Statistics of single molecule SERS signals: is there a Poisson distribution of intensities?

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This paper is aimed at clarifying the statistics of single molecule (SM) surface enhanced Raman scattering (SERS) signals. The argument of the possible existence of a Poisson distribution in the statistics of intensities in SM-SERS has been used many times in the last decade as a proof of single molecule detection. We show theoretically and experimentally that the conditions under which a Poisson distribution would be present are so unlikely to exist in a real system that there is no other option but to attribute the claims to poor statistical sampling. We believe the argument based on Poisson statistics should be dropped as a proof of single molecule detection in SERS.

1. Introduction and motivation

The quest for a reliable proof of single molecule (SM) surface enhanced Raman scattering (SERS) is an interesting story in its own right, which has been hampered by conflicting views and diverging interpretations. The principles of SM-SERS detection ought to be laid on proper foundations if they are going to be universally accepted and widely used. However, there are still conflicting viewpoints in SM-SERS; including the interpretation of basic experimental facts. In our opinion, the time is ripe for a critical revision of some of the original principles of SM-SERS. In doing so, we are not seeking to dismiss the important impact of the early pioneering works, but rather look at them by bringing together the dynamic understanding that has been the focus of more recent research. Hence, the main objective of this paper is a re-examination of previous assumptions and ideas in the milieu of SM-SERS and in the light of better experimental evidence and understanding. An overview of the early claims[1–3] and a précis of the main ideas based on ultra-low concentration measurements of SM-SERS have been given recently in ref. 4 where, by the same token, a new method to unambiguously identify SM-SERS signals was proposed. The method in ref. 4 is based on the use of two analytes (bi-analyte SERS; hereafter BiASERS) with distinct (and sufficiently different) Raman spectra and on the observation of *relative* spectral fluctuations in the statistics of SERS signals. The method overcomes several basic shortcomings with respect to the original ultra-low concentration approaches,[1–3] which render results that are in general of weak statistical significance,[4,5] and confront serious experimental challenges to ensure the accurate determination of dye concentrations.[6]

One central argument to some of the original claims of SM-SERS[2] is the observation of a *Poisson statistics* of SERS intensities at ultra-low concentrations. This has been re-emphasized in recent review articles[7–9] intended to summarize our state-of-the-art understanding. To make definite progress on these matters, a critical revision of the underlying concepts is required. We believe the claim that the intensities of SM-SERS signals follow Poisson statistics is basically incorrect, and based on a misconception of the nature of the enhancement distribution. The following sections are devoted to a theoretical/experimental justification of this claim.

We first dwell on general properties of Poisson statistics and study its sensitivity to simple perturbations. This introduction has the intention of developing a background of minimum conditions one would expect for a Poisson distribution to exist in SM-SERS. We then proceed to an experimental proof of the underlying concepts.

2. Poisson statistics and its connection to SM-SERS phenomena

2.1 Definition

The Poisson distribution for an integer random variable (X) is a discrete probability distribution of the form:

$$p(X = m) = \frac{(\mu)^m}{m!}\mathrm{e}^{-\mu}, \qquad (1)$$

where μ is the *mean value* of X (not an integer in general). The most common occurrence of Poisson distributions is when X represents the (integer) number of times a given event happens within a fixed time interval (all events must be independent and have the same fixed probability to occur per unit time).[10,11] The typical example is that of a call center, in which the number of calls are monitored over a fixed period of time.[10,11] Moreover, if $\mu \gg 1$, the Poisson distribution tends to a *Gaussian distribution* (with mean μ and variance μ); a consequence of the *central limit theorem*.[10,11] The crossover from a Poisson to a "Gaussian-like" distribution is explicitly shown in Fig. 1.

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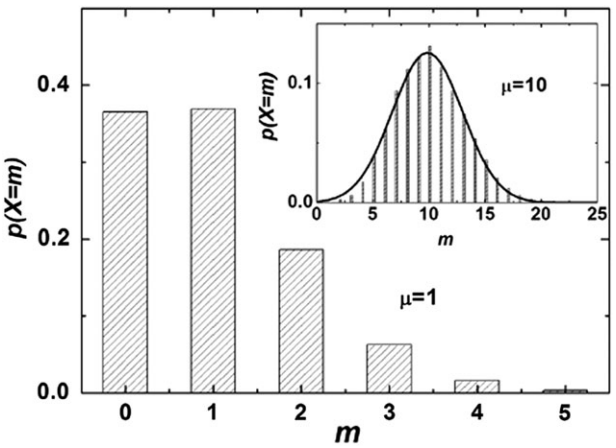

Fig. 1 Poisson distribution for $\mu = 1$. The inset shows the distribution for $\mu = 10$ superimposed with a Gaussian fit. For $\mu \gg 1$, there is a crossover from a Poisson to a Gaussian distribution (central limit theorem).

We now specialize the concepts and the discussion to the measurement of SM-SERS signals, with some emphasis on the case of colloidal liquids.

2.2 Sensitivity to an intensity distribution

Poisson statistics is inherently based on *counting* of events and loses its meaning by any perturbation on our ability to count. In the call center problem, for example, an event is "one call" and can be counted as such irrespective of its length, volume, content, *etc*. In SM-SERS, however, events are "counted" through a measurement of the *SERS intensity*. The SERS enhancement distribution (linking number of molecules with the measured intensity) becomes an inseparable part of the problem.

To pursue the analogy with the call center problem, the SM-SERS problem is equivalent to count the number of calls by measuring (for example) the intensity of sound they produce. If all the calls produce exactly the same "amount of sound", we can count them as such and the Poisson picture holds. If we have a distribution of sound intensities, however, we might not be able to distinguish two weaker-than-average calls from a single louder one.

When interpreting Poisson statistics in SM-SERS, the implicit assumption is that every molecule produces (in the detector) exactly the same SERS intensity. In our view, such an assumption cannot be justified. We specialize the following discussion for SM-SERS in liquids, but the arguments are not constrained to this case. Several factors conspire against the hypothesis of "quantized intensities". Among them (and in order of importance):

• The intrinsic extreme nature of SERS enhancement distributions at hot-spots,[5] in which a difference of a few nanometers in the position of the molecule can result in orders of magnitude difference in the enhancement. This is by far the overriding effect.

• The intrinsic random nature of Brownian diffusion of colloids and/or molecules in the scattering volume during the finite integration time, together with the (Gaussian) inhomogeneity of the beam itself and the anisotropy of the scattering volume.

• Intrinsic blinking or instabilities of the signal, including the possibility of photobleaching, desorption, laser heating effects, surface diffusion of dyes, *etc*.

• Radiation pressure and optical forces on the colloids or the molecule itself.[14]

• Nanometer-size changes in colloid cluster geometries, affecting plasmon resonance conditions.

• A whole list of additional problems including: rotational diffusion of the clusters, the polarization of the beam, and the "uniaxial" nature of the coupling of hot-spots with the incoming laser,[12,13] surface selection rules, *etc*.

The viewpoint that every single detected molecule will contribute with exactly the same amount of signal is an oversimplification that contradicts some very basic experimental and theoretical facts. A direct link between SERS intensity and number of molecules is virtually impossible in real systems; with the overriding factor being the first one; *i.e*. the extreme nature of the inhomogeneities in the electromagnetic enhancement distribution.

Intrinsic to claims of Poisson statistics in SM-SERS is the hope that all the factors listed above will cancel out or compensate in a way that provides a uniform intensity distribution. We believe this to be unrealistic in real systems.[2,15] The sources of variation in SM-SERS intensities are due to two origins: (i) a varying SERS intensity produced by the molecule, or (ii) our ability to detect "all" of the signal. It might be possible to invoke some compensation produced by a combination of photobleaching and/or optical forces[9] that might (hypothetically) ensure a uniform intensity. Even in such an unlikely situation, there are still the problems associated with detecting accurately this intensity; *i.e*. non-uniform excitation, molecule/colloid diffusion during integration time, *etc*...

A more pragmatic approach is to accept that there will be some variability in the intrinsic intensity of single molecule events, and explore how much of a variation we can have before the Poisson statistics picture is washed out. Some insight on this problem can be gained with simple modelling. Fig. 2 shows a basic example.

Consider the following *Gedankenexperiment*: the number of molecules follows a Poisson distribution with a given μ. We take here $\mu = 0.5$ in Fig. 2 to make the example relevant to the SM-SERS problem which is always in the limit $\mu < 1$. The molecules are measured through the intensity they produce. Under perfect conditions we consider that each molecule will produce an intensity of $I = 1$; *i.e*. the distribution is truly discrete and molecules can be counted through the intensity resulting in Poisson statistics. Consider now a mild distribution of intrinsic intensities around the same average $I = 1$. We consider in Fig. 2 the case of a Gaussian distribution around $I = 1$ (insets) for different values of the variance $\sigma = 0.1$, 0.3, and 0.5. We do a histogram of intensities obtained from 10^5 randomly generated events and try to recover the original Poisson statistics of the number of molecules. Note that all cases in Fig. 2 preserve the fact that the average intensity contributed by one molecule is $I = 1$. Fig. 2 is conclusive in the fact that a Poisson distribution picture is very quickly washed

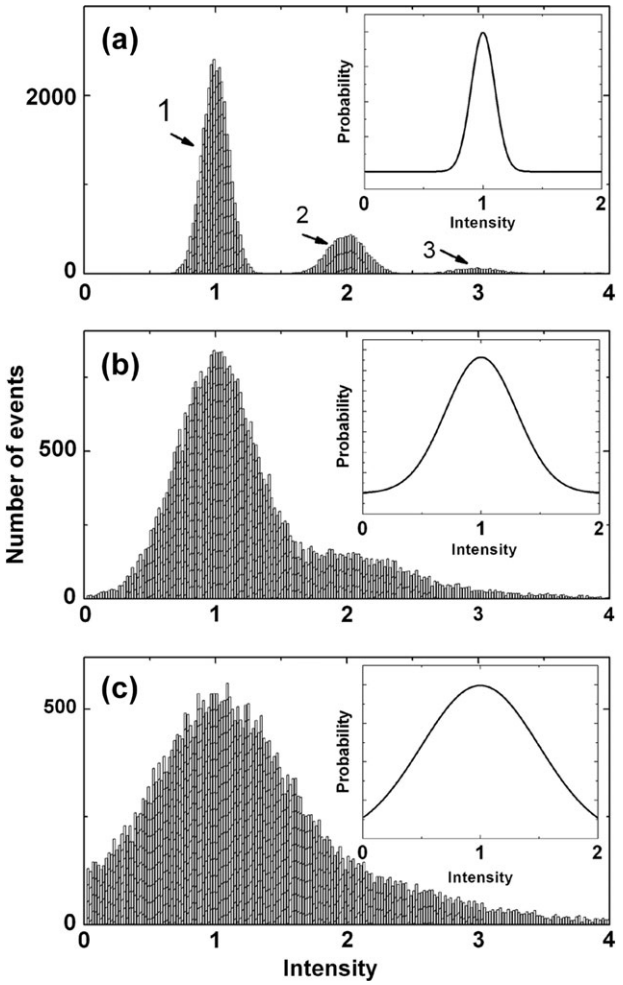

Fig. 2 Histograms of number of cases with a given intensity for three different scenarios of intensity distributions. In all examples, the number of molecules (10^5) follows a Poisson distribution (imposed) with $\mu = 0.5$, but each molecule contributes with an intensity taken from a Gaussian probability distribution centered at $I = 1$, with variance $\sigma = 0.1$ in (a), 0.3 in (b), and 0.5 in (c). We ignore in all histograms the $I = 0$ peak (which cannot be accurately measured in experiments due to an arbitrary cut-off in intensity at the noise level). The peaks representing 1, 2, and 3 molecules can be clearly seen in the histogram in (a). Note that as soon as the intensity distribution of individual molecules is spread over a region spanning a factor of roughly ~ 2 we loose the ability to count the molecules from the intensity and the Poisson statistics picture is completely lost. A Gaussian distribution with a spread within a factor of two among single molecule events in hot-spots is virtually impossible to achieve in real SERS substrates of any kind. See the text for further details.

out, even for the mildest forms of randomness in the intensity distribution like the ones considered here. Once the distribution of intensities of a single molecule is blurred within a factor of ~ 2 (which happens already for $\sigma \sim 0.3$) we loose the ability to count the number of molecules from the intensity.

It is important to note that the nature of the intensity distributions in real SM-SERS experiments is a lot more drastic than the ones considered here. This is where this paper makes contact with our previous study in ref. 5, which is devoted to the nature of the enhancement distribution in SM-SERS. We shall not repeat the details here, accordingly, but only say that the distributions of SM-SERS events are long-tail intensity distributions and the issue of sampling becomes overriding for any solid conclusion. A limited sampling of ~ 100 spectra, for example, will always produce a tail in the histogram with some "structure". This structure is not at all related to "counting molecules" but rather reflects the problem of sampling long-tail distributions. From here, the explanation of the experimental facts follows the lines of ref. 5, and we are forced to conclude that *all claims of Poisson statistics in SM-SERS are plain artifacts of the limited sampling*. We shall illustrate experimentally in the next section the problem of limited sampling for long-tail distributions of SM-SERS intensities.

To summarize the content of this section we point out that: (i) If the intensity of SM-SERS events is not well defined within a factor of ~ 2, the number of molecules cannot be counted. (ii) A factor of ~ 2 in reproducibility from molecule to molecule in different hot-spots is well beyond anything achievable in all the reported claims of Poisson statistics in the literature. (iii) The apparent structure in the histogram must therefore be attributed to the limited sampling of a long-tail distribution of intensities.

3. Experiments

The ultimate proof of the principles depicted in the previous section has to come from real data. This section shows how the "structure" in the histogram of SM-SERS signals can be a mere artifact of the limited sampling.

3.1 Data analysis issues

The main critique of previous analysis of experimental data apparently showing a Poisson distribution in SM-SERS is as follows:

• The ensembles taken are small (100–200 spectra). Binning of 100–200 values into ~ 20 bins is questionable by itself, and can only yield a reliable histogram with structures of ~ 3–4 peaks if the intensities are truly quantized with high precision. This is required simply due to the poor ratio of "values" *vs.* "bins". This argument works both ways: at such a values/bins ratio the observed structures can be caused by any random distribution in the values. The observed peaks are, at most, a natural consequence of the analysis. The poor sampling is, as we will show, the most critical issue. One cannot decide if the structure in the histogram is real or related to the poor sampling.

• The full width of the observed peaks in most of the reported histograms is ~ 3–4 bins; a different binning will significantly alter the histograms.

• Fitting 4 Gaussians (*i.e.* 12 free parameters) to a histogram consisting of ~ 20 data points will yield a fit of poor statistical significance. Moreover, fitting 4 independent Gaussians first and analyzing their intensities for a possible Poisson distribution later is flawed. One should include in the goodness of fit the equidistant positions of the peaks and the Poisson-related amplitudes for the Gaussians, which reduces drastically the number of free parameters. However, in the published histograms we found that the latter strategy does *not* yield good fits.

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• The problem of how low-level signals are dealt with is never sufficiently clarified in most reports. This is not a minor point for $\mu \sim 1$ where a large fraction of events will be "zero intensity events". The definition of the "zero molecule" peak in the distribution will always be arbitrary to a large extent due to a cut-off at the noise level. Unfortunately, the characterization of a Poisson distribution for $\mu \sim 1$ depends critically on the first two peaks.

3.2 Experimental results

We proceed with our experimental proof of some of these concepts. Our system is a LabRam Jobin Yvon confocal spectrometer attached to a BX1 Olympus microscope and a $\times 100$ immersion objective (numerical aperture of 1). We use the 633 nm line of a HeNe-laser as excitation. We used a standard sample of Lee and Meisel[15] Ag colloids with 10 mM KCl; equivalent to the conditions in ref. 2. The types of aggregated clusters formed in these colloids have been thoroughly studied elsewhere,[16] and we use crystal violet (CV) as the analyte (as in ref. 2). The criterion in ref. 2 was to choose a concentration such that there was less than one dye in the scattering volume (by concentration) on average. We know now that this number is to a large extent inconsequential, for the number of events depends on the *density of hot-spots* too. Even at much larger concentrations (~ 100 times) the spectral fluctuations are still dominated by SM events.[4] In fact, working at extremely low concentrations can be counterproductive, for the events become so sparse that the statistical significance is strongly reduced.

The scattering volume of our system is much smaller that the one in ref. 2, accounting for the different magnifications of the objectives ($\times 63$ *vs.* $\times 100$). We chose two different concentrations: (i) 0.1 nM of CV, producing an estimated average of one molecule in the scattering volume (similar to ref. 2), and (ii) 1 nM CV. The number of molecules producing a detectable SERS signal is in fact well below the average concentration, due to the convolution with the enhancement factor distribution (or equivalently: hot-spot density). 1 nM concentration corresponds to an estimated ~ 12 molecules per colloid, only a small fraction of which (less that one on average) will be at a hot-spot. Both results for 0.1 and 1 nM are essentially equivalent, but the statistics can be made more sound with less spectra in the latter, simply because we have more chances of populating the hot-spots and therefore detecting a SERS signal. We avoid, therefore, an excessive number of zero intensity events with the sample at 1 nM concentration.

Fig. 3 shows the basic result for the 1 nM sample. We take 3000 spectra (more than an order of magnitude compared to all previous reports) with 100 ms integration time and well times of 1 s. All the conditions are well within what we know is the single molecule regime.[4] Integration times of 100 msec minimize as much as possible problems with temporal averaging of multiple hot-spots and dwell times of 1 sec ensure statistically independent measurements in these colloids. Fig. 3a shows the histogram of intensities for the 800 cm^{-1} mode of CV for a subensemble of 100 spectra within the 3000. Similar results are obtained with any of the other main modes of CV (1170 or 1620 cm^{-1}). One could be tempted to assign a meaning to the "structure" observed in the histogram in terms of quantized intensities and countable molecules. The relative intensities of the peaks in fact do not follow a Poisson distribution, but this could possibly be corrected by adjusting the bin size and positions. An overinterpretation of the

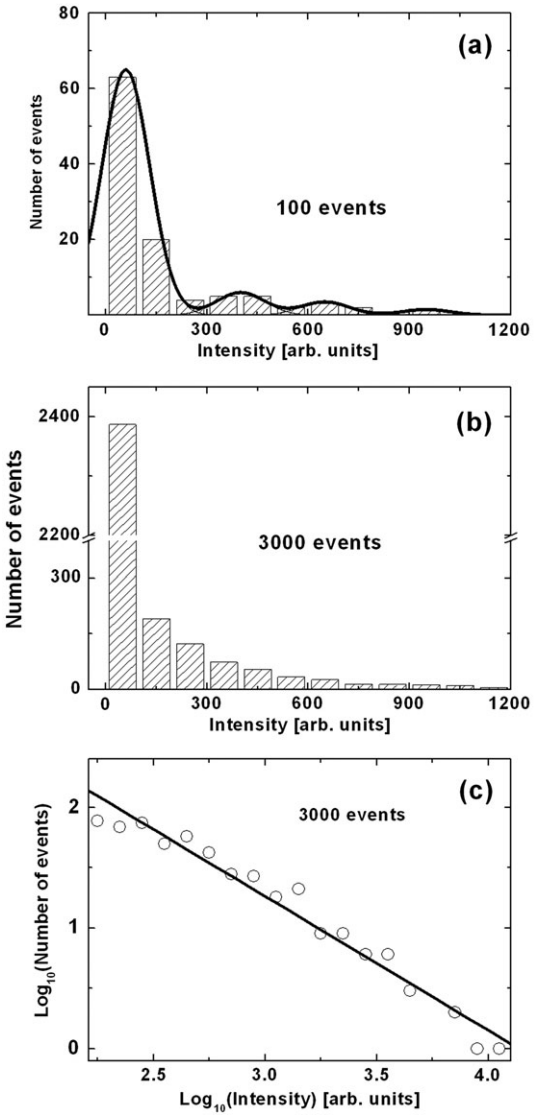

Fig. 3 (a) Histogram of number of events *vs.* intensity (800 cm^{-1} Raman mode of CV) for 100 spectra in a series of 3000. We show the raw histogram together with a possible interpretation in terms of "discrete" intensities. The relative intensities of the proposed peaks do not follow a Poisson distribution, but it could be argued that with better statistical sampling the peak intensities could be better resolved. However, increasing the sampled spectra to 3000 in exactly the same sample and experimental conditions and with the same binning produces the featureless tail-like distribution seen in (b). We plotted (a) and (b) in the same range of intensities (horizontal axis) to compare the regions where some "structure" could be postulated but, in fact, the histogram over 3000 events contains also much higher intensities. The proper way to visualize a tail-like distribution of intensities is in a log–log plot like (c) where the full data range is shown. As can be seen from this example, if sufficient sampling is used all structures in the distribution are washed out and we recover a continuous tail-like distribution of intensities. See the text for further details.

structures in the data could be done (as shown in the figure) if there are reasons to believe the features are real. Fig. 3b shows the histogram (with the same binning) of 3000 spectra. As can be seen in Fig. 3b the structure is washed out and a long-tail distribution with a sharp decay is obtained. On a log–log plot, as shown in Fig. 3c, it is possible to see that the intensity distribution actually resembles a long-tail distribution; which is in more logical accord with what is known about enhancement factors in hot-spots.[5] The long-tail distribution obtained in Fig. 3c is compatible with a power law distribution (or Pareto distribution with index $k \sim 1$ in this case).

The main result of this experimental section is that SERS signals in liquids at low analyte concentration (where single molecule SERS is indeed possible[4]) are *not* quantized, and claims of Poisson distributions are necessarily linked to deficient sampling.

4. Conclusions

In order to decide if a random variable (the SERS intensity in this case) follows a Poisson distribution we ought to be able to distinguish situations with 0, 1, 2, 3, ..., *etc*, molecules from the SERS intensity. This implies that the SERS signals from SM must be reproducible at least within a factor of two—this is a condition which is very hard to relax. Even a mild spread in the distribution of intensities is enough to wash out the Poisson statistics, as shown in this paper. Given the characteristics of hot-spots, which have been extensively studied elsewhere,[5] and the complex statistics of molecules on the surface of these colloidal clusters, a reproducibility within a factor of 2 is well beyond anything that is achievable. Even then, the non-uniformity of excitation and detection would wash out any countable features. We are forced to conclude that previous claims of a Poisson distribution for SM-SERS are attributable to a lack of statistical significance, a fact that has been stressed already in ref. 5, and do not represent by themselves a clear-cut proof of SM-SERS.

Contrary to assertions in ref. 7, which claim a widespread agreement on the existence of Poisson distributions in SM-SERS by several groups, we note that: ref. 17 and 18 only observed a "choppiness" in the statistics of SM-SERS as in the original report.[2] Ref. 17 only claims a "rough agreement with the experiment" for the Poisson distribution interpretation, and ref. 18 does not even mention the word "Poisson". With an improved understanding of this problem, it is safe to say that these observations are attributable to a deficient sampling (100 spectra in both cases). The same conclusion applies to ref. 19 and 20; only a rough agreement with a Poisson distribution is claimed in ref. 20, whereas ref. 19 does not attempt a fit. A simple inspection of the data in ref. 19 shows that it is most unlikely to be represented by a Poisson distribution, not only because of the relative intensity of the features but also from the fact that the peaks are not equally spaced in intensity. Both cases can again be attributed to insufficient sampling (100 spectra in ref. 20, and 200 spectra in ref. 19, respectively). In all these cases, a larger sampling should lead to "smooth" histograms with no countable features.

On a positive outlook, we know now that under the experimental conditions reported, for example, in ref. 2 (and in the present paper), a big fraction of the signals are indeed coming from single molecules with a very high probability.[4] We do not question the existence of the SM-SERS phenomenon, which has in fact been shown to be quite common at low (and even moderate) dye concentrations.[4,5] We question, however, the way in which it was and still is justified. We believe that claims of Poisson statistics based on plain intensity analysis (which have survived to the present date[7,8] despite evidence to the contrary) are simply not correct in the context of SM-SERS.

We hope our study here brings to a close one aspect of the much debated evidence for SM-SERS from Poisson statistics, and provides a solid step from where future developments can be built in this very active area of research.

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References

1 S. Nie and S. R. Emory, *Science*, 1997, **275**, 1102.
2 K. Kneipp, Y. Wang, H. Kneipp, L. T. Perelman, I. Itzkan, R. R. Dasari and M. S. Feld, *Phys. Rev. Lett.*, 1997, **78**, 1667.
3 S. R. Emory, S. Nie, K. Kneipp and G. R. Harrison, *Chimia*, 1999, **53**, 35.
4 E. C. Le Ru, M. Meyer and P. G. Etchegoin, *J. Phys. Chem. B*, 2006, **110**, 1944.
5 E. C. Le Ru, P. G. Etchegoin and M. Meyer, *J. Chem. Phys.*, 2006, **125**, 204701.
6 P. Hildebrandt and M. Stockburger, *J. Phys. Chem.*, 1984, **88**, 5935.
7 K. Kneipp and H. Kneipp, *Appl. Spectrosc.*, 2006, **60**, 322A.
8 K. Kneipp and H. Kneipp, in *Surface-Enhanced Raman Scattering: Physics and Applications (Topics in Applied Physics)*, ed. K. Kneipp, H. Kneipp and M. Moskovits, Springer Verlag, Berlin, 2006, vol. 103, p. 183.
9 K. Kneipp, H. Kneipp and J. Kneipp, *Acc. Chem. Res.*, 2006, **39**, 443.
10 E. Kreyszig, *Advanced Engineering Mathematics*, J. Wiley & Sons, New York, 1972.
11 C. Ruhla, *The Physics of Chance*, Oxford University Press, Oxford, 1992.
12 P. G. Etchegoin, C. Galloway and E. C. Le Ru, *Phys. Chem. Chem. Phys.*, 2006, **8**, 2624.
13 E. C. Le Ru, C. Galloway and P. G. Etchegoin, *Phys. Chem. Chem. Phys.*, 2006, **8**, 3083.
14 H. Xu and M. Käll, *Phys. Rev. Lett.*, 2002, **89**, 246802.
15 P. C. Lee and D. Meisel, *J. Phys. Chem. C*, 1982, **86**, 3391.
16 M. Meyer, E. C. Le Ru and P. G. Etchegoin, *J. Phys. Chem. B*, 2006, **110**, 6040.
17 C. C. Neacsu, J. Dreyer, N. Behr and M. B. Raschke, *Phys. Rev.*, 2006, **73**, 193406.
18 P. Etchegoin, R. C. Maher, L. F. Cohen, H. Hartigan, R. J. C. Brown, M. J. T. Milton and J. C. Gallop, *Chem. Phys. Lett.*, 2003, **375**, 84.
19 A. R. Bizzarri and S. Cannistraro, *Appl. Spectrosc.*, 2002, **56**, 1531.
20 Z. Zhou, G. Wang and Z. Xu, *Appl. Phys. Lett.*, 2006, **88**, 034104.

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