Anal. Chem. **2010,** *82,* 2888–2892

Resolving Single Molecules in Surface-Enhanced Raman Scattering within the Inhomogeneous Broadening of Raman Peaks

Pablo G. Etchegoin* and Eric C. Le Ru

The MacDiarmid Institute for Advanced Materials and Nanotechnology, School of Chemical and Physical Sciences, Victoria University of Wellington, P.O. Box 600, Wellington, New Zealand

We demonstrate both the observation of either a single or a few molecules resolved within the inhomogeneous broadening of a peak in surface-enhanced raman scattering (SERS). Our results demonstrate a fundamental aspect of spectroscopy and also a possible technique to learn more about the varying interactions that single molecules can have with a given SERS substrate. Resolving more than one molecule within the inhomogeneous broadening is only possible thanks to the combination of (i) high-resolution measurements, and (ii) low temperatures (to narrow down the intrinsic homogeneous broadening as much as possible). Besides being a textbook-like example of laser spectroscopy, this result provides yet another confirmation of single molecule sensitivity in SERS. We show specific experimental examples for these effects in single molecule SERS spectra of the molecules nile blue (NB) and rhodamine 800 (RH800). The possible physical origins of the fluctuations in terms of (i) interactions with the substrate, (ii) isotopic effects, or (iii) instrumental contributions, are explained and discussed.

The idea of detecting single molecules in surface-enhanced Raman scattering (SERS)[1,2] through frequency variations of single molecule spectra has been recently exploited[3] in combination with the bianalyte method[4,5] and isotopically edited molecules.[6−8] One of the most elementary concepts in laser spectroscopy[9] is that the line width of peaks (Raman for our case here) have homogeneous (internal) and inhomogeneous (external) contributions. The former has its origin in fundamental intramolecular interactions affecting the lifetime and dephasing of vibrations in molecules (anharmonic interations, for example)[9−13] and will exist even if the molecule were completely isolated in vacuo. The latter, on the contrary, has its origin in external perturbations contributing to (small) changes in the frequency and width of the peaks of individual molecules (forming part of an overall total measured population). These small changes are typically attributed to the diversity of similar (but not identical) conditions that molecules can experience in their interactions with a given environment. Additional contributions to the inhomogeneous broadening can also come from the presence of isotopologues in a given population, as we shall explain in more detail later. Hence, the resulting line width (for a collection of molecules) is the convolution of the homogeneous broadening with the distribution of (slightly) different external conditions (or isotopic variations) for each one of them. The concept of inhomogeneous broadening in a population of molecules underlies entire laser spectroscopy techniques (like "hole-burning",[9] for example), but in the limit of single-molecule detection it acquires an specially important meaning (vide infra). Furthermore, the intrinsic contributions to the line width described above are convoluted with the response function of the spectrometer, thus resulting in the "actual measured peak". At a formal level, we can say that the detection of a peak in a spectrometer is a double convolution problem: the homogeneous broadening convoluted with the inhomogeneous contributions to the line width (both being intrinsic properties of the sample), and this further convoluted with the response function of the spectrometer. The most fundamental natural line width of a peak is the homogeneous one (independent of external perturbations), and it is typically temperature dependent through anharmonic interactions. In standard spectroscopy in liquids, *solvation effects* (intermolecular interactions) are formally considered to be part of the inhomogeneous broadening (i.e., interaction with the environment). But in the case of single-molecule SERS spectroscopy the "environment" is the SERS substrate itself, and all slight variations in the conditions of the molecules come from the interactions with it (no intermolecular interactions).

* To whom correspondence should be addressed. E-mail: pablo.etchegoin@vuw.ac.nz.

(1) Le Ru, E. C.; Etchegoin, P. G. *Principles of Surface Enhanced Raman Spectroscopy and Related Plasmonic Effects*; Elsevier: Amsterdam, 2009.
(2) Aroca, R. F. *Surface-Enhanced Vibrational Spectroscopy*; John Wiley & Sons: Chichester, 2006.
(3) Dieringer, J. A.; II, R; B, L.; Scheidt, K. A.; Duyne, R. P. V. *J. Am. Chem. Soc.* **2007**, *129*, 16249.
(4) Le Ru, E. C.; Meyer, M.; Etchegoin, P. G. *J. Phys. Chem. B* **2006**, *110*, 1944.
(5) Etchegoin, P. G.; Meyer, M.; Blackie, E.; Le Ru, E. C. *Anal. Chem.* **2007**, *79*, 8411.
(6) Blackie, E.; Le Ru, E. C.; Meyer, M.; Timmer, M.; Burkett, B.; Northcote, P.; Etchegoin, P. G. *Phys. Chem. Chem. Phys.* **2008**, *10*, 4147.
(7) Zhang, D. M.; Xie, Y.; Deb, S. K.; Davisson, V. J.; Ben-Amotz, D. *Anal. Chem.* **2005**, *77*, 3563.
(8) Shirshendu, K. D.; Davis, B.; Ben-Amotz, D.; Davisson, V. *J. Appl. Spectrosc.* **2008**, *62*, 1001.
(9) Demtröder, W. *Laser Spectroscopy*; Springer: Berlin, 2002.
(10) Shelby, R. M.; Harris, C. B.; Cornelius, P. *J. Chem. Phys.* **1979**, *70*, 34.
(11) Kozich, V.; Dreyer, J.; Ashihara, S.; Werncke, W.; Elsaesser, T. *J. Chem. Phys.* **2006**, *125*, 074504.
(12) Zakaraya, M. G.; Maisuradze, G. G.; Ulstrup, J. *J. Raman Spectrosc.* **2005**, *20*, 359.
(13) Mukamel, S. *Principles of Non-Linear Optical Spectroscopy*; Oxford University Press: Oxford, 1995.

10.1021/ac9028888
Published on Web 03/08/2010

We dwell first momentarily on a few basic concepts around the idea of *frequency resolution*, before moving into the problem of single-molecule SERS detection. The response function of a spectrometer is the only characteristic we fully control. To some degree, it can also be changed by choosing different experimental conditions. For example, small frequency variations (of the order of ~0.5 cm^{-1}) in an observed Raman peak can be purely instrumental and due to the illumination (imaging) conditions on the entrance slit (in combination with the slit-width and the specific dispersion of the grating). A well-known fact in spectroscopy is that the resolution deteriorates with the size of the image on the entrance slit.[9] If the image is small (ideally a "point-like" source), the position of the peak will still depend on the exact location of the image (slightly "on" *or* "off" the optical axis), which—in turn—depends on the actual size of the entrance slit (i.e., how much of a departure from the axis is allowed by the actual size of the slit). Moreover, if the entrance slit of a spectrometer is kept as small as possible (to allow only on-axis signals to be detected), the "frequency wanderings" of the peaks due to the image position of a point source are avoided, and the true inhomogeneous broadening can be revealed. But this is naturally at the expense of a reduction in the throughput of the signal and in the statistics of detected single-molecule cases. In practice, a compromise needs to be reached. We believe there is (in general) an intrinsic risk in assigning all observed frequency shifts to the contribution of the inhomogeneous broadening from the sample only[3,14] unless the convolution with the instrumental response has been properly tested. Both effects can have otherwise similar consequences and it might be difficult to disentangle them. We shall take later definite steps toward minimizing any possible instrumental contributions to the signals thus revealing the true inhomogeneous broadening of the peak.

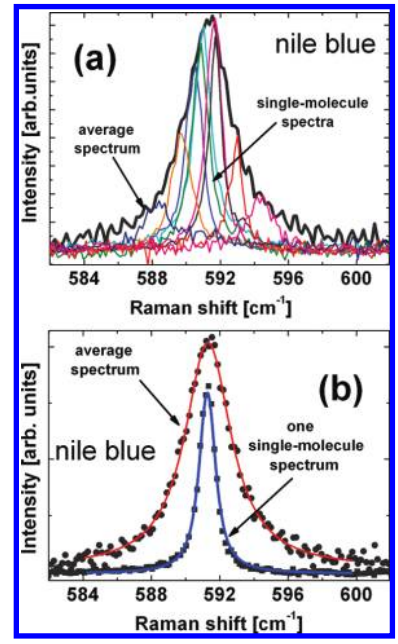

Figure 1. (a) Individual single-molecule events together with the average signal over 7500 spectra on a Raman map. We concentrate here on the ~590 cm^{-1} mode of nile blue (NB) at 633 nm laser excitation and 77 K, measured with a high resolution 2400 lines/mm grating. The intensity of the single molecule events have been rescaled to follow the average for visualization only, but they occur obviously with randomly varying intensities and frequencies that amount (on the whole) to the average spectrum. In (b) we show one individual (typical) single molecule event (approximately at the average frequency) and the average spectrum both fitted to Lorentzian lineshapes. The fwhm of the average is ~3.25 cm^{-1}, while typical single molecule events have fwhm's of ~1 cm^{-1}. This suggests that more than one molecule can be resolved simultaneously within the inhomogeneous broadening of the peak. This is indeed shown in Figure 2 for this particular mode.

EXPERIMENTAL RESULTS

In the limit of single molecule detection one should be able to distinguish the individual cases that are contributing to the average signal if the full-width at half-maximum (fwhm) of single molecule events is much "sharper" than the fwhm of the average (i.e., if the inhomogeneous broadening is a few times the homogeneous one). The best experimental conditions to observe these effects are (i) a grating with high resolution (to distinguish different cases of frequency wandering), and (ii) low temperatures. The latter ensures that, not only the peaks will be as "sharp" as possible (in terms of their intrinsic temperature-dependent homogeneous broadening), but also molecules will be as stable as possible on the substrate[15] (avoiding thermal diffusion and changes in position during the integration time of the measurement). This is shown in Figure 1(a) for measurements done on the ~590 cm^{-1} Raman mode of nile blue (NB). All experiments in this paper are done under similar experimental conditions used elsewhere[4,16] and we shall, accordingly, describe them only briefly here. Samples consist of 5 nM dye concentration with Ag colloids (Lee and Meisel,[17] with 10 mM KCl[18]) deposited on poly-L-lysine covered Si-wafers. The colloidal solution is siphoned off after ~15 min, leaving behind a sparse collection of small clusters that are dried and Raman mapped at 633 nm laser excitation (3 mW) on a LabRam (Jobin Yvon) confocal microscope (Olympus BX41) equipped with a ×100 superlong working distance (NA = 0.6) objective. Maps are typically 50 × 50 in size (1 μm step) with 1 s integration time, while the sample is held at 77 K on a Linkham cryostat for microscopy. The statistics is done over 7500 or 12 500 spectra (i.e., 3−5 maps) depending on the case. We have established before[16,19−21] that most of the signals obtained under these experimental conditions are coming from *single molecules*, with a smaller percentage of the spectra corresponding to a few (~ 2−5) molecules. The results in this paper provide a different (new) method of estimating how many molecules are actually contributing to the signal, which adds to the already many

(14) Schleifenbaum, F.; Blum, C.; Subramaniam, V.; Meixner, A. J. *Mol. Phys.* **2009**, *107*, 1923.
(15) Luo, Z.; Luo, Y.; Li, J.; Liu, K.; Fu, H.; Ma, Y.; Yao, J. *Chem. Commun.* **2009**, 1342.
(16) Etchegoin, P. G.; Lacharmoise, P. D.; Le Ru, E. C. *Anal. Chem.* **2009**, *81*, 682.
(17) Lee, P.; Meisel, D. *J. Phys. Chem.* **1982**, *86*, 3391.
(18) Meyer, M.; Le Ru, E. C.; Etchegoin, P. G. *J. Phys. Chem. B* **2006**, *110*, 6040.
(19) Galloway, C. M.; Le Ru, E. C.; Etchegoin, P. G. *Phys. Chem. Chem. Phys.* **2009**, *11*, 7372.
(20) Galloway, C.; Etchegoin, P. G.; Le Ru, E. C. *Phys. Rev. Lett.* **2009**, *103*, 063003.
(21) Blackie, E. J.; Le Ru, E. C.; Etchegoin, P. G. *J. Am. Chem. Soc.* **2009**, *131*, 14466.

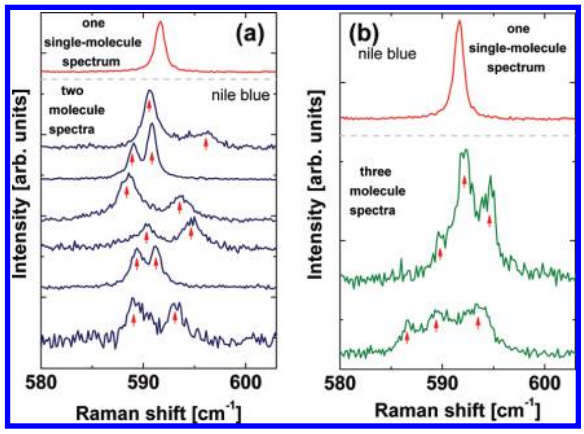

Figure 2. SERS signals with contributions from multiple molecules resolved within the inhomogeneous broadening of the average. At the top (in both plots) we show a typical single-molecule case on the same (horizontal) scale for comparison. (*a*) Signals with two molecules (clearly resolved) are relatively common at a concentration of 5 nM dye. Six examples with peaks at different positions and with different relative intensities and widths are explicitly shown. In (*b*) we show that it is also possible to see signals with contributions from three molecules. These latter cases are of course rarer than the former ones, but certainly expected to occur taking into account typical values for the homogeneous broadening of the peaks compared to the average (homogeneous + inhomogeneous) signal.

established proofs of single molecule SERS sensitivity known in the in the literature.[3,4,6,21−25]

Figure 1(a) shows that, indeed, under the right experimental conditions of resolution and "sharp" peaks, it is possible to unravel the origin of the inhomogeneous broadening of a peak (represented by the average spectrum in Figure 1). Several individual single molecule spectra are shown within the envelope of the average spectrum of 7500 cases. The single molecule spectra in Figure 1 are scaled in intensity to follow the average (for a more visual representation of the data), but the single molecule peaks appear in the range ∼584−596 cm^{-1} with similarly varying intensities. The average spectrum is then more a representation of the *frequency* (or rate) of their appearances rather than a measure of their characteristic intensities at different Raman shifts. We find indeed single molecule events of comparable intensity in the whole ∼584−596 cm^{-1} range; with the largest number of cases centered around ∼590 cm^{-1} (as revealed in the average). If both the average spectrum and one typical single molecule event are fitted (as shown in Figure 1(b)) we deduce that the FHWM of the latter is in a ratio of ∼3 with respect to the FHWM of the former. This ratio will obviously depend on the exact single molecule spectrum chosen for the comparison, but a value around ∼3 is certainly a characteristic one for many cases. This suggests that is it must be possible to resolve more than one molecule within the inhomogeneous broadening of the peak. This is, in fact, the case and it is explicitly shown in Figure 2 where several clear cases with two and three molecules are shown. Furthermore, it could be argued that the detection of multiple peaks (molecules) is expected to show that, indeed, the inhomogeneous broadening is being resolved. If multiple peaks are never observed (because the resolution of the spectrometer is not high enough or the intrinsic homogeneous broadening is not sharp enough) there is always the possibility that multiple peaks will be convoluted with the resolution of the spectrometer and will appear as small frequency shifts of slightly broader "single peaks". The main point we want to raise at this stage then is that it is possible under the present experimental conditions *to distinguish more than one molecule within the inhomogeneous broadening of the peak*. Arguably, this a very fundamental aspect of the origin of the average SERS signal coming from a population of molecules, which here is put into evidence at the single-molecule level. The reason why this has not been observed before[3] is the particular combination in our measurements of high resolution and low temperatures (that narrows the peaks down), together with a choice of a Raman mode with an intrinsically narrow homogeneous broadening (compared to the inhomogeneous one under SERS conditions). Last, but not least, the probe has to have an intrinsic Raman cross section which is large enough to allow single molecules to be detected with ease.[21]

The questions to address now are related to the physical origin of these frequency shifts. We insist at this stage that the reason for resolving at least two molecules within the envelope of the average can be in general the combination of (i) the intrinsic inhomogeneous broadening produced by the different environments of the two molecules (a property of the substrate), (ii) isotopic shifts (a property of the molecule, treated later), and (ii) the different *imaging* conditions achieved on the optical entrance plane of the spectrometer (i.e., the instrumental component).[26] We dedicate the rest of the paper to examine these several possible candidates for the observed shifts and discard some possible origins in specific cases at least.

We first address a property of the molecule itself: isotopic variations. Indeed, one possible (intrinsic) origin of frequency wanderings in single molecule spectra is the presence of naturally occurring isotopologues. If present, it could mean that the shifts are not related to the different external environments that single molecules experience, but rather to intrinsic isotopic versions of the same molecule. Isotopic effects (in particular the natural isotopic substitution of ^{12}C into ^{13}C) can be clearly observed in single molecule spectra of some specific types of vibrations; like the cyano-stretching mode in rhodamine 800.[27] In fact, the single-molecule isotopic shift of this latter case is well-understood[27] and results in large and easily measurable frequency shifts (even at low resolution and room temperature). The reason why this case is well understood rests on the fact that the cyano triple bond vibration (C≡N) is well localized and of high frequency (∼2230 cm^{-1}). This results in a well-defined eigenvector and the effect of natural isotopic substitutions (like $^{12}C \rightarrow {}^{13}C$) has a predictable effect on the reduced mass of the vibration and the frequency shift (∼53 cm^{-1}).[27] This is more difficult to predict for more complicated eigenvectors[1] with a participation of a larger number of atoms (like the 590 cm^{-1} mode of NB in Figure 1); in particular because the combinatorial problem of how different isotopic substitutions affect the reduced mass[1] is more difficult.

(22) Sawai, Y.; Takimoto, B.; Nabika, H.; Ajito, K.; Murakoshi, K. *J. Am. Chem. Soc.* **2007**, *129*, 1658.
(23) Goulet, P. J. G.; Aroca, R. F. *Anal. Chem.* **2007**, *79*, 2728.
(24) Pieczonka, N. P. W.; Aroca, R. F. *Chem. Soc. Rev.* **2008**, *37*, 946.
(25) Steidtner, J.; Pettinger, B. *Phys. Rev. Lett.* **2008**, *100*, 236101.
(26) Le Ru, E. C.; Etchegoin, P. G. *Chem. Phys. Lett.* **2004**, *396*, 393.
(27) Etchegoin, P. G.; Le Ru, E. C.; Meyer, M. *J. Am. Chem. Soc.* **2009**, *131*, 2713.

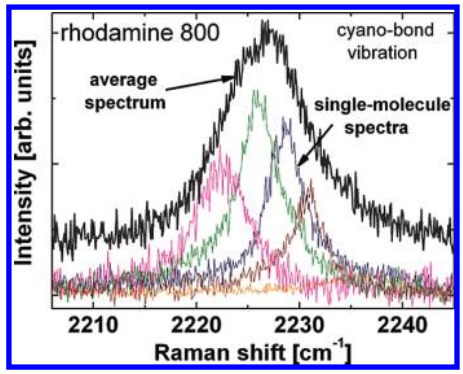

Figure 3. The equivalent of Figure 1 for the C≡N Raman active cyano bond (~2226 cm^{-1}) of rhodamine 800 (RH800). Like the data in Figure 1, spectra have been taken at 77 K with 633 nm laser excitation and 1 s integration time on a dry sample with Lee and Meisel Ag colloids (10 mM KCl[18]) on a poly-L-lysine covered Si-wafer and 5 nM dye concentration.[17] The average spectrum (over 7500 spectra) as well as several single-molecule spectra resolved within the inhomogeneous broadening of the average are shown. The average spectrum has been slightly shifted upward for clarity. The width of the single-molecule cases is a much larger fraction of the width of the average, meaning that it is more difficult to resolve multiple-molecule events except as overlapping peaks, as explicitly shown in Figure 4.

In Figure 3 we show data taken for the cyano-bond of rhodamine 800 (RH800) under the same experimental conditions of Figure 1. The fact that the cyano-bond is at ~2226 cm^{-1} with respect to the laser (as opposed to ~590 cm^{-1} for NB in Figure 1) implies that the resolution is increased even further for the same grating (2400 lines/mm). This is because the dispersion of optical gratings increases when going further into the near-infrared. Figure 3 shows again the average over 7500 spectra and a few individual single-molecule events. Note that, as a percentage change, the frequency wanderings in Figure 1 and Figure 3 are both equivalent to ~0.5% of the frequency. As before, it is still possible to observe different single-molecule situations resolved within the inhomogeneous broadening of the peak. However, single molecule spectra in this latter case have characteristic fwhm's which are now a larger fraction of the fwhm of the average (as can be perceived with a naked eye in the comparison between Figure 1 and Figure 3). Still, the results in Figure 3 and Figure 4 suggest that (natural) isotopic effects are unlikely to be fully responsible for the frequency wanderings, because in this case (if they occurred) they should be much larger (see the Supporting Information (SI) for a more in-depth discussion of isotopic effects in the cyano bond of RH800 and in the 590 cm^{-1} mode of NB). This experiment then goes some way toward suggesting that the existence of natural isotopologues cannot be all the time responsible for the frequency wandering observed experimentally. We regard this as an important conclussion of this study. But this leaves again the two initial candidates: (i) the intrinsic inhomogeneous broadening of the sample, or (ii) the response function of the spectrometer.

Regarding the spectrometer response, steps can be taken too to demonstrate that they cannot be the main contribution for the frequency wanderings either, at least in specific cases. This is done, for example, by comparing the average SERS signal obtained at two different confocal pinhole sizes, as shown in Figure

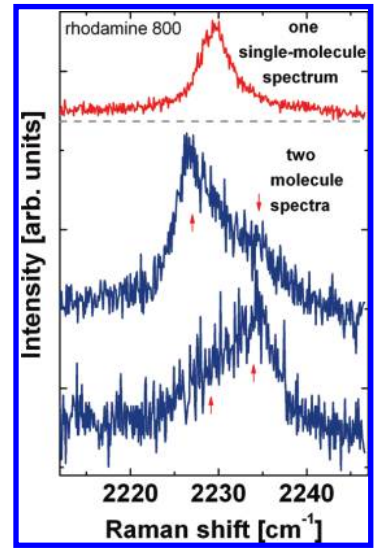

Figure 4. Multiple molecule spectra in the case of the cyano-bond of rhodamine 800 (Figure 3) appear as asymmetric peaks with overlapping contributions from single molecules. Up to two molecules can be distinguished fairly easily, with cases for more molecules appearing as broader featureless peaks. As in Figure 2, a single molecule event is shown at the top in the same (horizontal) scale for comparison.

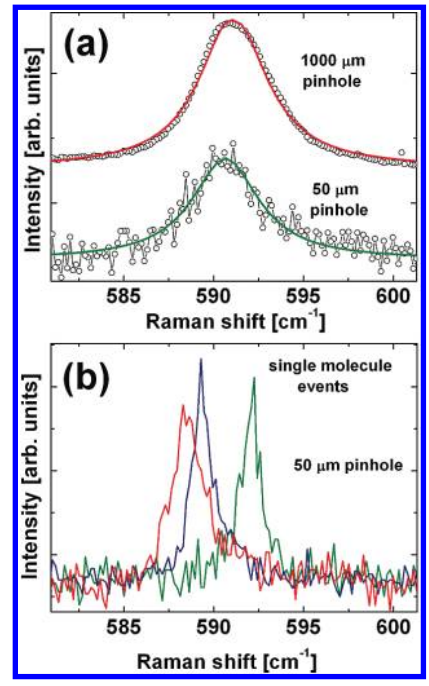

Figure 5. (a) Average signals (fitted to Lorenztians) for 15 000 spectra taken with a fully opened confocal pinhole (1000 μm),[28] and a much narrower pinhole (50 μm) that allows only a fraction of the illuminated area of the spot through the spectrometer. The number of single molecule cases detected in the latter case is smaller (only those single molecules that happened to be almost perfectly alingned with the optical axis of the spectrometer during the spatial map on the sample). Nevertheless, both average spectra render identical widths, showing that the frequency wandering of the peaks are not due to the instrumental convolution. In (b) we show a few examples of frequency wanderings of single molecule data taken with a 50 μm confocal pinhole.

5. Closing the confocal pinhole of the microscope in our system means that we are monitoring the smallest possible "point-like"

source at the entrance slit of the spectrometer. In Figure 5 we show that the average signals obtained with the pinhole fully opened (1000 μm) and that obtained with a much smaller (50 μm) one are the same. The number of single molecule cases detected with the smalller pinhole are naturally less (only those molecules that are almost perfectly aligned with the optical axis of the collecting optics) and that results in a noisier average signal (see Figure 5(a)). But the fact that there is no narrowing of the average peak over many events shows that the frequency shifts are not a problem of the imaging at the entrance slit but rather an intrinsic property of the sample; that is, the true inhomogeneous broadening of the molecules caused by their slightly different environments.

CONCLUSIONS

If we did not know (through a different technique, like the bianalyte SERS method[4,6,21]) that the conditions used for our experiments here are such that single-molecule SERS is achieved, it would be difficult to use the evidence of resolving peaks within the inhomogeneous broadening as hard evidence for single molecule sensitivity. This is because it is conceivable that situations involving larger numbers of molecules (in a very inhomogeneous SERS substrates with many distributed hot-spots) can produce "similar" effects.[26] However, the opposite is true: if we know that these experiments are in the single-molecule SERS limit (as we do from a large variety of previous experimental information[4,6,21]), then it is *indeed* expected that single molecules should be resolvable within the inhomogeneous broadening of the signal, and that situations with more than one molecule being resolved should exist. In fact, this latter situation represents the real demonstration of resolving the inhomogeneous broadening. If only frequency wanderings are observed (never with more than one peak) it is most likely that single molecule contributions are not being resolved and many multiple molecule events might appear as "frequency shifts" simply because the resolution is not high enough to differentiate the situation from a *real* frequency shift of a single molecule.

Overall, the results in this paper are therefore more a confirmation of what is actually expected, rather than a proof of single-molecule sensitivity in SERS. Notwithstanding, it is pleasing to see the expected fundamental aspects falling into place in those situations where they are expected. We have strived to combine experimental and instrumental conditions to suggest that two obvious candidates (like isotopic shifts and/or the spectrometer response) cannot be fully responsible for the observed frequency wanderings. The experimental evidence shown here adds considerable weight around to the idea that the real inhomogeneous broadening of single molecules can be resolved. Furthermore, it is conceivable that we might be able to learn in the future more about the characteristics of the molecular interaction with the environment at the single molecule level. The study of the statistics within the inhomogeneous broadening of different peaks of the same molecule could be particularly interesting; for they might reveal details associated with different moieties of the molecule interacting with the surrounding environment (the metallic surface). Single-molecule SERS is now in a mature stage of development to start answering subtler questions than the mere demonstration of single molecule sensitivity. We believe the results discussed in this paper certainly bestow a hint of the potential laying ahead for single-molecule SERS spectroscopy.

ACKNOWLEDGMENT

Both authors are indebted to the Royal Society of New Zealand for partial support through a Marsden Grant.

SUPPORTING INFORMATION AVAILABLE

Further information including two figures is available. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review December 18, 2009. Accepted February 26, 2010.

AC9028888

(28) Le Ru, E. C.; Blackie, E.; Meyer, M.; Etchegoin, P. G. *J. Chem. Phys. C* **2007**, *111*, 13794.

Supplementary information for "Resolving single molecules in SERS within the inhomogeneous broadening of Raman peaks"

Pablo G. Etchegoin and Eric C. Le Ru

ISOTOPIC SHIFTS OF THE CYANO BOND IN RHODAMINE 800

The structure of rhonamine 800 (inset of Fig. S1) contains a cyano bond (C≡N) with a well localized vibration at $\sim 2226\,\mathrm{cm}^{-1}$. This vibration is Raman active, and the fact that its eigenvector is well localized makes the prediction of isotopic effects simpler. In Ref. [1], for example, we studied the evidence (in single molecule SERS spectra) for the presence of natural isotopic substitutions of the type $^{12}\mathrm{C} \rightarrow {}^{13}\mathrm{C}$ in the carbon of the cyano bond. The latter results in a large (and easily measurable) softening of the vibration by $\sim 53\,\mathrm{cm}^{-1}$, which has its origin in both: the large intrinsic frequency of the vibration, and the localized nature of the eigenvector [1]. In simple terms, a difference of one unit mass in a localized vibration produces a much more important change in its *reduced mass* when compared to a vibration with an extended eigenvector. This results then in a much more important change in the frequency. A frequency shift of $\sim 53\,\mathrm{cm}^{-1}$ is not obviously considered as a change within the inhomogeneous broadening, but rather represents a completely new vibration. In general, however, isotopic shifts will be a lot smaller ($< 1\,\mathrm{cm}^{-1}$), and this is the reason why we need to deal with them here in order to either discard them, or attribute them as the reason for the small frequency wandering within the inhomogeneous broadening observed in Figs. 1 and 3 of the main text.

One particular question one should address for RH800 is the possible effect of an isotopic change in the *core* structure of the dye. The cyano bond is not completely isolated but rather anchored to the main core of the structure through a carbon (inset of Fig. S1). There are a lot more chances ($\sim 30\%$) to replace one of the carbons in the main core of the structure by $^{13}\mathrm{C}$ than there is to replace the carbon in the cyano bond itself ($\sim 1\%$). In Fig. S1 we show the predicted isotopic spread of RH800 which comes (predominantely) from the $^{12}\mathrm{C} \rightarrow {}^{13}\mathrm{C}$ substitution. For example, there is a $\sim 30\%$ chance of finding a RH800 molecule in which one $^{12}\mathrm{C}$ in the core structure has been replaced by a $^{13}\mathrm{C}$ (ignoring the single carbon in the cyano bond for the time being). Even if the core structure of the molecule (acting as a "counterbalance" to the cyano bond) has a small participation in the eigenvector of the $\sim 2226\,\mathrm{cm}^{-1}$ vibration through its anchoring point, a change of (say) $\sim 1\%$ in the reduced mass can result in a change of $\sim 0.5\%$ in the frequency of the mode, which will already explain a wandering of $\sim 10\,\mathrm{cm}^{-1}$ in the frequency (as seen in Fig. 3 of the main text).

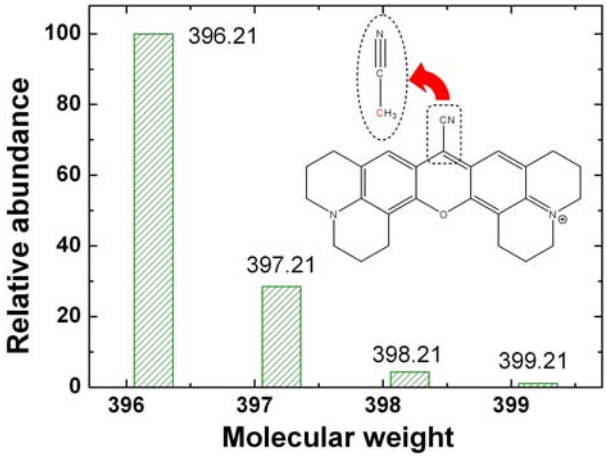

FIG. S1: Histogram of relative abundances of different (natural) isotopic versions of RH800. The variations are (mainly) caused by the natural isotopic substitution $^{12}\mathrm{C}(98.9\%) \rightarrow {}^{13}\mathrm{C}(1.1\%)$ and the fact that carbon is the most abundant atom in the structure. To study the possible effect on the cyano bond of isotopic substitutions in the core of the structure, it is possible to "simplify" the structure by a smaller molecule modelling the immediate chemical environment of the cyano bond. This is also shown in the inset. The "red carbon" in this simplified molecule can be assinged an equivalent mass that mimics the presence the the rest of the structure. The molecule can then be solved with density functional theory [1, 2] and the corresponding isotopic shifts can be calculated. Changes in isotopic mass in the core of the structure do not result in frequency changed of the cyano bond larger that $\sim 3\,10^{-3}\,\mathrm{cm}^{-1}$. See the text for further details.

Two facts give a hint that residual isotopic effects from the core of the structure are not a plausible explanation for the frequency wandering of the cyano bond. Firstly, isotopic effects go *always* in the same direction (softening) and this will lead to an asymmetric average peak that is not in agreement with the experimental observation. Furthermore, one can add an additional layer of confidence on this conclusion by performing a *Density Functional Theory* (DFT) calculation of the

vibrations and gain a reasonable estimate of how much a typical residual isotopic shift can be in reality. We have done this explicitly for the cyano bond of RH800, and we summarize briefly the results here.

One possible way to simplify the calculation is to work with an *equivalent molecule*, as shown in the inset of Fig. S1. The "anchoring carbon" of the cyano group into the core structure of the dye is replaced by a CH_3 group with a "heavy carbon" (red atom in the inset of Fig. S1). The CH_3 group respects the required valence towards the cyano group and –by attributing an artificially large mass to the anchoring carbon– we can model the effect on the cyano bond vibration of the rest of the molecular structure. All the combinatorial possibilities of replacing one ^{12}C in the core of the structure by one ^{13}C (leading to the $\sim$ 30% most abundant isotopologues in Fig. S1) are then replaced by a change in one unit mass in the "heavy" anchoring carbon. In this way, the calculation is orders of magnitude faster and more accurate (as far as the description of the local environment of the cyano bond is concerned).

Discounting the atomic weight of one carbon, one nitrogen, and three hydrogens (in the equivalent molecule) from the total molecular weight of the real molecule, we conclude that the "heavy" carbon should weigh: 367.21. Ignoring the isotopic substitutions in the cyano bond itself, the four peaks in Fig. S1 are represented by "heavy carbons" of masses: 367.21, 368.21, 369.21, and 370.21. The fact that the equivalent molecule is small, results in important computational advantages; we can afford, for example, an extensive Pople basis set [3]: 6-311++G(d,p) with the widely used B3LYP [4, 5] energy functional (as implemented by *Gaussian03* [6]). In this conditions, a DFT calculation of the vibrational frequency of the cyano bond for the different masses of the equivalent molecules results in the following frequencies: 2352.2072, 2352.2064, 2352.2055, and 2352.2047 cm^{-1}, respectively. In general, absolute frequencies in DFT are not expected to be as accurate as differential changes (with respect to changes in mass, for example). Not only that the changes in frequency of the cyano bond are all in the same direction (i.e. softening), as expected, but also they are never larger than $\sim 3\,10^{-3}\,\mathrm{cm}^{-1}$ (which is well within anything that can be resolved with the spectrometer). Hence, we are forced to conclude that residual isotopic variations in the different isotopologues of RH800 are unlikely to be the physical reason for the frequency wandering observed in Fig. 3 of the main text. Direct isotopic substitutions in the carbon or nitrogen of the cyano bond are not only less likely (in probabilistic terms) but also lead to much larger shifts that are easily detectable [1], as explained before. Therefore, if the instrumental component is discarded, the only possible reason for the frequency wandering is the *real inhomogeneous broadening* of the peak, representing single molecules in slightly different situations in the sample.

ISOTOPIC SHIFTS OF THE 590 cm^{-1} MODE OF NILE BLUE

The 590 cm^{-1} mode of Nile Blue (NB) is, arguably, more difficult to study because its eigenvector is spread over the structure of the molecule and therefore we have no option but to resort to an actual calculation including all the atoms. This is beyond our computational capabilities for DFT (with a reasonably large basis set for the atomic orbitals). Still, the qualitative picture can be obtained from semi-empirical electronic methods like AM1 [7] even if the exact frequencies are not perfectly matched to experimental values. In Fig. S2(a) we show the basic structure of NB together with its different natural isotopic contents. There are 20 carbon atoms in NB ($C_{20}N_3OH_{20}$). The $\sim$ 22% second most abundant version of the molecule in the histogram of Fig. S2(a) comes predominately from the substitution of one ^{12}C atom in the structure by one ^{13}C (which has a natural isotopic abundance of 1.1%, as pointed out in the previous section). But there are 20 different places where this substitution can occur, and each of them affects the frequency of the 590 cm^{-1} Raman active mode in a slightly different way.

Figure S2(b) shows the calculated frequency for 21 different structures of NB in which none or only one ^{12}C atom is replaced by ^{13}C. Absolute frequency values do not come up right within semi-empirical methods like AM1 by a margin of $\sim$ 5% (the actual predicted frequency of the 590 cm^{-1} mode is 620.3 cm^{-1}). This is a well known shortcoming of these methods which also exists in more sophisticated versions of electronic calculations like DFT, and it is normally solved by an empirical scaling factor (see Ref. [8] for more details). We scaled all the frequencies by a factor of 0.9516 to make them match experimental values. The peak labelled as "all ^{12}C" in Fig. S2(b) corresponds to the most abundant version of the molecule where all the 20 carbons are ^{12}C. The other peaks in Fig. S2(b) correspond to situation where a single $^{12}\mathrm{C}\rightarrow{}^{13}\mathrm{C}$ substitution has occurred. Each individual case with a ^{13}C substitution has a relatively low probability of occurring ($\sim$ 1%). As it can be appreciated from the calculation, different substitutions produce different shifts, due to the particular participation of a specific site in the eigenvector of the mode [1]. The even less probable situation of double substitutions (mainly responsible for the third peak in the histogram in Fig. S2(a)) will have more possible combinations (i.e. 20!/(2!18!)=190 cases in total) and will have different effects on the frequency depending on which pair of atoms is chosen. But

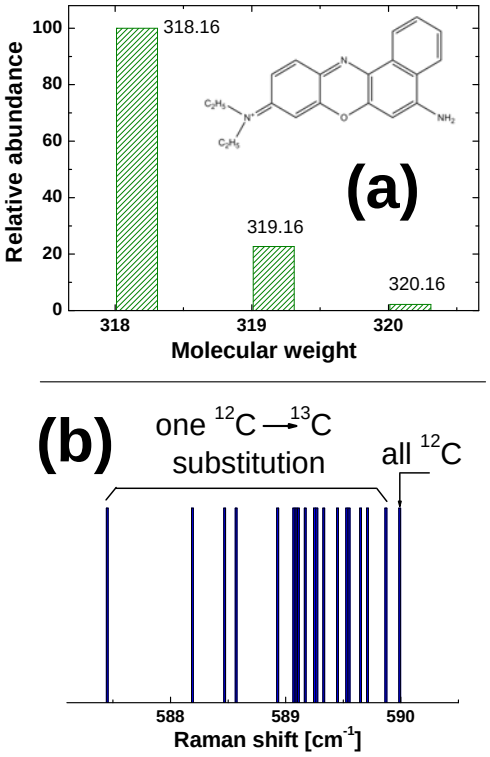

FIG. S2: (a) Histogram of relative abundances of different (natural) isotopic versions of NB. As before (in Fig. S1), the variations are (mainly) caused by the natural isotopic substitution ^{12}C(98.9%) $\rightarrow^{13}$ C(1.1%). There are 20 different carbons in NB (inset) and substitutions in different sites of the structure produce different effects on the 590 cm^{-1} Raman active mode, due to the different participation of specific sites in the eigenvector of the mode. The frequency shifts calculated for the 20 different possible isotopic substitutions ^{12}C $\rightarrow^{13}$ C with the AM1 method [7] are shown in (b). Each individual case has a probability of occurring of 1.1% (the natural abundance of ^{13}C). The main conclusion we draw from this calculation is the well known fact that isotopic substitutions can only soften the frequency and could only justify an asymmetric lineshape contribution to the inhomogeneous broadening, which is not seen experimentally. The interaction with the environment is most likely to be the dominant origin of the inhomogeneous broadening in single molecule spectra.

the effect *will always be a softening of the mode*, and this is the main conclusion we would like to rescue from these simulations. Undoubtedly, isotopic substitutions by themselves could only justify asymmetric lineshapes with single molecule cases ($\sim 20\%$ for NB) appearing as softenings of the frequency by different amounts. This is not observed experimentally, leading to the conclusion that the frequency wanderings are most likely affected by "environmental" contributions (the interaction with the SERS substrate). Isotopic variations are surely convoluted with the effect of the environment but, by themselves, they would be insufficient to explain the experimental data. Last, but not least, if isotopic substitutions were the main effect for frequency shifts observed in single molecule spectra, the probability of observing two or three different molecules simultaneously (as seen in Fig. 2 of the main paper) with different isotopic substitutions would be truly negligible ($\sim 10^{-4}$ and $\sim 10^{-6}$ for two and three molecules, respectively). Experimentally, it is actually not too difficult to find these cases.

[1] P. G. Etchegoin, E. C. Le Ru, and M. Meyer, J. Am. Chem. Soc. **131**, 2713 (2009).

[2] E. C. Le Ru and P. G. Etchegoin, *Principles of Surface Enhanced Raman Spectroscopy and Related Plasmonic Effects* (Elsevier, Amsterdam, 2009).

[3] R. Krishnan, J. S. Binkley, R. Seeger, and J. A. Pople, J. Chem. Phys. **72**, 650 (1980).

[4] A. D. Becke, J. Chem. Phys. **98**, 5648 (1993).

[5] C. Lee, W. Yang, and R. G. Parr, Phys. Rev. B **37**, 785 (1988).

[6] M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, K. N. Kudin, T. Vreven, J. C. Burant, et al., *Gaussian 03, Revision C.02*, Gaussian, Inc., Wallingford, CT, 2004.

[7] M. J. S. Dewar, E. G. Zoebisch, E. F. Healy, and J. J. P. Stewart, J. Am. Chem. Soc. **107**, 3902 (1985).

[8] R. F. Aroca, *Surface-Enhanced Vibrational Spectroscopy* (John Wiley & Sons, Chichester, 2006).