Squaraines as unique reporters for SERRS multiplexing†

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Modified anilino squaraine dyes provide unique SERRS spectra that can be identified at low concentrations within any mixture of current reporters using longer, biologically compatible wavelengths of excitation.

Surface enhanced resonance Raman scattering (SERRS) is a highly sensitive spectroscopic technique that is finding an increasing number of applications in biodiagnostics including gene probes,[1,2] and DNA detection.[3] The technique is flexible and with controlled chemistry can be performed in solution,[4] or at a nanostructured surface.[5] Although a range of dye labels exist, there is a growing need to develop reporter molecules that are easily identifiable within a multiplexed analysis, allowing the maximum number of individual species to be identified, releasing the full potential of the technique. A further advantage of SERRS is that the excitation wavelength (λ_{ex}) can be selected anywhere in the UV to near-infrared range and wavelength selectivity can be observed using some combinations of dyes.[6] Herein, we present the application to SERRS of squaraine dyes (SQ) that report unique spectra that can be identified in any current mixture of SERRS labels, using longer wavelength red and near-infrared (NIR) λ_{ex}.

"Squaraine dyes", so termed by Schmidt,[7] were first synthesised by Trebs and Jacob in 1965.[8] A number of derivates of this class of cyanine dyes have been reported.[9,10] These materials have since found applications as xerographic photoreceptors,[11] cation detectors,[12] long-wavelength fluorescence reporters[13,14] and in non-linear optics.[15] The general structure is shown below in Fig. 1. The quadrupolar donor–π-acceptor–π-donor structure is characterised by the central C_4O_2 cyclobutadione bridge comprising an electron-deficient Hückel ring. The overall structure can be represented in a number of forms and the charge is considered to be largely delocalised over the molecule. This structure of this class of dye materials has been the subject of theoretical studies, principally to examine unconventional nonlinear optical properties.[16] Bigelow and Freund used MNDO and CNDO/s methods to model the aniline derivative **SQ1** and found that a polar medium stabilised the quinoid form.[17]

The SERRS spectra of a number of known reporter molecules (attached to oligonucleotides) along with 2,4-bis{4-[(6-hydroxyhexyl)methylamino]phenyl}squaraine (**SQ2**) are shown below in Fig. 2. It is notable that **SQ2** can be resolved by eye from the 10 other major dye reporters at longer wavelengths. Examination of the spectra from these materials, including xanthenes, cyanines[18]

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and azo type dyes,[19] reveals that no current reporter displays a strongly allowed band at higher energy than the "graphitic" band at 1600–1650 cm^{-1}. We have examined a wide range of SERRS reporters and the region of the spectrum beyond this from 1650 to 2000 cm^{-1} is almost totally free of bands. Group vibrations with energies corresponding to this region are mainly those of carbonyl groups. In infrared (IR) spectroscopy the selection rules generally dictate strong absorbance bands for carbonyl groups due to the large change in permanent dipole during vibrational modes. Conversely, in Raman spectroscopy the carbonyl band is weaker or generally not observed due to a small polarizability change during the vibration (or rather the gradient at the midpoint of the vibration). Therefore, the presence of a strong carbonyl SERRS band from the squaraine dye is remarkable, in itself. Practical investigation of the reproducibility of this vibrational structure reveals that it is strong and consistent in a range of SERRS experimental conditions.

A theoretical study using density functional theory (DFT), (Gaussian '03) was undertaken to further understand the appearance of the spectrum and the effect of substitution on the molecule. Initial geometry optimisation calculations were performed on **SQ1** using the G03 computational package and the HF/6-31G* and B3lyp/6-311g** combinations. The optimized
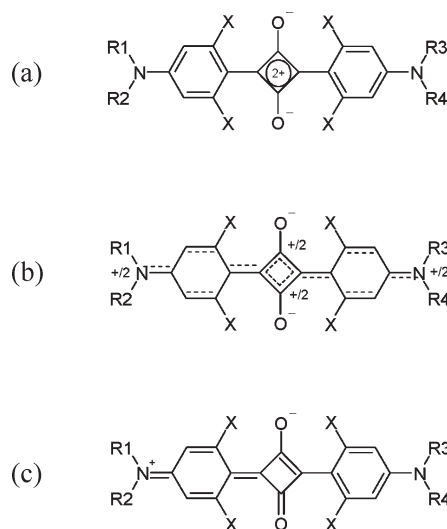

Fig. 1 Structures for bis(4-alkylaminophenyl)squaraine derived dyes: (a) fully aromatic, (b) centrosymmetric partially quinoid form, (c) non-centrosymmetric form with quinoid and aromatic regions: **SQ1**: R1 = R2 = R3 = R4 = CH3, X = H; **SQ2**: R1 = R3 = CH3, R2 = R4 = C6H12OH, X = H; **SQ3**: R1 = R2 = R3 = R4 = C3H6CH3, X = H; **SQ4**: R1 = R2 = R3 = R4 = C3H6CH3, X = H; **SQ5**: X = OH, amino groups are replaced by OH.
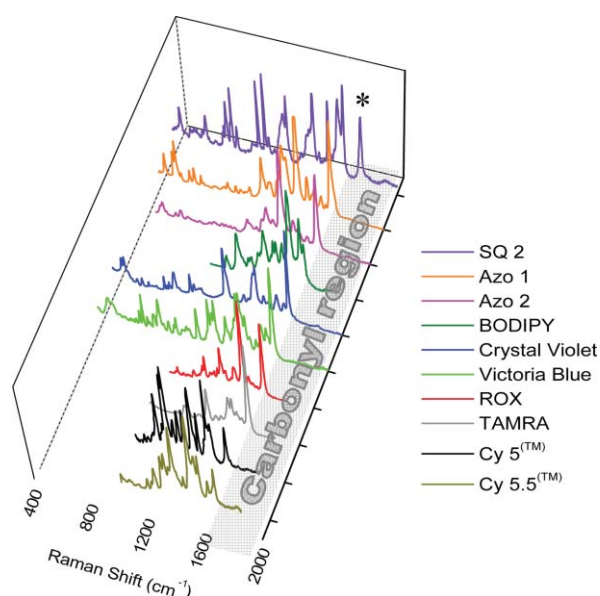
Fig. 2 SERRS spectra (using Ag nanoparticles) of a range of dyes effective at longer wavelengths (*e.g.* 632.8, 785 nm λ_{ex}). The unique band C_4O_2* is shown within this context. Note: Azo 1 and Azo 2[20] are resonant in the green ($\sim$500 nm) but remain effective SERRS reporters in the red due to strong interactions with silver surfaces.

bond lengths and angles were in agreement with those reported previously[17] and the molecule was found to possess D_{2h} symmetry. Frequency calculations were then performed using various DFT methods to see which method could give the best agreement with the experimentally observed carbonyl band at 1740 cm^{-1}. The best agreement is obtained with the BP86 method. A further two calculations were then performed using slightly bigger basis sets, namely the 6-311++G** basis set and the 6-311+G(2d,p) basis set.

The C_2O_2 group is highly delocalised. When this group vibrates both symmetrically and asymmetrically a band is not observed in the IR spectra. This effect is characteristic of squaraines and is often used to confirm that the dye has been synthesised successfully, as squaric acid has strong carbonyl lines in the IR. In the Raman spectrum the totally asymmetric C_4O_2 mode is not observed due to the effect of the selection rules. However, the symmetric C_2O_2 mode results in a large change in polarizability and thus the strong Raman band at 1740 cm^{-1}. We have studied a series of aniline squaraine dyes (**SQ1–5**) and have found that the symmetric carbonyl vibration is isolated and always present, despite modification of the *N*-alkyl groups. Strong spectral lines originating from carbonyl groups are rare but not unheard of in SERS spectroscopy, with cocaine a notable example. However, it is important to consider that, in the case of the squaraine, the line is resonance enhanced as it is closely associated with the chromophore. Indeed, theoretical studies indicate that up to 80% of the charge transfer occurs within the central C_4O_2 group.[18] Thus, overlap of the λ_{ex} with a suitable vibronic state would be expected to lead to a large *B*-term enhancement of the observed spectrum. In this computational study, the Raman intensity calculated for the C_4O_2 (1740 cm^{-1}) vibration is relatively small when compared to the principle "dye backbone" lines. In order for both lines to appear in equal intensity (as is observed) a secondary resonance would be required. *A*-term resonance enhancement is

normally observed in small, highly symmetric molecules, but could explain the increase in intensity observed for the C_4O_2 group given the high level of symmetry and charge transfer occurring in this region.

An additional factor in achieving strong SERRS spectra is the effect of surface selection rules. SERRS is extremely dependant on the orientation of the molecule with respect to the oscillating electric field at the surface. Additionally, if a molecule is chemically bonded to the metal surface the symmetry group may change and thus affect the intensities of the associated spectral lines. However, study of the solid-state resonance Raman (RR) spectra in a KBr disc reveals that the intensity of the C_4O_2 bands is significantly weaker in the solid state than in the SERRS analysis. Honeybourne has proposed that the observed non-linear optical properties from squaraines are derived from charge distortion by the proximity of another polar molecule in a T-shaped aggregate.[16] This effect could explain the loss of symmetry and thus the lesser intensity of the band in the solid state. The strongest signals in this study were recorded when the aggregating agents poly-L-lysine and spermine were used (as is typical in many SERRS studies).[4,18] In this study it was necessary to modify the general structure of the dye (to **SQ2**) in order to observe the strongest signals- comparable in sensitivity to other long-wavelength reporter dyes.[18] The aggregating agent may interact with the $C_6H_{12}OH$ chains in **SQ2** in order to bring the dye to the nanoparticle surface. The basic anilino squaraine structure is highly organic and modification in this manner also improves solubility in the polar SERRS environment. Spectra could be recorded in this manner with a full range of nanoparticle preparations including citrate-reduced silver and gold (Fig. 3) In the timescale of the SERRS experiments at 632.8 and 785 nm no loss of signal due to photo degradation was observed.

It is notable that squaraine dyes generally based on derivatives of 2,3,3-trimethylindolenine[14] also act as efficient SERRS reporters at longer wavelengths, although they do not exhibit strong resonance enhanced carbonyl lines. This is likely to be due to the lower symmetry class that these molecules belong to. Despite this, these compounds have very strong electronic transitions at longer wavelengths ($\varepsilon \sim 200\,000$) and provide information-rich
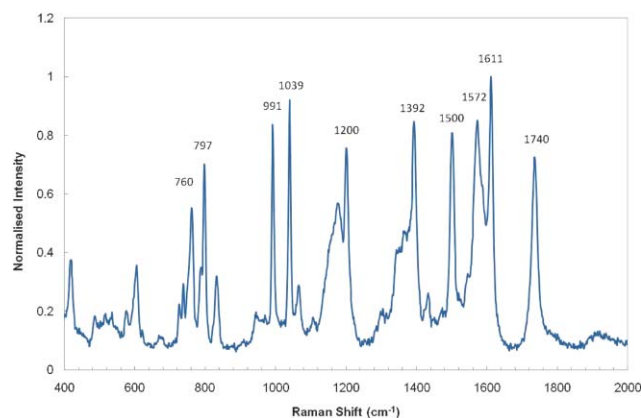

Fig. 3 SERRS spectrum of **SQ2** (Ag nanoparticles, $\lambda_{max} = 406$ nm). The symmetric C_4O_2 (1740 cm^{-1}) vibration can be described as an extension along the C–O$^-$ bond combined with simultaneous shortening of the C–C bonds within the cyclobutene ring in a "breathing" fashion (A$_g$ symmetry). The full band assignment is reported in the ESI.†
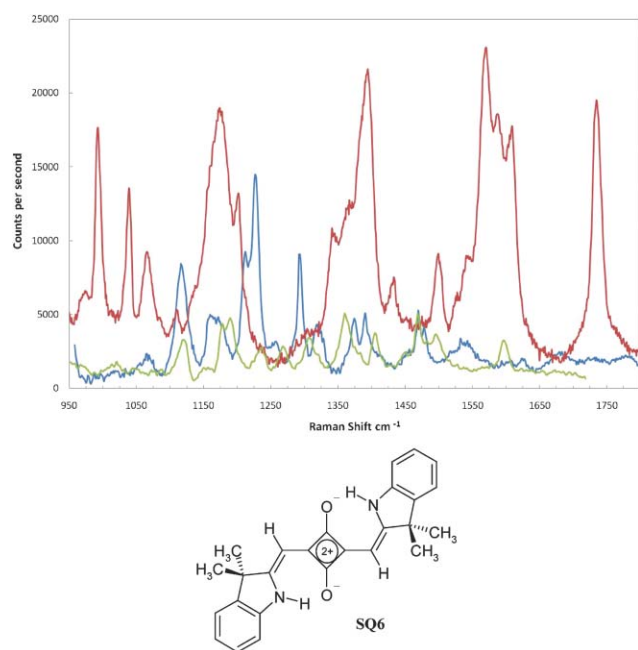
Fig. 4 Direct SERRS intensity comparison on Klarite™ gold surface (20 µW, 632.8 nm, 1 s): (a) red **SQ2**, (b) blue 2,4-bis[(3,3-dimethylindolin-2-ylidine)methyl]squaraine **SQ6**, (c) green: Cy5™.

SERRS spectra that possess a number of vibrational bands in the region 400–1300 cm^{-1} making these compounds excellent candidates for SERRS multiplexing.† These compounds are currently being developed as longer wavelength fluorescent labels in proteomics[20] thus the application to SERRS is significant both in terms of sensitivity and potential simultaneous multiple target identification. For further information on the synthesis (and application to proteomics) of these types of dyes readers are referred to the work of Lackowicz and Terpetschnig.[14,21]

A direct comparison of the SERRS intensity from **SQ2**, **SQ6** and Cy5™ is shown in Fig. 4. The dyes were deposited from standard solutions onto a commercially available SERRS substrate (Klarite™) that has been shown to have even enhancement across the active area.[5]

In conclusion, we have shown how strong SERRS spectra can be obtained from the squaraine chromophore. Sensitivity using 632.8 nm λ_{ex} is equal to or better than comparable cyanine dyes.[18] Reporters such as these yield strong unique spectra that are extremely distinctive, allowing facile detection within any real sample matrix. To the best of our knowledge this is the first report of such a vibration, encoded within a strongly SERRS active molecule. This application significantly expands the range of reporters resonant at longer, biologically compatible, wavelengths and will allow this rapidly developing technique to reach its potential in multiplexed analysis.

Notes and references

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