are negatively charged. The result is that good surface adsorption of the DNA dyes cannot be readily achieved.

New probe molecules were therefore designed for the SERRS approach. In one approach, a tail of propargyl amines was added to the DNA chain containing the fluorophore. These propargyl groups exist with the amine in the protonated form at neutral pH and consequently add a tail of positive charge to the DNA chain. In an alternative approach, a number of positively charged dyes were added at the end of the chain. Another advantage of SERRS is illustrated by this approach since the problem of fluorescence quenching which can arise with multiple fluorescent tags does not apply to SERRS. Consequently, the use of multiple dye labels closely spaced together on one end of the chain, to increase signal strength while leaving the rest of the probe sequence for molecular biology, is more effective with SERRS than fluorescence. The addition of these probes to colloid did produce some evidence of surface enhanced resonance Raman scattering but the technique was ineffective in that it was very irreproducible. A second problem had to be overcome in that the negative charge on the DNA was preventing effective aggregation of a DNA/silver colloidal cluster. To overcome this, the DNA charge reduction agent spermine was added. It proved to be effective both in reducing DNA charge and as an aggregating agent for the silver particles. Thus, the addition of the specially designed tails and spermine generate effective surface adsorption and therefore allow detection by SERRS. The limits of sensitivity obtained in this experiment are comparable to those of fluorescence, or better. Figure 4(a) illustrates the probe tails designed for the two approaches described above and Figure 4(b) illustrates the ability of SERRS to discriminate between the two tags. These are early examples and expansion of the chemistry will provide many more examples of multiplexing, using methodologies unique to SERRS.

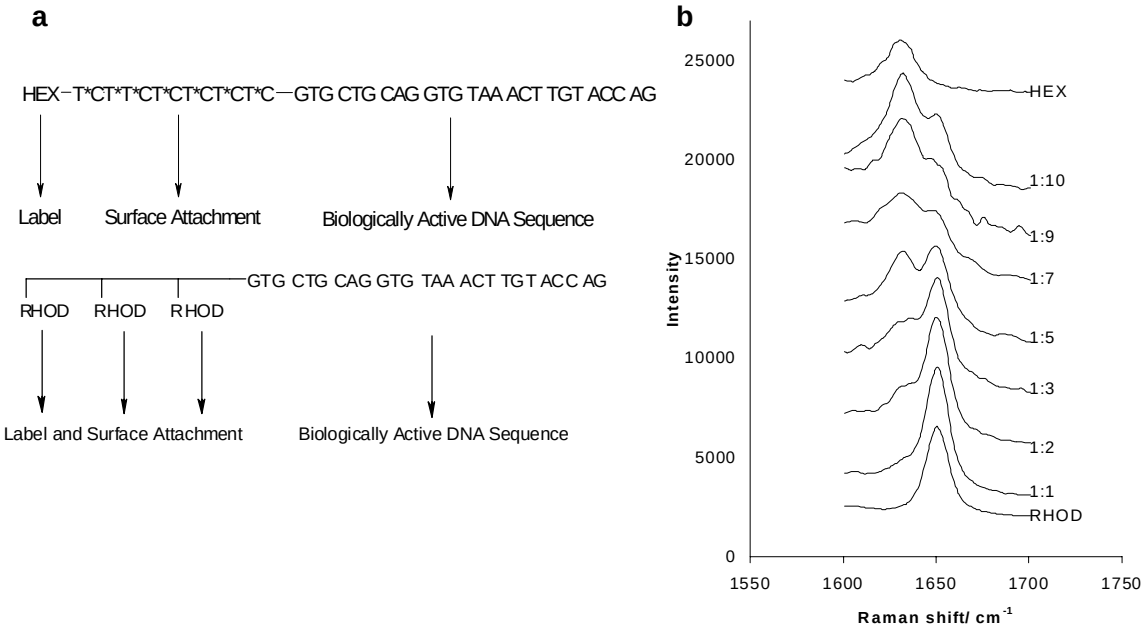

Figure 4. (a) Design of the probe tails used for DNA detection by SERRS—HEX and RHOD are dye labels—and (b) SERRS of mixtures of the two probes at a range of ratios, RHOD:HEX.

3.2 Protein Analysis

SERRS can also be applied effectively in protein analysis. In this case, interest is mainly in the nature of the chromophore in the protein rather than in the quantitation of the amount of protein. This can be illustrated with reference to P-450 proteins that contain a haem chromophore as part of their structure. By using laser excitation of the correct frequency to be resonant with one of the adsorption bands of this chromophore, the resonance effect enhances only the vibrational spectrum from the haem. This provides selective information concerning the active pocket. The effect of substrate addition can be seen by following oxidation and spin state markers. This information can be obtained by resonance Raman spectroscopy

and this technique is preferred, where practical, for studies of protein function, since adsorption of a protein on a metal surface can cause deformation and change in activity. However, there are specific advantages in SERRS over resonance. Firstly, some of the key proteins tended to fluoresce and in these circumstances, the fluorescence quenching of SERRS is very effective. This applies to some of the P-450s although protein purification can also overcome some of these problems. However, resonance also uses higher powers and requires more concentrated samples and consequently the effect of the exciting radiation on the protein can be much greater. Additionally, the adsorption of proteins on metal surfaces is of specific importance in biotechnology and in some occasions the effect of adsorption is not key to the application required. Thus, in the protein analysis field, SERRS still has a specific niche. This is strengthened by the much greater sensitivity that it offers compared to resonance Raman scattering. A typical SERRS spectrum of P-450 is indicated in Figure 5 and the oxidation state marker band, ν_4, is indicated. With different excitation wavelengths the spin state, ν_{10}, marker band is clearly resolved. We have demonstrated previously that even for substrates that do not directly complex with the iron, there is an allosteric effect on the pocket that can be reflected particularly in the vinyl groups present on the haems. In a further extension of this work, we have indicated the advantage of SERRS in selective nitration of the tyrosine groups of proteins. In this case, the sharp nature of the signals provides an indication of whether the nitrated tyrosine is present in a hydrophilic or hydrophobic environment.[7]

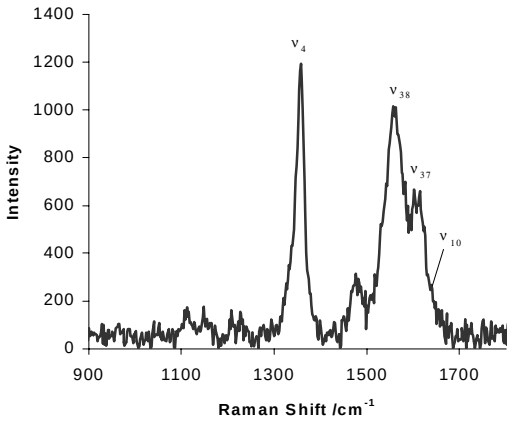

Figure 5. SERRS of P-450 on aggregated silver colloid, collected with excitation at 457.9 nm .

4. CONCLUSIONS

As a technique, SERRS is now well enough understood and a number of chemical methodologies have been introduced to control a number problematic variations to allow semi-quantitative, or quantitative, measurements to be made. This is achieved by firstly ensuring strong surface adsorption. Furthermore, the analyte should preferably have a molecular absorbance, and an exciting frequency close to the maximum should be chosen to provide the maximum sensitivity. The understanding of the effect is still primitive, but at a practical level SERRS can be manipulated in a controlled fashion to provide useful results from a number of analytes, including DNA and proteins.

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