Reproducible SERRS from structured gold surfaces†

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Metallic substrates with ordered spherical cavities have been shown to be very effective for surface-enhanced Raman scattering (SERS) and can be fabricated reproducibly using electrodeposition. The sensitivity of detection is increased by several orders of magnitude by using surface-enhanced resonance Raman scattering (SERRS). In this report we demonstrate SERRS for the first time on electrodeposited gold films templated with colloidal spheres and demonstrate the reproducibility of the response. We also obtain a direct comparison between SERRS and SERS by choosing two dyes, Cy5™ and Cy3™, which are similar in structure but differ in their excitation maxima, such that one is resonant and the other non-resonant with our laser excitation. As expected, the resonant enhancement is found to be of the order of 10^3 over and above that for SERS. The net SERRS enhancements are shown to be of the order of 10^9. We also find that the resonant enhancement profile of the different peaks for the chromophore follows the plasmonic resonance absorption spectrum obtained for the structured surface.

Introduction

After its first observation in 1974 by Fleischmann *et al.*,[1] the phenomenon of surface-enhanced Raman scattering (SERS) was recognised by Albrecht and Creighton[2] and Jeanmarie and Van Duyne.[3] Surface-enhanced resonance Raman scattering (SERRS) was demonstrated by Jeanmarie and Van Duyne[3] in the same paper. The authors noted the extremely low laser powers required for acquiring the surface Raman spectra under the conditions of molecular resonance with the laser. When the incident laser is in resonance with an electronic transition of a molecule in proximity to a surface-enhancing substrate, the cross-section of the molecule is greatly enhanced and SERRS is observed. SERS itself is a very sensitive and selective technique for detecting surface species. SERS enhancements of the order of 10^6 or greater are routinely reported. The SERS and resonance effect are multiplicative and can give overall intensity enhancements in the range of 10^4 to 10^8 for SERRS.[4–6] Thus, use of SERRS raises the sensitivity further by several orders of magnitude, offering the possibility of single-molecule detection.[7,8]

The extreme sensitivity of SERRS, giving molecule-specific information, is unparalleled amongst analytical techniques. This makes it ideal for the study of biomolecules. Thus SERRS has been used to study heme proteins,[9] to study the mechanism of electron transfer in cytochromes,[10] and more recently for trace detection of DNA[11,12] and enzyme activity.[13] The sensitivity of SERRS exceeds that of fluorescence, which is currently the most prolific technique employed for studying biomolecules, as proven theoretically[14] and experimentally.[15] SER(R)S has another immense advantage over fluorescence in that the signals have extremely small line-widths compared to the broad spectra of fluorescence emission; and hence it is possible, using SER(R)S labels, to carry out multiplexed detection.[16]

Despite more than 30 years of vigorous activity, a complete understanding of the enhancement mechanism of SER(R)S and wide commercial application has remained elusive. The bane of understanding and commercial exploitation has been, primarily, the irreproducibility of the substrates for SER(R)S. The bulk of the work on SER(R)S has been carried out using electrochemically-roughened silver electrodes and colloidal silver nanoparticles. Over the last decade researchers have focused on discovering and improving methods for fabricating reproducible substrates for SER(R)S. Amongst these, vapour-deposited silver films[17] and silver colloids[18] have been the most heavily researched. There are relatively few reports[12,19] of SERRS on gold substrates. Nevertheless, gold is attractive as a substrate because, although the surface enhancements are $\sim$10 times lower than for comparable silver surfaces, gold surfaces are more stable than their silver counterparts. Contrary to claims, most substrates are inherently not reproducible. Also, in most of the techniques there is no direct control over the formation of the metallic structures.

In the past few years, we have demonstrated our ability to fabricate nanostructured metallic films using colloidal templated electrodeposition.[20] These have been proven to generate tunable plasmon resonances[21] for SERS.[22,23] We have recently shown the tunability of our substrates for SERS even with a near-infrared source (1064 nm laser).[22] We have also demonstrated SERS on our substrates fabricated with palladium and platinum,[24] proving the flexibility of our technique. In our

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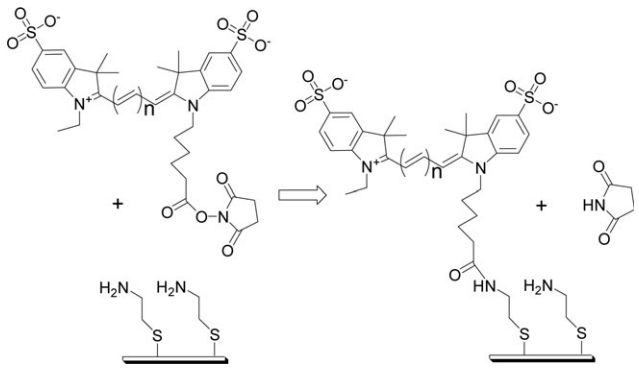
† Electronic supplementary information (ESI) available: Absorption spectrum of both the dyes (Fig. S1), a representative cyclic voltammogram (Fig. S2) and a typical SEM image (Fig. S3) of the structure. See DOI: 10.1039/b712144j



Scheme 1 Attachment of the dyes to the surface. NHS esters of the dyes readily react with primary amines resulting in amide bond formation. For Cy3™, $n = 1$ while for Cy5™, $n = 2$.

view, electrodeposition offers direct control over the formation of structured metallic films and has significant advantages over other techniques commonly employed to fabricate substrates for SER(R)S. Although SERS has been studied on inverse opals[25,26] by other researchers as well, there has been no report of utilizing them for SERRS.

In this paper, we report for the first time SERRS on electrodeposited ordered-spherical-cavity gold films and show the reproducibility of signals on them. We compare the enhancement provided by SERRS over and above that from SERS by using two molecules from the family of indocarbocyanine dyes. Cy3™ and Cy5™ were chosen as they are almost identical in structure, with the linking bridge in Cy5™ being one ethylene unit longer (Scheme 1). As a result, Cy5™ is resonant with the 633 nm laser excitation used in this study while Cy3™ is not. In our experiments the dyes are covalently attached to the gold surface through a flexible linker and for both dyes the surface coverage was measured electrochemically. This allowed us to calculate the corresponding enhancement factors.

Experimental

All reagents used were analytical grade and obtained from Sigma-Aldrich, unless stated otherwise.

Preparation of structured substrates

Substrates were prepared following the previously published method of Bartlett *et al.*[27] Substrates were prepared by templated electrodeposition on flat, conductive surfaces prepared by thermal vapour deposition of a 10 nm layer of chromium, followed by 200 nm of gold, onto glass slides. These gold surfaces were treated with a 10 mM ethanolic solution of cysteamine for at least 48 h before use. Uniform polystyrene spheres (1 wt% aqueous solutions obtained from Duke Scientific Corp.) were assembled on the treated gold surfaces by evaporative self-assembly in a thin-layer cell under conditions such that a single monolayer of hexagonally-close-packed spheres was obtained. Electrodeposition of gold through this template was carried out at constant potential from a commercial cyanide-free gold-plating solution (Tech. Gold 25, Technic Inc.) containing a proprietary plating additive (ECF 60, Technic Inc.) to give a bright finish. After electrodeposition the polymer spheres were removed by dissolving in DMF. This resulted in substrates with a hexagonal array of interconnected sphere-segment voids of uniform diameter. The depth of the voids can be precisely controlled through the charge passed during the electrodeposition.

In the current study, substrates were prepared with a sphere diameter, D, of 600 nm. Substrates were prepared in which the film height was 450 nm, that is, $0.75D$ (relative to sphere diameter). The 600 nm void films have been well studied and have distinct plasmon resonances at $0.75D$ film thickness corresponding to our 633 nm laser.[21,28] Hence, it was chosen for the study reported in this paper. The film heights are as estimated from the pore-mouth diameters of the voids determined by examining their SEM images.† The method has been earlier described by Bartlett *et al.*[27]

Surface immobilization of molecules

After cleaning, the substrates were dipped in an ethanolic solution of cysteamine (10 mM) for 24 h. The substrates were then washed and dried under argon. We used the well-known chemistry of *N*-hydroxysuccinamide esters (NHS esters) employed in immobilizing biomolecules. NHS esters of Cy3™ and Cy5™ were purchased from GE Healthcare Life Sciences. The substrates with the cysteamine attached to the gold surfaces were treated overnight in the dark with aqueous solutions of the dyes made in pH 8.1 phosphate buffer. Subsequently they were thoroughly rinsed with buffer and dried under argon. For valid direct comparisons, the same substrates were split into two for immobilizing each dye molecule. Since there is only a slight difference in their molecular structure and size, we expect a negligible difference in their surface chemistry and, hence, their coverage.

Surface coverage determination

We used cyclic voltammetry to determine the surface coverage of the immobilized dye molecules on the gold substrates. The charge under the oxidation peak was determined from the cyclic voltammogram† obtained by scanning the potential from 0 to 1.1 V *versus* a saturated calomel electrode (SCE) as the reference and a platinum mesh electrode as the counter. Anhydrous acetonitrile (kept under nitrogen) with 0.3 M tetrabutylammonium tetrafluoroborate (TBATFB) was used as the electrolyte. The solution was purged with high-purity argon (BOC gases) for at least 20 min before carrying out a measurement. During the measurement the electrochemical cell was blanketed with argon. The irreversible oxidation peak has been attributed to the radical cation formation in cyanine dyes.[29] Thereafter, the dyes are postulated to dimerize and/or deprotonate in an ECE (electrochemical–chemical–electrochemical) type of electrochemical reaction.

Absorption measurements

The absorption spectra of the dyes† were recorded in water using a PerkinElmer lambda 10 spectrometer with a 1 nm resolution at a scan speed of 240 lines per minute (medium scan speed).

Reflection measurements

The reflectivity of samples prepared as described above was studied using a BX51TRF Olympus microscope illuminated with an incoherent white light source coupled to an Ocean Optics (300–1000 nm spectral range) spectrometer. The spectra were recorded in the normal incidence mode. A 20× objective with a numerical aperture of 0.40 was used to record the spectra. All spectra were normalized with respect to that recorded on flat gold deposited on glass slides by vapour deposition. A CCD camera (Olympus DP2) mounted on the microscope enabled the simultaneous recording of optical images of the area whose spectrum was being monitored.

Raman measurements

All Raman spectra were collected using a Renishaw Raman 2000 system equipped with a 633 nm He–Ne laser with a maximum power of 2.8 mW, measured on the sample. In resonant SERS the power had to be decreased to 1% of the maximum. Spectra were collected using the extended scanning mode from 200 to 3200 cm^{-1} and 10 s accumulation time, with the single scan over the desired range taking 1 min to collect for recording normal SERS spectra. A 50× objective was used, providing a <1 μm spot on the sample. The slit width was kept at 50 μm, which provided a spectral resolution of 4 cm^{-1}. For recording solution spectra, a 20× objective was used under a static scan of 1 s for resonance Raman and an extended scan of 15 s for normal Raman. The laser power for resonance Raman was half that used for normal Raman measurements for solution spectra.

Results and discussion

The absorption maxima of Cy3™ and Cy5™ were determined by recording their spectra in solution. As expected, Cy5™ had a maximum at 647 nm while that of Cy3™ was at 549 nm (see ESI†). Thus, Cy5™ is resonant with the laser and should show SERRS, while Cy3™ should only display SERS with the 633 nm laser of the Raman spectrometer used. We prepared a series of samples with the same height, and hence morphology, and split them into two. The immobilization steps were then carried out on the identical surfaces. A simple energy minimization revealed a difference of 1 Å in the radii of the two molecules. Thus, the surface coverage of the molecules is expected to be approximately the same, albeit slightly in favour of the non-resonant molecule. On recording the Raman spectra with a 633 nm laser on our substrate, the signal was found to be much more intense for Cy5™ as compared to Cy3™ and the laser power had to be reduced to 28 μW which was 100 times less than that used for recording spectra of Cy3™. Spectra recorded for both molecules at similar surface coverages on topologically-similar positions on the substrates are shown in Fig. 1. Cy5™ shows SERRS having a molecular resonance with the 633 nm laser excitation in addition to the usual plasmonic resonances on these substrates at this thickness ($0.75D$).[21]

There is a one-to-one correspondence between most of the bands for the chromophore between both the indocarbocyanine dyes, with the bands showing the expected shifts due to the difference in the number of methine bonds. The signals for Raman bands are enhanced around 1000 times in the case of SERRS *vis-à-vis* SERS as measured on our substrates. Apart from an increase in the intensity of the peaks we also see an increase in the background. This background is significantly smaller (with the maximum of the background around 300 counts s^{-1} mW^{-1}) for the clean gold surface. The intensity maxima of the backgrounds increase by $\sim$200 times between the spectra for Cy5™ and Cy3™. The increase in background confirms the effect of the molecule on SER(R)S spectra. In this case the background increase observed for Cy5™ compared with Cy3™ is attributed to the resonant enhancement. The Raman band assignments are based on the work of Sato and coworkers on carbocyanine dyes.[30] With the attachment scheme employed here, where the chromophore is connected to the surface through a 10-atom-long linker, the shift in Raman peaks for the chromophore was found to be negligible ($\sim$1 cm^{-1}).

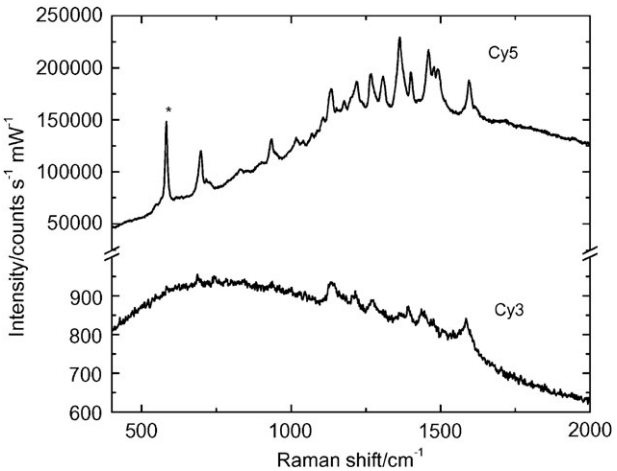


Fig. 1 SERRS spectrum of Cy5™ and SERS spectrum of Cy3™ recorded at structurally-identical positions on the ordered-spherical-cavity gold substrates. The spectra are presented without background correction and smoothing but have been normalized with respect to laser power and collection time.[31]

Further, on comparing the SERRS enhancements over and above that of SERS, for Cy5™ and Cy3™ respectively, we find that the different bands are enhanced to varying degrees. Moreover, this variation of resonance enhancement for different peaks is only observed on the structured surface and not in solution. On overlaying the plasmonic resonance spectrum acquired from reflectance measurement on the structure, we see a correlation between the resonance enhancements for different peaks and the plasmon resonance. The resonance enhancement, defined as the ratio of the band intensity for resonant Cy5™ to that for non-resonant Cy3™, and the plasmon resonance spectrum are presented in Fig. 2.[31] The peak intensities have been calculated after background subtraction. This observation implies an electromagnetic mechanism for the resonantly-enhanced scattered fields as well. We postulate that the extent of coupling of the outcoming scattered radiation from the electronically-excited molecule also contributes to the SERRS intensities observed. Therefore, the resonance enhancements of SERRS over SERS are higher for

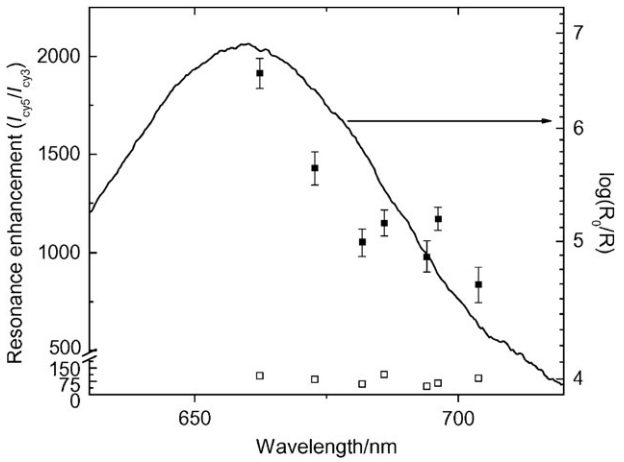


Fig. 2 Resonance enhancement (defined as I_{Cy5}/I_{Cy3} for the peaks after background correction) comparing SERRS peaks of Cy5™ with SERS peaks of Cy3™ recorded on 600 nm sphere templated gold films with 450 nm film thickness (■) and in solution (□). The inverted reflectance spectrum for the structured film has been shown for comparison. The maximum corresponds to the maximum absorption on the substrate.

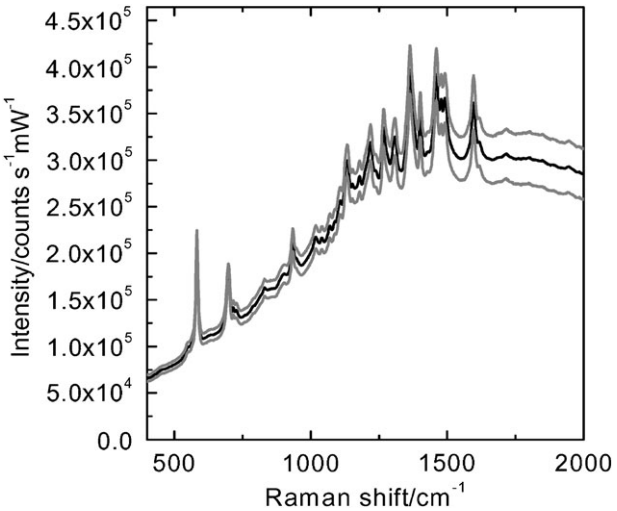


Fig. 3 Reproducibility of SERRS spectra. The average spectrum is shown in black while the grey ones correspond to ±1 standard deviation. The average spectrum has been calculated from 30 spectra recorded on three separately-fabricated substrates having identical morphology.

the Stokes-shifted peaks closer to plasmonic resonance compared with those which are farther away. The dependence of SERRS enhancements on the exciting wavelength has been well studied.[32] However, the resonance dependence of outgoing scattered radiation for the peaks of the same molecule has been seldom reported. The effect of resonance of the incoming laser with molecular excitation contributes much more than the outgoing resonances towards the total resonance enhancement. The extent of coupling of the scattered field in SERRS, that is the outgoing resonances, with plasmons on the substrate contributes to the enhancement only a factor of 2 to 3 over and above the contribution due to molecular resonance. This is estimated by comparing the maximum resonance enhancement, which ties in with the outgoing plasmon maximum, to the resonance enhancement observed far away from the plasmon maximum, where it reaches a baseline level (see Fig. 2). The error bars (~6%) for the enhancement ratios have been calculated from 18 measurements at different locations on a single substrate (split into two for Cy3™ and Cy5™).

To further demonstrate the reproducibility of our electrodeposited ordered-spherical-cavity substrates and the SERRS signals on them, three substrates were fabricated having the same height (and hence, morphology). Cy5™ was attached following Scheme 1 and SERRS signals were recorded on each from 10 different locations by translating a distance of 10–50 microns. The reproducibility data is presented in Table 1 and the spectra displayed in Fig. 3. The errors for SERRS peaks only vary between 5 and 10%, which is better than any report in literature for substrates and compares well with the work on silver colloids by Keir *et al.*, who report a process improvement for fabrication of silver colloids and obtain a variation of 6%.[18] However, our ordered-spherical-cavity substrates, besides being more reproducible than colloids, can be used as electrodes, are therefore suitable for electrochemical SERS, and are also much more stable and portable than colloidal suspensions.

For quantitative determination of absolute enhancement factors, the surface coverage of the dyes was determined by electrochemical means. The area under the peak for the one-electron oxidation of the dyes leading to the formation of a radical cation was determined (see ESI†). The oxidation is irreversible leading to dimerization and/or deprotonation of the cyanine dyes.[29] The irreversibility was also confirmed by a decrease in the SER(R)S intensities of the peaks observed after oxidation. In the case of Cy5™, we could see no shift in the peaks associated with –C═N– as SERRS is highly selective towards resonant molecules and perhaps the oxidation destroys the conjugation of the chromophore rendering it non-resonant with 633 nm laser, or destroys the molecule itself.

Table 1 Reproducibility data for different SERRS peaks[a]

	SERRS peak/cm^{-1}						
	1595	1460	1219	1134	934	697	583
Mean intensity/counts s^{-1} mW^{-1}	63 054	50 054	42 912	42 164	35 267	46 565	11 1691
Standard deviation/counts s^{-1} mW^{-1}	5686	4236	2607	2892	2191	2423	5624
% Error	**9.4**	**8.5**	**6.1**	**6.9**	**6.2**	**5.2**	**5.0**

[a] Peak intensities have been calculated after background subtraction from 30 spectra recorded on three substrates with identical morphologies.

Nevertheless, cyclic voltammetric experiments yielded a surface coverage of 4.8×10^{-11} mol cm^{-2} (2.9×10^{13} molecules cm^{-2}) for Cy5™. This corresponds to a partial coverage of around 20% compared to a well-packed monolayer. Using this surface coverage we obtain an enhancement factor of 5.2×10^6 for the most intensely enhanced peak (583 cm^{-1}) with respect to the resonance Raman spectra in solution. The enhancement factor has been calculated following the accepted method described in the literature[22,33] for a confocal system and using the electrochemically determined coverage of dye molecules on the surface. However, it should be pointed out that it was extremely difficult to acquire reasonable solution spectra owing to the huge fluorescence of Cy5™ on excitation with the 633 nm laser and that this could only be achieved by careful optimization of the dilution, collection time and confocal volume. Considering the fact that resonant Raman itself could be at least 1000 times stronger than non-resonant Raman, the colloidal templated gold substrates we have employed in this study are expected to give enhancements of the order of $\sim 10^9$. On comparing the 1590 cm^{-1} peak of Cy5™ with the corresponding peak in Cy3™, we obtained an absolute enhancement factor of 1.6×10^8 for SERRS over normal Raman.

Conclusions

In this study we have demonstrated SERRS for the first time on ordered-spherical-cavity gold substrates templated by colloidal crystals. We find the average enhancements due to resonance to be of the order of 10^3 by comparing two homologous dye molecules, one resonant and the other non-resonant with the laser excitation used. The net enhancements are found to be $\sim 10^9$ for SERRS over normal Raman. We have also demonstrated the reproducibility of signals from our substrates. Hence, our study proves the feasibility of reproducible assays utilizing SERRS analysis employing such ordered-spherical-cavity substrates.

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