Statistical analysis of intensity fluctuations in single molecule SERS spectra

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We have investigated the fluctuations of the intensity and the line intermittency in the surface enhanced Raman spectra of a single iron–protoporphyrin IX molecule. A statistical analysis has revealed a high correlation between the intensity of each frequency couple in the spectrum. Removal of the continuum background has led to a suppression of the correlation at those frequencies where no Raman lines are present. Conversely, we have observed the persistence of a strong correlation at the intensities corresponding to the vibrational modes of the molecule. Further evidence of correlation between the intensities and the background signal indicates that the background is involved in the enhancement mechanism. Moreover, analysis of the Raman line intermittency reveals a random activation of the different molecular vibrational modes. These results can be generally put into relationship to the presence of two different contributions to the intensity fluctuations: one, strictly related to the continuum background, and affecting the whole spectrum, and another one which selectively acts on the various vibrational modes of the molecule.

Introduction

Surface enhanced Raman spectroscopy (SERS) is a powerful microanalytical technique which may couple single molecule (SM) detection capability with a high content of chemical information.[1–4] Such a capability can, on one hand, be exploited for applicative aims, *e.g.* in nanomedicine or nano-technology.[5–7] On the other, it allows one to investigate some fundamental aspects of molecular dynamics usually obscured in conventional Raman experiments.[8–11]

The huge enhancement in SERS spectra, occurring when a molecule is adsorbed on a rough metal surface, is generally assumed to arise from two, likely cooperating, mechanisms: an electromagnetic (EM) local field enhancement, associated with surface plasmon oscillations, and a so-called chemical effect requiring a tight interaction of the molecule with the metal surface.[1,2] The chemical contribution might involve charge transfer (CT) excitations between the molecule and the metal.[12–14]

SERS spectra share with other spectroscopies, such as fluorescence, the appearance of drastic intensity fluctuations and blinking (line intermittency) when the SM regime is approached.[8,11,15,16] The richness of SERS signals makes the investigation of such a temporal variability extremely rewarding.[9,17,18] Indeed, the temporal evolution of SM SERS spectra reflects different processes occurring at the molecule–metal interface with a strong dependence on the molecular nanoenvironment. In particular, they may involve a gating of the

vibrational modes of the molecule, whose temporal emission has been recently shown to follow Levy statistics; being indicative of a complex temporal behaviour.[19] Furthermore, SERS spectra are always characterized by the presence of a continuous background, which is underneath the Raman lines; such a background, whose origin is still debated, continuously changes in time, modulating the intensity of the spectrum at all the frequencies.[8,20]

On such a basis, a statistical analysis of the temporal variability in SM SERS spectra could help in the elucidation of the processes occurring within a single molecule placed at a rough metal surface and interacting with light. Actually, it could allow one to extract dynamical information encoded in the spectra, and to clarify the mechanisms underlying the SERS processes. Here, we focus our attention on the correlation properties of both the intensity fluctuations and the intermittency of the Raman lines by analyzing sequences of several SERS spectra, each one with 1 s integration time. As the target molecule, we have chosen iron–protoporphyrin IX (FePP), an organic molecule widely studied by SERS even at SM level,[10,20,21] which also has some relevance in applicative schemes.[22,23]

A cross-correlation analysis has been performed on the intensity fluctuations detected at several couples of frequencies selected within the whole spectrum. We found a strong correlation in the intensity fluctuations for each frequency couple. Such a result points to a common origin for the intensity fluctuations of intensities at all the frequencies. Instead, upon removing the background from each spectrum, high correlation values only persist in correspondence with the frequencies of the main vibrational modes of the FePP molecule. This indicates that the background not only affects the

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Raman intensity of the whole spectrum, but it is also involved in the enhancement process of the Raman signal. Furthermore, a correlation analysis in the line appearance evidenced a random activation of the different modes which probably follow independent activation channels.

Materials and methods

Solutions of colloidal silver particles were prepared by standard citrate reduction of $AgNO_3$ (Sigma) by the procedure of Lee and Meisel;[24] the concentration of silver particles being about 10^{-11} M, corresponding to about 7×10^{12} particles per litre. As activation agent, NaCl was added to reach the final concentration of 0.25 mM (for more details see ref. 25).

Fe–protoporphyrin IX (FePP) (Sigma) was dissolved into a KOH solution 0.5 M at pH = 12 at a concentration of 5×10^{-6} M. An aliquot of successive dilutions of this solution was incubated with silver colloidal suspension for 5 h at room temperature to obtain a concentration of 10^{-11} M with an approximate 1 : 1 ratio between molecules and colloidal particles. A droplet (about 10 μl) of the solution was deposited onto a glass slide of area 15×15 mm^2.

SERS spectra from FePP were collected with a confocal Jobin-Yvon SuperLabram equipped with a 514.5 nm radiation line of an Argon laser. The illumination and back-scattering collection system consists of a confocal microscope coupled to a single grating spectrometer (300 mm focal length spectrograph with a 1800 grooves mm^{-1} grating) and a liquid nitrogen cooled back illuminated CCD detector. The microscope objective was 100× with NA = 0.9, producing a laser spot size of about 1 μm in diameter. The spectral resolution is lower than 5 cm^{-1}. The laser power was been kept below 2 mW; for each spectra series having been checked that the Raman fingerprint of FePP were preserved.[26]

Active sites, characterized by an intense Raman scattering, were searched by manually scanning the sample under the microscope objective of the Raman equipment; sites where only noise is registered were discarded. Sequences of SERS spectra were acquired in an automatic way by recording each spectrum with 1 s integration time, followed by a deadtime of 1 s; each sequence was constituted by 600 spectra. Spectra series with deadtime in the range of 1–5 s were also acquired as a control.

A preliminary analysis was performed by checking the vibrational modes in the spectrum derived by summing all the spectra in the sequence. Only sequences characterized by summed spectra clearly showing the vibrational fingerprints of FePP were taken into account for successive investigations.

The total noise N_t of a SERS signal can be determined as the root of the sum of the square noise components: $N_t = [(N_R)^2 + (N_{SN})^2 + (N_D)^2]^{1/2}$, where the readout noise N_R, and the dark charge noise N_D, are specified by the chip manufacturer; N_{SN} is the shot noise associated with the signal and whose amplitude is given by the square root of the measured signal. For our CCD chip, N_R was 5 electrons rms and N_D one electron pixel^{-1} h^{-1}. The CCD chip had, at a wavelength of 514.5 nm, a quantum efficiency of about 0.92 with one count per two collected photons. In a typical SM SERS experiment, for a rate of about 100–500 counts per second, a total noise,

dominated by the shot noise, of about 10–22 counts for a 1 s integration time can be evaluated.[3,10] Under the assumption of shot noise, the spread of the Raman intensity around the average value has been usually described in terms of a Poisson distribution with a standard deviation equal to N_t.[3] However, a recent revisit of the statistics of SM SERS spectra argues against such a conclusion.[27]

Results and discussion

Fig. 1A shows the temporal intensity trend of a sequence of 600 SERS spectra from a bright site of a dry drop of silver colloidal solution incubated with FePP at a concentration of 10^{-11} M; each spectrum having been collected with 1 s integration time. Drastic intensity fluctuations can be observed in the region between 1250–1650 cm^{-1}, in which the main vibrational modes of FePP are located (see the continuous curve in Fig. 1B). On the other hand, significant intensity
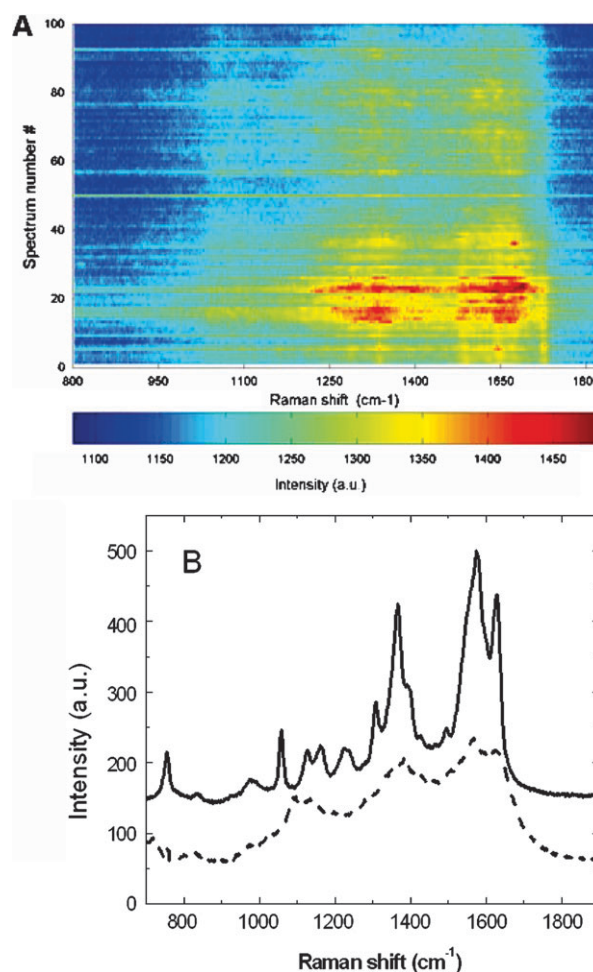

Fig. 1 (A) Two-dimensional intensity trajectory of SERS spectra with 1 s integration time of immobilized silver colloidal particles incubated with FePP at a concentration of 10^{-11} M. (B) Continuous line: spectrum from a dry drop of FePP in bulk (10^{-6}) M in alkaline aqueous with 100 s integration time (for a complete attribution of the bands see ref. 21). Dashed line: spectrum as obtained by summing 600 SERS spectra of FePP at 10^{-11} M with 1 s of integration time. The spectral resolution is less than 5 cm^{-1}.
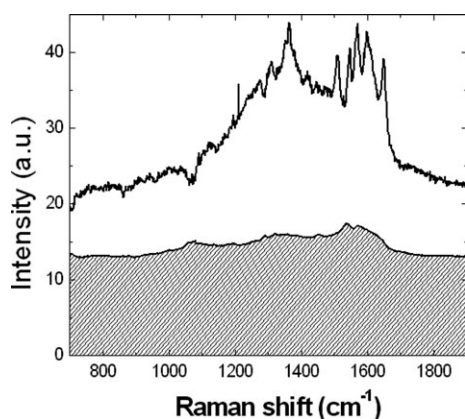
Fig. 2 Standard deviation (continuous line) and shot noise (dashed region), as a function of the Raman shift, as derived from a sequence of 600 SERS spectra.

Fig. 3 Squares: spectrum number, for eight frequencies, corresponding to the main FePP vibrational modes, at which a line (*i.e.* a peak) overcoming a threshold of 2σ above the noise level is detected for in FePP at 10^{-11} M. Continuous line: spectrum as obtained by summing 600 SERS spectra of FePP at 10^{-11} M with l s of integration time.

fluctuations also appear in correspondence with the frequencies at which no Raman lines are detected (see *e.g.* the 1000 and 1800 cm^{-1} regions). Intensity fluctuations are a common feature of SERS spectra in the SM regime,[8,9,11,15] even if their presence does not guarantee that only a SM is observed; in other words, the possibility of having more than one emitting molecule in the laser spot cannot be ruled out. Generally, intensity fluctuations progressively disappear when spectra with longer integration times are taken into account. On the other hand, the overall spectral features of FePP can be recovered by summing several SERS spectra (see the dashed curve in Fig. 1B); this being equivalent to recording a spectrum with a long integration time (ergodic case).[10]

Fig. 2 shows the standard deviations of the intensity, as a function of the Raman shift, evaluated from the sequence of 600 spectra (continuous line); the corresponding shot noise of the intensity (see Materials and methods) are also shown (dashed region in Fig. 2). The standard deviation values are significantly higher than those of shot noise at all the frequencies; this indicating that the intensity is affected by additional phenomena.

As already mentioned, SERS spectra in the SM regime also reveal an intermittency in the line appearance, *i.e.* lines corresponding to the molecular vibrational modes randomly appear from spectrum to spectrum. Such an aspect is evidenced in Fig. 3 which shows the spectrum number in the sequence (squares), at which each of the analyzed lines appear. It has been assumed that a line is detected when the intensity at the corresponding frequency, within a range of 5 cm^{-1}, overcomes a threshold of 2σ above the noise level. All the analyzed lines are characterized by an intermittent behaviour in the sequence; *i.e.* the molecule randomly switches from a bright (on) state to a dark (off) state under continuous laser excitation for a specific vibrational mode.

Now, we would like to address the question of whether there is some interplay between the intensity fluctuations and the appearance intermittency of the Raman lines in the different regions of the spectrum. We have performed a statistical analysis of the Raman intensity applied to sequences of SERS spectra by performing a cross-correlation analysis between the intensities in different regions of the spectrum. The covariance
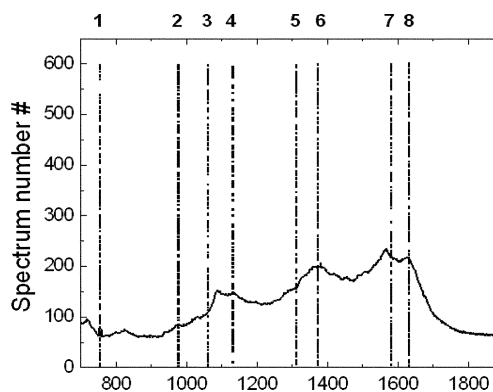
$\sigma_{\nu1\nu2}$ between the intensities at the two frequencies ν_1 and ν_2, at a 0 time delay has been evaluated by:[17]

$$\sigma_{\nu_1\nu_2} = \frac{1}{N}\sum_i [I_i(\nu_1) - \langle I(\nu_1)\rangle][I_i(\nu_2) - \langle I(\nu_2)\rangle] \qquad (1)$$

where $I_i(\nu_j)$ ($j = 1, 2$) is the Raman intensity at the wavelength ν_j at the spectrum number j; $\langle I(\nu_j)\rangle$ is the Raman intensity, at the wavelength ν_j, averaged over all the spectra series of the measurement. Covariance values provide information about the correlation between the intensity fluctuations at two different frequencies. Accordingly, high values for $\sigma_{\nu1\nu2}$ indicate that the intensity at ν_1 fluctuates, around the average value, together with the intensity at ν_2.

A typical two-dimensional (2D) covariance map of the intensity from 600 SERS spectra of FePP in the SM regime is shown in Fig. 4A; for comparison a 2D covariance map as obtained for FePP in bulk is also shown (see Fig. 4B). Interestingly, while the covariance map of FePP in bulk appears very homogenous with low values at all the regions, an inhomogeneous covariance map is observed for FePP at the SM level. Markedly higher values are detected at the points corresponding to the crossing frequencies of the main FePP vibrational modes (1000–1600 cm^{-1}). Covariance maps with similar features have been registered from SERS spectra series of other sites of the sample. These results are similar to those obtained for the intensity fluctuations in the SM SERS spectra of rhodamine.[17]

Furthermore, covariance maps registered at different laser power, in the 0.5–2 mW range, revealed the same features; accordingly photodegradation or thermal effects can be ruled out in the used power range.[26]

Since highly correlated intensity fluctuations appear even at frequencies where no Raman lines are located, it can be inferred that the background is involved in the line intensity modulation. In this respect, we mention that the intensity fluctuations correlation, observed in a single rhodamine molecule, has been ascribed to the continuum background arising from bare silver colloids and fluctuating in intensity.[17] To provide some grounding to such a hypothesis, we decided to
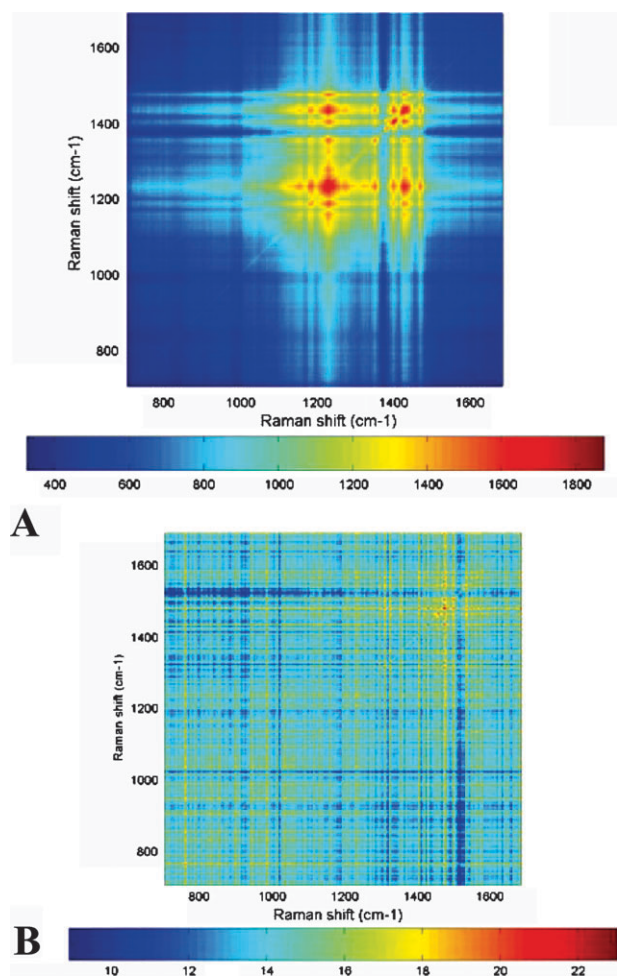
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Fig. 5 Covariance map of intensity fluctuations (see eqn (1)) of FePP at a concentration of 10^{-11} M; the map having average values upon removing the background from each spectrum of the series, which is the same as in Fig. 4A.

Fig. 4 A: Covariance map of intensity fluctuations (see eqn (1)) of FePP at a concentration of 10^{-11} M; the map having an average values of about 800 (c/s)^2. B: of FePP in bulk. The maps have been obtained by applying eqn (1) and averaging over 600 SERS spectra, recorded in sequence with 1 s integration time; the map having an average value less than 18 (c/s)^2.

perform a correlation analysis, after removing the background signal from the SERS spectra. The resulting 2D covariance maps obtained after removing the continuum background from each spectrum of the analyzed sequence are shown in Fig. 5. In particular, the value obtained by averaging the intensity at 1800–1850 cm^{-1} has been subtracted from the intensity at the different analyzed frequencies. By comparing the map in Fig. 5 with that in Fig. 4A, we note a drastic reduction of the covariance values in most parts of the map. Almost zero values are observed in the regions where only the background signal is present. Furthermore, high covariance values persist at the points of the map corresponding to the frequencies of the main FePP vibrational modes. An analysis of the covariance maps from different spectra series has provided evidence of similar patterns; this being indicative of the covariance not being strongly dependent on the specific accessibility of the Raman vibrational modes. In addition, we found that high covariance has been detected in correspondence with the Raman lines; some relationship between that line intensity and the covariance can be then hypothesized.
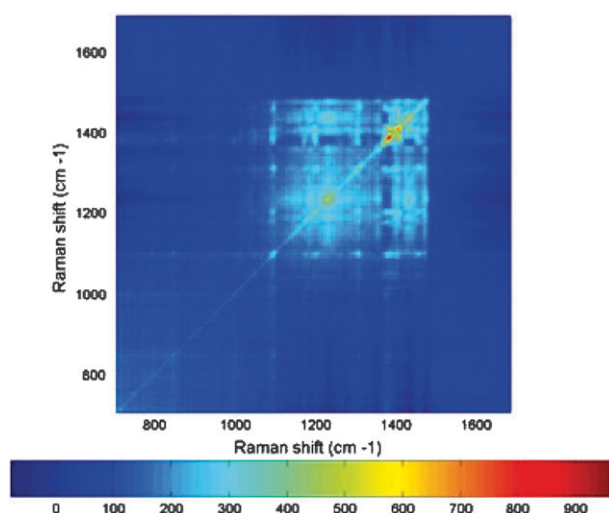
These results clearly point out that the observed correlation in the intensity fluctuations cannot be simply attributed to the background signal. Instead, such a persistence reflects the presence of a mechanism which simultaneously affects the intensity of the different Raman lines in the spectrum.

The background has been hypothesized to be due to Raman inelastic scattering processes deriving from electron–hole excitation in a nanostructured metal surface under light illumination.[13] In particular, it could arise from a CT between the metal and the adsorbed molecule: under light illumination, an electron can be occasionally pushed out from the metal by filling a molecular orbital of the adsorbed molecule with available π^* orbitals.[2] In our case, FePP, characterized by the presence of π^* orbitals, can receive an electron from the metal giving rise to an electronic coupling between the molecule and the metal. This is supported by the evidence of a continuous switching between the Fe^{2+} and Fe^{3+} oxidation states in SM SERS measurements of FePP on silver nanoparticles.[10] On the other hand, the crucial role played by the CT mechanism in SM SERS spectra has been recently demonstrated by analyzing a system in which the EM enhancement mechanism was ruled out.[28] We can therefore assume that the CT process could trigger the enhancement mechanism.[2] Such a CT process could both modulate the intensity fluctuations of the whole spectrum contributing to the continuum background, and can yield a selective activation of the molecular vibrational modes.

To investigate whether the appearance of the different lines in a SM spectrum is correlated in time, we have performed a different kind of analysis by focusing our attention on the lines. In particular, we have applied eqn (1) by assuming that the intensity $I_i(n)$ is one or zero depending on whether, at the analyzed frequency, a line is detected or not (see also the legend of Table 1). Table 1 reports the covariance values for each couple of lines, after averaging over a sequence of 600 spectra. The diagonal points in the map correspond to the occurrence of the line in the analyzed sequence, while the

Table 1 Covariance values of the line appearance in the SERS spectra of FePP at a concentration of 10^{-11} M, obtained by applying eqn (1) in which the intensity $I_i(n)$ has been taken as one or zero depending on whether, at the analyzed frequency, a line is detected or not, respectively

Frequency /cm^{-1}	(1) 760	(2) 980	(3) 1055	(4) 1150	(5) 1310	(6) 1370	(7) 1580	(8) 1630
(1) 760	145	43	37	28	33	45	30	38
(2) 980		160	33	32	37	52	43	38
(3) 1055			130	24	30	43	31	38
(4) 1150				132	34	32	27	37
(5) 1310					140	40	32	44
(6) 1370						169	40	45
(7) 1580							146	45
(8) 1630								185

out-of-diagonal points give the covariance values. For the diagonal points in the map, we found values between 130 and 185, with an average of 150. By taking into account the fact that 600 spectra constitute our series, the latter value corresponds to an average occurrence of about 25% for the simultaneous appearance of two different lines in the spectrum; similar values having been registered for other sequences of spectra. From the out of diagonal points, covariance values ranging between 30–55 with an average value of about 45 have been observed. By taking into account that values between zero (no correlation at all) and the number of times for a line detection (diagonal values) are expected, these results indicates a low level of correlation for line appearance. Accordingly, the simultaneous activation of the analyzed FePP vibrational modes are generally characterized by a low correlation. More specifically, some modes are almost independent, while other ones could be partially correlated. For instance, we have previously demonstrated that the lines 1363 cm^{-1} and 1375 cm^{-1}, related to the ν_4 band marking the iron oxidation state,[21] are alternately detected during the measurements.[10] On the other hand, a rather high correlation has been observed in the pyrrole ring vibrational modes.

The emerging picture for the behaviour of a molecule adsorbed on a rough metal surface under laser illumination is rather involved. The drastic temporal fluctuations in the Raman signals are characterized by different features. One contribution simultaneously affects all the vibrational modes of the molecule and it is likely strictly related to the enhancement process; the other one selectively activating one or more vibrational modes of the molecules. In this respect, a statistical analysis is an extremely useful tool helping to disclose the large amount of information encoded in the temporal evolution of SM SERS spectra.

Conclusions

Our results show that covariance analysis of SERS signals from samples in the SM regime can provide information on the SERS mechanisms as well as on the processes occurring at the SM level. We found that the fluctuating background plays a crucial role in the intensity fluctuations likely to be acting on the enhancement process. At the same time the evidence that the vibrational modes of the molecule substantially follow independent activation channels indicates a complexity in the SM SERS emission. A statistical analysis of the SERS signals from a SM sample can help to extract information on the behaviour of SM on the rough metal surface by opening new perspectives in understanding the mechanisms acting on molecules adsorbed on nanostructured metal surfaces.

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