A Statistical Criterion for Evaluating the Single-Molecule Character of SERS Signals†

Johanna E. Bohn, Eric C. Le Ru, and Pablo G. Etchegoin*

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

Received: September 17, 2009; Revised Manuscript Received: November 4, 2009

A method based on the study of the statistical characteristics of SERS signals is proposed to identify regimes in which most of the spectra are primarily single-molecule in nature. Once this regime is identified, the signals can be taken (with some statistical confidence) to represent single-molecule spectra and can be used to infer properties of the substrate (including its single-molecule SERS enhancement factor distribution) that would be otherwise washed out by ensemble-averaging. The method proposed here extends and complements the bianalyte SERS technique to situations in which a suitable "partner" molecule might be difficult to find or synthesize. Specific examples of the method are shown, and its advantages and limitations are discussed.

I. Introduction

Single molecule surface-enhanced Raman scattering (SM-SERS) has now been conclusively demonstrated through a variety of different methods.[1–3] In most cases, the single-molecule character of an individual spectrum cannot be decided in isolation, but rather, it can be inferred from the statistical nature of many examples taken in a SERS substrate with certain characteristics. Therefore, central to the idea of a credible demonstration of SM-SERS is the underlying topic of *sampling* and its link to the statistical occurrence of signals. When a new SERS substrate is studied, extremely rare events at ultralow concentrations cannot be trusted per se as a demonstration of SM-SERS sensitivity, for their "rarity" can be attributed to a long list of equally rare circumstances that do not necessarily involve single molecules.[4] This latter statement is particularly true in colloidal systems, which are notorious for their inhomogeneities. The bianalyte SERS technique[1] was precisely aimed at solving the problem of the reliability of the single-molecule character of the signals in a statistical sense. This is achieved by providing a contrast signal to the one we want to detect and studying their relative occurrences. The method has been further perfected recently by the use of isotopically edited molecules,[5,6] thus providing pairs of analytes with nominally identical chemical properties but different SERS spectra. Far from being exceptional, isotopically edited probes for SERS are becoming of age[7] and have been available, in fact, for quite some time.[8] Under some restricting circumstances, even the natural isotopic spread of certain molecules can be used to identify single molecule spectra.[9]

Notwithstanding, an important question remains at this stage: if we have a large number of spectra of a single dye in a given SERS substrate, is there something in the signal statistics itself that can help us to decide whether they are primarily single molecule in nature (or not)? This point transcends a mere academic question, for it applies directly to potential real experimental situations of interest. There can be more than one reason why it is very difficult, in some cases, to find a suitable bianalyte partner for a given molecule. For example, it could be difficult (for chemical reasons) to obtain isotopologues of the same molecule; that is, the ideal bianalyte partner.[5,6] It could also happen that, even if isotopologues (or natural isotopic variations of the same molecule[9]) are available, they do not result in important or easily resolvable spectral differences in their Raman spectra.[7] There is always, of course, the alternative of using two different molecules; which is how the original bianalyte method was demonstrated in the first place.[1,10] The proviso here is that this might result in slight differences in the surface chemistries of the probes and their interactions with the metal surfaces responsible for the SERS enhancements. Although this is not a serious limitation for the bianalyte SERS technique in situations when we can choose the analytes at will, it could potentially pose a problem if we cannot select the probes arbitrarily due to the constraints, chemical or otherwise, of a specific application. It should also be noted that the idea of trying to infer the single molecule character of the spectra from the statistics of a single analyte present in the sample is what drove the original claims of Poisson distributions.[11] The latter, however, relies on the unrealistic assumption of quantized intensities for single molecules, an assumption that is not supported by the experimental evidence.[12,13]

Accordingly, it is generally not easy to prove the single molecule character of signals of a single dye, and this should be the underlying general message of this study. Having a method that attempts to describe it, at least in some statistical or approximate sense, and is based on the signals of only one molecule is obviously of interest and actually goes back to some of the unresolved problems in the original claims of single molecule SERS.[11,13] This can provide then an additional criterion to the more straightforward situation posed by the bianalyte SERS technique—where single molecule events can be readily identified—but for situations in which we have to rely (for experimental reasons) on only one molecule being present in the statistics. It is the purpose of this paper to show how such a method can work in theory and in practice and propose a possible statistical criterion to quantify the "single-molecule character" of a certain sequence of spectra.

We start with a simple description of the expected phenomenology of the statistics in the next section and then move on

† Part of the "Martin Moskovits Festschrift".

* Corresponding author. E-mail: Pablo.Etchegoin@vuw.ac.nz.

10.1021/jp908990v
Published on Web 12/15/2009

to a systematic characterization of it based on a proposed method of analysis, including explicit experimental examples.

Methods to pinpoint SM-SERS signals (in a colloidal liquid, for example) have one specific task in mind: to isolate the maximum number of SM signals with maximum statistical reliability. This is the aim of the bianalyte SERS method mentioned before.[1,5,6] Thereafter, the concentration of the analyte is not the important parameter, except for the initial "tuning" to observe SM-SERS. If the concentration is too low, most signals are most likely SM-SERS events, but their statistics suffers from poor sampling and becomes therefore unreliable. On the contrary, if the concentration is too high, the statistics is not an issue, but only a very small fraction of the events will actually be SM in nature. Between these two extremes, there is a range for optimum concentrations to gain the maximum number of SM events while keeping a reliable sampling. Once this concentration is decided according to the experimental conditions (which include also the integration time, scattering volume, laser power, photostability of the probes, etc.[4]), the emphasis is from there in the understanding of the SM-SERS cases. This paper is basically about how to find that region.

II. General Properties of the Enhancement at Hot Spots

We need to highlight first a few very general concepts of the enhancement factor at hot spots and some properties of the expected statistics to kick-start the discussion. Let us go one step "backward" and reflect on the effect of concentration on the statistics of SERS signals of only one type of analyte. We take the case of SERS signals from colloidal systems (either in liquid or dry forms, as in refs 1, 6, and 10) as a paradigm, but the conclusions are not at all limited to these cases and would apply equally well to the statistics of signals obtained on any other type of substrate capable of producing enough SERS enhancement ($\sim 10^8-10^{10}$) to observe single molecules.[21]

The following points are completely general for any situation involving single molecule SERS signals amid the presence of hot-spots:

(1) Single molecule signals are directly linked to the presence of hot spots, providing SERS enhancement factors in the range of $\sim 10^8-10^{10}$ for typical experimental conditions.[4] Enhancement factors in this wide range are typically enough to observe single molecules of standard resonant (or preresonant) dyes.[4]

(2) Hot-spots are linked to extreme spatial variations in the enhancement factor; that is, they are highly localized regions in space where the enhancement can vary by an order of magnitude by moving a few nanometers away from the maximum.[14] Examples of the spatial variation of the enhancement in typical hot-spot geometries abound in the literature.[4,12,14] As a result of this, the probability of having a specific value for the enhancement for molecules distributed at random (in a region where there are hot-spots) turns out to be a long-tail (power-law) distribution.[12,15,16] Different models have been proposed in the literature to represent this distribution,[12,15] but the exact details are not important for the forthcoming discussion.

(3) Depending on the experimental setup and SERS substrate, the number—or more precisely, the number density (per area or volume)—of hot spots suitable for SM-SERS may vary dramatically and is generally unknown. At high density (for example, with a low-magnification objective), every SERS event will probe several hot spots (which may or may not contain a molecule and therefore may or may not produce a SERS signal). At the other extreme of low hot-spot density (for example, in diluted colloidal solutions or when the plasmon resonance condition rarely matched the incident wavelength), most SERS events will exhibit a negligible signal, independently of the number of molecules. Therefore, the sparsity of SERS signals is not in itself a criterion for the SM-SERS nature of signals. It may be a result of many-molecule SERS signals originating from a low-hot-spot-density substrate. It is this convolution between hot-spot density and concentration of molecules per hot spot (both unknown in most cases) that renders the identification of the SM-SERS regime so difficult. The bianalyte SERS method[1] provides a solution to this problem. We here study how the statistics of events for one type of molecule may provide another approach.

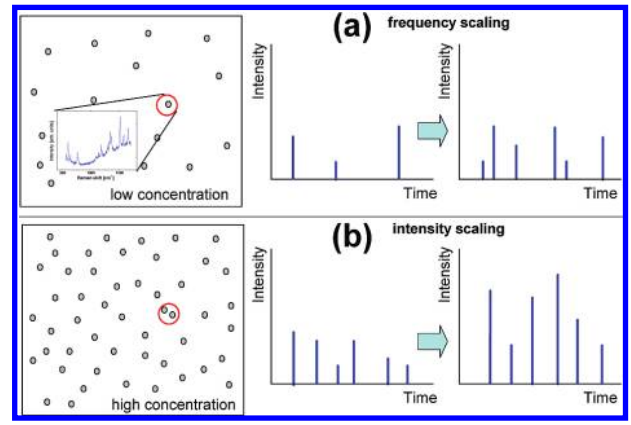

Figure 1. Schematic representation of a statistical criterion for single molecule SERS statistics. In part a, we represent the situation at low concentration: a laser (with a given spot-size area represented by the red circle on the left) scans a SERS substrate. As a function of time, we observe SERS signals (presumably from single molecules) at different positions (blue spectrum on the left). Signals appear with a certain frequency, with intensities spanning a certain range. If the signals are truly from single molecules, the statistics of intensities is mapping the average enhancement factor (EF) distribution of the SERS substrate (in the range of EFs large enough to see single molecules; i.e., EF $\sim 10^8 - 10^{10}$). If the concentration is doubled, we expect to observe (in the statistical sense) the same distribution of intensities but twice as often. In part b, in contrast, we represent the situation at high concentrations. The fact that signals at different points are being produced by more than one molecule means that upon doubling of the concentration, we expect (statistically speaking) not the frequency of the events but their span in intensity to be affected. The situations of frequency or intensity scaling upon a concentration increase are schematically represented by the plots on the right.

III. A Criterion for Single-Molecule Statistics of One Dye

With these basic general elements in mind, we can introduce the simple statistical ideas underpinning our method. The tasks are, hence, to: (i) describe the expected statistics by using a minimum set of assumptions; (ii) relate the phenomenology to what is seen experimentally, and (iii) grasp the essential elements that are needed to enact a criterion for the single-molecule character of the signals.

A. Principle of the Method. The basic idea underpinning our method is a very simple statistical concept, schematically represented in Figure 1. We imagine a SERS substrate with places of high enhancement (hot spots) at different positions and a given concentration of molecules spread over the substrate. We give an example here for a fixed planar substrate, but the ideas apply equally well to the statistics of signals on any other SERS substrate capable of sustaining hot spots. The laser spot (red circle in Figure 1) monitors a certain area where there might be zero, one, or more hot spots and on each, one or more molecules with enough SERS enhancement to be seen as a single molecule. At low concentrations, signals will necessarily be

sparse and will be (primarily) single-molecule in nature. But as explained, sparsity by itself is not a proof of single-molecule detection.[1] We expect the following phenomenology to be true as a function of concentration:

(1) If we assume for the time-being that the signals are, indeed, single-molecule in nature (a fact to be confirmed later), we are basically mapping the enhancement factor distribution of the substrate using one molecule at a time (at places with high enhancements; enough to see single molecules). If we double the concentration and repeat the experiment, we should basically see the same type of distribution of intensities that we saw before, but twice as often. In other words, statistically speaking, we will observe the same span of intensities corresponding to single molecules probing the enhancement distribution of the SERS substrate, but have twice the chance of observing the different types of events. In this case, a doubling of the concentration affects the frequency with which the sparse events happen, but not their intrinsic statistical span of intensities. This is schematically represented in Figure 1a (plots on the right). We call this regime *frequency scaling*.

(2) At much higher concentrations, the signals from the sampling at different points are contributed by more than one molecule in the area covered by the laser spot. In particular, every hot spot contains several molecules. This is depicted in simplified terms in Figure 1b. Signals are less sparse in this case but may still be sparse because of a low hot-spot density. The concept of "frequency of occurrence" of the events and their scaling with concentration loses its meaning in this limit, for signals occur from the same places (the active hot spots) before and after doubling the concentration. However (one more time in a statistical sense), a doubling of the concentration should result in a doubling of the intensities in the distribution of events. In simple terms, the sampling with double the concentration must be equivalent to the sampling of the initial one with twice as many molecules per hot spot. We call this regime *intensity scaling*.

It is important to re-emphasize at this stage that: (i) single molecule signals at low concentrations (Figure 1a) are not quantized in intensity. This relatively simple point has a long history of disagreements in the claims of SM-SERS[13] that go back to the original reports on single-molecule SERS. Instead, intensities span over a range that corresponds to enhancement factors in the typical window $\sim 10^8-10^{10}$.[4,12,15] Likewise (and because of the same reason), a doubling in the number of molecules in Figure 1b at each spot does not mean that the intensity at that particular spot will double exactly. The intensity is convoluted with the details of the spatial variations of the enhancement factor distribution (i.e., where the molecules actually land). When we talk about a doubling, we are talking about a property of the distribution of intensities, not of individual events taken in isolation. Some spots will give less than double the signal; others will give more (upon doubling the concentration). In the statistical sense, the intensity is doubled. The concepts used here are, therefore, not at all based on the (unrealistic) hypothesis of single-molecule intensity quantization.[13]

B. Practical Implementation on a Model Example. Equipped with this elementary statistical idea, we head toward establishing an experimental protocol to demonstrate how they actually work in reality and to transform the method in a possible criterion to establish a regime for single-molecule SERS signals. The interplay between the effects on the frequency or intensity of the events is precisely the characteristic that we want to capture in the analysis.

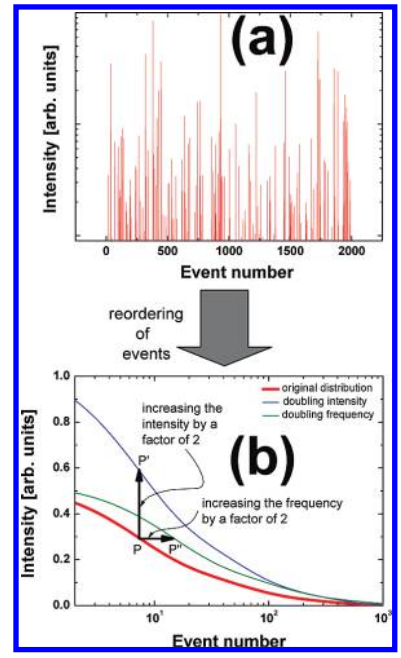

Figure 2. The raw statistics of SERS intensities at a given concentration, $c_{initial}$ (shown schematically in part a), is rearranged in decreasing order of intensities (red curve in part b). From here, two distributions are generated to predict what will happen at a concentration c_{final} ($= rc_{initial}$). We show in the figure the specific example for a concentration ratio $r = 2$. In the distribution that accounts for the effect on the intensity, every point P has the same number of events but with twice (r in general) the intensity (i.e., it moves to point P′). On the other hand, in the distribution accounting for the change in the frequency of the events, each point remains at the same intensity but it happens now twice (r in general) as often (i.e., it moves to point P″). The green and blue distributions are called I_{freq} and I_{inten}, respectively. An actual measurement at twice the concentration is then plotted with these data. Depending on whether I_{freq} or I_{inten} represents better the actual experimental result, we are in a regime in which single or many molecules dominate the statistics.

The sampling of a large number of single molecule cases in SERS results typically in "spike-like" data, schematically shown in Figure 2a, where the intensities of the different events are plotted as a function of an arbitrary "event number" (which simply counts them). If we find a way to process the data that will reveal whether increasing the concentration by a factor c results (in the statistical sense) in an increase of the "number of spikes" by a factor of c or of their intensities (by the same factor), we have basically solved the problem. An analysis tool to process the statistics of SERS signals cannot rely on many intermediate processing details if it is going to be of any use. The use of histograms, for example, should be avoided at this stage. Histograms can be notoriously unreliable when it comes to issues of binning, cut-offs, etc. A criterion to establish the single molecule nature of the statistics cannot rely on how the binning of intensities is done, for example. If the data processing is too sophisticated, it is unlikely to be universally applicable. Our specific processing method here comes directly out of the data, with a minimum of manipulation that does not rely on the criterion and decisions of the user. With the aid of Figure 2, we highlight the main steps of the proposed method in what follows:

(1) We start with the raw sequence of data of SERS events, as depicted in Figure 2a. Here, we take, for example, the case of simulated single molecule spectra under the enhancement factor of a model hot spot.[12,16] Extensive details on how these

simulations are done can be found in the Supporting Information of ref 10. Here, we take only the final outcome as a starting point to explain the method with clean and controllable data.

(2) As a first step, the events are ordered by decreasing intensities, thus obtaining the distribution in Figure 2b (red curve). Experimentally, the larger the number of spectra collected (sampling), the smoother this distribution will be. We found for our examples that a number of spectra on the order of $\sim 10^3$ produces already reasonable results (see the next section), but larger numbers might have to be considered in other cases. We typically use between 4000 and 6000 spectra to obtain good results.

(3) There are a small number of spectra that contribute with the highest intensities, and this drops relatively fast. This is a direct manifestation of the well-established long-tail nature of the enhancement distribution at hot spots,[12] in which a small number of cases contribute to a measurable fraction of the intensity. It is convenient, therefore, to plot the distribution on a semilog plot (as done in Figure 2b), to expand the distribution horizontally and improve the visualization of the results. The largest number of events will be in a long tail of low intensity events.

(4) Once the distribution at a given concentration is obtained from the experiment, we generate two new distributions directly from it to predict the possible scenarios of what would happen if the concentration is further increased. We show in Figure 2b the specific example of doubling the concentration, but the same logic will apply for any other increase by a factor r ($= c_{\text{final}}/c_{\text{initial}}$). Suppose we look at events of a certain intensity in the original distribution (point P on the red curve, for example). If we double the concentration and this results primarily in a statistical doubling of the intensity of the spikes, the resulting distribution should look exactly like the original one but multiplied by a factor of 2 (from P to P′ in Figure 2b). If, on the other hand, doubling the concentration does not affect the intensity of the spikes but rather increases their frequency of occurrence, this means that there should be twice as many events of the same intensity and therefore the distribution moves *horizontally* by a factor of 2 (from point P to point P″ in Figure 2b).

(5) In this manner, we generate two new distributions from the original one which measure (in the statistical sense) whether the main effect of doubling the concentration results in a doubling of the number of events (with the same span of intensities) or in a doubling of their distribution of intensities. We shall call these two distributions I_{freq} and I_{inten}, respectively. Both distributions are obtained by elementary operations from the original one (vide infra). The method can be applied, obviously, to any arbitrary concentration ratio $r = c_{\text{final}}/c_{\text{initial}}$ by either multiplying the intensity by a factor of r (to obtain I_{inten}), or multiplying the abscissa by a factor of r (to gain I_{freq}).

The strategy of the method is then rather simple: An actual experiment is performed at the increased concentration, c_{final}. The events are organized in decreasing order of intensity to obtain $I_{c_{\text{final}}}$. The measurement is done with the same sampling and number of spectra of the original one at c_{initial}. From the experimental point of view, it is easy to obtain I_{inten} from $I_{c_{\text{initial}}}$ by simply multiplying the intensity by r; that is, $I_{\text{inten}} = rI_{c_{\text{initial}}}$. Furthermore, I_{freq} is easily obtained by plotting $I_{c_{\text{initial}}}$ in terms of "$r \times$ event number" on the abscissa (i.e., by stretching the horizontal axis by a factor r). We show a specific experimental implementation of these concepts in the next section.

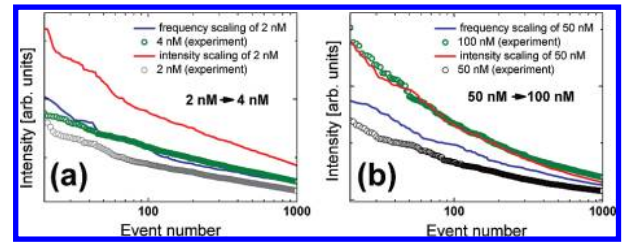

Figure 3. A basic example of frequency (a) or intensity (b) scaling of the distribution of SERS intensities in a Ag colloidal aggregated solution. In (a), a series of 4000 spectra at 2 nM concentration are ordered in decreasing intensity (gray circles). From this experimental distribution, two other distributions are generated: (*i*) a frequency scaled one by a factor of 2 (blue line), and (*ii*) an intensity scaled one, also by a factor of 2 (red line). The results are compared afterward with the experimental distribution for a concentration of 4 nM (green circles). In (b), the same procedure is carried out but for an initial and final concentration of 50 and 100 nM, respectively. As can be readily appreciated, the experimental intensity distribution in (a) at 4 nM is much better described by a *frequency scaling* from the 2 nM result, while the opposite happens between 50 and 100 nM. This suggests, therefore, that most signals at $\sim$ 2 nM concentration are mostly single molecule in nature, while multiple molecules in each event are contributing to the statistics at 50 nM.

IV. Experimental Results

Experiments were performed under the exact conditions of ref 6 for the dye RH6M-d_4.[6] Accordingly, we shall keep details to a bare minimum. The SERS substrates consisted of a Lee & Meisel[17] citrate reduced Ag colloid at 10 mM KCl.[18] SERS spectra were gained at a fixed integration time of 0.2 s with a $\times$100 objective index-matched to water (NA = 1) and the 633 nm line of a HeNe laser at 3 mW. Spectra were collected with a Jobin-Yvon LabRam spectrometer equipped with a notch filter and a N_2-cooled CCD camera.

Initially, we show in Figure 3 a proof of principle of the method. Two cases are shown to exemplify the limits of frequency or intensity scaling of the statistics of single-molecule events. Two experimental intensity distributions are compared in Figure 3a for 2 and 4 nM concentration of the dye, whereas Figure 3b shows the equivalent data for 50 and 100 nM, respectively. The intensities of the events are here obtained by principal component analysis of a single (fingerprint) peak of the molecule (the $\sim$600 cm^{-1} mode in this case), but any other reliable method may be used to extract SERS intensities. As can be clearly appreciated from the data in Figure 3, it is perfectly possible to distinguish the two limiting cases in which frequency or intensity scaling dominates. Hence, this implies that at $\sim$2 nM, the vast majority of signals were single-molecule in nature, whereas contributions to the intensity of each event from more than one molecule must be present in the second case.

Several points are important to note:

(1) The first few events in the intensity distribution (the highest intensities) are always bound to be "inaccurate". The very top values of the EF (for long-tail distributions of EFs[12]) are always the most difficult to sample because of their intrinsic statistical sparsity.[16]

(2) The end-tail of the distribution, on the other hand, are the weakest events. There are plenty of them, but their unreliability is now not a matter of sampling but rather an issue with signal-to-noise ratio.

(3) Between the first few events with the largest intensities (and poor sampling) and the last events in the low-intensity tail of the distribution, we find the most representative values to characterize the statistical properties. This is the reason why

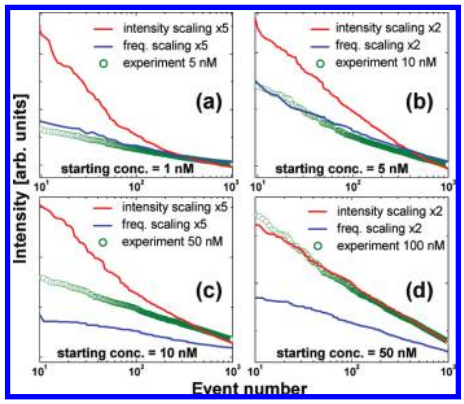

Figure 4. Experimental results for RH6M-d_4 comparing four different pairs of concentrations. From 1 to 5 nM (a) and 5 to 10 nM (b), the distributions at the higher concentration can be represented primarily by an increase in the frequency of events, without affecting their statistical intensity span. In comparison, trying to deduce the distribution at 50 nM from that at 10 nM in part c shows that the result is somewhere between a frequency and intensity scaling; i.e., we are in the transition from single- to many-molecule signals. Finally, trying to deduce the statistics of the 100 nM case from that at 50 nM shows that the main effect is in the intensity span of the events, which now scales with the concentration ratio. This regime can be treated as a "many-molecules range", and only a small minority of signals will be truly single-molecule in nature.

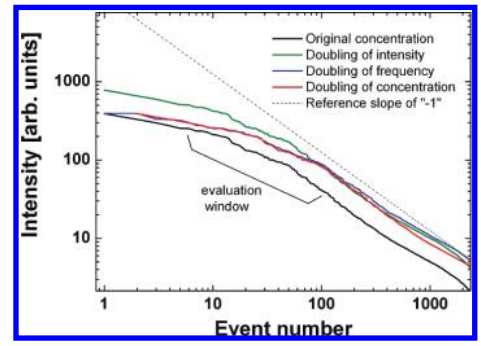

Figure 5. An initial small concentration (black) produces a (single-molecule) distribution that contains a $1/N$ dependence. This is explicitly revealed in a log−log scale here (unlike Figures 3−4) by a region with a slope of " −1 " (an explicit dashed line with a slope of −1 is shown for reference). From the original distribution, we generate both an intensity-scaled (green) and a frequency-scaled (blue) one (as in Figure 2b). This is then compared to a simulation of what would happen in a distribution at twice the concentration (red). The distribution at twice the concentration matches really well the frequency-scaled one until the slope becomes −1 and a scaling in frequency or intensity produces the same result (seen here as a convergence of the green, blue, and red curves). Still, the method is applicable in the window defined by the first few events (which are always statistically less reliable) and the region with a slope of −1. This is labeled as *evaluation window* in the figure.

we present in Figure 3 a window between event number 20 and 1000 (for a sampling of 4000 spectra in total). This window contains events that have enough cases and enough intensity to be statistically reliable and representative of the distribution as a whole. This window of statistical reliability depends obviously on the experimental setup and type of SERS substrate. In general, it can be decided by simple visual inspection of the data.

A key point of the method proposed here, therefore, is that it creates a distribution that is dependent neither on the statistics of the highest intensity events (with poor sampling) nor on that of the lowest intensity ones (that can be compromised by signal-to-noise considerations). Instead, the method of reordering + frequency or intensity scaling manages to compare a "middle ground" of intensities that is more representative of the overall enhancement distribution (because it is statistically reliable) and where concentration scaling (in frequency or intensity) is expected to work.

It is also possible to observe the transition region between the two regimes of frequency and intensity scaling, as well as scaling by more than a factor of 2. All these facts are illustrated with further experimental data for RH6M-d_4[6] (under the same experimental conditions) in Figure 4. We measured five different concentrations of RH6M-d_4: 1, 5, 10, 50, and 100 nM. We assessed the scaling properties four times between pairs of concentrations: 1:5; 5:10; 10:50, and 50:100. This is done to avoid jumps in concentration ratios larger than 5. For clarity, we plot only I_{freq} and I_{inten}, deduced from the lower concentration of each pair (red and blue solid lines), together with the experimental result for the higher concentration (green circles). In other words, the curve with green circles should always be contained between the red and blue lines. Figure 4 shows different tests carried out for the different pairs of concentrations stated above. In Figure 4a, for example, we start with the intensity distribution of the 1 nM sample and generate I_{inten} (red curve) and I_{freq} (blue curve) for $r = 5$ using the procedure described in the previous section. We then obtain experimentally the distribution for a 5 nM sample (green circles) and plot it on the same graph. The same is done for the other three pairs of concentrations in Figure 4b, c, and d. It is clear that, despite the (expected) experimental imperfections, going from 1 to 5 nM results mainly in a 5-fold increase in the frequency of events but not an increase in their statistical span of intensities. The same happens when going from 5 to 10 nM (Figure 4b). By the time we compare the 10 and 50 nM runs, we obtain a distribution that sits somewhere between the effects of frequency and intensity scaling (Figure 4c). This is a clear sign of a transition region between two types of statistics dominated by a single or many molecules. Furthermore, when going from 50 to 100 nM (Figure 4d), we find that the distribution at the higher concentration can be entirely described as an effect in the statistical values of the intensities. Therefore, Figure 4 reveals how the test we propose here works in practice. It also shows that it is possible to distinguish a regime of single-molecule signals dominating the statistics.

V. Potential Problems

In all these results, it is very important that the scaling of the average signals with concentration is as accurate as possible before analyzing the statistics. If it is not, this might be revealing an experimental problem with the distribution of dyes over the sample. The method works as expected if we can ensure that the population of dyes has been spread evenly throughout the sample and the average intensity, accordingly, scales with concentration. An evenly distributed dye population might be difficult to achieve (in a reproducible way) for a variety of experimental reasons that have to be analyzed on a case-by-case basis. Dilution problems of dyes at low concentrations (produced by wall adsorption or unwanted charged surfaces) is always a challenging task.[19] In that sense, it is always recommendable to talk about nominal (intended) concentrations rather than real ones, which could be affected by the experimental procedures in the sample preparation. In the same category is the actual mixing of the dyes with the substrate, which can result in a localized or uniform distribution, depending on how it is done. This is, accordingly, an experimental issue that needs to be resolved in each case for a specific substrate.

Another (more fundamental) limitation is if the distribution of intensities (for example, in Figure 2) follows a $\sim 1/N$ dependence (where N is the event number). The technical reason for this is that once the distribution goes like $\sim 1/N$, an intensity scaling by a factor of c is indistinguishable from a frequency scaling by the same factor. This is, indeed, the case in many samples. An example is explicitly shown in Figure 5 with simulated data. The exact nature of the distribution depends on the particular characteristics of the enhancement factor distribution for a given sample. In the case of colloidal liquids, it depends (in our experience) on factors such as the specific sample and its particular state of aggregation, fine details of the particle size distribution, etc. Figure 5 shows that if the distribution has portions in which a $\sim 1/N$ dependence is observed, it is impossible to distinguish frequency from intensity scaling. Still (in our experience), this is not the case for the entire distribution, and there is in many cases an "evaluation window", which could contain as many as ~ 100 events (as in Figure 5) in which the criterion is still valid. Accordingly, the window of statistical reliability, where the test can be applied, has to be decided according to the specific substrate under use.

Ultimately, we could claim there is no real substitution for having a contrast signal in situ (as in the bianalyte technique), but the results of this paper show that (with some limitations) it is possible to enact a procedure to distinguish single-molecule statistics, which is revealed visually in the distribution of intensities.

VI. Discussion and Conclusion

By combining the information of the different cases presented above, we can conclude that it is possible to establish a regime in which single molecule events (of one dye) dominate the statistics. This is despite the presence of several limitations (both experimental and fundamental) that might hamper the application in completely general cases. Needless to say, these conclusions are valid in the statistical sense, and the safest practice is to take the lowest of these concentrations without compromising too much the sampling. A comparison between the method proposed here and the predictions of the bianalyte SERS method is interesting. Although there is some degree of arbitrariness in the exact value of the crossover (generated, for example, by the arbitrary definition of the noise level in the bianalyte SERS method[10]), it is possible to claim that the two criteria are in very good agreement. At 5 nM concentration with two probes (RH6M and RH6M-d_4[6]), $\sim 87\%$ of the spectra are identified as "single-molecule", with the number dropping to $\sim 75\%$ at 10 nM and $\sim 20\%$ at 50 nM. Accordingly, it is possible to claim that the transition from single- to many-molecule statistics occurs broadly speaking around ~ 10nM. These values make sense, of course, for the exact experimental conditions we are using. They would change, for example, if the integration time, substrate, or scattering volume is changed. The method proposed here can also be potentially used in the analysis of single-molecule SERS statisitics gained from the Langmuir–Blodgett technique, developed by Aroca and co-workers.[2,20]

From a purely practical point of view, having a method that can help us pinpoint a regime of single-molecule-dominated statistics is very important for more than one reason. Arguably, one of the main problems that SERS suffered from for decades was the lack of a reliable tool to quantify the number of molecules contributing to the signal. The convolution of the concentration of the analyte with the spatial distribution of the enhancement that has extreme variations (typical of hot spots) made this quantification difficult. The method proposed here complements and supports the bianalyte SERS technique. With the benefit of hindsight, it could have been used also (originally) as a proof to demonstrate the existence of single-molecule signals without any unrealistic assumption of quantized intensities. Moreover, we believe that the method of analyzing the statistics of intensities, as in Figure 2, might have more general applications than the one shown here, including the experimental determination of the enhancement factor distribution itself (which can be shown to be proportional to the inverse of the derivative of the distributions used here, like that in Figure 2). Many practical applications of the technique proposed here for the determination of different properties of SERS substrates by single-molecule sampling can be readily envisioned.

References and Notes

(1) Le Ru, E. C.; Meyer, M.; Etchegoin, P. G. *J. Phys. Chem. B* **2006**, *110*, 1944–1948.
(2) Goulet, P. J. G.; Aroca, R. F. *Anal. Chem.* **2007**, *79*, 2728–2734.
(3) Steidtner, J.; Pettinger, B. *Phys. Rev. Lett.* **2008**, *100*, 236101.
(4) Etchegoin, P. G.; Le Ru, E. C. *Phys. Chem. Chem. Phys.* **2008**, *10*, 6079–6089.
(5) Dieringer, J. A.; Lettan, R. B., II; Scheidt, K. A.; Van Duyne, R. P. *J. Am. Chem. Soc.* **2007**, *129*, 16249–16256.
(6) Blackie, E.; Le Ru, E. C.; Meyer, M.; Timmer, M.; Burkett, B.; Northcote, P.; Etchegoin, P. G. *Phys. Chem. Chem. Phys.* **2008**, *10*, 4147–4153.
(7) Deb, S. K.; Davis, B.; Ben-Amotz, D.; Davison, V. *J. Appl. Spectrosc.* **2008**, *62*, 1001.
(8) Zhang, D.; Xie, Y.; Deb, S. K.; Davison, V. J.; Ben-Amotz, D. *Anal. Chem.* **2005**, *77*, 3563.
(9) Etchegoin, P. G.; Meyer, M.; Le Ru, E. C. *J. Am. Chem. Soc.* **2009**, *131*, 2713–2716.
(10) Etchegoin, P. G.; Meyer, M.; Blackie, E.; Le Ru, E. C. *Anal. Chem.* **2007**, *79*, 8411–8415.
(11) Kneipp, K.; Wang, Y.; Kneipp, H.; Perelman, L. T.; Itzkan, I.; Dasari, R. R.; Feld, M. S. *Phys. Rev. Lett.* **1997**, *78*, 1667.
(12) Le Ru, E. C.; Etchegoin, P. G.; Meyer, M. *J. Chem. Phys.* **2006**, *125*, 204701.
(13) Etchegoin, P. G.; Meyer, M.; Le Ru, E. C. *Phys. Chem. Chem. Phys.* **2007**, *9*, 3606.
(14) Le Ru, E. C.; Etchegoin, P. G. *Principles of Surface-Enhanced Raman Spectroscopy and Related Plasmonic Effects*; Elsevier: Amsterdam, 2009.
(15) Fang, Y.; Seong, N.-H.; Dlott, D. D. *Science* **2008**, *321*, 388.
(16) Le Ru, E. C.; Etchegoin, P. G. *J. Chem. Phys.* **2009**, *130*, 181101.
(17) Lee, P. C.; Meisel, D. *J. Phys. Chem.* **1982**, *86*, 3391–3395.
(18) Meyer, M.; Le Ru, E. C.; Etchegoin, P. G. *J. Phys. Chem. B* **2006**, *110*, 6040–6047.
(19) Hildebrandt, P.; Stockburger, M. *J. Phys. Chem.* **1984**, *88*, 5935.
(20) Pieczonka, N. P. W.; Aroca, R. F. *Chem. Soc. Rev.* **2008**, *37*, 946.
(21) Standard dyes used as probes in SERS will normally have vibrations with differential Raman cross sections in the range of $\sim 10^{-26}-10^{-28}$ cm^2/sr. Dyes with cross sections in the $\sim 10^{-26}-10^{-27}$ cm^2/sr can easily be observed with SERS enhancement factors (EFs) on the order of $\sim 10^8$, whereas molecules in the range of $10^{-28}-10^{-29}$ cm^2/sr need some of the highest available EFs at $\sim 10^{10}$ for standard experimental conditions. See ref 4 for a more complete discussion of this point.

JP908990V