Single-Molecule Surface-Enhanced Raman Spectroscopy

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Abstract

A general overview of the field of single-molecule (SM) surface-enhanced Raman spectroscopy (SERS) as it stands today is provided. After years of debates on the basic aspects of SM-SERS, the technique is emerging as a well-established subfield of spectroscopy and SERS. SM-SERS is allowing the observation of subtle spectroscopic phenomena that were not hitherto accessible. Examples of the latter are natural isotopic substitutions in single molecules, observation of the true homogeneous broadening of Raman peaks, Raman excitation profiles of individual molecules, and SM electrochemistry. With background examples of the contributions produced by our group, properly interleaved with results by other practitioners in the field, we present some of the latest developments and promising new leads in this new field of spectroscopy.

1. INTRODUCTION AND OVERVIEW

1.1. Origins

With reports from two independent groups in 1997 (1–4) the field of single-molecule (SM) surface-enhanced Raman scattering (SERS) was brought to life. It is now a well-established subfield of SERS as well as within the broader scope of SM laser spectroscopy, which had traditionally been dominated by fluorescence (5–13). In fact, SERS has been compared with fluorescence as a viable competing tool for SM optical studies since the early 2000s (14–16). The SERS effect (17, 18) has a longer and distinguished trajectory that goes back to its controversial origins in the mid-1970s (19–25). Traditionally, SERS has dwelt in controversies and differing opinions. Many pioneers of the technique have held the view (in many cases, correct) that most of the fundamentals of the technique had been established and understood in the 1980s (23). Still, longstanding controversies regarding some of the most fundamental aspects such as the chemical versus electromagnetic enhancement (26, 27), the role of resonance effects (28), or the actual magnitude of the SERS enhancement factor (EF) (29–31) continued to plague the field well into the late 1990s. Hence, the suggestion in 1997 (1, 2) that SERS could, indeed, detect single molecules had two main effects in the field. On the one hand, it opened another debate on the interpretation, and even the reality, of the observations (32–34). On the other hand, and perhaps more importantly, it reinvigorated a field that had spent a few years in a "latent" state: Suddenly, SERS was another possible tool in the field of SM laser spectroscopy.

1.2. The Development of SM-SERS

1.2.1. The low-concentration approach. The initial reports on SM-SERS were based on the idea of ultralow-concentration studies (1, 2, 35–38). However, researchers soon found serious limitations in terms of statistical soundness. The idea is fairly simple: Microscope objectives with standard magnifications (times 100, for example) can have relatively small scattering volumes [of the order of ~10 fL for an immersion objective (30)]. If one dilutes the concentration of dye so that there is on average one (or less than one) molecule in the scattering volume, the only signals that are observed are attributed to SM cases. The idea has its origins in SM fluorescence where the concept of dilution beyond the critical concentration of one molecule in the scattering volume is routinely used. This concentration is achieved (typically) in the picomolar range. This simple idea, however, does not translate easily to the case of SERS, where molecules need to be adsorbed on a metallic substrate, which typically results in a highly nonuniform molecular density. For example, in a colloidal solution, many molecules can be absorbed on each metallic nanoparticle, even at picomolar analyte concentrations, if the particle concentration is also low. Moreover, the dynamics of the individual colloids become important, e.g., how many particles go through the scattering volume during the integration time. The analyte concentration required for SM-SERS cannot, therefore, be straightforwardly estimated as in SM fluorescence studies. It is a complex convolution of many factors including colloid concentration (and, indirectly, their size and polydispersity), adsorption efficiency of the analyte onto the metal, integration time, colloid diffusion time, and scattering-volume size.

1.2.2. Hot spots and the enhancement factor distribution. Perhaps even more fundamental to the problem is the fact that SM-SERS is always associated with the presence of hot spots, which are highly localized regions (39), typically at gap junctions between metallic nanostructures (38, 40, 41), where the EF for Raman signals can be as high as $\sim 10^9$–10^{11} (35, 39, 42). A key

point that is missing in the low-concentration approach is that only a very small fraction of the molecules can produce a measurable Raman signal (i.e., only those molecules located at hot spots). Therefore, if the concentration is of the order of one molecule in the scattering volume, the statistics of signals becomes very sparse and consequently unreliable. SM spectra can be obtained with much sounder statistics at higher concentrations (43). What matters is, in fact, the convolution of the analyte surface concentration with the spatial localization of the enhancement distribution, which can be quite extreme (39, 44–46). As a result, the low-concentration approach leaves room for skepticism because the rarity of the SM-SERS events may be a consequence of the equally rare inhomogeneities in the sample. This is a problem that is particularly acute in the case of colloidal liquids [such as, for example, Lee & Meisel colloids (47)], which are notorious for their inhomogeneities. Adding to the initial debate regarding the reality of the SM-SERS phenomenon were claims of "quantized intensities" that led to the concept of Poisson distributions in the statistics of SM-SERS signals (2). Even though these claims have survived for approximately ten years, they have never been independently confirmed and are based on unrealistic assumptions about the nature of the EF at hot spots (uniform enhancement) (31, 39, 48). Only if all molecules at hot spots produce exactly the same SERS signal is it possible to count the number of molecules through a histogram of intensities. This situation has never been shown to be applicable in real samples. In fact, the original claims of Poisson distributions may be attributed to insufficient sampling of signals (48).

1.2.3. The bi-analyte approach. Sampling is an important aspect in SM-SERS, as the extreme nature of the localization of hot spots leads typically to long-tail distributions for the SERS EF, which are particularly difficult to sample to obtain sound statistics (39). Because only a small fraction of the molecules are observable (i.e., those at hot spots), to improve the statistics it is desirable to perform SM-SERS experiments at larger concentrations than the ones used in the low-concentration approach, ideally targeting a final surface concentration of exactly one molecule per hot spot (or correspondingly less if more than one hot spot is probed within the scattering volume). Such concentrations may be as much as ~100 times larger than the ones used in the original trials, and these typically lie in the nanomolar range. Estimating a priori which exact concentration is necessary is even more difficult, as it requires—in principle—a knowledge of the EF distribution [i.e., the probability for a randomly adsorbed molecule to experience a given EF (39)]. For this reason, an experimental demonstration of the SM nature of SERS signals is paramount to ensure the credibility of SM-SERS experiments. The bi-analyte technique, introduced in 2006 (49), solves this problem by using two distinguishable SERS analytes. The SM nature of SERS events can then be assessed experimentally, at least in a statistical sense. The bi-analyte method has been studied in detail (50) and further perfected with the use of isotopically edited molecules (51–53). Thanks to its wide-ranging applicability (it is applicable to most, if not all, SM-SERS situations), the bi-analyte method provides a relatively simple and general approach to assess the concentration at which the largest number of SM-SERS events can be sampled. With such improved statistics, the SM-SERS phenomenology and, in particular, the role of hot spots can be studied in detail. In addition, SM-SERS can then be used as a tool to study not only the SERS effect, but also other properties that are not accessible in conventional experiments on an ensemble of molecules. Several studies along this line have recently appeared, and this new trend is certainly likely to develop further: Recent examples include SM-SERS studies of the inhomogeneous broadening of Raman peaks (51, 54, 55) or of the electrochemical properties of single molecules (56, 57) (also see Section 5 below).

1.3. Review Outline

In this review, we first discuss some important general features of SM-SERS detection, with a particular emphasis on the SERS EF and the nature and role of hot spots. We then briefly review the most important experimental approaches to the problem. The remainder of the review is dedicated to possible applications of SM-SERS, following two main streams: first, as a tool to further our understanding of the SERS effect or related effects such as surface-enhanced fluorescence (SEF) and, second, as a new promising tool to investigate indirectly properties of molecules that are accessible at the SM level but are hidden in measurements of an ensemble of molecules.

2. SOME IMPORTANT FEATURES OF SM-SERS

Thanks to the steady research efforts invested toward understanding SM-SERS over the past decade, the conditions under which SM-SERS can be observed have become increasingly well understood. These have been discussed in full length in past reviews (31, 58); accordingly, we summarize here only the most important features, with a particular emphasis on the latest developments.

2.1. Experimental Estimation of the SM-SERS Enhancement Factor

The EF is central to the problem of SM-SERS detection (30), as it is to SERS in general. Its magnitude has also been at the center of much controversy, partly because of the use of widely varying definitions by different authors. Various possible definitions of the SERS EF are discussed extensively in Reference 30. We believe that the most general definitions of the SERS EF should be based on a comparison of the SERS signal with that of the same molecule in normal Raman conditions. In this way, the EF is (in a first approximation, ignoring chemical effects, for example) an intrinsic property of the SERS substrate, independent of the analyte chosen to measure it. This is a very important point, as the intrinsic (non-SERS) Raman cross-sections of typical SERS analytes can vary by as much as six orders of magnitude, typically from $\sim 10^{-24}$ cm^2/sr for a dye at resonance to $\sim 10^{-30}$ cm^2/sr for a small nonresonant molecule. Failing to normalize by this intrinsic cross-section has resulted in large discrepancies in the SERS EF, which have lingered in the literature to this day. For example, the original reports claimed that SERS EF of the order of $\sim 10^{14}$–10^{15} were necessary for SM detection. This fueled a large research effort to explain such large EFs theoretically (59, 60), and the failure of the electromagnetic model to account for these was often quoted as a demonstration of the existence of the chemical enhancement. It has since been realized (30, 31) that the SERS EFs for single molecules are of the order of $\sim 10^9$–10^{10}; the additional factor of 10^5 is attributed to the resonance Raman enhancement of the bare cross-section (53) when comparing with a (typical) nonresonant molecule. This point is now fairly well understood and the "myth" of SERS enhancements of the order of $\sim 10^{14}$–10^{15} is nothing but one of the many deadends that the field has visited in its historical development.

However, much uncertainty remains, as reflected by the large variations in the SM-SERS EF (and incidentally average SERS EF) that are quoted in the literature. It is important to realize that measuring experimentally the SM-SERS EF is a difficult task in general and should involve the following:

- Careful characterization of the normal Raman cross-section of the molecule of interest at the same excitation wavelength: Measurements in solution should be preferred because the concentration can be reliably characterized, provided that the analyte is soluble at the required concentration. This step may be difficult, or even impossible, for resonant molecules

that fluoresce. As a result, the choice of resonant target analyte may be restricted to those for which the cross-sections have been measured, e.g., crystal violet (30, 61) or rhodamine 6G (62). Large errors may occur at this step if, for example, the analyte is measured at a concentration well above its solubility limit or if a powder is used as reference.

- Experimental confirmation, for example, using the bi-analyte method, that the spectra used to estimate the SERS EF are SM spectra: Without this, there is always a possibility that many molecules are being measured and that the obtained SERS EF is dramatically overestimated.
- Detailed characterization of the scattering volume, for example, as described in Reference 30.
- Critical analysis of the dynamical effects occurring during the integration time, including—in particular—nanoparticle diffusion for colloidal solutions and photobleaching for resonant and preresonant molecules: This is, again, the potential source of large errors. For example, if an SM-SERS is acquired over 100 ms, but, in reality, the molecule photobleached after only 10 ms, then the corresponding SERS EF would be underestimated by a factor of ten (63).

It is clear from this brief list that many things can go wrong when estimating an SM-SERS EF, and credible estimates should include a detailed discussion of the points highlighted above. Unfortunately, many reports of SM-SERS EF do not provide enough details to assess critically the validity of their estimates. In our opinion, there is probably a tendency to overestimate the real SM-SERS EF, but this could be verified only if full details are systematically provided.

2.2. Which Enhancement Factor for SM-SERS?

With these clarifications in mind, we can attempt to answer an important question: Which SERS EF is necessary for SM detection? The answer is (not surprisingly) highly molecule dependent. For example, for the same nominal EF, a resonant molecule would be expected to give a SERS signal up to $\sim 10^6$ times stronger than a small nonresonant molecule. The answer also depends on many additional experimental factors such as integration time, laser power density, the objective's numerical aperture, etc., and it is not always possible to vary these at will. For instance, the maximum useful integration time for SM-SERS detection in colloidal solutions is limited by the diffusion time of the nanoparticles in the scattering volume. For resonant molecules, photobleaching also limits the effective integration time (or laser power) that can be used (63). Furthermore, as previously mentioned, SERS EF estimates in the literature are often subject to large uncertainties or errors. With these caveats in mind, we can estimate a few orders of magnitude. Arguably, the easiest case is that of a dye or molecule in preresonant conditions (such as rhodamine 6G excited at 633 nm). To some degree, this avoids problems associated with photobleaching at resonance, while retaining a normal (non-SERS) Raman cross-section that is not too low [typically $\sim 10^{-27}$ cm^2/sr (30)]. In such conditions, SM-SERS can be observed with SERS EFs in the range of 10^9–10^{10} on both colloidal and dry SERS substrates (30). A minimum SERS EF of $\sim 10^7$–10^8 under optimized conditions may be inferred in this case (58) from typical experimental conditions. Assuming this can be appropriately scaled to other situations, we can write a rule-of-thumb estimate for the minimum SERS EF for SM detection as

$$\mathrm{EF}_{\mathrm{SM\text{-}SERS}}^{\mathrm{Min}} \sim \frac{d\sigma/d\Omega}{10^{-19}\ \mathrm{cm}^2/\mathrm{sr}}. \quad (1)$$

Applying this rule to a small nonresonant molecule with a typical $d\sigma/d\Omega \sim 10^{-30}$ cm^2/sr, we obtain a minimum SERS EF of $\sim 10^{11}$. This is, indeed, what has been measured in recent experiments of SM-SERS of nonresonant molecules (64, 65), which include important biological

examples such as adenine. This also shows that, in principle, any molecule can be detected via SERS at the SM level, although achieving it remains an experimental challenge. On the opposite end of the spectrum, the minimum EF for a dye at resonance would be of the order of $\sim 10^5$. Such a low EF (by SM-SERS standards) may be achievable on most SERS substrates including island films (66) and single elongated nanoparticles (60, 67, 68), which means that SM-SERS is not necessarily—as often assumed—constrained to gap-containing SERS substrates. Pushing the limits of this prediction even further, we find that Equation 1 predicts that SM detection would be possible without any enhancement (EF = 1) for a molecule with a Raman cross-section of the order of $\sim 10^{-19}$ cm^2/sr. Although this is too big for the standard dyes that are used as canonical SERS probes, it is nevertheless the order of magnitude of the Raman cross-section of carbon nanotubes, which can be studied via Raman spectroscopy at the single-nanotube level (69, 70) without any SERS enhancement.

Overall, the rule-of-thumb estimate given by Equation 1 provides a reasonable ballpark figure for the minimum SERS EF required for SM detection. Nevertheless, its applicability should be assessed on a case by case basis, and there will be natural exceptions. For example, for an analyte experiencing a large chemical enhancement, the minimum SERS EF for SM detection will be correspondingly reduced. Moreover, for any specific example, it is likely that careful optimization of the optical setup and experimental parameters would reduce even further the minimum detection limit.

2.3. Hot-Spot Localization and Enhancement Factor Distribution

From the previous section, it is clear that large SERS EFs, of at least $\sim 10^8$–10^9 in most situations, are desirable for SM-SERS detection. Many SERS substrates are in principle capable of supporting such large EFs, including single elongated metal nanoparticles (60, 67, 68), but most of the SM-SERS studies so far have used gap-containing substrates, typically (but not exclusively) formed by aggregating individual colloidal particles (38, 40, 71, 72). The electromagnetic SERS EF can reach $\sim 10^{11}$ in the gap of such structures at the localized surface plasmon resonance, and this is well understood from classical electromagnetic theory (35, 39, 42, 73–79). Larger predicted SERS EFs have been occasionally reported (80, 81) in very specific geometries. In all cases, large enhancements are invariably associated with a very strong localization of the highest SERS EF in a very small region on the surface. As intimated above, the region around the point of largest enhancement is called the hot spot, and the SERS EF typically drops by orders of magnitude over a few nanometers when moving away from the maximum (39). This is a very general feature of electromagnetic hot spots, and a few examples are given in the EM calculations presented in **Figure 1**, with additional explanations in the caption. In fact, there is no known metallic structure to date that would provide a large SERS EF (say $\sim 10^8$) everywhere on the surface over a large area. The fast drop of the EF from the hot spot can usually be described by a long-tail distribution of the surface SERS EF, and this is what produces the peculiar statistical properties of SM-SERS signals, which are heavily dominated by large fluctuations and sampling problems (39).

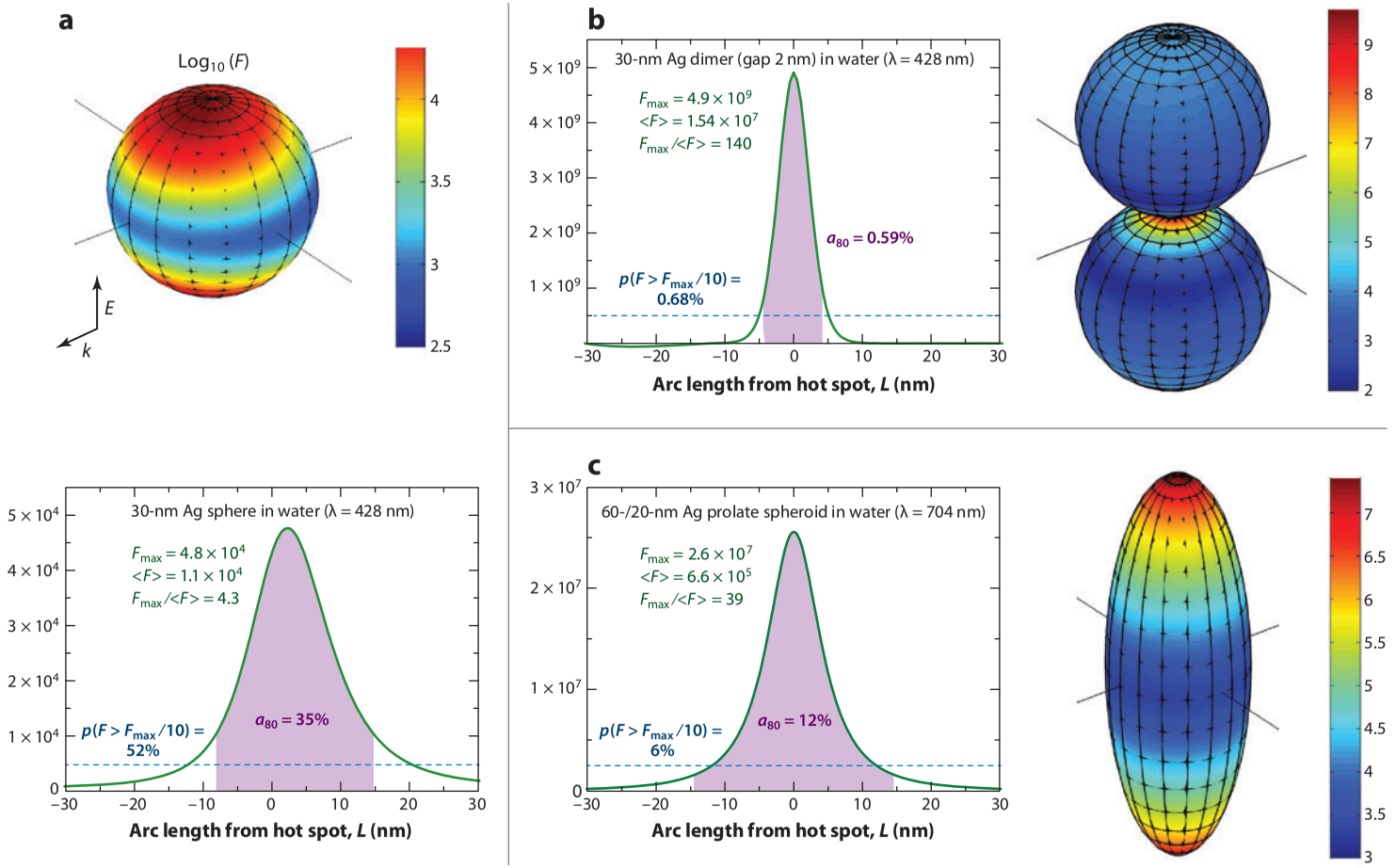
The hot spots in a given SERS substrate may be characterized by their strength, their size, and their frequency of occurrence. The latter is more a property of the substrate as a whole; for example, it is typically governed by the nanoparticle concentration for colloidal solutions. The strength of the hot spot can be related to the maximum SERS EF. In the examples shown in **Figure 1**, the sphere exhibits a rather weak hot spot with a maximum SERS EF of $\approx 5 \times 10^4$ at resonance. The elongated particle has a maximum SERS EF of $F_{\max} \approx 3 \times 10^7$, in principle sufficient for SM detection. This could be even larger for more pointy shapes or higher aspect-ratio particles (68). Large maximum SERS EF are also typical of interacting particles with nanometer

gaps; for example, $F_{max} \approx 5 \times 10^9$ for the silver dimer shown in **Figure 1** (gap of 2 nm). The hot-spot strength is then strongly dependent on the exact gap dimensions (39).

There are several ways to characterize hot-spot sizes, but most are more suited to theoretical calculations than actual experimental measurements. For example, the hot-spot size can be defined as the area within which the SERS EF [in the $|E|^4$-approximation (82), for example] is larger than a prescribed value (say $\sim 10^6$) to observe single resonant molecules. In the examples shown in **Figure 1**, this would be 0 nm^2 for the sphere, 750 nm^2 for each of the two hot spots of the spheroid, and 500 nm^2 on the surface of each particle close to the gap of the dimer. However, it is often more revealing to consider the degree of hot-spot localization (i.e., its size with respect to the total surface area) rather than its actual size. To illustrate this, we provide below, using the examples of **Figure 1**, a list (nonexhaustive) of potential metrics for the degree of hot-spot localization (which indirectly also characterize the hot-spot size):

- We first consider the area on the surface where the SERS EF is larger than $\sim 10^6$, but now normalize it with respect to the total surface area available for molecular adsorption (assumed random). We then obtain $p(F > 10^6) \approx 11.9\%$ for the spheroid and $p(F > 10^6) \approx 4.6\%$ for the dimer.
- The metric above, however, ignores the large difference in maximum EF in the two cases. As an alternative, we consider the relative area where the SERS EF has not decreased by more than a given factor, say 10, compared with its maximum value. For example, we have $p(F > F_{max}/10) \approx 52\%$ for the sphere shown in **Figure 1**, $p(F > F_{max}/10) \approx 6.1\%$ for the spheroid, and $p(F > F_{max}/10) \approx 0.68\%$ only for the dimer. This metric emphasizes spectacularly the extreme localization in gap-containing structures.
- The hot-spot localization and the associated extreme long-tail distributions of SERS EF can also be evidenced in the ratio of maximum to average SERS EF (39). They are, for example, $F_{max}/\langle F \rangle \approx 4$ for the sphere, 40 for the spheroid, and 140 for the dimer. In the latter case, this means that one molecule located at the best spot will emit a SERS signal as strong as 140 molecules at the average EF.
- Finally, we may further relate the localization to the average SERS intensities by considering (39) the relative area a_{80} from which, say 80%, of the total SERS signal originates (assuming random adsorption). This region is shown schematically as the dashed area in the plots of **Figure 1**. We obtain $a_{80} \approx 35\%$ for the sphere, 12% for the spheroid, and 0.6% only for the dimer, showing again the extreme localization in the case of the dimer.

The specific values quoted above are provided to fix ideas and depend on the exact geometries. However, a strong localization always occurs when large SERS EFs are present, for example, in elongated particles or gap-containing substrate, the latter of which is particularly affected. Moreover, regardless of the exact definition (which is more amenable to theoretical comparisons among substrates than anything else) of hot-spot size or localization, as a rule of thumb, the SERS EF at the hot spot varies substantially over distances of the order of a few molecular dimensions, as small as ~ 1 nm. This implies that small molecular motions produced by surface diffusion, laser-heating effects, etc., can alter the SM-SERS signals by an order of magnitude (39). This extreme sensitivity to position is at the heart of the long-tail nature of the enhancement distribution around hot spots, and it is one of the main reasons for their peculiar (fluctuation-dominated) statistics as well as for why quantized SM-SERS intensities (2, 83) (which imply that all detected single molecules have exactly the same EF) are such an unrealistic prospect in any current real experiment. The almost unavoidable hot-spot localization effect must always be taken into account when designing or analyzing SM-SERS experiments.



3. EXPERIMENTAL APPROACHES TO SM-SERS

3.1. Overview of the Problems

From a purely experimental point of view, we can arbitrarily divide the main bottlenecks toward SM-SERS detection into the two following main problems: (*a*) placing the molecule where it can be detected (i.e., at hot spots) and (*b*) observation, analysis, and interpretation of the signals once the SM-SERS conditions have been achieved. The former problem has historically been tackled by "brute force" random adsorption over SERS substrates that contain hot spots of some sort. One could argue that this is a fairly inefficient way of solving the problem, but we still lack the (nano)tools to control precisely the position of molecules on a substrate. For this problem alone, there is much room for improvements that may not be related to SERS only. Despite a few isolated attempts to control the process of adsorption of the molecules on SERS substrates—which include methods as varied as electrostatic interactions (84, 85), Langmuir-Blodgett films (86–90), or chemically controlled aggregation (91)—our tools are still too "macroscopic" to achieve the ideal goal of being able to position one given molecule in a given hot spot with nanometer precision. By contrast, the second goal of observing and interpreting the signals finds a fundamental limitation in the original approaches of ultralow concentrations. The classification of sparse events as opposed to rare ones, in the sense of "anomalous," is the main limitation here. The bi-analyte SERS method has helped researchers to overcome that limitation by making the statistics more reliable, but the limiting factor remains in many cases the poly-dispersity and/or nonuniformity of substrates, which makes the interpretation of signals reliant on parameters that we do not control fully. The "holy grail" of SM-SERS detection would enable us to control simultaneously both of the problems stated at the beginning of this subsection and with arbitrary precision. We are still some way off from that objective, but some preliminary studies in this direction have recently appeared (92).

3.2. Low Concentrations

Despite all the limitations mentioned at ultralow concentrations, a few attempts have been undertaken to pursue the idea of doing SM-SERS at low (rather than ultralow) concentrations in situations where we can distinguish SM signals with some reliability (93). In this sense, the technique of Langmuir-Blodgett films developed by Aroca and coworkers (86–90) represented a major

Figure 1

Examples of calculated enhancement factor (EF) distributions around hot spots for three model surface-enhanced Raman spectroscopy (SERS) substrates immersed in water: (*a*) a silver sphere (radius 30 nm), (*b*) a silver dimer of spheres (radius 30 nm, gap 2 nm), and (*c*) a silver prolate spheroid (radii 20 and 60 nm) representative of elongated or tip-containing nanoparticles. Calculations are performed using Mie theory (17), generalized Mie theory (39), and the *T*-matrix method (68), respectively. The excitation wavelength is taken at the dipolar localized surface plasmon resonance, which provides the maximum SERS EF. The logarithmic false color maps show graphically the surface SERS EF distribution [in the $|E|^4$-approximation (82)]. The plots next to them provide a more quantitative visualization of the hot-spot localization effect, showing the SERS EF in the plane of incidence as a function of arc length, L (along the surface). The most important characteristics of the hot spot are indicated in each case: Maximum SERS EF, F_{max}, surface-averaged SERS EF, $\langle F \rangle$ (over the entire particle), and other hot-spot localization metrics as discussed in the text. These plots demonstrate how extreme the SERS EF distribution can be, especially when large SERS EFs are present. For example, in the case of a dimer, the SERS EF decreases by a factor of 10 for a molecule that is only 5 nm away from the point of the largest EF.

step forward, by achieving much more credible control over the spatial distribution of the dye over the surface of the SERS substrate and, therefore, of the actual surface coverage. The statistics of signals still suffer because of hot-spot localization, but it is possible in some cases to infer the conditions of SM-SERS observation through a combination of concentration studies and statistical analysis (93). Nevertheless, studies at low and even ultralow concentrations are still pursued as a viable route for SM-SERS. A recent example is the SM detection of fullerenes (C_{60}) (94). The objections to the statistical soundness of these observations are the same as those applied to the original studies more than a decade ago. Overall, it is always more difficult to demonstrate SM-SERS at low concentrations without the natural statistical contrast provided by the bi-analyte method (49), whose latest developments are briefly summarized in the next section.

3.3. Bi-Analyte Techniques

The bi-analyte SERS method for SM detection was introduced (49) to remedy some of the problems associated with the low-concentration approach, notably the poor statistics of SM-SERS signals. The method must normally be aided by suitable statistical analysis of the fluctuations (50, 95). As a relatively simple contrast method to demonstrate the SM nature of the signals, the bi-analyte SERS method is fairly universal and applicable to a wide variety of different situations. A few alternative options were initially proposed to distinguish the SM nature of SERS signals, but they relied too heavily on ultralow concentrations (96). The method has since been perfected with the use of isotopically substituted bi-analyte partners (51–53, 64, 97). Studies have now been reported for different isotopic forms of rhodamine 6G (51, 52) and crystal violet (53). There are several advantages to having sound statistics to infer results from SM-SERS experiments. For example, once the signals are identified as coming from single molecules, they can be normalized with respect to a reference to obtain the EF (30) (see below for further examples of its application). Utilizing the best aspects of both approaches, Aroca and coworkers combined the Langmuir-Blodgett approach with the bi-analyte SERS method (31, 66). More recent developments in the technique include the simultaneous use of two dyes that can be spatially resolved (98). A full-length explanation of the many aspects and benefits of the bi-analyte SERS technique for SM detection is given in Reference 58.

3.4. Substrate Improvements

At a more fundamental level, researchers pursuing mainly nanoengineering or self-assembly routes have made serious attempts to control the EF of SM-SERS (99–103). A well-controlled SERS EF would drastically facilitate the assessment of the SM nature of SERS signals and the interpretation of SM-SERS experiments. The EF at an SM level has, however, always been notoriously difficult to control. Whether these efforts will succeed in controlling SM-SERS with uniform enhancement remains to be seen. Some recent highlights in this field involve attempts to produce controllable dimers (99, 104, 105), which can provide (in principle) a fixed known enhancement at the hot spot. Despite the various limitations that still exist, it is not difficult to envision further improvements in the substrate characteristics for SM-SERS, perhaps drawing further resources from areas such as micro/nanofluidics (106), laser optical forces (107, 108), self-organization (109, 110), surface functionalization (91), or other recent advances in the nanosciences (111, 112).

3.5. Tip-Enhanced Raman Spectroscopy

The bi-analyte method and other advances discussed above do not address the question of how to position the molecule where it can be observed (i.e., at the hot spot). This is a difficult problem and

is currently at the forefront of SM-SERS research. Tip-enhanced Raman spectroscopy (TERS) has provided for some time a viable, although technically challenging, approach to this issue. Pettinger and coworkers have contributed a great deal to the field of TERS, yielding impressive demonstrations of the power of the technique (including TERS microscopy) (113). For further details on TERS, see Reference 114, in which Pettinger provides a comprehensive review of SM-SERS and -TERS (114), including the historical aspects of the origin of TERS. As with many other aspects of SM-SERS, the TERS technique is still under development. Although special implements are required to view single molecules using TERS, the future of this technique looks promising.

3.6. Detection of Every Single Molecule

The TERS approach proposes to build the SERS substrate around the target molecule. Recently, some attempts using chemistry or self-assembly have been undertaken to implement this approach in an easier setting: For example, nanodumbbells have been used for SM-SERS detection with controllable gaps (115, 116). Using a different approach, we have recently investigated the possibility of site-selective adsorption of the target analyte at the hot spot for SM-SERS detection (92). Such methods in principle would permit the detection of every single target analyte in solution at the SM level via SERS. Although these approaches may not be completely universal for any given analyte, it does show the wide variety of methods that are being tried to gain control over the main two problems mentioned associated with SM-SERS (see Section 3.1): the ability to position the molecules at hot spots and the ability to measure and understand the origin and significance of the signal. It will be interesting to see what these new approaches bring to the field of SM-SERS.

4. SM-SERS APPLICATIONS: FUNDAMENTAL UNDERSTANDING OF SERS AND RELATED EFFECTS

Given the progress made over the past decade or so, the field of SM-SERS has moved from the initial stage of proof of principle to a more mature state where applications of the technique can be seriously envisaged and investigated (even while some fundamentals remain to be understood). The applications of SM-SERS fall into two broad categories. The first aims to further the fundamental understanding of the SERS effect. This includes, for example, studies of SM-SERS EFs at hot spots or of SEF in SM-SERS conditions (see below for additional examples). The second uses SM-SERS as a tool, to indirectly study other properties of single molecules that would not be accessible in ensemble measurements. This category is, therefore, more concerned with the molecular Raman spectroscopy of single molecules, whereby the SERS effect provides the amplification to see the signal. Recent examples of this approach are discussed in Section 5 (see below). In Sections 4 and 5, we emphasize some of the work contributed by our group to the field of SM-SERS, which is mainly based on the bi-analyte SERS technique, but we strive to do this in the context of contributions from other groups, which are mentioned where appropriate.

4.1. SM-SERS Studies of the SERS Phenomenon

An increasing number of papers try to exploit the SM nature of the signals to uncover microscopic details of the SERS phenomenon, particularly in relation to hot spots. As a result, the bi-analyte SERS method has provided clear proof of the hot-spot localization effect (49) and of its consequence for the statistics of SM-SERS signals. It has also allowed for rigorous estimates of the

SM-SERS EFs (30) and, to some degree, of its distribution (50). It remains, in our opinion, the most reliable approach for such fundamental studies.

In addition, SM studies have also shown that the measured maximum SM-SERS EF may be artificially affected by photobleaching in some instances and, therefore, may depend on the exciting laser power density (63). Thus, ensuring that this effect is negligible when estimating SM-SERS EF is important.

As an example of more recent work, Park et al. (117) utilized SM-SERS to study what they interpret as charge-transfer contributions to the SERS enhancement of single molecules. In an unrelated development, Stranahan et al. (45) introduced the idea of detection of SERS signals with super resolution by using the (still poorly understood) SERS-blinking effect (118–120) and subsequent image processing. Reference 45 in particular reports the study of diffusion processes of single molecules around hot spots, which provide an interesting development that may reveal some poorly understood aspects of SM-SERS. One of the (still) not fully resolved issues in SM-SERS is the nature and origin of "blinking," which is repeatedly mentioned in the literature as a microscopic source of fluctuations (45, 121, 122). However, the naming of this concept results in a semantic problem: "Blinking" comes from fluorescence spectroscopy, where an electron can be trapped in a triplet state in a molecule and thus can quench the fluorescence emission. No such analog exists for Raman, which is an instantaneous process and cannot be "turned off." Blinking in SERS may be related in some instances to thermal diffusion of a single molecule in and out of hot spots, where changes in position of a few nanometers can make a huge difference to the SERS intensity (45). However, minute changes in the geometry of the substrate (e.g., the gap between two particles) could also lead to similar drastic changes in SERS intensities. In this case, blinking would be observed even in many-molecule conditions. Until such effects are excluded, it is still not clear whether blinking is a defining characteristics of SM-SERS. More studies are needed to clarify this issue. Our understanding is that blinking is not a defining aspect of SM-SERS, for there are countless counterexamples where large fluctuations in the signals can arise in many-molecule experiments.

In our opinion, other aspects of the study of the Raman effect on single molecules remain controversial: for example, orientational effects and Raman tensors of single molecules (123) at hot spots (124), where the potential contributions of local field polarization effects (76) and intrinsic resonant Raman tensor changes have not been fully elucidated. The phenomenology of SM orientation on metal surfaces under SERS conditions is currently far from settled (125, 126).

4.2. Nonresonant Molecules

Developing the ability to detect nonresonant molecules with SERS at the SM level was a long-standing ambition in the field and was pursued immediately after the first claims of SM-SERS (127). Given that most biological molecules fall into this category, such a development acquires special importance. Thus, the ability to detect single nucleotide bases or single amino acids becomes appealing for various applications. Using the concept of isotopic substitutions to create bi-analyte partners, SM-SERS detection of adenine (and isotopically substituted adenine) was recently demonstrated (64). Other types of nonresonant molecules (not necessarily biological) have also been detected (117, 128). The conditions required for SM-SERS detection of nonresonant molecules present greater challenges than those encountered when resonant or preresonant molecules are used (as in the majority of SM-SERS studies to date). Contamination issues become especially problematic (64), as any contaminant is expected to have a SERS signal comparable to or even larger than the nonresonant target analyte. Further development of nonresonant SM-SERS must address this issue.

4.3. Ultrafast Dynamics and Surface-Enhanced Fluorescence

SEF is one of the most specialized topics studied with the aid of SM-SERS, and it has wide-ranging consequences for the understanding of subtle aspects of the electromagnetic EF in SERS. Raman peaks under SERS conditions are affected by the dispersion of the plasmon resonance (125, 129–132), and this affects the estimation of the SERS cross sections. The dispersion of the resonance is also responsible for the modification of the SEF background that is normally present simultaneously with the Raman peaks (133–135). The origin of the "Raman background" under SERS conditions (not only SM-SERS) has been a frequent topic of discussion (136, 137). References 134 and 135, for instance, show that an interpretation of the Raman background as SEF provides a consistent picture of the light emission of resonant or preresonant molecules on metallic surfaces. These studies also highlighted the unavoidable nonradiative component of the SERS cross section, providing a natural explanation for the apparent discrepancy between the so-called pumping and SERS cross sections, which can also be retrieved from SM-SERS experiments (138). Overall, SM-SERS studies of this type have allowed for the observation of unique phenomena that would be impossible to monitor otherwise. The results of Reference 135, for example, imply the observation of fluorescence emission from a single molecule that happens only a few femtoseconds after excitation.

4.4. Brief Overview of Other Developments

We mention here a few additional interesting developments that use SM-SERS to understand the SERS effect. The following studies, however, are at a very early stage of development, and it is difficult to predict what importance they will have. Studies of SERS—possibly down to the SM level—have been reported using graphene as a substrate (139). To obtain a new level of understanding, current efforts also aim to extend the normal scope of SM-SERS studies, including to nonlinear optical effects (140) and electron transfer mechanisms at the SM level (141). Ingenious combinations of SM-SERS detection with other techniques, such as optical traps (142), as well as attempts to extend SM-SERS to other types of related spectroscopies such as coherent anti-Stokes Raman scattering (143) are also appearing more often in the literature. Although it is too soon to tell, some of these concepts/studies may become crucial to understand specific aspects of SM-SERS.

5. SM-SERS APPLICATIONS: STUDYING OTHER PROPERTIES OF SINGLE MOLECULES

As defined above, the second type of SM-SERS applications aims at using the SM-SERS technique as a tool to understand aspects of Raman scattering. Here, we briefly review a few salient examples from our own contributions in the context of work done by other groups.

5.1. Raman Properties of Single Molecules

One of the most salient characteristics of SM-SERS is that we can measure optical properties that are not affected by inhomogeneous broadening. This is a fundamental aspect of Raman spectroscopy that SM-SERS has been able to unveil, as exemplified by the determination of the resonance excitation spectrum of a single molecule performed by Van Duyne and coworkers (144). Another example of the same idea, is the observation of the homogeneous broadening of Raman peaks in SM-SERS spectra (55) (see **Figure 2** with further explanations in the caption). Last but

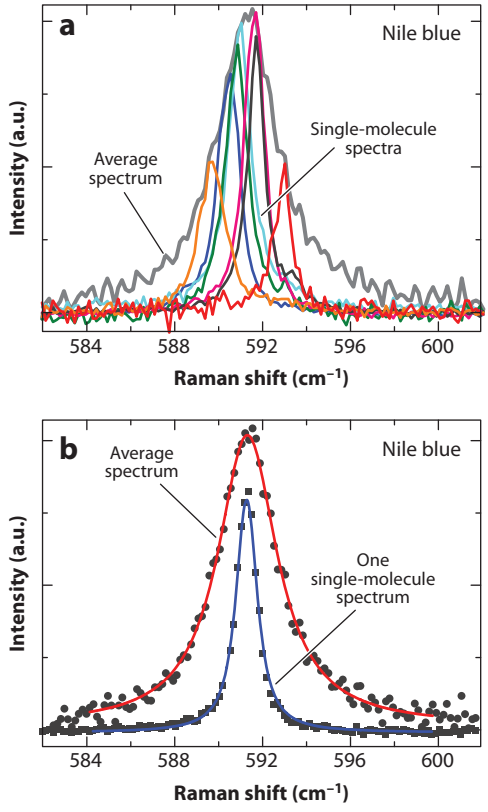


Figure 2

(*a*) Average signal (over 7,500 spectra) on a Raman map (at 77-K, 633-nm excitation) together with different single-molecule (SM) events in the same map. The 590-cm^{-1} mode of Nile blue is measured here with a high-resolution 2,400 lines/mm grating. The SM events are rescaled to follow the average for visualization purposes only. (*b*) Comparison of one individual (typical) SM event (at approximately the average frequency) with the average spectrum. The full width at half maximum (FWHM) of individual SM-SERS events is approximately one-third of the FWHM of the average. This sometimes allows the observation of several SM events simultaneously within the envelope of the average. See Reference 55 for further details. Copyright American Chemical Society, reproduced with permission.

not least, special spectral properties of molecules can be observed with SM-SERS. For example, natural isotopic substitutions can be detected (with some limitations) (54). The example shown in **Figure 3** (from Reference 54) implies a unique situation where a change of one unit mass, in one atom, in one molecule, is directly observed by Raman spectroscopy. Although many of these are textbook-like examples of spectroscopy with no immediate application, they demonstrate the exquisite level of sophistication that SM-SERS, as a tool, has reached. Furthermore, there is the hope that SM-SERS studies will eventually shed some light into subtle aspects of biological processes (145–148). We believe it is only a matter of time before this possibility becomes a reality.

5.2. Monitoring Other Properties of Single Molecules via SM-SERS

Other interesting developments that are branching out from SM-SERS as a technique are worth mentioning. One notable example is the study of SM conductance and/or SM heating properties

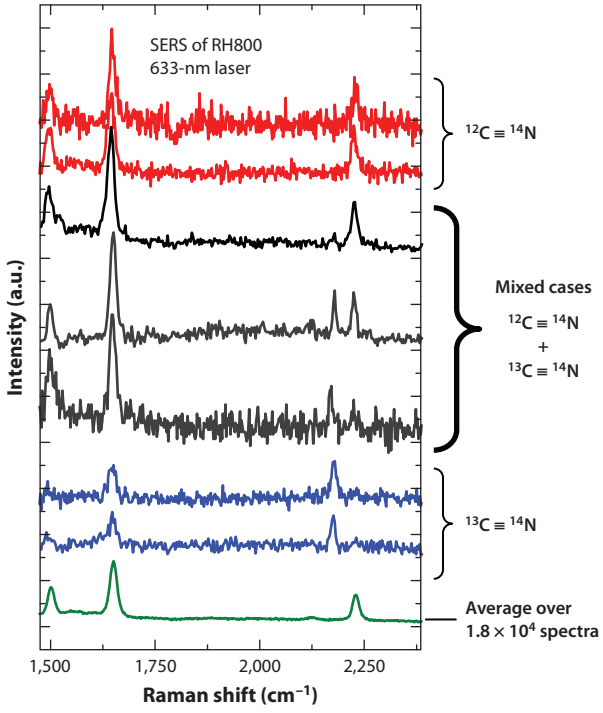


Figure 3

Single-molecule surface-enhanced Raman spectroscopy (SM-SERS) spectra of rhodamine 800 (RH800) in solution [Lee & Meisel (47) Ag colloids]. (*Red spectra*) Normal Raman spectra displaying the $^{12}C{\equiv}^{14}N$ cyano bond at 2,230 cm^{-1}. (*Black spectra*) Mixed cases where there are at least two molecules in the spectra: one with a normal $^{12}C{\equiv}^{14}N$ bond and one in which the carbon atom of the cyano bond has been replaced by ^{13}C (thus resulting in a softening of ~56 cm^{-1} for the cyano bond vibration). (*Blue spectra*) SM-SERS for molecules with $^{13}C{\equiv}^{14}N$ only. (*Green spectra*) Average (18,000 spectra) spectrum. The 1,650 cm^{-1} mode of RH800 remains unchanged in all cases. The SM-SERS spectra for $^{13}C{\equiv}^{14}N$ (*blue*) imply the detection of a change of one unit mass in one atom ($^{12}C \rightarrow {}^{13}C$) in one molecule (RH800). See Reference 54 for further details. Copyright American Chemical Society, reproduced with permission.

(149, 150). These experiments combine two techniques that would have been unthinkable even a few years ago: simultaneous transport and Raman properties at the SM level. Last, but not least, the idea of controlling SM-SERS signals through electrochemical means (151) is particularly attractive at the SM level (56), as it can provide a glimpse of electrochemical phenomena that cannot be observed in ensemble averages. Reference 57, for example, demonstrated the step-like nature of the oxidation/reduction process at a SM level (using the bi-analyte technique) following an underlying potential in a specially prepared cell that combines the simultaneous conditions to perform electrochemistry and SM-SERS. As can be appreciated in **Figure 4**, it is possible to observe the step-like nature of the oxidation/reduction process of individual molecules. Depending on the local redox potential of the molecule being observed, the oxidation/reduction points are not the same for different molecules. The statistics over several SM events, by contrast, recovers the macroscopic electrochemical behavior of the ensemble average measured at much higher molecule concentrations (57). In this manner, aspects of the electrochemistry of single molecules that had never been observed before become accessible. We believe these results open interesting future possibilities, in particular in connection to the redox processes of biologically relevant molecules.

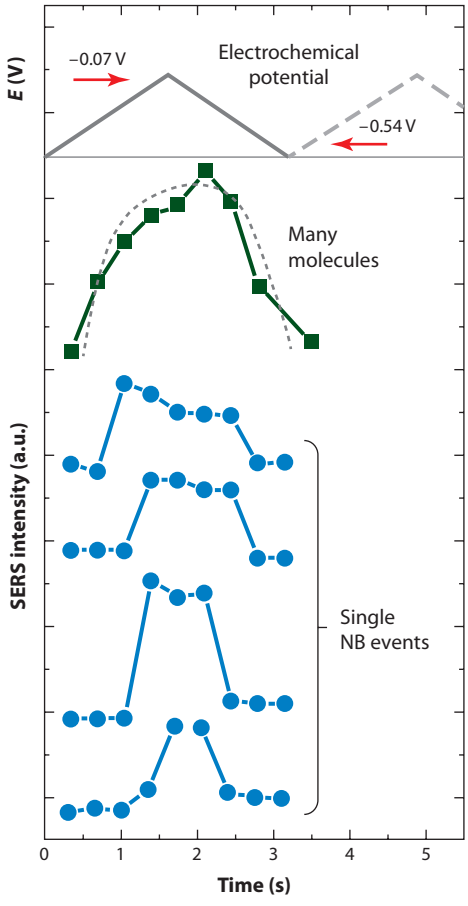


Figure 4

Redox properties of different single-molecule surface-enhanced Raman spectroscopy (SM-SERS) events monitored with the intensity of the 590 cm^{-1} mode of Nile Blue (NB) in an electrochemical cell (from Reference 57). The experiment uses the bi-analyte SERS method (49) with RH6G and NB as partners, but it only concentrates on SM-SERS signals from NB that are affected by the electrochemical potential (*shown at the top*). The Raman signal from many molecules (*green*), measured at much higher concentrations, displays a smooth intensity profile (dome-like) that follows the electrochemical potential. On the contrary, signals from SM display sharp transitions between oxidation/reduction; these are triggered at different times along the cycle depending on the local redox potential being experienced by that particular molecule. A histogram of the distribution of SM oxidation and reduction events with the applied potential over many molecules recovers the standard voltammogram for NB (57). This is an example of new phenomenon that is available only for observations due to developments in the field of SM-SERS. See Reference 57 for further details. Copyright American Chemical Society, reproduced with permission.

6. CONCLUSIONS

Single-molecule SERS is still "history in the making." The long list of more than 100 references noted within the Literature Cited section of this article is far from complete, and it is unavoidably biased toward our taste and preferences. But they represent only the tip of the iceberg of a field that has grown enormously from its humble beginnings and heated debates begun just over a decade ago. We believe SM-SERS will soon enter a "mature" stage (if it is not already there),

where the reality of SM-SERS will be taken for granted: At such a stage, SM-SERS will be used to better understand the SERS effect and to unveil new phenomena that have remained out of reach before with measurements of ensemble-averaged signals. An example of the type of possible future study is the electrochemistry of single molecules mentioned in the previous section, but the list is likely to enlarge very quickly in the next few years and may develop into further subfields. It is interesting to realize (with the benefit of hindsight) that some of the conditions used to observe SM-SERS today were available, in principle, more than 20 years ago. Many SERS spectra taken at the time were probably, in retrospect, SM-SERS spectra. But it has taken much time and a great deal of ingenuity to unveil effects that have been literally in front of our eyes for quite a while. It is our hope that the future path of SM-SERS as a technique will be equally surprising and unpredictable when viewed in retrospect.

DISCLOSURE STATEMENT

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Errata

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