

# Single molecule analysis by surfaced-enhanced Raman scattering†

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Our main objective in this *tutorial review* is to provide insight into some of the questions surrounding single molecule detection (SMD) using surface-enhanced Raman scattering (SERS) and surface-enhanced resonance Raman scattering (SERRS). Discovered thirty years ago, SERS is now a powerful analytical tool, strongly tied to plasmonics, a field that encompasses and profits from the optical enhancement found in nanostructures that support localized plasmon excitations. The spectrum of the single molecule carries the quantum fingerprints of the system modulated by the molecule–nanostructure interactions and the electronic resonances that may result under laser excitation. This information is embedded in vibrational band parameters. The dynamics and the molecular environment will affect the bandwidth of the observed Raman bands. In addition, the localized surface plasmon resonances (LSPR) empower the nanostructure with a number of optical properties that will also leave their mark on the observed inelastic scattering process. Therefore, controlling size, shape and the formation of the aggregation state (or fractality) of certain metallic nanostructures becomes a main task for experimental SERS/SERRS. This molecule–nanostructure coupling may, inevitably, lead to spectral fluctuations, increase photobleaching or photochemistry. An attempt is made here to guide the interpretation of this wealth of information when approaching the single molecule regime.

## Introduction

Single molecule detection is the science of identifying  $1/N_A$  ( $1.66 \times 10^{-24}$ ) mol or 1.66 yoctomol of a substance. For analytical chemistry of solutions, this can mean acquiring the spectroscopic signal arising from a *ca.* 10 picoliter volume of a  $10^{-10}$  molar solution of the analyte.

Detecting the spectroscopic signal from such a small fraction requires extraordinary absorption or emission properties of the analyte. The straightforward approach is to probe a distinct molecular absorption (different from the background solid, liquid or monolayer matrix), by pumping an electronic transition of the analyte and monitor the resulting fluorescence. Most of the work on single molecule spectroscopy has

been done using this technique.<sup>1</sup> The success of this method indicates that the emission process is highly efficient; in fact the cross section for chromophores with large quantum yields can be as high as  $10^{-16}$  cm<sup>2</sup> per molecule ( $10^{-2}$  nm<sup>2</sup> per molecule), allowing the acquisition of SM fluorescence spectra with good signal to noise ratio (SNR). However, the molecular information one obtains from fluorescence, at room temperature, is very limited. Raman scattering is an alternative optical spectroscopy that provides a vibrational spectrum that is far richer in information.<sup>2</sup> Unfortunately, the inelastic scattering from molecules, in contrast, is a very inefficient process, with cross sections as low as  $10^{-30}$  cm<sup>2</sup> per molecule ( $10^{-16}$  nm<sup>2</sup> per molecule).

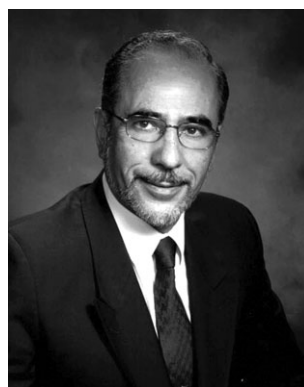
There is another class of materials with scattering cross sections that notably surpass those of fluorescence, metal nanoparticles. For instance, 30 nm diameter spheres of Ag, Au, Cu and Al may have scattering cross section between  $10^{-12}$  and  $10^{-13}$  cm<sup>2</sup> when excited at their localized surface

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plasmon resonance.<sup>3,4</sup> This extremely efficient process is, of course, elastic scattering (Mie scattering).

The cross section is a property of the molecule or the nanostructure that carries the dimension of  $\text{cm}^2$ , and can be defined as the proportionality factor between the incident beam  $I_0$  (a flux of photons  $\text{s}^{-1} \text{cm}^{-2}$ ) and the scattered light  $I_{m,n}$  (photons  $\text{s}^{-1}$ ), where, in the case of a molecule,  $m$  and  $n$  are molecular quantum states. For inelastic scattering, the relations are:

$$\begin{aligned} I_{m,n} &= \sigma_{\text{RS}} I_0 \\ I_{m,n} &= \sigma_{\text{RR}} I_0 \\ I_{m,n} &= \sigma_{\text{SERS}} I_0 \\ I_{m,n} &= \sigma_{\text{SERRS}} I_0 \end{aligned} \quad (1)$$

where  $I_{m,n}$  is the total amount of scattered light (scattering sphere including all polarizations).

The dispersion of the Raman cross section reaches a maximum when the laser frequency of excitation is in full resonance with that of a molecular electronic transition, a process known as resonance Raman scattering (RRS). Under such conditions cross sections as high as *ca.*  $10^{-24} \text{cm}^2$  per molecule have been measured.<sup>2</sup> Typical fluorescent probes in single molecule fluorescence (SMF) have cross sections of at least  $10^{-17} \text{cm}^2$ , and this is generally taken as the threshold that needs to be surpassed for SMD. It can be seen then that even in the event of the best RRS systems, we are still seven orders of magnitude away.

The discovery three decades ago of surface-enhanced Raman scattering (see Kerker,<sup>5</sup> p. 3), brought to light the link between the strong elastic scattering by metal nanostructures and the weak inelastic scattering by molecules. An excellent sampling of the history and prehistory of SERS/SERRS can be found in Kerker's book.<sup>5</sup> The SERS/SERRS experiments show unequivocally that giant enhancements (several orders of magnitude) of the inelastic scattering of molecules adsorbed at, or near, the surface, are observed for metal nanostructures which sustain localized surface plasmon resonances. These include metal colloids, metal island films and roughened electrodes. It also became clear with time that the "surface enhancement" was indeed a "nanostructure mediated enhancement", and SERS substrate fabrication is now a fast developing branch of nanotechnology.<sup>6,7</sup> This signal amplification could be attributed primarily to the electromagnetic (EM) mechanism, and enhancement factors (EF) for isolated metallic particles were predicted to be from  $10^3$  to  $10^7$  (see Barber<sup>5</sup> page 475 and Kerker<sup>5</sup> page 417).

A comprehensive discussion of the SERS cross sections, as well as, definitions, magnitude and guide to the measurement of EFs has been just published,<sup>8</sup> and this is an important reference source for communicating SERS/SERRS results. For the purpose of our discussion, the EF is simply the ratio of two cross sections:

$$\text{EF} = \frac{\sigma_{\text{SERS}}}{\sigma_{\text{RS}}} \text{ or } \text{EF} = \frac{\sigma_{\text{SERS}}}{\sigma_{\text{RRS}}} \quad (2)$$

In retrospect, based on the calculated EFs, enhanced RRS (SERRS) could have qualified for single molecule spectroscopy. In 1997, the first two independent reports on SMD by

enhanced Raman scattering were published.<sup>9,10</sup> One of these studies was indeed a clear case of SERRS, where the laser excitation frequency (514.5 nm) was in resonance with both the surface plasmon of the silver colloids and the molecular absorption of rhodamine 6G.<sup>9</sup> Nevertheless, the authors went on to claim EFs of the order of  $10^{14}$  to  $10^{15}$ . However, in the second report,<sup>10</sup> although the analyte was also a dye which strongly absorbs in the visible (crystal violet), the laser frequency used (830 nm) was outside the high intensity region of the molecular absorption band, thus demonstrating that it is also possible to detect a non-resonant single-molecule (SERS) on aggregated colloidal Ag. The authors pointed out that the effective cross sections that would explain their SMD were at least  $10^{-17}$ – $10^{-16} \text{cm}^2$  per molecule. For a molecule such as this, outside the resonance absorption of the dye the Raman cross section would be *ca.*  $10^{-28} \text{cm}^2$  per molecule, based on that value, only an EF of  $10^{11}$  would be needed for SMD.

Over 10 years have passed since these initial reports, and a vast body of evidence has confirmed the validity of SM-SERS and SM-SERRS. The following sections include a discussion of the main elements involved in SMD, beginning with a look at the type of nanostructures and molecules that have been studied to date. This is followed by a description of the SMD experiment and the characteristics of the spectrum of a single molecule.

### Nanostructures for SMD

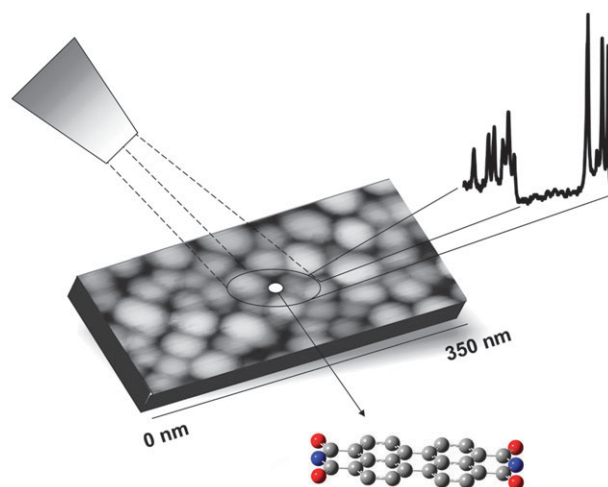
The seminal observations by Kneipp *et al.*<sup>10</sup> and Nie and Emory,<sup>9</sup> not only demonstrated that it is possible to achieve single-molecule detection using SERS/SERRS; but they raised several important questions about the unique role of "hot particles" that later evolved into "hot spots" in aggregated nanostructures.<sup>11,12</sup> The concept of hot spots captures the realization that local electric fields in aggregated nanostructures supporting LSPR may display large spatial fluctuations. In some nanometric spatial locations (hot spots) the local electric field may exceed the applied field by several orders of magnitude. Hot spots have become the "holy grail" of SERS investigations, with an ongoing quest for fabrication methods that produce nanostructures containing hot spots for use in SERS/SERRS applications<sup>13</sup> spurring a whole body of research onto itself. In addition, the natural link between SERS and LSPR has attracted expertise from the field of plasmonics. As it has come to be understood, surface-plasmon based photonics or *plasmonics* is a term used to identify a branch of photonics that harness the properties of surface plasmons, connecting photonics and electronics.<sup>14</sup> Surface plasmons provide the opportunity to confine light to very small dimensions and the basic support comes from the physics of surface plasmons that deals with the properties of electromagnetic fields on the nanometric spatial scale and femtosecond times. Therefore, research on SMD using SERS/SERRS is intrinsically connected with developments in plasmonics. There is an extensive body of literature that could be included here to illustrate this interconnection; however, a few details should be sufficient in the context of the present review. To begin, efficient near field molecule-nanostructure coupling and energy transfer mediated by surface plasmons has been demonstrated.<sup>15a</sup> In addition, for an

isolated gold nanoparticle the homogeneous linewidth of the surface plasmon resonance has been measured to be  $\sim 160$  meV, which in the time domain corresponds to 8 femtoseconds,<sup>15b</sup> while vibrational lifetimes in molecules are in the picosecond range. Although the field enhancement distribution around a single particle of silver and gold varies considerably with the particle shape,<sup>16</sup> the hot spots seems to occur at localized areas of intense EM fields that exist in nanostructured thin metallic films and clusters of colloidal metal nanoparticles, not strictly organized matter. The interstitial spatial location of enhanced field has been extensively investigated using the two sphere model, a model that is mathematically tractable and provides a good physical insight into the effect of the EM enhancement and the coupling with the molecular excitations.<sup>17</sup> The more efficient Ag or Au aggregates, or island films seem to add two very important features for practical SMD: a spatial distribution of hot spots and the ability to measure SM-SERS using a non-resonant molecule with a broad range of excitation wavelengths.<sup>18</sup> The spatial concentration of field enhancement in silver island films is clearly presented in the work of Shubin *et al.*,<sup>19</sup> where the inhomogeneity length scales (oblate grains of silver) were much smaller than the wavelength of the incident radiation, a condition required for SMD using SERS. The presence of multiple resonances with highly location-dependent frequencies have been captured in near-field images<sup>20</sup> of nanostructures used for SMD (*i.e.* colloidal aggregates), and they can provide a rationale for the peculiar features observed in the spectrum of the single molecule. The experimental evidence we have today, strongly suggest that SMD under non-resonance Raman conditions requires the presence of hot spots.

Recent reports provide experimental evidence that SMD can also be achieved under RR conditions using gold or silver tips whose apex has a nanometric radius of curvature. This approach, known as tip-enhanced resonance Raman scattering (TERRS), is the inverse of other SERS experiments by having the enhancing substrate approach the analyte.<sup>21,22</sup> In a recent TERRS experiment from Pettinger's group,<sup>21</sup> approximately five molecules of the dye malachite green isothiocyanate on Au(111) were probed by an STM gold tip with a curvatures of 20 nm onto which a laser beam (632.8 nm) is focused. Similarly, single molecule tip-enhanced resonance Raman spectra from brilliant cresyl blue adsorbed on a planar Au surface was obtained using a modified Ag tip.<sup>22</sup> Theoretical models and experimental evidence suggest that the tip behaves as a single particle, and estimations of the field enhancement indicate that the EF from such a structure may not alone be enough for SMD SERS experiments.<sup>17</sup> Indeed, the reported TERRS results benefit from the resonance Raman effect (malachite green or brilliant cresyl blue are used), and the required EM enhancement for SMD could be only of the order of  $10^7$  or less. Single molecule TERRS seems as this point in time unlikely.

In summary, SMD is today strongly linked to LSPR supporting nanostructures with the following connotations:

1. Nanostructures for SMD detection are entirely restricted to silver and gold, or their composites.
2. The most successful nanostructures for SMD are aggregates (disordered matter and fractals) where hot spots are



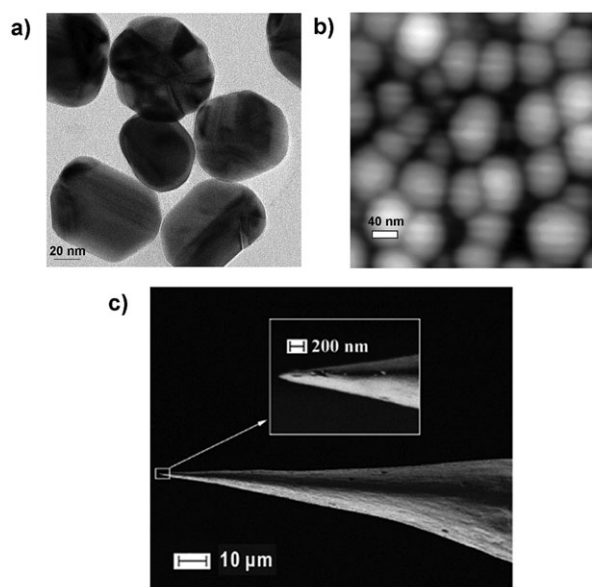
**Fig. 1** The fundamental components for surface-enhanced Raman microscopy.

realized. There has been, and continues to be, a healthy discussion in the literature regarding the extent of EM enhancement at hot spots and on the enhancement necessary to achieve SMD.<sup>8,11,23</sup>

3. The acceptance of hot spots brings about the inevitable conclusion that in a SERS experiment there is an unequal contribution of the molecules to the observed SERS signal, where the lion share of the signal must come from molecules on hot spots.

4. Tip-enhanced resonance Raman scattering approaches SMD by taking advantage of the RRS effect and the EM enhancement that can be provided by the tip ( $10^6$ – $10^7$ ).<sup>21</sup>

5. In addition, the observations may carry the signature of the chemical interactions molecule–nanostructure, electronic



**Fig. 2** A sampling of the types of nanostructures that have demonstrated SM sensitivity. (a) TEM of Colloidal Silver nanoparticles. (b) AFM of evaporated silver island film (c) TEM of a silver STM tip. (Reprinted in part with permission from ref. 22. Copyright 2007 American Chemical Society.)

interactions that can modulate the observed frequencies and relative intensities and also change the absolute Raman cross section. The set of these factors is commonly discussed under the umbrella of “chemical effects”.<sup>4</sup>

6. For clarity, it is necessary to distinguish two regimes for SERS/SERRS observations: (i) the ensemble average SERS/SERRS with well defined and time independent frequencies, bandwidth (full width at half maximum–FWHM) and relative intensities. (ii) SERS/SERRS obtained when the statistical average breaks down and the number of scatterers contributing to the recorded spectrum approach the limit of one, the regime of ultrasensitive detection and SMD. Frequencies, FWHM and relative intensities are modulated by the molecular environment (the molecular sensor). Nanofabrication of silver nanoparticles of different shape using wet chemistry,<sup>24</sup> or advanced lithographic techniques that allows control over particle-shape, size, and inter-particle separations, such as nanosphere lithography<sup>25</sup> and electron-beam lithography, can produce excellent substrates for ensemble average SERS, but not for SM-SERS.

### The molecules of SM-SERRS and SERS

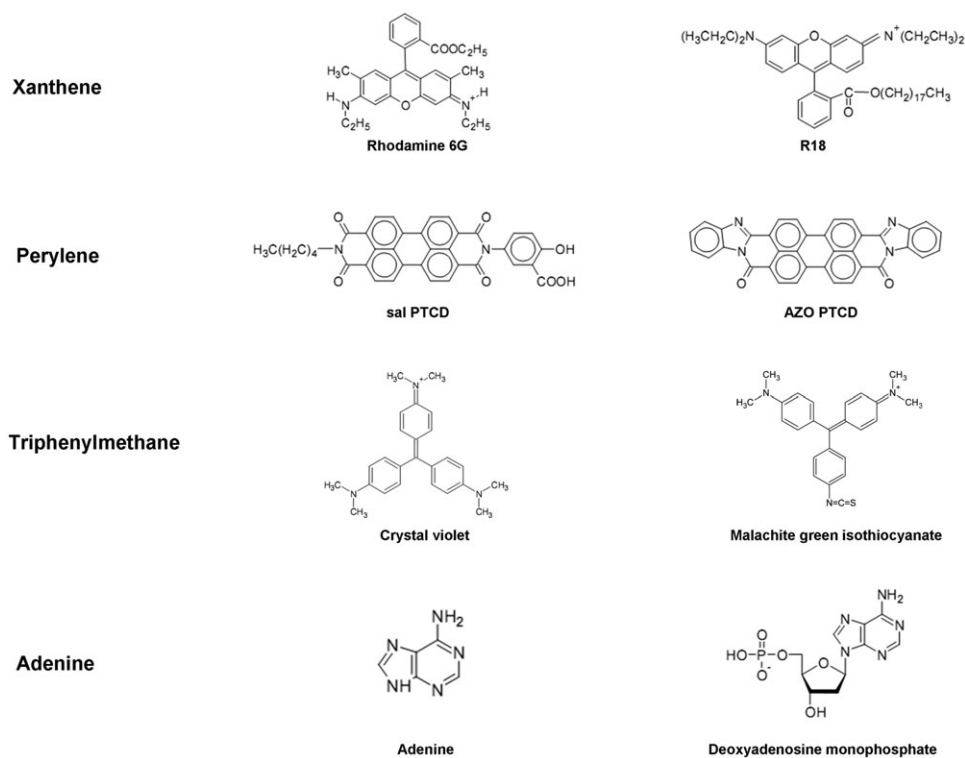
Apart from the TERRS experiments, all of the data available speak to the need for hot spots in SMD using SERS/SERRS, *i.e.*, for the detection of a single molecule’s vibrational fingerprint, it is necessary (not sufficient) to couple the target vibrating molecule to surface plasmon supporting nanostructures capable of producing spatially distributed enhanced electric fields and multiple plasmon resonances. Here we will briefly review the coupling molecules that have thus far been reported in SMD experiments. The structure for a handful of these molecules for which SMD has been reported is given in Fig. 3. One finds at least 50 experimental reports on SMD†. Out of these 50, fifteen are results obtained with Rhodamine 6G,<sup>9,26</sup> including deuterated R6G,<sup>27</sup> seven are derivatives of the 3,4,9,10 perylene tetracarboxylic acid diimide (PTCDI),<sup>28</sup> five are on porphyrin containing molecules,<sup>29</sup> four are on crystal violet,<sup>10,23</sup> two cyanine derivatives,<sup>23</sup> two on malachite green (one using TERRS<sup>21</sup>), two on adenine, also deoxyguanosine monophosphate and deoxyadenosine monophosphate,<sup>30</sup> Horseradish peroxidase,<sup>31</sup> green fluorescent protein,<sup>32</sup> and fluorescent protein allophycocyanin,<sup>33</sup> DNA bases,<sup>23</sup> Enkephalin (peptide),<sup>34</sup> fluorescein,<sup>35</sup> brilliant cresyl blue (TERRS<sup>22</sup>), thionine,<sup>36</sup> and *para*-mercaptoaniline.<sup>37</sup> An excellent review with references to the original SMD work is available.<sup>14</sup> The common denominator to all these molecules is the presence of a “soft” highly polarizable moiety such as an aromatic, or groups containing electron rich heteroatoms, such as sulfur. Correspondingly, these molecules possess sizable Raman scattering cross sections.

Raman instruments are generally equipped with laser lines in the visible (488 to 785 nm); thereby the majority of detected molecules for SMD are dyes that strongly absorb in this region of the electromagnetic spectrum, profiting from the large cross section of RRS (as high as  $10^{-24}$  cm<sup>2</sup> per molecule). Similarly, charge-transfer complexes that absorb in the visible will also be very active SERR scatterers. However, the same puzzle that was there at the beginning of SERS is there for SMD: why do

we see the enhanced Raman scattering from adsorbed pyridine molecules; but not from the background water in the electrochemical experiments? It would seem that the explanation given then is the same for SMD, the difference in the scattering cross section! For instance, the difference between the scattering cross section of  $\beta$ -carotene and water at 514.5 nm excitation in the 1500–1600 cm<sup>-1</sup> Stokes spectral region is  $\sim 10^8$ . Therefore, a modest enhancement of  $10^6$ – $10^7$ , would make possible the SMD of the dye, while the water molecules in the background will be silent. In practical analytical spectroscopy, this large variation in cross sections is well known and incorporated in many analytical protocols. The large fluorescent cross section of chromophores can be a nuisance in Raman spectroscopy, where negligible amounts of any fluorescent material can overwhelm the signal of the Raman scattering from the bulk. Contrary, this can be beneficial, for a small amount of a dye can be easily picked out of a mixture with non-absorbing materials by RRS. SMD profits from these differences and at the same time the experimentalist can identify interferences arising from impurities with electronic resonances in the region of excitation, such as those from the ubiquitous carbon background<sup>38</sup> due to carbon resonances in the visible. There are groups of molecules that have vibrational modes with a very small Raman cross section, such as, aliphatic hydrocarbons, aliphatic alcohols, fatty acids, *etc.*, and it is unlikely that the SMD of methanol using SERS will be reported any time soon! In our own work, we take advantage of this property by fabricating two dimensional structures using the Langmuir–Blodgett (LB) technique, where the matrix is a fatty acid (arachidic acid or stearic acid) doped with a dye.<sup>28</sup> The dyes are dispersed in a mixed Langmuir monolayer of a fatty acid that readily forms high quality LB monolayers on silver island films which have been evaporated onto glass substrates, at varying concentration levels, down to a single monomer of dye within the probed surface area illuminated by the objective.

SMD using SERS (not SERRS) has been recently discussed by K. Kneipp and H. Kneipp.<sup>23</sup> Nevertheless, they used visible dyes and near-infrared excitation (830 nm), away from their main electronic absorption band. Most notable is their use of pseudoisocyanine (PIC) and 5 M methanol, with an average of one PIC molecule adsorbed onto a Ag nanoaggregate and  $10^{14}$  methanol molecules in the probed volume. Since the SERS signals from a single PIC molecule appear at the same signal intensity level as that of the non-enhanced Raman signal of the  $10^{14}$  methanol molecules, an apparent SERS enhancement factor of fourteen orders of magnitude is obtained. However, the RS cross section of PIC may be several orders of magnitude, higher than that of methanol and, correspondingly, the EF would be orders of magnitude smaller as shown for the case of crystal violet.<sup>8</sup>

A daunting challenge to SM-SERS is that at very low concentrations, SMD critically depends on the structure of the molecule for its selective adsorption, particularly, onto hot spots. This is the question Vosgroene and Meixner<sup>39</sup> have explored in a SERRS study on a series of xanthene molecules (a rhodamine family of molecules). They found that for several rhodamine dyes the minimal detectable concentration critically depends on the structure of the molecule, for instance,



**Fig. 3** A collection of molecules used in SMD by SERS/SERRS.

they observed that “Shielding of the partially positively charged amino groups requires two to three orders of magnitude higher concentrations.” These observations highlight the fundamental role of molecular adsorption in SERS/SERRS experiments, whether you are working in the ensemble average or ultrasensitive detection.

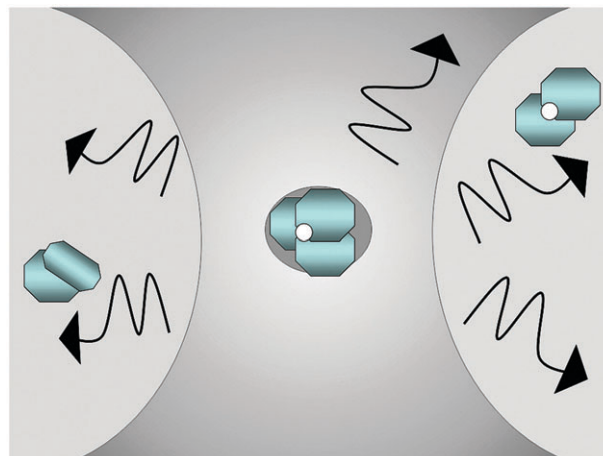
In summary, although the ensemble SERS/SERRS has been observed for all types of molecular systems, granted with modest EFs, SMD has only been observed for a very limited selection of chemical structures, in particular, molecules containing “soft” moieties. Though SMD by SERS has been demonstrated for a small number of cases, exploiting the additional enhancement of RRS (double resonance) is the most accessible technique, allowing SMD to be achieved with a larger selection of substrates of Ag or Au, as well as with the use of TERRS.

### The SM experiment

The bulk of the work reported on SMD has been carried out with aggregated colloids of silver and gold, nanostructured surfaces of silver and gold prepared by vapour deposition, and modified STM Ag and Au tips. From the point of view of the laser excitation, there are two distinct groups of experimental conditions; excitation in the visible, and excitation in the near-infrared (830 nm). Experiments in the visible using colloids and island films are carried out using very low energy densities, on the order of  $\mu\text{W } \mu\text{m}^{-2}$  or  $\text{nW } \mu\text{m}^{-2}$ , in order to avoid photobleaching and carbon interferences. However, using near-infrared excitation, the energy densities reported are of the order of  $100 \text{ mW } \mu\text{m}^{-2}$ . The latter's large difference in energy densities more than compensates for the decrease in

the magnitude of the molecular cross section as we move into the near-IR region of the spectrum. Since plasmon multi-resonances in Ag or Au aggregates allow for hot spots in the visible and the near-IR spectral regions, the advantages of working in the near-IR are several: it is possible to work with much higher energy densities, processes such as photodissociation and photobleaching are far less likely, interferences from contaminants which are resonant in the visible; such as graphitic carbon, are avoided. In addition, the detected signal is reported to show little fluctuation in contrast to those acquired with visible excitation.

Regardless of the type of nanostructure used, the methodology of the SMD is similar. Two key elements are needed: the



**Fig. 4** The combination of small probe area and dilute concentration of the target molecule is necessary for SMD experiments.

limiting of the probe volume and the dilution of the analyte to such levels that there are only a few molecules in the probed area. The latter has been made possible by the advent of high throughput micro-Raman spectrometers with excellent quantum efficiency CCD detectors which have ushered in a new era of Raman analysis. In fact, it has been Raman microscopy's ability to probe such small areas and volumes that has made SMD possible. Developments in this direction are ongoing, as evidenced by the combining of scanning probe microscopy and the detection of the inelastic light scattering in TERRS.

For solutions (colloids) and island films this volume reduction is facilitated by the use of objectives with high numerical aperture values with collection volumes in the range of nanoliters to femtoliters. In conjunction with limited probe volumes, the use of extremely dilute concentrations of the target analyte is also a characteristic of SM-SERS. Typical concentrations used are on the order of  $10^{-11}$  to  $10^{-14}$  M, with the target molecule dispersed in a solvent or matrix of molecules with a low Raman cross section, such as water, methanol or a fatty acid.

For samples such as LB monolayers on island films and other immobilized nanostructures, the probed surface area is on the order of  $1 \mu\text{m}^2$ . In TERRS experiments, sub-wavelength resolution is achieved through the extent of near field at the apex of the metal tip. The combination of dilute solutions with Raman microscopy leads to experimental conditions where, on average, there are only a very few molecules fixed to, or in proximity of the enhancing substrate within the scattering/probed volume.

Herein lays one of the greatest challenges to SMD through SERS. Unlike SM fluorescence which relies on an intrinsic property of the molecule (quantum efficiency), SM-SERS is facilitated by the plasmonics of a substrate. Further complicating matters, the areas of enhancement necessary for SM-SERRS compose a very small percentage of the enhancing nanostructure surface. As a result the probability of a target molecule residing in a hot spot is rare. There is, at present, no method to create reproducible, well characterized structures that produce the enhancement needed for non-resonant SM-SERS. While there have been great strides with nanolithography, the fabricated structures fail to support hot spots for SM detection. This elusive nature of SM-SERS sensitive substrate is a major challenge and has been christened as the "SERS uncertainty principle".<sup>40</sup>

Experimentally then, outside the work of Kneipp *et al.* using excitation in the near-IR, SMD studies have used excitation lines in the visible that are close to, or coincide with, a molecule's electronic resonance so as to benefit from the increased Raman cross section associated with resonance Raman scattering. While this increase in scattering efficiency improves the limits of detection, it brings additional complexity to the SM-SERRS experiment, including the increased chances of photochemistry and photodegradation.

It is important to consider the geometry of the experiment, for this too may affect the observed spectra. Two additional experimental parameters that fall under this category are: the molecular orientation on the surface of the metal nanostructure (surface selection rules), and the polarization (direction of the electric field) of the incident radiation. There is a great deal

of work demonstrating the dependence of not only the field enhancement, but also spectral properties such as relative peak intensities, on the polarization of the exciting electric field. Due to the complex plasmonics of SM sensitive substrates it is virtually impossible to know *a priori* the polarization for maximum enhancement. When the laser polarization does coincide with that of a hot spot, maximum enhancement is achieved.

Regardless of the substrate used, these experimental variables have an impact on the success or failure in the detection of a SM-SERS signal. A more in depth discussion of these issues can be found in the literature.<sup>38,41,42</sup>

### LB SM-SERRS: An example of the ensemble to single molecule

The Langmuir–Blodgett technique is based on the fact that certain classes of molecules, such as amphiphilic molecules with hydrophilic "heads" and hydrophobic tails, will organize themselves on the surface of a subphase (typically water) in a single molecular layer. This layer can be carefully transferred to a substrate through a controlled deposition as shown in the cartoon of Fig. 5. The advantage of LB films is that they allow control of molecular architecture such as orientation and intermolecular distances. The limitation of the LB approach is that very few molecules can be used in the fabrication of LB monolayers. For molecules that can be incorporated into LB monolayers, the concentration of target analytes can be controlled with a great deal of certainty. This can be accomplished by reducing the concentration of target analyte in a spectrally "inert" matrix (a molecule with a very low Raman and SERS cross section compared to the target analyte), typically a fatty acid such as arachidic acid. The use of monomolecular films reduces the

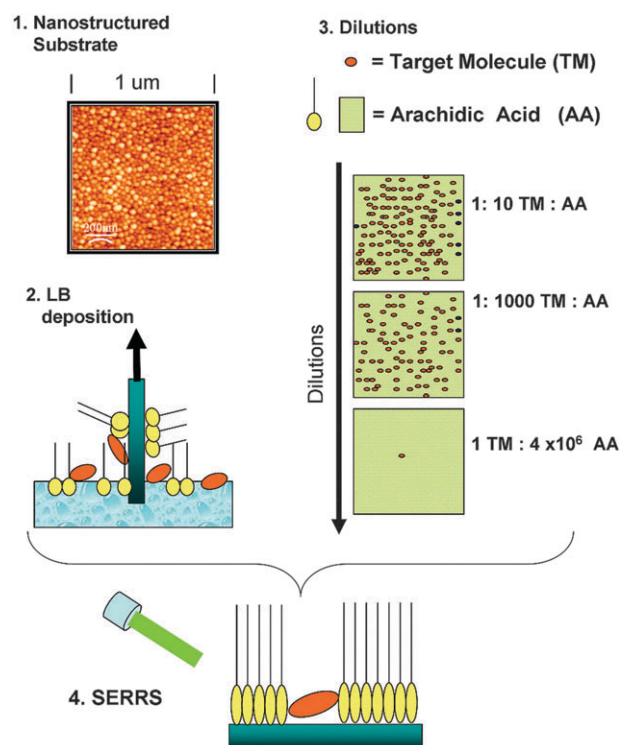
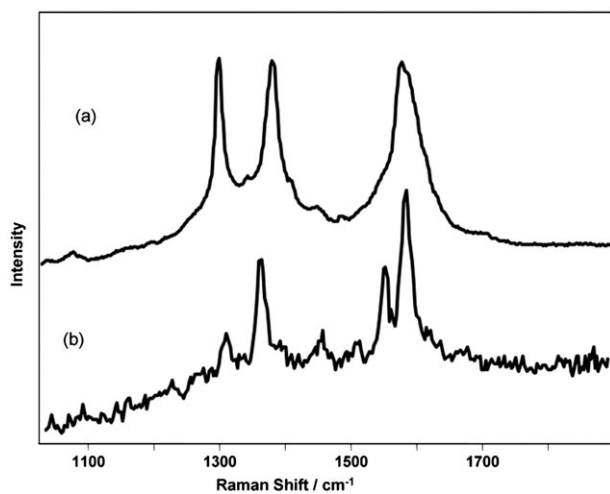


Fig. 5 A schematic of the Langmuir–Blodgett approach to SM-SERRS using island films.



**Fig. 6** A Comparison between the ensemble SERRS (a) to that of a single molecules (b). The target molecule is *N*-pentyl excited with 514.5 nm, from Ag/Au island film.

probe volume to a probe area. The reduction from 3D to 2D greatly reduces the ambiguity of analyte concentration.

The substrates used in these studies are metal evaporated island films of silver or silver/gold mixture. The resultant films are composed of nanoparticles with a range of diameters 40–80 nm's, with a particle density  $\sim 500$  particles per  $\mu\text{m}^2$ . The advantages of films such as these are the multi-resonances the surface provides. As such they can be used with a variety of molecules with excitations throughout the visible. When this method is coupled with the spatial resolution and sensitivity of Raman microscopy, the systematic analysis of the breakdown of the SERRS signal from ensemble to SM is possible. Our group has successfully applied this technique to the detection of the SM-SERRS signal of several chemical systems. In particular we have been able to investigate for the first time the spectral features of overtones and combinations at the single molecule level.<sup>43</sup>

The LB films are constructed to have on average between  $2 \times 10^6$  to 1 molecule in the probe area which for our system is *ca.*  $1 \mu\text{m}^2$  using a  $50\times$  objective with 514.5 nm excitation. The analytes used in our studies all absorb *ca.* 500 nm, hence under resonance Raman conditions, therefore the laser power at the sample is kept low ( $< 20 \mu\text{W} \mu\text{m}^{-2}$ ) for all measurements to avoid photodissociation. In Fig. 6, the differences between the ensemble SERRS measurement taken from a sample with a surface density of  $10^6$  molecules per square micrometre, to that of a SM can be seen. The most prominent change is in the bandwidth of the peaks. The source of the broadening seen in the ensemble spectrum is not well understood but may be a result of the many surface sites contributing to the signal. Other characteristics that differentiate the two different regimes are in the changes seen in the spectra collected from different locations on the surface. In the case of the ensemble, the characteristic SERRS spectra are virtually uniform spot to spot. This breaks down when surface coverage approaches 1000 to 100 molecule per square micrometer. At these regimes, spectral variations are seen from spot to spot, with differences in relative intensities, bandwidth and frequency recorded.

The statistical nature of the SERRS mechanism is demonstrably by the systematic Raman mapping of the surface at the various surface coverage's in conjunction with extending the approach to bi-analyte studies as presented in Fig. 7. With the bi-analyte approach, the recording of the fingerprint spectrum of the one or the other of the targets, but never together, at low surface concentrations, gives further evidence that it is indeed a SM-SERRS spectrum being recorded.

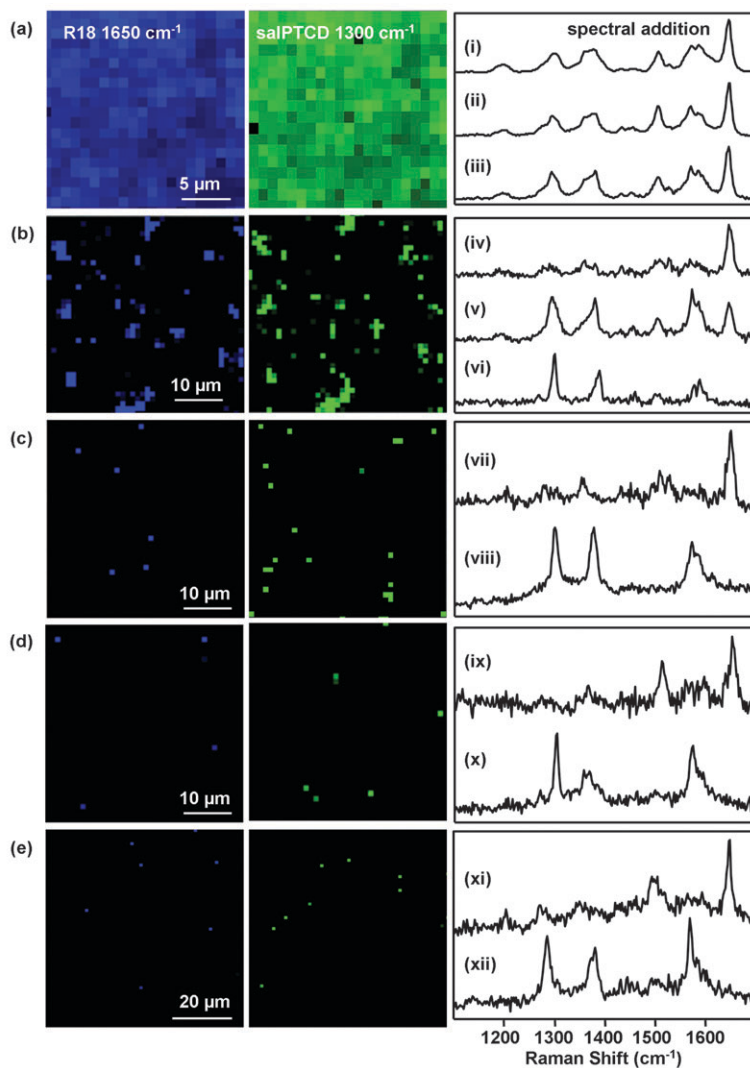
### The spectrum of a single molecule

Unlike SM fluorescence, where the quantum nature of the system is manifested in properties of the measured signal, at present there is not a single unique qualifier to verify, without a shadow of a doubt, that it is indeed a SM signal being recorded. Instead, the validity of the SM claim is supported by a collection of evidence. It should be noted that the behavior or characteristics of the SM signal is very dependent on the method of sampling, which makes attempts to establish an encompassing set of criteria difficult. The impact of different measurement configurations is most readily seen in the "statistics" and "fluctuations" seen in SM studies. As an example, static experiments such immobilized nanoparticles and TERS are very susceptible to photo-driven processes that can affect the molecule-metal interactions which can give rise to fluctuations in the SERRS signal.

Again there is no uniform behavior as these changes are strongly dependent on the type of molecular system being studied and the degree of chemical interaction between the molecule and the metal. So the question everyone is asking; What is the evidence supporting the claim that the recorded spectrum emanated from a single molecule? The most common signature of the SMD is fluctuations of the signal; meaning changes in the frequency, bandwidth, relative intensity and, sometimes, an "on and off" signal (blinking). Fluctuations have been observed in all the SMD SERRS, including TERS, experiments. These variations can be seen under constant illumination and collection as well as in variation between different SM spectra collected for the same system. It has been suggested (Futamata<sup>26</sup>) that temporal fluctuations in SM-SERRS (blinking) is likely due to a thermal diffusion of adsorbate molecules from the hot spot to other ordinary sites. Recently, a statistical analysis of the temporal evolution of SM-SERS spectra has been reported that seems to provide further information on the factors determining temporal fluctuations.<sup>29</sup> Here, photodynamics (photobleaching, photo-reactions or photodesorption) plays a crucial role in the observing of on-and-off signals. Unfortunately, these types of fluctuations seem to be more a signature of the statistic breakdown than of a single molecule event.

### Conclusion

Single molecule detection using SERS/SERRS and nano-structures is still at an early stage of scientific development, holding enormous wealth for potential applications. SMD using the double resonance of SERRS is the most accessible methodology, and molecular dyes are obvious targets for single molecule studies. There is also irrefutable experimental evidence that SMD can be achieved with

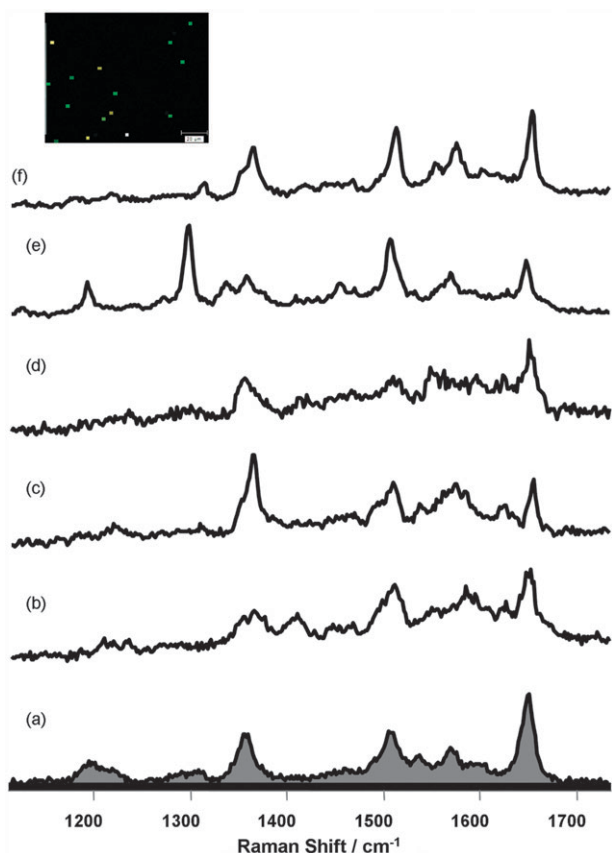


**Fig. 7** 2D SERS mapping signal for a bi-analyte system. Maps are based on the characteristic band for each dye, R18 and salPTCD, at concentrations of (a)  $4 \times 10^4$ ; (b)  $10^3$ ; (c)  $10^2$ ; (d) 10; and (e) 1 molecule(s) per  $\mu\text{m}^2$ . (Reprinted with permission from ref. 28. Copyright 2007 American Chemical Society.)

molecular dyes; but with excitation frequencies far from the dye's absorption band (for instance, 830 nm), *i.e.*, SMD using SERS. It is broadly accepted that SMD expresses dependence between two independent variables: the hot spots and the molecule. Since hot spots are frequency dependent spatial locations of highly enhanced electric field in complex nanostructures (aggregates or fractals), the adsorption of the molecule precisely into that spot has a probability that depends on several experimental variables such as: nature of the adsorption, surface charge and molecular charge distribution, adsorption kinetics, surface diffusion and temperature. Assuming that the "hot spot-molecule" is a necessary duality for SMD, the sufficiency of a molecule's scattering cross section is determined by the enhancement factor of the hot spot. The EF at a hot spot is a lift greater than  $10^6$ . The EF of  $10^6$  can be seen as the upper limit of the "average SERS enhancement" with well determined, time independent, vibrational characteristics. With the high throughput Raman instrumentation and high sensitivity

detectors the "tip of the iceberg" detected in SMD of strongly absorbing dyes using SERS could be as low as  $10^6$ . For SERS experiments, where the radiation is out of resonance with the molecule, the EF needed could be *ca.*  $10^{11}$ . SMD detection at the low end of EF (less than  $10^8$ ) has been reported using gold tips in TERS experiments and, among many others, including our own work using Langmuir-Blodgett monolayers doped with dye on silver island films. The high end of EF is that of the SMD using excitation outside the absorption band. These soft molecules normally have vibrational modes with high cross sections that could be detected at the single molecule level with EF in the  $10^{11}$  range. The use of a bi-analyte experiment as proof of SMD proposed by Etchegoin's group<sup>8</sup> requires the assumption that the molecule resides in a hot spot with EF in the high end, so that the signal will stand out. The same bi-analyte approach implemented with two dyes in an LB monolayer clearly proves that, at the single molecule level, each one of them is detected separately confirming a localized enhancement for each





**Fig. 8** SM-SERRS spectra collected from a mixed LB film of the TRITC dye–arachidic acid with a surface coverage of 1 molecule per  $\mu\text{m}^2$  deposited on a silver island film. The insert map was generated from the component analysis using the reference ensemble spectrum (a). The spectra (b)–(f) are sampling of the SM spectra detected from the 1500 hundred collected with 514.5 nm excitation.

molecule. However, in the latter case, the EF does not have to be at the higher end.

The future of SM-SERS/SERRS will demand from experimentalists and theorists a great deal of effort to clear up all the details in SM spectra and the taming of hot spots in nanostructures. The pursuit of SMD for applications in ultrasensitive chemical analysis justifies the effort. However, the most important and fascinating implications and applications may come from the advancement of our knowledge of the coupling between molecules and surface plasmons, the consequences of helping to connect the photonics with the electronics through plasmons that can be manipulated by their coupling with single molecules. The interaction of molecular systems with confined electromagnetic waves in metallic nanostructures is clearly the main subject of study and will no doubt be the source of new knowledge in SM-SERS and SM-SERRS.

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