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Influence of Photostability on Single-Molecule Surface Enhanced Raman Scattering Enhancement Factors

P. G. Etchegoin,*†‡ P. D. Lacharmeise,§ and E. C. Le Ru*†‡

The MacDiarmid Institute for Advanced Materials and Nanotechnology and School of Chemical and Physical Sciences, Victoria University of Wellington, PO Box 600, Wellington 6041, New Zealand, and Instituto de Ciencia de Materiales de Barcelona-CSIC, Esfera UAB, 08193 Bellaterra, Spain

Experimental determinations of enhancement factors in Surface Enhanced Raman Scattering (SERS) are intimately intertwined with the photostability of the probes. We study the effect of the limited photostability in single-molecule SERS (SM-SERS) events and show explicitly how this may result in a large under-estimation of the SERS enhancement factors (EFs) obtained experimentally. To this end, we use the bianalyte technique with isotopically edited probes to provide the best-case scenario for the isolation of single molecule events, and study the statistics of EFs at different incident laser powers. When photo-bleaching stops playing an important role within the integration time used to capture the spectra, SM-SERS EFs approach an upper bound, which is in agreement with estimations of the EFs within the electromagnetic theory of SERS enhancements. Our results reinforce, in addition, the fact that the highest SM-SERS EFs observed experimentally are typically of the order of $\sim 10^{10}$.

Surface Enhanced Raman Scattering (SERS)\(^1\)−\(^3\) has experienced a renaissance in the last ten years,\(^4\) and the topic of single-molecule SERS (SM-SERS)\(^5\) is responsible to a large degree for this renewed interest in the technique. Notwithstanding, several basic aspects of SM-SERS are still under intense discussion. Among them, the topic of single-molecule SERS enhancement factors has seen substantial disagreement among different practitioners of the technique.\(^6\) The time is ripe though to make progress with carefully designed experiments to test the different hypotheses and, by the same token, to settle conceptual and quantitative inaccuracies that have plagued the field for many years. This paper is devoted to a specific topic, to wit: SM-SERS enhancement factors (EFs) and a demonstration of how they depend on the experimental conditions under which they are measured. The paper presents what we believe is a clear demonstration of a statement that has been mentioned many times in the literature (but without any extensive experimental evidence for SM-SERS conditions), namely, that “the experimentally determined EFs are limited by (and only make sense within) the constraints imposed by the photo-stability of the analyte”.\(^7,8\) The connection between what is actually measured experimentally and the theoretical predictions of SERS-EFs is also discussed and clarified. Nonetheless, it is advantageous (for the sake of clarity) to briefly summarize first a few concepts around the issue of SM-SERS EFs; this is done in the forthcoming section as an overview to the main topic of this paper.

Overview. Let us first refresh two very basic concepts on single-molecule SERS EFs before proceeding further: (i) If we solve the electromagnetic problem at different wavelengths for a given SERS substrate and we evaluate the EF at a point (for example in the $|E|^4$-approximation),\(^3,9\) we obtain a figure for the SM-SERS enhancement that is independent of the probe and represents the most objective characterization of the performance of the substrate at this point. Typical estimates of SM-SERS EFs show that maximum values of $\sim 10^{11}$ can be expected at hot-spots from purely electromagnetic (EM) contributions,\(^10,11\) with values of the order of $10^7−10^8$ being already enough to observe single molecules\(^5\) under typical experimental conditions. (ii) If a molecule is now added to a specific position and the SERS EF is measured, several modifications may arise. The local electromagnetic environment can modify the Raman polarization of the molecule (the self-reaction effect)\(^3,12\) and, in addition, direct electronic interactions between the molecule and the metallic SERS substrate (with different levels of strengths) can arise.\(^12\) All these contributions can be absorbed into what is normally classified as “chemical” enhancement.\(^3\) Moreover, orientation and polarization effects\(^14\) (i.e., surface selection rules),\(^15,16\) may further affect the SM-SERS EF. Both chemical enhancement

References:


‡ To whom correspondence should be addressed. E-mail: pablo.etchegoin@vuw.ac.nz (P.G.E.), eric.leru@vuw.ac.nz (E.C.L.R.).

† The MacDiarmid Institute for Advanced Materials and Nanotechnology.

‡ Victoria University of Wellington.

§ Instituto de Ciencia de Materiales de Barcelona-CSIC.

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and surface selection rules are molecule-dependent but are in most cases secondary.

One of the main problems with SERS EFs (and part of the reason for the historical disparity in the different claims) is the fact that there is not a single EF definition for a start. This has been extensively studied in ref 6 but can be summarized in a nutshell by the fact that the SERS signal is produced by the convolution of the EF distribution (which can have extreme spatial variations) and the distribution of molecules at different sites. The principal limitation to obtain an EF in SERS is the difficulty in estimating the effective number of molecules that are actually contributing to the signal or, equivalently, what is the convolution between the spatial variations of the EF in a given SERS substrate and the distribution of molecules on it. This problem has had two main consequences in the historical development of the technique: for a start, it contributed to the wide range of estimates (by several orders of magnitude) for SERS EFs that delayed a consensus on what was actually being measured (as extensively discussed in ref 6) but, in addition, it prevented researchers from realizing that single molecule SERS is actually a lot more common than what was originally thought. Arguably, this is perhaps the most salient contribution of the bianalyte SERS technique, namely, a reliable and simple-to-use way to both identify single molecule SERS situations while gaining copious amount of statistics for events that happen on substrates that are typically uncontrollable in their microscopic details (colloidal solutions, for example). Once a reliable method is introduced to determine (at least in some statistical sense) the number of molecules contributing to the SERS signal, a quantification of the EF can follow (if the bare Raman differential cross section of different modes of the probe are known). It is only relatively recently that single-molecule SERS-EFs have been estimated in this way (with reliable experimental tools) and shown to be in agreement with typical EM theoretical predictions.

However, there are still a number of experimental issues to be considered. An almost-always-ignored aspect in the determination of SERS EFs is the intrinsic photostability of the probe. Actually, in the formal definitions of the different SERS EFs (single molecule EFs or otherwise), it is explicitly stated that photobleaching must be avoided (by an appropriate choice of experimental conditions) for the definitions to be valid. After all, the photostability is a property that is specific to the probe under use, and it is hence not directly related to the main contribution to the SERS EF, which is mainly electromagnetic and would exist even if the probe were not there. Experimentally, on the other hand, factors like the photostability of the probe do play a role in the determination of the EFs and can account in many cases for differences by factors of the order of \(10^2-10^3\), as we shall show later. Moreover, because the photobleaching mechanisms are likely to be intimately linked to the actual EM enhancements felt by the probe, it may not be straightforward to disentangle the two effects.

Still, the photostability of the probe may definitively limit the measured enhancement. This results in a laser power-dependent SM-SERS EFs instead of a self-normalizing property independent of power (as it should be if the probe were ideal); photobleaching should therefore be excluded or it should be taken into account explicitly if a physical interpretation of the measured EFs is sought. It is important to realize that excluding/avoiding photobleaching may not be as straightforward as it is under non-SERS conditions. Even non-resonant molecules have been shown to photobleach efficiently under SERS conditions. The importance/presence of photobleaching under given experimental conditions should therefore ideally be assessed experimentally, and one of the aims of this work is to demonstrate one method of doing this. From the purely practical point of view of determining the EFs experimentally, we could say that the photostability of the probe plays ostensibly a much more important role than the chemical contribution in the assessment of the overall enhancement, for it can account for differences in the measured values of \(10^2-10^3\) (depending on how the experiment is carried out). This is a factor whose presence and effect on the experimentally determined EFs cannot possibly be ignored if, for example, evidence for a chemical component to the enhancement (beyond an electromagnetic prediction) is searched for. It represents also a fundamental aspect of the SERS problem not in the idealized predictions of electromagnetic EFs from calculations but rather in the chemical reality and the fact that all SERS probes (dyes) with the highest intrinsic Raman cross sections (that allow easy experimentation of single molecule SERS conditions) are to one degree or another photosensitive.

**EXPERIMENTAL SECTION**

The basic aim of our experiments is to isolate a large number of single molecule events and obtain their respective SM-SERS EFs. This is then repeated for different incident powers (1/10th and 1/100th of the maximum) while keeping the same integration time and experimental conditions. Hence, the main aim is to observe how the photostability of the probes affect the SM-SERS EFs values and to compare these with one another and with theoretical estimates.

We perform the experiments in two different systems. The first one is the standard Lee & Meisel citrate-reduced Ag colloid solution. This is done using a \(5 \times 100\) immersion objective as depicted in Figure 1a. The second system consists of dry colloidal clusters (also Ag Lee & Meisel Ag colloids) deposited sparsely onto a Si wafer with a thin (grafting) layer of polylysine, as schematically depicted in Figure 1b. The sample preparation is identical to that used in ref 17, and, therefore, details are kept to a minimum here. For the identification of single molecule events in the statistics, we use the bianalyte SERS method with isotopically edited dyes, as described in ref 23. Isotopic editing

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produces the best possible probes for bianalyte SERS by providing two molecules that have identical chemical properties but, at the same time, distinguishable Raman features. We use the deuterated and normal versions of a methyl-ester derivative of Rhodamine 6G (hereafter d4-RH6M and RH6M, respectively), which have been fully characterized for their normal and SERS properties in ref 23. The knowledge of the normal Raman differential cross sections is necessary if absolute SM-SERS enhancements are to be quantified. The statistics of signals and separation of SM-SERS events is performed by means of the Modified Principal Component Analysis method (MPCA) developed in ref 18 and used also in ref 23. Note that this tool is not necessary, in general, and other equivalent methods of analysis can be used with identical outcomes.

In fact, at some basic level, the effect studied in the paper can be observed by simple visual inspection of the data. We demonstrate this explicitly in Figure 2, where we show a sequence of 500 spectra taken in the colloidal solution at 3.8 mW and the same situation for 0.38 mW with the SERS intensities multiplied by a factor of 10. Both results in Figures 2a,b are plotted with the same vertical scale. Even with a limited sampling like this, it is possible to develop a visual idea of the maximum signals we can obtain (in the statistical sense) at a given incident power. Figures 2a,b show qualitatively that (i) the number of events at 0.38 mW become sparser (as expected), for many events that would have been visible at ten times more incident power are now lost below the noise level, and (ii) some events at 0.38 mW are (when normalized by the power) stronger than those observed at 3.8 mW. In other words, this suggests that higher SMEFs are visible at a lower incident power because they are not limited by the photostability of the probes.

Nonetheless, the disadvantages of stopping at a visual inspection of the data are obvious: we cannot decide easily which spectra were truly single-molecule in nature, we cannot quantify the actual SM-SERS EF of each event, and we cannot gain enough statistics for the conclusions in situations where, for example, 12000 spectra have to be analyzed. The MPCA method provides an easy and unbiased way (no fits or modeling of peak shapes required) to gain the statistics and, more importantly, extract those spectra that are catalogued as “single molecule events”. Moreover, the single-molecule EF (SMEF) of any given event can be calculated from a careful characterization of the scattering volume and by comparison to a reference compound of known Raman differential cross sections (da/dΩ). For the measurements in liquid with the immersion objective we use as reference 2-bromo-2-methylpropane (2B2MP) as in ref 6 while for the ×100 air objective in ref 6 while for the ×100 air objective in the dry maps we use the signal of nitrogen gas at normal pressure and temperature. Further details on how we extract the single molecule events and obtain their EFs are given in the Supporting Information.

For the rest of the paper, accordingly, we shall assume that we have the experimental and analysis tools to identify single molecule SERS events and put a number to their EFs. The rest...
of the discussion is therefore centered around the effect on the SM-SERS EF statistics at varying incident laser powers.

DATA ANALYSIS CONSIDERATIONS

It is convenient for the presentation of the results to pause and understand first what is expected from the statistics of SM-SERS EFs at varying laser powers. This is summarized in what follows, in conjunction with Figure 3.

• The main experimental limitation in real measurements of SM-SERS EFs is the presence of a “cutoff”, or minimum detectable signal above the noise, for a given integration time. Any SM-SERS event below this “cutoff” will not be detected and therefore be absent from the statistics. Moreover, if photostability is an issue for a given incident laser power, there may also be a maximum SM-SERS EF beyond which photobleaching will inevitably occur within the integration time. In saying this, we implicitly assume that the probability of photobleaching is directly correlated with the EM enhancement felt by the probe. The validity of this assumption will be justified qualitatively by the experimental results, but it is also based on previous studies of the link between SERS enhancement and photostability.7,8

• In an idealized situation where there is no minimum cutoff and no photostability problems, the statistical nature of the EF of single molecule events should be the same for all powers, that is, the SMEF is a self-normalizing quantity. For example, if we isolate a certain number of SM-SERS events (with the bianalyte technique), evaluate their corresponding SMEFs, and plot them as a function of an arbitrary “event index” (that simply counts them), we would observe a statistical spread of EFs that should look the same irrespective of the actual power being used. This spread is directly link to the EF probability distribution.11 This is schematically represented in Figure 3a.

• If we now take into account a fixed cutoff below which the signal is buried in the noise, we then no longer see many SM-SERS events (those with a low SMEF that results in a SERS signal below the noise). Note that the cutoff is basically the noise level on the CCD detector at a fixed integration time, that is, it does not depend on power and is the same for all cases. Accordingly, as the power is reduced, many events will fall below this threshold and will disappear from the statistics of EFs. This is schematically depicted in Figure 3b. For a fixed sampling size, the actual number of events contributing to the statistics decreases with decreasing power, for many disappear under the cutoff. The maximum EFs, however, should still be the same for all the cases if there is no limitation imposed by photobleaching. In this case, the measured maximum EFs would be a faithful representation of the maximum SMEF of the substrate.

• If we now add simultaneously the effect of a cutoff and the limitations of photostability, we obtain the situation depicted in Figure 3c. When the power is increased, the intensity of events with the largest SMEFs is reduced because photobleaching of the probe occurs during the integration time. The maximum measured EFs are therefore reduced. There is now a “rooftop” in the statistics of EFs, which is not intrinsic to the substrate but rather has to do with the limited photostability (within the integration time) of the probe that is used to measure it. This is schematically shown in Figure 3c. Note that by decreasing the integration time, it would be possible to recover some of these events (i.e., increase the “rooftop level”) because the probes would be less likely to photobleach within this shorter time. This would, however, be at the expense of loosing many low-intensity events, and therefore statistics on the low-EF side, because of the noise-related cutoff.
An interesting additional question is what happens to the average signals (rescaled by power) when some of the events at the highest enhancements are being lost with increasing power. This will obviously depend on the relative contribution of the large-EF events to the average SERS signal. We shall show later that experiments performed in colloidal solutions (Figure 1a) or dry samples (Figure 1b) produce slightly different answers for this question, and we shall return to this specific topic in the discussion.

With these concepts in mind (Figure 3), we can present rapidly the experimental results in colloidal solutions and dry samples. Figure 4a shows the average signals of a RH6M/d4-RH6M sample in solution under the experimental conditions delineated in the Experimental Section. The average spectra are rescaled by power to facilitate a direct comparison. From the bianalyte spectra producing the averages in Figure 4a, the single molecule events are isolated, their signals quantified, and the corresponding single-molecule EFs calculated. The statistics of SM-SERS EFs for different incident powers (each one differing from the previous one by an order of magnitude) is shown in Figure 4b for the liquid sample. Both single molecule events of RH6 M and d4-RH6M are considered for the statistics of SM-SERS EFs. The equivalent situation for the dry maps (average signals scaled by power, and statistics of SM-SERS EFs) is shown in Figure 5a,b. To compensate partially for the lower number of SM-SERS events seen at lower powers (because of the presence of a cutoff at the noise level), the sampling (number of spectra) is increased at lower powers to regain more reliable statistics. The rest of the paper is devoted to an analysis of the experimental data in the light of what is known for SM-SERS EFs.

DISCUSSION AND CONCLUSIONS

The most salient aspects of the data displayed in Figures 4 and 5 can be summarized as follows:

- The statistical spread of single molecule EFs for both colloidal liquids and dry samples shows an obvious power dependence in their characteristic values which can be readily observed in Figures 4b and 5b. The SM-SERS EFs display the expected behavior for the situation where both a cutoff at the noise level and a maximum EF compatible with the incident power and related to the photostability of the probes are at play, as discussed earlier and shown schematically in Figure 3c. The rooftop threshold associated with photobleaching is here not as well-defined as in the idealized model of Figure 3c. This arises from the combined results of (i) the stochastic nature of the photo-
bleaching mechanisms, (ii) the limited sampling of large events, and (iii) the (so-far unresolved) details of the exact link between photobleaching and local field enhancement.

- The effect of photobleaching at 5 mW incident power in fact becomes extreme in the dry samples. This is evident in the average spectrum (inset of Figure 5a), which contains “amorphous carbon-like” features resulting from the photo-decomposition of molecules.25,26 Similarly, many individual spectra exhibit these “amorphous carbon-like” features and cannot be labeled as single-molecule events. They therefore disappear from the SERS EFs statistics in Figure 5b, which explain the smaller than expected number of SM-SERS event at 5 mW. The larger prominence of photobleaching at high power in the dry sample compared to the liquid sample can be understood simply because the photostability of the probes is (as a rule of thumb) increased in solution for reasons to do, among other things, with the reduced oxygen content of the environment. This situation where photobleaching is directly evident in the modification of the SERS spectrum corresponds to the “easy” case. The effect of photostability is then obvious in the experimental spectra and it is clear that any calculated SM-SERS EFs are affected by it.

- Of more interest to us here are the other cases (the three incident powers in liquids and the two lowest incident powers for dry samples). In such cases, the average spectra do not show any “amorphous carbon-like” features, and they, moreover, seem to scale with incident power, that is, there is no evidence of photobleaching in the average SERS spectra. However, the statistics of SM-SERS EFs clearly reveal that photobleaching does play a role in these cases and strongly affects the measured maximum SM-SERS EFs. This effect is completely hidden unless a power-dependence study is carried out. Even in this case, one cannot rely only on a study of the average SERS intensity to assess the importance or not of photobleaching. This is one of the most important conclusions of this study.

- The maximum EFs measured here for SM-SERS events is slightly below $\sim 10^{10}$ (in liquid). Smaller maximum values, by as much as two orders of magnitude, are measured as a result of photobleaching when the power is increased from the minimum value. The maximum enhancements seen on dry samples also seem to be slightly below what is observed in liquids. This may be due to the fact that the EM properties of liquid and dry samples are obviously not the same. It can also be attributed to the reduced photostability of the probes in air rather than in water (in the presence of a higher concentration of oxygen). The results for the lowest power used here for dry samples could therefore still be susceptible to a small amount of photobleaching.

Still, the scaling of the average signals with power is striking, especially given the fact that photobleaching does affect the largest SM-SERS events, as evidenced by the SM-SERS EF statistics. The explanation for this effect lies in the convolution of the EF distribution and the exact mechanisms by which the probability of photobleaching is linked to the SM-SERS EF felt by the molecule. Both of these are still subject to much uncertainty, and it is therefore difficult to elaborate. Our results indicate the following:

- Photobleaching does affect more those molecules that are subject to a larger field enhancement (and therefore have a larger SERS EF).

- At large powers (and/or long integration times), photobleaching is clearly evident in the average SERS spectrum as a reduced average intensity and the appearance of “amorphous carbon-like” features.

- At intermediate powers, only a small proportion of molecules are subject to photobleaching, the ones with the highest SERS EF. This reduces their apparent (measured) SM-SERS EF and therefore their contribution to the average spectrum. This contribution, however, appears to be negligible compared to that of the molecules not subject to photobleaching, that is, it only affects the top-end of the EF distribution.

Even though it is not the main topic of this paper, we mention in passing that the EM-SERS EFs statistics observed in liquid solutions could in principle also be the result of a different physical mechanism, namely the radiation pressure27–29 induced by the laser beam on the colloidal clusters in the scattering volume. If this force is sufficiently large, the clusters may be “pushed” away from the scattering volume over a time scale smaller than the integration time. Because radiation pressure is power-dependent, such an effect would result in a similar statistics for the EM-SERS EFs (although strictly speaking, the reduction in SMEFs with increasing power should affect all SMEFs and not only the largest ones as is the case for photobleaching). The difficulties in obtaining large sample statistics for large SMEF events, and the uncertainties on the exact photobleaching mechanism in SERS conditions, make it difficult to distinguish between the two effects. However, given the similarities between the liquid and dry sample results in our experiments, we believe that this effect is secondary here, that is, the molecule photobleach faster than the radiation-pressure induced residence time of the clusters. We cannot, however, exclude it rigorously. It is also an important effect to bear in mind for SM-SERS EF experiments in liquids with more photostable analytes.

The results in this paper show why it is important to adopt an experimental approach to the question of whether the photostability of the probes (or more “exotic” effects like radiation pressure) play a role for the determination of SM-SERS EFs. Moreover, the values of the SM-SERS EF obtained here are compatible with theoretical estimates of maximum EFs for Ag colloids in water.3 EFs in the range $\sim 10^{10} – 10^{11}$ are among the highest observed experimentally when they are normalized by the intrinsic bare cross sections of the probes and when the number of molecules contributing to the signals are quantified properly.

Decreasing incident laser powers should result eventually in a SERS EF that is not affected by photobleaching within the integration time and, therefore, in the intrinsic EF of the SERS substrate. This could, however, result in a prohibitively large number of spectra to gain sufficient statistics. For example, decreasing the power by another factor of 10 in our experiments could require $\sim 10^6$ spectra to gain a similar statistical confidence. Another interesting possibility should be the study of the EF as a function of ambient conditions that can affect
photobleaching (replacing oxygen by argon, for example). We believe that the EF values at the lowest laser power we are using here are actually very close to (or already in) “saturation” and reflect mainly the intrinsic maximum enhancement in these substrates. This opinion is based on both a handful of individual spectra gained at lower incident powers and on theoretical expectations for the maximum enhancement. While this is a matter of discussion in the specialized literature at the moment, we believe at this particular point in time that there is no credible experimental evidence to date for SM-SERS EFs above $\sim 10^{10}-10^{11}$.

Finally, as mentioned in the introduction, the idea that the experimentally determined SERS EFs are limited by (and only make sense within) the constraints imposed by the photostability of the probes has been exploited before, and this “flaw” of the probes can, actually, be used as an advantage to map out the EFs distribution.$^{7,8}$ Our paper extends here these ideas to a situation where they directly apply to single molecule conditions and shows explicitly how they can play a dominant role in the discussion of how much the SERS EF for single molecules actually is. Failing to test for the presence/absence of laser intensity scaling of the EFs can already account for discrepancies in single-molecule SERS EFs of the order of $\sim 10^2-10^3$, as explicitly demonstrated in this study. This is obviously important for any attempt to reconcile the experimentally determined values with theoretical predictions for the enhancement.

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SUPPORTING INFORMATION AVAILABLE

Further details are given in Figures S1–S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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