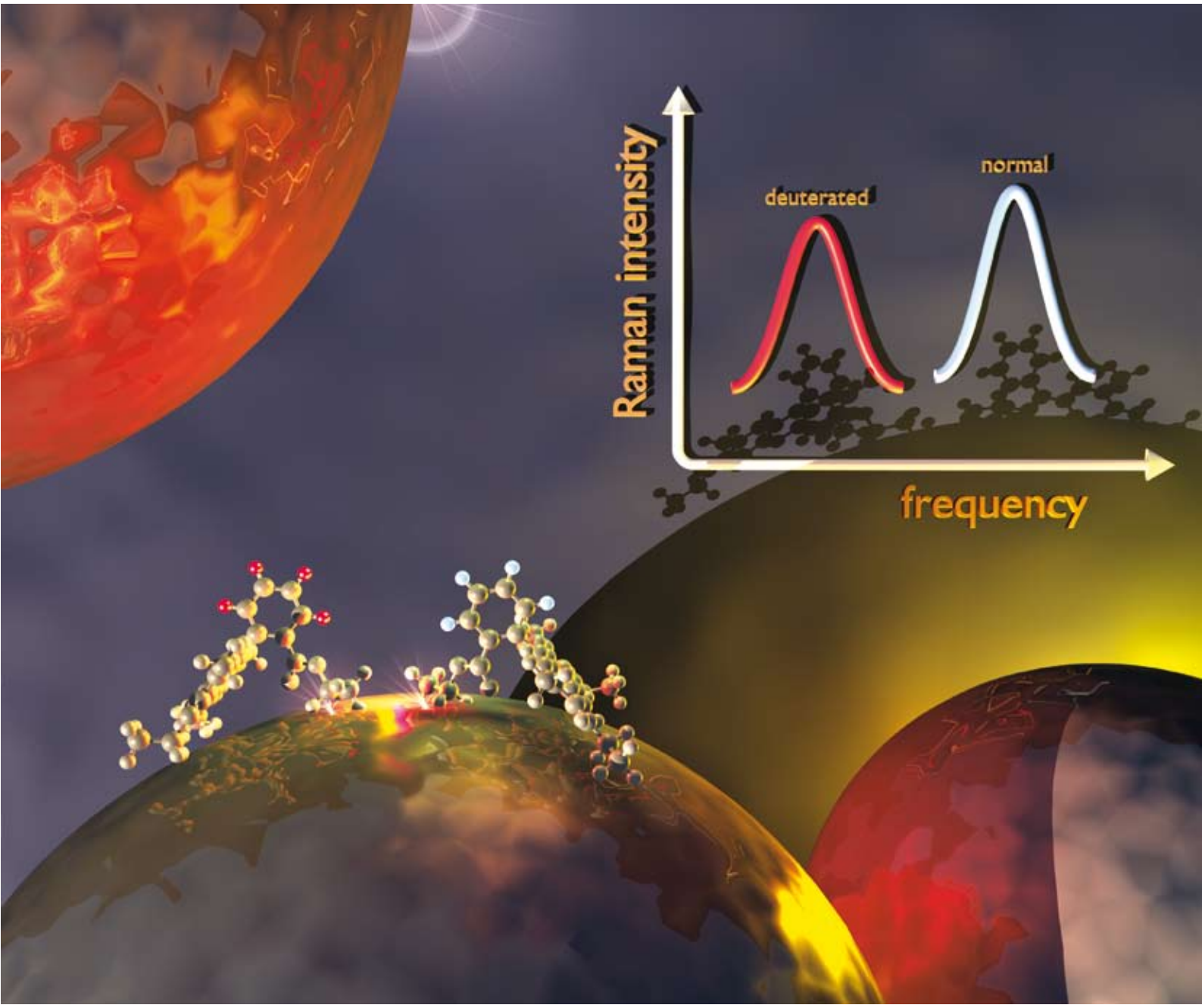
PCCP

Physical Chemistry Chemical Physics

www.rsc.org/pccp

Volume 10 | Number 28 | 28 July 2008 | Pages 4069–4200



ISSN 1463-9076

1463-9076(2008)10:28;1-X

Bi-analyte SERS with isotopically edited dyes

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Received 4th March 2008, Accepted 21st April 2008
First published as an Advance Article on the web 3rd June 2008
DOI: 10.1039/b803738h

Isotopically substituted rhodamine dyes provide ideal probes for the study of single-molecule surface enhanced Raman scattering (SM-SERS) events through multiple-analyte techniques. Isotopic editing should, in principle, provide probes that have identical chemical properties (and surface chemistries); while exhibiting at the same time distinct Raman features which enable us to identify single-molecule SERS events. We present here a specific example of two-analyte SM-SERS based on the isotopic substitution of a methyl ester rhodamine dye. The dyes are carefully characterized (in both standard and SERS conditions) to confirm experimentally their similar chemical properties. We then demonstrate their utility for bi-analyte SERS (BiASERS) experiments and, as an example, highlight the transition from a single, to a few, to many molecules in the statistics of SM-SERS signals.

I. Introduction

The detection of single-molecule surface enhanced Raman scattering (SM-SERS) has recently entered a new phase in its development with the introduction of multiple-analyte techniques[1–5] to identify, categorize, and classify single molecule events in SERS. The technique (together with the mathematical groundwork based on a modification of principal component analysis[6] (PCA) for a fast and unbiased analysis of the data) has recently been explained in full length in ref. 7, which will serve, accordingly, as a basis for the discussion of the results presented here. Multiple analyte techniques, like the bi-analyte SERS (BiASERS) method proposed in ref. 1, work basically as a contrast method to observe the statistics of single molecule events related to one dye in the background of the signals produced by the other (or others). Hence, the method provides an additional degree of freedom with respect to plain intensity fluctuations[8] to decide on the ''single molecule character'' of the signal. It has, for example, been used for an accurate estimation of single-molecule SERS enhancement factors.[4] Underlying the comprehension of SM-SERS spectra is the extreme nature of the statistics of single molecules events in-and-around the so-called electromagnetic (EM) hot-spots, which typically display a long-tail distribution of enhancements; a topic studied in full detail in ref. 9. In a recent development, Dieringer *et al.*[5] moved the BiASERS technique to a new level of sophistication with the introduction of isotopic editing. We believe this to be an important development in the field and our work here builds on this previous work. In what follows, we provide a brief overview of the present status, an outlook into the application of isotopic editing for SM-SERS, and justification for its importance.

From a purely experimental point of view, there are many variables that can be optimized in multiple analytes techniques for SM-SERS to pin down single molecule events more efficiently and simplify the interpretation. The nature of the probes stands out as one of the most important first steps to a successful implementation of the concept. As a case in point: in the two analyte version of the technique (BiASERS) studied in ref. 1–3 and 7 one would ideally like to study (for example) two molecules that have *different* SERS spectra but *identical* chemical properties. In particular, one would like the *surface chemistry* of the probes (in connection with their interaction with the metal substrate producing the SERS enhancement; typically silver or gold) to be as similar as possible; if not identical. One would also like to compare single molecule fluctuations of SERS peaks that have very similar SERS cross sections (and should therefore have a similar resonance condition with the excitation wavelength). Otherwise, the statistics of single molecule events in SERS could conceivably be biased towards one dye (or the other), depending on the different ''sticking'' properties to the metal surface, which in turn affects the assumed nominal concentrations for the statistical analysis of the results. This results in relatively strong constraints for the selection of the probes, and one usually has to recourse to a compromise. In the previous reports of BiASERS experiments, a compromise was achieved among the desired properties by means of specific dyes. In ref. 1 the ''partner probes'' were a benzotriazole dye[10] and Rhodamine 6G (RH6G). Ref. 3, on the other hand, used *n*-pentyl-5-salicylimidoperylene and octadecylrhodamine B together with the technique of Langmuir–Blodgett films, while ref. 2 made use of the combination of the two related probes 4,4′-bipyridine and 2,2′-bipyridine. More recently[4,7], Nile Blue (NB) and RH6G have also been used as successful BiASERS partners for SM-SERS. While all of these combinations proved the multiple analyte concept was a successful indicator of SM-SERS, one

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could argue that the combination of 4,4′-bipyridine and 2,2′-bipyridine in ref. 2 is perhaps the best among this list, in the sense that it uses two probes which are the closest in both resonance conditions and surface chemistry.

Isotopic editing—with a long and well established tradition in spectroscopy—appears as one of the best solutions to this problem. If isotopic editing of a useful SERS probe (with high SERS cross sections) results in a spectrum with distinguishable features compared to the standard (unedited) version of the same molecule, we have then two probes that—for all practical purposes—should have exactly the same chemical properties but can still be distinguished by their SERS signals. This is particularly important from the point of view of the *surface chemistry* of the probes.

It is interesting to note that isotopic editing is actually not a new concept in SERS either. Zhang and coworkers[11] have already demonstrated recently the usefulness of isotopic editing for analytical studies in SERS, but have not extended the study to the SM-SERS regime (despite the fact that all the ingredients were already present in their study). As mentioned earlier, Dieringer and coworkers[5,12] have also recently suggested and applied the concept of isotope editing to bi-analyte SERS experiments. Arguably, the results in ref. 5 will establish new standards on the study of single molecule SERS conditions in years to come.

In order to produce quantitative studies of single molecules in the future, an important initial step is the *full* characterization of the molecules themselves. In particular, it is not always possible to simply assume the Raman cross sections of isotopically shifted modes to be exactly the same as the unedited counterparts; because oscillator strength redistributions, changes in linewidths, and even splitting of modes (or appearance of new modes) can occur. It is also possible to test experimentally (rather than assume) that the isotopic substitution effectively does not change the surface chemistry or binding to the surface. This can be done through the comparison of enhancement factors (measured independently under identical experimental conditions) for modes that are *not* affected by the isotopic substitution in the edited and unedited versions of the molecule.

Along these lines, we show in this paper an explicit implementation of isotopic editing for BiASERS in solution, together with a full experimental verification of these assumptions. The isotopic editing is achieved in a methyl ester version of Rhodamine 6G (instead of the ethyl ester version used in ref. 5), which we shall call RH6M hereafter (shown in Fig. 1). The structure of rhodamine is broadly characterized by a three-ring chromophore (xanthen moiety) and a phenyl side-chain, which is in a different plane from the main backbone. The isotopic editing is performed by replacing the four hydrogens of the phenyl moiety with deuterium, thus resulting in d4-RH6M. We shall show the usefulness of these probes as BiASERS partners for SM-SERS and provide a full characterization of them (including fluorescence, absorption, NMR, and bare Raman cross sections) for future use and reference as standard isotopic probes.

II. Chemical synthesis

(i) Synthesis and chemical characterization of 3,6-bis(ethylamino)-9-[2-(methoxycarbonyl)phenyl] xanthylium (RH6M):

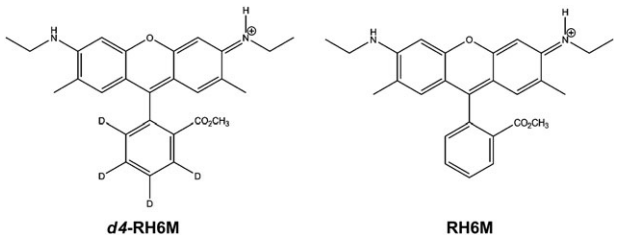

Fig. 1 Deuterated (left) and normal (right) versions of a methyl ester version of Rhodamine 6G (hereafter d4-RH6M and RH6M, respectively). The only difference with the isotopic rhodamines studied in ref. 12 is the methyl group (instead of ethyl) attached to the phenyl moiety. Isotopic editing is achieved as in ref. 11 by replacing the four hydrogens of the phenyl moiety by deuteriums.

All chemicals were obtained from Sigma-Aldrich and used without further purification. Following the procedure of Aburada,[17] phthalic anhydride (0.20 g; 1.35 mmol, 99+%) and *o*-ethylamino-*p*-cresol (0.41 g; 2.70 mmol, 95%) were dissolved in the high boiling point solvent 1,2-dichlorobenzene (5 mL, bp 180.5 °C) in a flask equipped with a stirring bar, a reverse Dean–Stark apparatus and a condenser. After refluxing overnight, the reaction mixture was cooled to 50 °C, and sodium hydroxide (36 mg; 0.9 mmol) and dimethylsulfate (0.265 mL; 2.8 mmol) were added. The resulting mixture was then heated at 90–100 °C for 5 h. In the workup, the reaction mixture was diluted with water (8 mL) and concentrated *in vacuo* (at 90 °C) to remove 1,2-dichlorobenzene by azeotropic distillation. This procedure was performed twice with water and once with methanol to ensure complete removal of the original solvent. The product was evaporated to dryness to give the product as a red residue (84% yield). Recrystallization from a mixture of conc. hydrochloric acid (1 mL) and distilled water (1 mL) yielded metallic green needles. The structure and purity were confirmed by mass spectroscopy and ^{1}H and ^{1}3C NMR spectroscopy (including COSY, HMBC, and HSQC). ^{1}H and ^{13}C NMR spectra were run on 300 MHz and 500 MHz Varian spectrometers, respectively. Mass spectra were acquired on a Q-Tof mass spectrometer with electrospray ionization. The samples were run in the positive V mode, and were calibrated by sodium formate for the mass range 0–1000 Da (Q-Tof Premier, Waters Corp.). ^{1}H NMR (300MHz, $CDCl_3$): $\delta = 1.41$ (t, $J = 7.3$ Hz, 6H), 2.29 (s, 6H), 3.55 (q, $J = 7.3$ Hz, 4H), 3.66 (s, 3H), 6.69 (s, 1H), 6.71 (s, 1H), 7.28 (dd, $J = 7.4$ Hz, $J = 1.5$ Hz, 1H), 7.74 (dt, $J = 7.4$ Hz, $J = 1.5$ Hz, 1H), 7.80 (dt, $J = 7.4$ Hz, $J = 1.5$ Hz, 1H), 8.31 (dd, $J = 7.4$ Hz, $J = 1.5$ Hz, 1H) ppm. ^{13}C NMR (500 MHz, $CDCl_3$): $\delta = 165.4$, 157.0, 156.6, 134.4, 133.0, 131.3, 130.2, 129.8, 128.4, 126.3, 113.7, 94.2, 52.6, 38.7, 18.5, 13.9 ppm. HRMS [M]+ Calcd 429.2178; found 429.2174. UV-Vis (H_2O): $\lambda_{max} = 526$ nm.

(ii) Synthesis and chemical characterization of 3,6-bis-(ethylamino)-9-[2-(methoxycarbonyl)-d4-phenyl] xanthylium (d4-RH6M): The deuterated version of Rhodamine 6M was synthesized according to the above procedure using d4-phthalic anhydride (0.21 g; 1.35 mmol, 98 at.% D). Mass spectroscopy and NMR data again confirmed the target structure. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.40$ (t, $J = 7.2$ Hz, 6H), 2.28 (s, 6H), 3.56 (t, $J = 7.2$ Hz, 4H), 3.66 (s, 3H), 6.64 (s, 1H), 6.70 (s, 1H) ppm. ^{13}C NMR (500 MHz, $CDCl_3$): $\delta = 165.4$, 157.0,

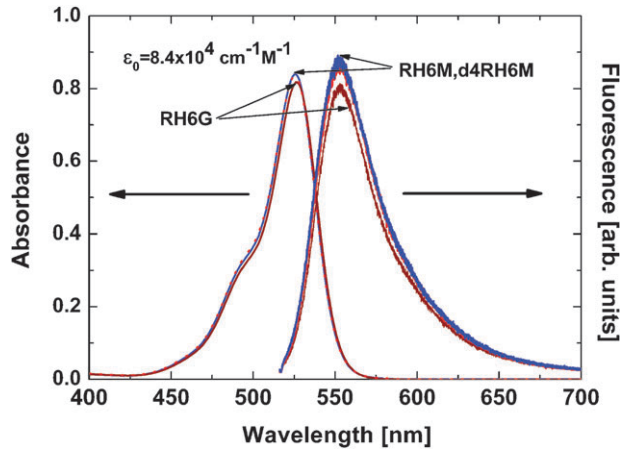

Fig. 2 UV/Vis extinction and fluorescence spectra of RH6G, RH6M, and d4-RH6M. RH6G and d4-RH6M were measured at 100 μM while RH6M was measured at 90 μM and is scaled for comparison. The d4-RH6M and RH6M data are effectively overlapping in the figure.

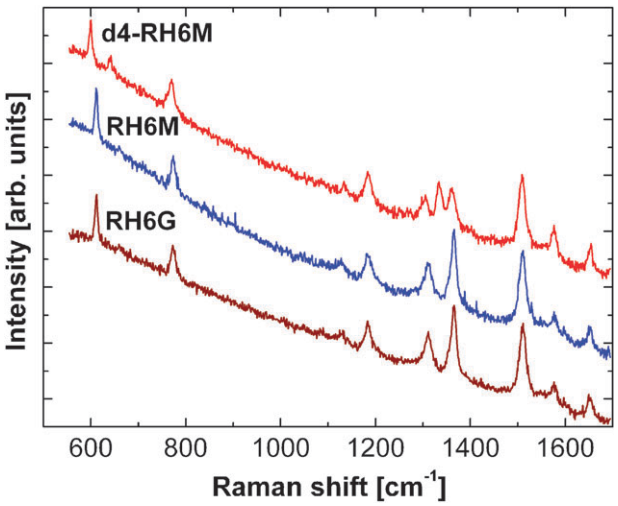

Fig. 3 Raman spectra (displaced vertically for clarity) of RH6G, RH6M, and d4-RH6M. RH6G and d4-RH6M were measured at 100 μM concentration, while RH6M was measured at 90 μM. A water background spectrum taken under the same conditions was subtracted from each spectrum.

156.5, 134.3, 129.7, 128.4, 126.2, 113.6, 94.0, 52.5, 38.6, 18.5, 13.9 ppm. HRMS [M]+ Calcd 433.2429; found 433.2425. UV-Vis (H_2O): $\lambda_{max} = 526$ nm.

III. Optical characterization

A Fluorescence and extinction measurements

It is important to first assess whether the RH6M and d4-RH6M dyes have the same basic optical characteristics in water, and that both are consistent with the more commonly used ethyl ester analogue RH6G. To this end, we conducted extinction and fluorescence measurements for the dyes under identical conditions. Fig. 2 shows the extinction spectra of RH6M and d4-RH6M along with RH6G for comparison. It can be seen that the spectra are essentially identical, with decadic molar extinction coefficients of around 8.4×10^4 cm^{-1} M^{-1} at $\lambda_{max} = 526$ nm. Similarly, fluorescence measurements (also shown in Fig. 2) taken at 514 nm excitation show RH6M, d4-RH6M and RH6G exhibit equivalent fluorescence over the measurement range (within 5%).

B Bare (non-SERS) Raman spectra and cross sections

Before using the dyes for SERS it is critical to first examine them under *normal* Raman conditions. This allows us to compare the dyes without the confusion of possible adsorption/SERS effects and is a necessary first step for measuring the SERS enhancement factors. As has been stated in detail in ref. 4, failure to account for the bare Raman cross sections can lead to considerable errors in the estimation of the SERS enhancement factors.

For the Raman measurements, a Jobin-Yvon LabRam microscope with a ×100 Olympus water immersion objective (NA = 1.0) was used for excitation and collection in a standard backscattering configuration. Excitation was provided by a 633 nm HeNe laser, with a power of ~2.3 mW at the sample. Prior to each measurement, the reference 2-bromo-2-methylpropane (2B2MP) was measured under the same experimental conditions to provide a standard for estimation of the cross sections.[4] Cross sections are determined with respect to the 516 cm^{-1} peak of 2B2MP, which has an absolute differential Raman cross section of 5.4×10^{-30} cm^{2}sr^{-1}.[4] Along with the RH6M and d4-RH6M dyes, RH6G was measured for comparison. The Raman spectra after subtraction of a water background taken under the same conditions are shown in Fig. 3. All spectra were acquired with three acquisitions of 600 s. For the cross-section analysis, peak areas were determined by a fit with pseudo-Voigt functions. Table 1 shows the bare Raman cross sections of the RH6G, RH6M and d4-RH6M dyes in solution.

Looking at the spectra of the three dyes we can see that the RH6G and RH6M spectra are identical. This agreement is to be expected given that RH6M is simply a methyl ester analogue of the ethyl ester RH6G. In contrast, the deuterated dye d4-RH6M is obviously different from its non-deuterated analogue RH6M, with extra peaks present in the 600–650 cm^{-1} and 1300–1380 cm^{-1} regions. These distinct spectral regions are the key to using these dyes in the BiASERS method, for they provide unique fingerprints to identify single-molecule events (in contrast to mixed events).

Focusing now on the estimated cross sections for RH6M and d4-RH6M given in Table 1, we can see that, apart from the obvious spectral differences in the regions 600–650 cm^{-1} and 1300–1380 cm^{-1}, the cross sections of the other modes are consistent. In fact, the spectral differences in these two regions can be seen as a redistribution of the oscillator strength of the vibrational modes. For example, the 612 cm^{-1} mode of RH6M is replaced by two peaks in the case of d4-RH6M, at 600 and 641 cm^{-1}, and the cross section of the 612 cm^{-1} peak of RH6M is approximately equal to the combined cross sections of these two peaks in d4-RH6M. Similarly, in the 1300–1380 cm^{-1} region, the total area of the two peaks in the RH6M spectra is similar to that of the three peaks in the d4-RH6M spectra. In summary, measurement of the Raman (non-SERS) spectra and cross sections of the three dyes allows

Table 1 Comparison of experimental non-SERS differential Raman cross-sections for RH6G, RH6M, and d4-RH6M along with previously measured values from ref. 4 for RH6G in parentheses (the discrepancy for the 1652 cm^{-1} peak of RH6G is a result of not subtracting the water background in ref. 4; the current results are therefore more reliable)

	RH6G	RH6M	d4-RH6M	
$\bar{\nu}_I$ cm^{-1}	$\frac{d\sigma}{d\Omega}$ cm^2 sr^{-1}	$\frac{d\sigma}{d\Omega}$ cm^2 sr^{-1}	$\bar{\nu}_I$ cm^{-1}	$\frac{d\sigma}{d\Omega}$ cm^2 sr^{-1}
612	0.49 (0.67) $\times 10^{-27}$	0.52×10^{-27}	600	0.36×10^{-27}
			641	0.15
774	0.65 (0.76)	0.60	770	0.52
1185	0.70 (0.60)	0.63	1185	0.63
1311	0.99 (1.00)	0.94	1303	0.51
1364	1.81 (1.80)	1.57	1335	0.68
			1361	0.90
1510	2.00 (2.40)	1.87	1509	1.65
1652	0.57 (1.20)	0.52	1653	0.55

us to conclude that: (i) the RH6M dye is equivalent (within experimental error) to the more typically used RH6G dye; and (ii) RH6M and d4-RH6M have equivalent Raman cross sections for most modes (apart from isotopically modified ones in the 600–650 cm^{-1} and 1300–1380 cm^{-1} regions). These modified regions can be used to distinguish spectroscopically the two dyes in a BiASERS experiment. The Raman peaks in the other (non-modified) regions are ideal for assessing the similarity of the chemical properties of the two dyes, in particular in terms of surface chemistry (adsorption efficiency and orientation).

C Average SERS properties. In addition to the dyes having identical non-SERS properties, it is also imperative that they behave the same under SERS conditions, in particular that they stick to the SERS substrate to the same degree and in the same manner. Thus, before progressing to BiASERS measurements, the dyes were measured separately under SERS conditions in the many-molecule regime. This enables us to characterize further some important aspects of these dyes (such as adsorption properties) without the statistical complications of the single-molecule regime.

Both dyes were measured at a concentration of 25 nM using freshly prepared Lee & Meisel colloids as described in ref. 4. In this study, the analytical enhancement factor (AEF) was used as an estimate for the SERS enhancement of each mode. As described in detail in ref. 4, the AEF is simply the ratio of the average cross sections estimated under SERS and non-SERS conditions, corrected for concentration. For SERS measurements, the colloidal solution was mixed with a 20 mM KCl solution in a 50%/50% proportion (thus achieving a 10 mM KCl concentration in the final sample). The long-term stability and characteristic clusters present in this system have been studied elsewhere.[13] Next, a small volume of the dye is added to obtain a final dye concentration of 25 nM. Measurements were carried out using the same setup as described for the bare Raman measurements and intensities were compared to the 2B2MP reference measured after each dye. A total of 60 spectra were measured for an integration time of 10 s each in order to wash out any intensity fluctuations associated with the polydispersity of the colloidal SERS substrate. The estimated average AEFs are given in Table 2.

Table 2 Comparison of experimentally-measured analytical enhancement factors (AEFs) for RH6G, RH6M and d4-RH6M. Note that these AEFs are specific to the SERS substrate and preparation conditions (which are here the same for the three molecules). Note that the relatively larger AEFs measured for the 1651 cm^{-1} peaks may be an artifact due to the difficulty in fitting this peak in the bare non-SERS Raman spectra. When the peak is a doublet, the frequencies of the second components are given in parentheses

			d4-RH6M	
$\bar{\nu}_I$ cm^{-1}	RH6G AEF	RH6M AEF	$\bar{\nu}_I$ cm^{-1}	AEF
612	1.3×10^6	1.2×10^6	600	1.3×10^6
			640 (631)	1.4
768–775	0.9	0.9	764 (777)	1.2
1181 (1198)	0.7	0.8	1181	1.1
1312 (1292)	0.7	0.7	1301 (1290)	1.4
1363 (1349)	0.7	0.8	1332	1.4
			1357	1.1
1511	1.0	1.0	1511	1.2
1651	1.5	1.6	1651	1.5

Two features of the AEF data are apparent. The first is that, for each dye, the AEFs are similar throughout all vibrational modes. The second is that among all three dyes the AEFs are similar, indicating that the dyes behave the same (chemically) under SERS conditions. Isotopic editing can result in subtle differences in surface chemistries in general. Kinetic isotope effects[14] (resulting in different activation energies; *i.e.* different "sticking" properties to the surface) are an example of the latter. This can be the case in particular if the isotopically substituted moiety of the molecule has an important participation in the surface binding. In general, therefore, it is important to check for the identical analytical SERS properties of the dyes, rather than just assume them. The fact that the RH6M and d4-RH6M dyes exhibit similar AEFs under SERS conditions is a strong indication that (i) they have the same adsorption efficiency or affinity to the surface, (ii) any chemical contribution to the SERS enhancement is of the same order of magnitude (note that this is not believed to be present in this particular system[4]). In short, RH6M and d4-RH6M seem to be chemically identical (in terms of their adsorption properties) and this is an important prerequisite for their use as partners in BiASERS.

IV. Single molecule SERS experiments

Once the two probes (d4-RH6M/RH6M) with identical chemistries have been produced by isotopic editing, one can then apply the BiASERS concept. BiASERS experiments with d4-RH6M/RH6M mixtures have been performed under the same experimental conditions as in the previous section, but only 5 nM concentration for each dye. The rest of the experimental details can be found in ref. 7 and the characterization of the scattering volume of our system can be found in full length in ref. 4.

To analyze these results, it is first necessary to identify the spectral regions that are more promising for determining the statistics of SM-events. There are at least two basic criteria that need to be followed: (i) the reference peaks of both dyes

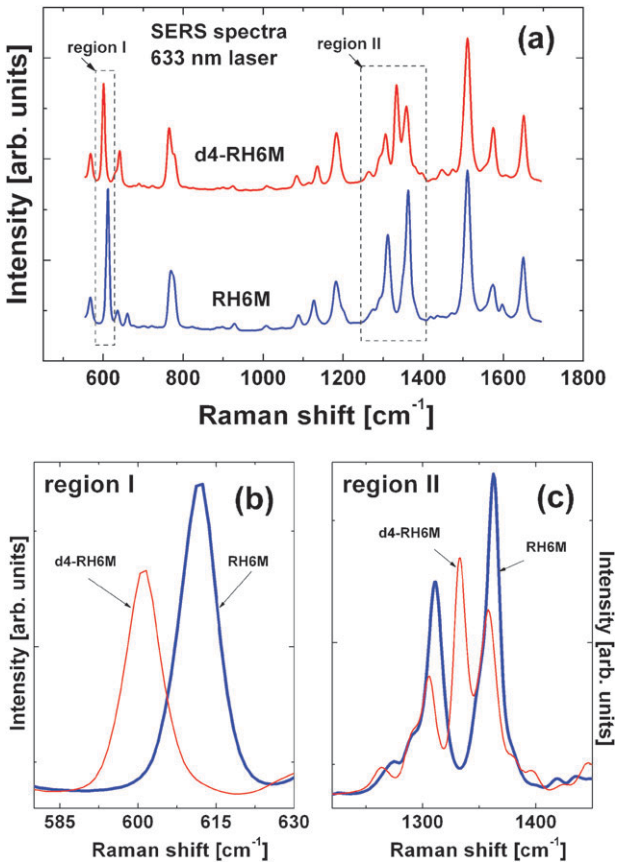

Fig. 4 SERS spectra of d4-RH6M (top) and RH6M (bottom) at 100 μM concentration in Lee & Meisel Ag colloids[15] with 10 mM KCl. The measurements correspond to the average of 10^3 spectra with 1 s integration time. Region I and region II show distinct spectral differences produced by isotopic substitution; both regions are zoomed into in the bottom plots (b) and (c), respectively. The isotopic shift of the $\sim$612 cm^{-1} mode of RH6M to $\sim$600 cm^{-1} can be easily seen in (b), while the corresponding doublet in region II in RH6M splits into a triplet shown in (c).

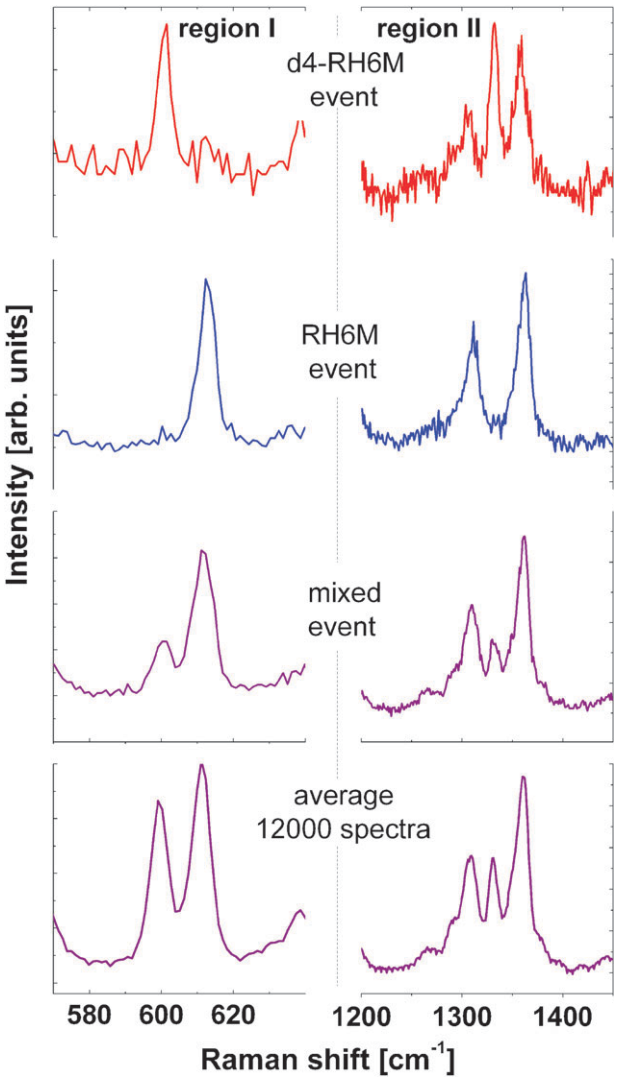

Fig. 5 Single molecule SERS events for d4-RH6M and RH6M together with a mixed d4-RH6M/RH6M event. The average spectrum for 12×10^3 spectra taken at different integration times is also shown at the bottom. The three topmost spectra (for both regions) with SM-SERS events were taken on a sample with Lee & Meisel Ag colloids, 10 mM KCl, 5 nM d4-RH6M + 5 nM RH6M, 633 nm laser excitation (3 mW), ×100 immersion objective (200 μm confocal pinhole[4]) and integration time $\tau = 0.1$ s. Note that the "mixed" event is not the same as the average spectrum at the bottom, a situation that is expected if a few molecules (at least one of each type) are not subject to the same single-molecule enhancement factor (one may be closest to the hot-spot, or they could be located at different hot-spots in a small colloidal cluster).

should be sufficiently distinct from one to the other to allow the correlations in intensity among different regions to be clearly separated, and (ii) the peaks for the two dyes should ideally not be too far away in energy ($\sim$200 cm^{-1} at most) from each other, to avoid unnecessary problems with the dispersion of the resonance[16] during analysis. Fig. 4 shows the two possible regions in the SERS spectra of the individual dyes that satisfy these criteria and can therefore be used for the analysis of the BiASERS spectra. Indeed, Fig. 5 shows three selected cases (out of 10^3) of Raman spectra taken with 0.1 s integration time where characteristic signatures of a single d4-RH6M, single RH6M, or mixed event (d4-RH6M/RH6M) can be easily observed simultaneously in both regions I and II, of the spectra. Fig. 5 also shows the average over 12×10^3 spectra consisting of 3×10^3 taken at $\tau = 0.1$ s integration time, 3×10^3 at $\tau = 0.2$ s, 3×10^3 at $\tau = 0.5$ s, and 3×10^3 at $\tau = 1$ s. The different integration times are used here to study the transition from a single, to a few, to many molecules induced by the natural averaging in liquids introduced by Brownian motion, as we now explain.

The data analysis for each set of 3×10^3 events was carried out with the modified principal component analysis (MPCA) method[7] applied to region II. Note that it is not essential to use the MPCA method for the analysis of BiASERS experiments, and we will therefore only focus on the final results. More details on this method can be found in the supplementary information of ref. 7. The results are displayed in Fig. 6, where we concentrate on the three integration times that best exemplify the crossover among the statistics of a single, a few, and many molecule events, respectively. Fig. 6 plots the

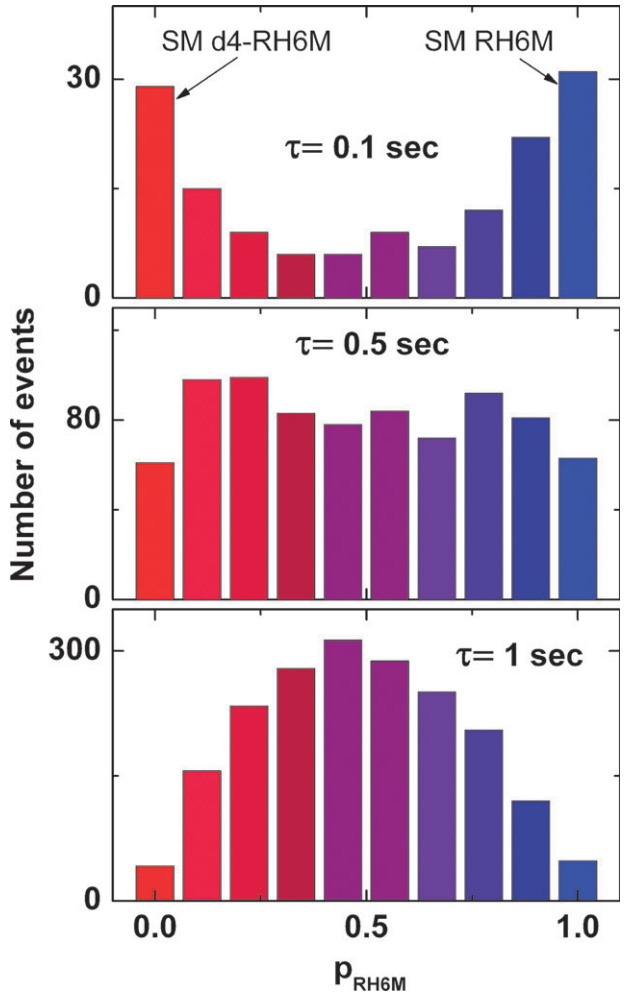

Fig. 6 Histograms of the relative contribution of one dye (p_{RH6M} in this case) to the total signal (as defined in ref. 1 and 7). $P_{RH6M} = 0$ (= 1) means that the signal is a pure d4-RH6M (RH6M) event. Weak signal events below a threshold (fixed by the noise level) are withdrawn from the statistics following the procedure of ref. 7. The histograms of events for integration times of $\tau = 0.1$, 0.5, and 1 sec are explicitly shown at the top, middle, and bottom, respectively. For $\tau = 0.1$ s there are only 146 events (out of 3000) above the noise level, while for $\tau = 0.5$ and 1 s there are 810 and 1936, respectively. The statistics for $\tau = 0.1$ s is dominated by single molecule events of either d4-RH6M or RH6M, with a few mixed events in between. The histogram for $\tau = 1$ is mainly dominated by mixed signals.

histograms for the relative contribution of one dye (RH6M in this case) to the total signal, which we call p_{RH6M}. $p_{RH6M} = 0$ (= 1) means a pure d4-RH6M (RH6M) signal. At integration times of $\tau = 0.1$ s only $\sim$146 events (out of 3000) exhibit a sufficiently high signal-to-noise ratio for a meaningful analysis. These signals are dominated by single-molecule events with either $p_{RH6M} \approx 0$ or $p_{RH6M} \approx 1$ with a small number of "mixed" events in between. By contrast, at $\tau = 1$ s, there are 1936 events above the noise level and the spectra are mostly dominated by mixed signals. Arguably, the example in Fig. 6 is one of the cleanest experimental examples reported so far demonstrating the crossover from a single-molecule to a many-molecule regime in SERS. We observe a gradual transition, which is induced in this particular case by the averaging effect of the integration time combined with Brownian motion in the liquid. Such a transition will depend (in general) on the characteristics of the experimental conditions at hand (dry/wet samples, scattering volume, density of hot-spots, *etc.*) While this is not the main topic here, we also note that in the change of statistics from one case to another in Fig. 6, there is information on the dynamics of the colloid clusters producing the averaging of the signals.

V. Conclusion

The results reported in this paper present the full characterization of an important SERS probe, RH6M, and its isotopically edited analogue, d4-RH6M, which can be used hereafter as BiASERS "partners" for further single molecule SERS studies. The relatively large SERS cross sections of the probes together with their identical chemistries and surface interactions with the metal substrate make them ideal probes to test SM-SERS contributions in different experiments. This was illustrated here for a BiASERS study in colloidal solution as a function of integration time. These results highlight another important aspect of SM-SERS: it is not enough to have two different SERS probes with identical chemical properties to carry out BiASERS experiments. In addition to the probes, there are parameters that need to be tailored for the observation of single molecule statistics and these include: (i) the scattering volume of the collecting optics (which will define the dye concentration level that can be used), (ii) the integration time (in connection with the intrinsic diffusion times in the sample), (iii) the nature of the sample (freely diffusing in liquid, static, *etc.*). Fig. 6, for example, represents one of the simplest examples of how the statistics can change from a single-molecule regime (but sparse, with only 146 out of 3000 events) to a many-molecule regime with less sparse statistics (1936 out of 3000), only by sampling over a longer time in a sample that changes continuously (due to Brownian motion).

The different aspects that can be learned from SM-SERS studies using multiple analyte techniques are still in their infancy. But from one perspective or another, it is clear that isotopic editing will provide a wealth of information in years to come on SM-SERS statistics with the best possible probes for the task. The thorough characterization of the non-SERS and SERS properties of two such probes (RH6M and d4-RH6M), together with the demonstration of their use in a simple BiASERS experiment, attempts to contribute to that endeavor.

Acknowledgements

PGE and ECLR acknowledge partial support for this research by the Royal Society of New Zealand (RSNZ), through a Marsden Grant.

References

1. E. C. Le Ru, M. Meyer and P. G. Etchegoin, *J. Phys. Chem. B*, 2006, **110**, 1944.
2. Y. Sawai, B. Takimoto, H. Nabika, K. Ajito and K. Murakoshi, *J. Am. Chem. Soc.*, 2007, **129**, 1658.

3. P. J. G. Goulet and R. F. Aroca, *Anal. Chem.*, 2007, **79**, 2728.
4. E. C. Le Ru, E. Blackie, M. Meyer and P. G. Etchegoin, *J. Chem. Phys. C*, 2007, **111**, 13794.
5. J. A. Dieringer, R. B. Lettan II, K. A. Scheidt and R. P. Van Duyne, *J. Am. Chem. Soc.*, 2007, **129**, 16249.
6. I. T. Jolliffe, *Principal Component Analysis*, Springer, Berlin, 2nd edn, 2002.
7. P. G. Etchegoin, M. Meyer, E. Blackie and E. C. Le Ru, *Anal. Chem.*, 2007, **79**, 8411; see also the supplementary information provided with the paper.
8. P. G. Etchegoin, M. Meyer and E. C. Le Ru, *Phys. Chem. Chem. Phys.*, 2007, **9**, 3006.
9. E. C. Le Ru, P. G. Etchegoin and M. Meyer, *J. Chem. Phys.*, 2006, **125**, 204701.
10. D. Graham, C. McLaughlin, G. McAnally, J. C. Jones, P. C. White and W. E. Smith, *Chem. Commun.*, 1998, 1187.
11. D. Zhang, Y. Xie, S. K. Deb, V. J. Davison and D. Ben-Amotz, *Anal. Chem.*, 2005, **77**, 3563.
12. R. P. Van Duyne, Proceeding of the *International Conference on Raman Spectroscopy (Book of Abstracts)*, Yokohama, Japan, 2006, p. 3.
13. M. Meyer, E. C. Le Ru and P. G. Etchegoin, *J. Phys. Chem. B*, 2006, **110**, 6040.
14. C. Papp, T. Fuhrmann, B. Tränkenschuh, R. Denecke and H. P. Steinrück, *Chem. Phys. Lett.*, 2007, **442**, 176.
15. P. C. Lee and D. Meisel, *J. Phys. Chem.*, 1982, **86**, 3391.
16. E. C. Le Ru, M. Dalley and P. G. Etchegoin, *Curr. Appl. Phys.*, 2006, **6**, 411.
17. K. Abudara and M. Akagi, *Eur. Pat.*, EP0468821, 1992.