Surface-Enhanced Resonance Raman Spectroscopy Signals from Single Myoglobin Molecules

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The extremely large cross-section available from metallic surface enhancement has been exploited to investigate the Raman spectrum of heme myoglobin adsorbed on silver colloidal nanoparticles at very low concentrations. The study has been performed on particles both in solution and immobilized onto a polymer-coated glass surface. In both the cases, we have observed striking temporal fluctuations in the surface-enhanced resonance Raman spectroscopy (SERRS) spectra collected at short times. A statistical analysis of the temporal intensity fluctuations and of the associated correlations of the Raman signals has allowed us to verify that the single molecule limit is approached. The possible connections of these fluctuations with the entanglement of the biomolecule within the local minima of its rough energy landscape is discussed.

Index Headings: **Surface-enhanced resonance Raman spectroscopy; Single molecule; Silver colloids; Myoglobin.**

INTRODUCTION

The development of ultra-sensitive spectroscopic techniques now makes possible the detection and the identification of single inorganic and organic molecules.[1–5] This opportunity opens new perspectives into the knowledge of the fundamental dynamic processes at the single molecule level. In particular, it appears to be extremely promising for the investigation of protein systems whose dynamics exhibit a large variety of motions regulating the biological functionality.[6,7] Indeed, protein dynamics spans a wide range both in the spatial and the temporal scale: from fast, local motions of atoms or groups of atoms, to slow collective motions involving a large portion of the macromolecule. A protein macromolecule during its function continuously samples many slightly different states, local minima of the protein energy landscape, called conformational substates (CS).[8] Therefore, a spectroscopic response coming from an ensemble of biomolecules would be inhomogeneously broadened as a consequence of the conformational heterogeneity of the ensemble.[9–11] By approaching the single molecule level, phenomena usually hidden due to ensemble averaging can be directly observed and, in addition, the real dynamic behavior of the biomolecule can be followed.[12,13]

Besides providing otherwise inaccessible information on protein systems, single molecule detection could open exciting new possibilities in many areas, e.g., single embedded molecules can provide a wealth of information on the local properties of complex materials such as biological membranes,[14] living cells,[15] and organic polymers.[16] In addition, single molecule detection and manipulation represent necessary steps to build biosensors and electronic nanobiodevices[17,18] as well as to reach DNA sequencing without gene amplification.[19] However, applications in these emerging fields require, in addition to a high sensitivity detection, a detailed knowledge of the temporal behavior of single molecules and a great ability in controlling the spatial location of these molecules. For such a purpose, many different spectroscopic approaches can be used; one of these is Raman spectroscopy, which, providing a fingerprint of the molecular structure, is a valuable tool for the identification of chemical species. The extremely low efficiency of Raman spectroscopy can be overcome by using the dramatic enhancement of the cross-section (up to 10^{14} times) as reached by adsorbing the target molecules onto nanometer–sized metallic particles or onto rough metallic islands (surface-enhanced Raman spectroscopy (SERS)).[20] A further increase of the sensitivity can sometimes be obtained by coupling SERS with resonance Raman spectroscopy (SERRS). The giant SERS and SERRS cross-sections have been generally attributed to two, likely cooperating, effects: the enhancement of the local electromagnetic (EM) effect close to the metallic surface through interaction with surface plasmon excitations, and the enhancement of the Raman scattering due to specific interactions of the adsorbed molecule with the metal surface (charge transfer (CT) effect).[21–23] The capabilities of SERS and SERRS have been recently exploited to investigate the vibrational properties of organic molecules[22–26] and proteins[3,27] at the single molecule level.

Here we investigate the SERRS spectra of myoglobin (Mb) at the very low concentration of 10^{-11} M, adsorbed on silver nanoparticles about 70 nm in diameter. Myoglobin is a monomeric protein, containing a single heme group, that has assumed a paradigmatic role through several theoretical and experimental approaches in the general investigation of the structure-dynamics-function relationship in proteins.[28] The vibrations of the biologically active prosthetic heme group are selectively enhanced, by adsorption on metal nanoparticles, over modes from the protein backbone, as recently shown in a SERRS study on the four-heme-containing hemoglobin.[3] With Mb, while such a property is exploited to approach the single molecule detection, one does not have to deal with additional heterogeneities that might arise from the presence of more than one Raman active heme group, which could make the interpretation of the temporal behavior more involved.

Two different approaches have been followed. First, we have analyzed the SERRS spectra of Mb silver colloidal particles in solution by controlling the number of particles in the scattering volume. Second, the Raman spectra of located brightly emitting colloidal particles immobilized at a very low concentration onto a polymer-

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coated glass surface have been analyzed. In both the cases, significant fluctuations of the Raman intensity have been observed in the different regions of the vibrational spectrum; such a phenomenon has generally been attributed to a deviation from the ensemble average.[2,3] A statistical analysis of the intensity fluctuations of some significant Raman lines for both diffusing and immobilized particles has allowed us to put into evidence that in both cases, the single molecule limit is reached within our experimental conditions. In addition, a detailed analysis of the SERRS spectra of the immobilized Mb colloidal sample, in which the same molecule is followed in time, has revealed that a significant correlation appears in the fluctuations of the Raman signals collected in the different regions of the spectra. Such a result could offer some new insight in understanding the origin of the temporal fluctuations in the single molecule SERRS spectra. Actually, the possibility that the observed fluctuations, reminiscent of those recently observed by molecular dynamics (MD) simulation on a single biomolecule,[29] could reflect the entanglement of the protein into the local minima of the energy landscape during its dynamic evolution is discussed.

EXPERIMENTAL

Sample Preparation and Apparatus. Solutions of colloidal silver were prepared by standard citrate reduction of $AgNO_3$ (Sigma) by following the procedure of Lee and Meisel.[30] As activation agent, NaCl was added to reach the final concentration of 0.25 mM. The colloidal optical extinction spectrum reveals the same features reported in the literature.[3,21] Accordingly, the concentration of silver particles can be estimated to be about 10^{-11} M, corresponding to about 7×10^{12} particles per liter.

Purified horse Mb (Sigma) was dissolved into a phosphate buffer solution at pH 6.8 at a concentration of 1 mM. An aliquot of successive dilutions of this solution was incubated with silver colloidal suspension for 5 h at room temperature to obtain a final Mb concentration of 10^{-11} M with a ratio of 1:1 between molecules and colloids.

A droplet, with an approximate volume of 10 μL, of this solution has been deposited onto a glass slide of area 15×15 mm^2 coated with polymerized 3-aminopropyltriethoxysilane (APES, Sigma). The glass-slide coating, which is able to bind Ag colloids through bonds between the metal particles and the amino-group of APES, was assembled by following the procedure in Ref. 31: cleaned glass slides (3:1 $H_2SO_4 : H_2O_2$, incubated for 30 min), was immersed in a solution of 1 mL of APES in 15 mL of chloroform for 3 min. Last, the slides were rinsed with chloroform upon removal and rinsed twice with MilliQ H_2O.

A characterization of the silver colloidal particles was performed by use of atomic force microscopy (AFM). A Nanoscope IIIA (Digital Instruments) in tapping mode was used to obtain the AFM images of the immobilized samples under ambient conditions. The colloids consist of single spherical and rod-shaped particles and aggregates of two, three, and four up to many particles (see Fig. 1); the heterogeneous size and shape particle distribution was characterized by an average diameter size of about 70 nm.

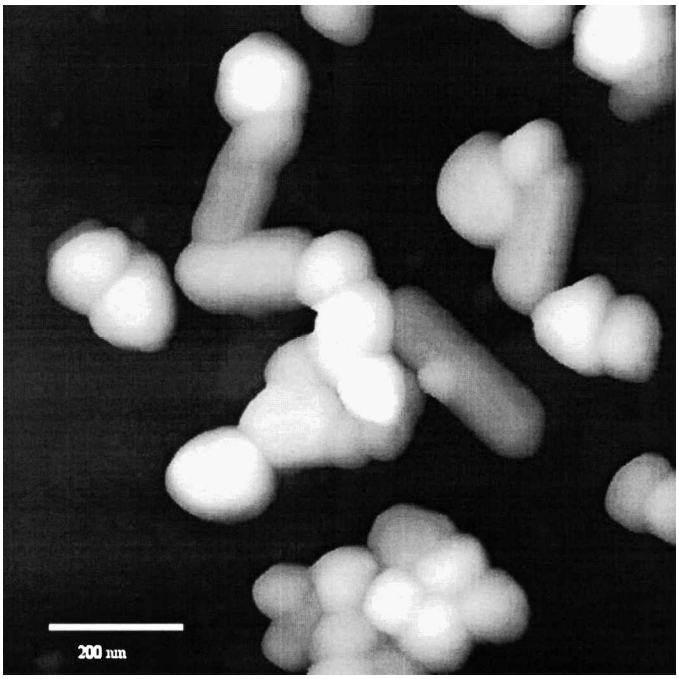

FIG. 1. A tapping mode atomic force microscopy (TM-AFM) image of silver colloidal nanoparticles immobilized on a polymerized glass surface. The colloidal particles have been incubated with 0.25 mM of NaCl. The heights of the aggregate range from 30 to 90 nm. The scan rate is 0.35 Hz.

The SERRS spectra were recorded through a Jobin-Yvon Labram confocal system by exciting with the 632.8 nm radiation line provided by a HeNe 15 mW laser. The microscope (equipped with a 100× objective with NA = 0.9) is confocally coupled to a 300-mm-focal-length spectrograph with an 1800 grooves/mm grating (optimized in the red). A charge-coupled device (CCD) detector, Peltier cooled to 223 K, was employed to record the data. The spectral resolution is lower than 5 cm^{-1}. Liquid samples were analyzed by directly dipping the objective into the solution. The laser spot size is about 1 μm^2 in diameter and the scattering volume is about 5 pL.

Signal and Noise Analysis. In order to discuss the experimental results, it is worthwhile to estimate the signal-to-noise level that we expect in our measurements. The CCD chip has, at a wavelength of 632 nm, a quantum efficiency of about 0.45, and it produces one count per three collected photons.

Generally, the total noise N_t of a signal can be determined as the root sum of squares of the different noise components: $N_t = [N_{SN}^2 + N_R^2 + N_D^2]^{1/2}$ where the read out 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 is 4 electrons rms and N_D is one electron/pixel/min. In a typical single molecule SERRS experiment, a rate of about 50–300 counts/s are usually detected by taking into account the raw data, which include a ground due to a residual fluorescence of the sample. In these conditions, the dominant noise source is the inevitable shot noise associated with the signal itself. Accordingly, a total

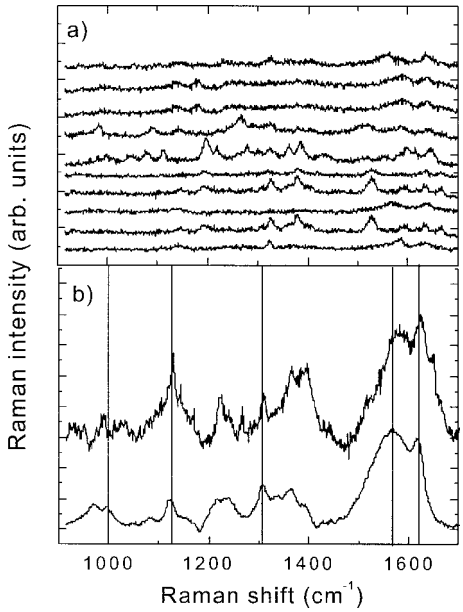

FIG. 2. (**a**) SERRS spectra, as obtained with 1 s of integration time and 60 s between two successive measurements, of silver colloidal solution incubated with Mb at a concentration of 10^{-11} M (protein to Ag particle ratio of 1:1). The spectra have been collected by direct immersion of the microscope objective into the solution. The scattering volume is about 5 pL. (**b**) Upper curve: spectrum obtained by summing 200 SERRS spectra, each one recorded with 1 s of integration time, of Mb silver colloidal solution; lower curve: Raman spectrum of pure Mb, in phosphate buffer at pH = 6.8, at a concentration of 10^{-4} M obtained with an integration time of 30 s. The laser wavelength is 632.8 nm and the incident power is 15 mW. The vertical lines mark the 1000, 1121, 1308, 1570, and 1620 cm^{-1} bands, respectively.

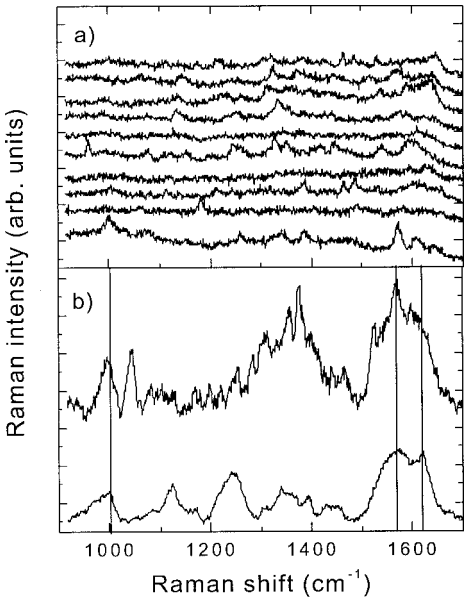

FIG. 3. (**a**) SERRS spectra as obtained with 1 s of integration time and 60 s between two successive measurements, from immobilized silver colloidal solution incubated with Mb at a concentration of 10^{-11} M (protein to Ag particle ratio of 1:1). The laser spot size is 1 μm in diameter. (**b**) Upper curve: spectrum obtained by summing 100 SERRS spectra, each one recorded with 1 s of integration time, from immobilized Mb silver colloidal particles; lower curve: Raman spectrum of dry Mb obtained with an integration time of 30 s. The laser wavelength is 632.8 nm and the incident power is 15 mW. The vertical lines mark the 1000, 1570, and 1620 cm^{-1} bands, respectively.

noise of about 7–18 counts is expected for measurements obtained for an integration time of one second.

As long as the shot noise is dominant, a Poisson distribution with a standard deviation equal to N_t, is expected to describe the spread of the Raman intensity around the average value. However, because of the quite large number of Raman events, a Gaussian distribution with a half-height width σ given by N_t generally well-approximates the intensity spread of a Raman signal.[24]

RESULTS AND DISCUSSION

Surface-Enhanced Resonance Raman Spectroscopy Spectra of Myoglobin at a Very Low Concentration. Figure 2a shows a selected collection of SERRS spectra from silver colloids incubated with Mb at a concentration of 10^{-11} M with an approximate ratio of 1:1 between protein molecules and colloidal particles. The spectra were collected by direct immersion of the microscope objective into the colloidal solution with 1 s of integration time and about 60 s from one measurement to the next. The spectra are characterized by a great variability in both frequency and intensity of the Raman signal from one spectrum to another. On the contrary, a stable, well-characterized spectrum is observed when a pure Mb solution at 10^{-4} M concentration is investigated (Fig. 2b, lower curve). The latter spectrum is well known and arises from a $\pi \rightarrow \pi^*$ optical transition in the heme group resulting in a resonantly enhanced Raman spectrum dominated by in-plane porphyrin ring modes.[33] In particular, the bands at about 1000 and 1121 cm^{-1} correspond to the C_β–vinyl and C_β–methyl stretching in the heme group; the band at about 1308 cm^{-1} is an in-plane vinyl bending; finally, the most marked bands at around 1570 and 1620 cm^{-1}, correspond to in-plane skeletal vibrations of the heme and to two $\nu(C_1 = C_b)$ modes of the vinyl in the heme group, respectively (see the marker-mode lines in Fig. 2b).

However, if several distinct SERRS spectra of the type shown in Fig. 2a are summed up or, equivalently, the spectra are collected for longer integration times, the main vibrational features of the stable, time-invariant spectrum of Mb at 10^{-4} M are attained (see Fig. 2b, upper curve); at the same time, the temporal variability of the resulting spectra drastically decreases.

A similar behavior is observed in the SERRS spectra of the immobilized particles. Figure 3a shows a series of selected SERRS spectra from silver particles incubated with Mb at a concentration of 10^{-11} M immobilized into a polymer-coated glass surface. These SERRS spectra are recorded in sequence with 1 s of integration time and about 60 s from one measurement to the next, after scanning the sample under the microscope and locating brightly emitting colloidal particles. In this connection, we note that an average number of about 0.4 Mb molecules is expected to be present in the laser-illuminated spot (see Sample Preparation section). Again, the spectra have been found to fluctuate with time in both the intensity and position of the different vibrational modes; a similar trend has been observed in the SERRS spectra of other protein systems.[3,27] On the other hand, when the spectra obtained by a sum of many SERRS spectra or, equivalently, SERRS spectra collected for a longer time

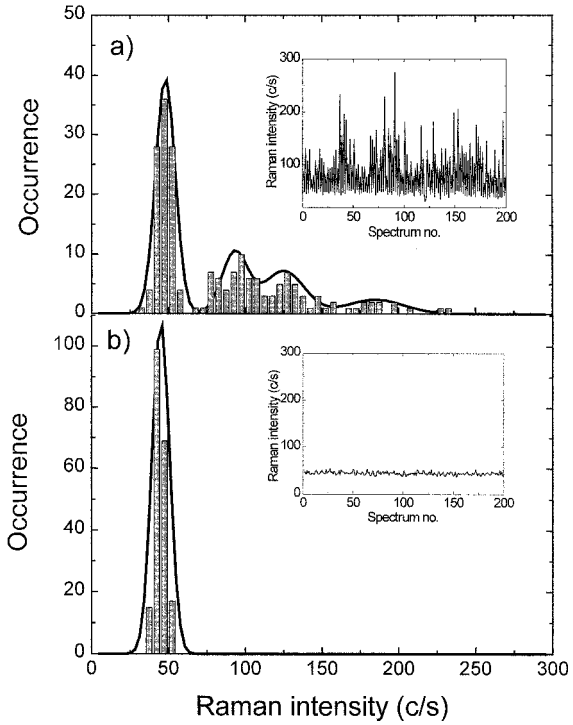

FIG. 4. Statistical analysis of the Raman intensity detected at 1620 cm^{-1} for 200 SERRS spectra from: (**a**) Mb silver colloidal solution; the solid line is a superposition of four Gaussian curves whose peaks reflect the probability of finding 0, 1, 2, and 3 molecules, respectively, in the scattering volume; (**b**) silver colloidal solution; the solid line is a Gaussian fit to the data. The histograms have been performed by using, in both cases, 30 bins whose width is 10 c/s, corresponding to about 3% of the maximum of the Mb colloidal sample. Inset: Raman intensity as extracted at 1620 cm^{-1} for 200 SERRS spectra. The analysis has been performed directly on the raw data.

are taken into account, they show the main vibrational modes of the standard Raman spectra of dry Mb, whose features are practically the same of those of hydrated Mb. Actually, Fig. 3b shows a SERRS spectrum obtained by summing 100 SERRS spectra (upper curve) and, for comparison, the Raman spectrum of dry pure Mb (Fig. 3b, lower curve). Some of the peaks detected in the SERRS Mb spectrum can be visualized in the dry Mb spectrum (see the marker-mode lines in Fig. 3b at about 1000, 1570, and 1620 cm^{-1}, respectively).

Analysis of the SERRS Spectra from Samples in Solution. To closely investigate the fluctuations in the SERRS spectra, we have focused our attention on the 1620 cm^{-1} line, which represents a peculiar and intense marker of the heme group. The Raman intensity of this line for a collection of 200 SERRS spectra from both the Mb-containing colloidal solution and the bare colloidal solution reveals a wide variability (see the insets of Fig. 4a and Fig. 4b, respectively); this variability is more marked for the Mb colloidal sample in comparison with the pure colloidal sample. Figure 4a shows a plot of the occurrence of the Raman intensity of this line related to the former sample. The resulting distribution appears multimodal and a fit (see the continuous line in Fig. 4a) with a superposition of four Gaussian shape distributions indicates the presence of four peaks whose occurrence progressively decreases as long as higher Raman intensities are taken into account. The largest number of events, represented by the first peak, is recorded at an intensity of about 50 c/s. A similar trend has been observed for crystal violet dissolved in a silver colloidal solution.[24] When the same plot is drawn for the colloidal solution in the absence of Mb, a monomodal distribution, centered at an intensity of 50 c/s, is, instead, obtained (see the continuous line in Fig. 4b). The full width at half-maximum (FWHM) of this distribution (about 14 c/s) is in good agreement with that expected from the CCD noise level (about 13 c/s, see Signal Noise Analysis section). It should be noted that the position as well as the width of this distribution are quite similar to those characterizing the first peak in the multimodal distribution of the Mb colloidal solution (50 c/s and 15 c/s, respectively, in Fig. 4a). It can therefore be inferred that the first peak in the multimodal distribution arises from the ground signal as due to the sample containing no Mb.

The observed trend in Fig. 4a can be interpreted in terms of detection of a different number of hot SERRS centers during the measurements. In particular, as due to diffusion of metal particles, 0, 1, 2, and 3 emitting Raman centers can be found in the scattering volume; the occurrence, and hence, the probability, of finding hot emitting particles progressively decreases with the number of particles. In this connection, we note that the Raman intensity distributions are substantially equispaced. Such a result finds a close correspondence with that occurring for crystal violet silver colloidal samples in the single molecule limit.[24] Accordingly, an estimation of the average number of hot SERRS centers in the scattering volume can be made in our case. By extracting from the curve in Fig. 4a the probability of finding 0, 1, 2, and 3 hot Raman particles, an average number of 0.65 particles in the scattering volume can be calculated. Since in our experimental conditions, 30 Mb molecules, on average, are expected to be present in the scattering volume, it comes out that the hot Raman centers represent a percentage of about 2% of the total; such an estimation is in good agreement with evaluations in Refs. 3, 24, and 25. This means that a large portion of the molecules that are likely to be present in the scattering volume are not emitting during the detection time. The occurrence of such extremely rare events can be explained by taking into account the idea that single molecule SERRS signals are suggested to arise from target molecules adsorbed at some particular sites possibly located at the junction between two silver nanoparticles;[3,25] the EM and the CT effects are assumed to be relevant only for those chemisorbed molecules interacting with ballistic electrons in the silver particles at these particular sites.[21,25]

Let us now estimate the time τ that a single colloidal particle (with an average diameter of about 70 nm) spends in the probed volume. Under the assumption that colloidal particles follow a Brownian motion, by using a diffusion coefficient of 3×10^{-12} m^2/s,[24,32] a residence time of about 5 s in the scattering volume can be determined. Longer times should be expected for dimers, trimers, and larger aggregates. It turns out that in two successive measurements (separated by about 60 s), signals arising from different particles are plausibly expected to be collected. Nevertheless, it should be remarked that a few very seldom events characterized by an extremely large Raman signal, persisting over long times, have been

detected during our measurements. These events, which are not included in our final results, likely correspond to very large colloidal aggregates with several hot SERRS centers.

In summary, our data point out a marked deviation from a single mode distribution and a large spread for the line intensity of Mb colloidal solution at a concentration of 10^{-11} M. These findings, in agreement with what was observed in Ref. 24, suggest that in our measurements, SERRS signals from single molecules are detected. It should be pointed out that, even if a few tens of Mb molecules are expected to be present in the scattering volume, the restrictive conditions required to obtain large enhanced Raman signals are satisfied only for a small portion of molecules. Therefore, it can be assumed that, under our experimental conditions, the single molecule limit has been reached.

In principle, the detection of signals in the single molecule limit could offer the exciting possibility of investigating whether the vibrational features of a single Mb protein molecule deviate from those of the ensemble. However, the diffusion-based selection of the molecule under investigation means that different molecules are studied during the course of the experiment (due to molecules entering and leaving the scattering volume). Therefore, the actual time evolution of a specific single molecule cannot be followed. In this respect, a more convenient approach might be represented by the study of immobilized particles.

Analysis of the SERRS Spectra from Immobilized Samples. Also in this case, we have started our analysis by repeatedly registering the intensity of the 1620 cm^{-1} Raman line from bright spots selected by scanning the sample in the x–y plane with the objective of the microscope. The fact that bright spots represent quite rare events corresponds with the low number, previously estimated, of Mb molecules inside the laser spot and, in addition, with the evidence that only about 2% of Mb molecules likely present in the laser spot are SERRS active. For both the Mb colloidal and pure colloidal immobilized samples, the Raman signal fluctuates considerably when 100 individual spectra, each registered at an integration time of 1 s, are taken into account (see insets of Figs. 5a and 5b, respectively). The variation of the Raman intensity from one spectrum to the next is generally less marked in comparison with that observed in the sample in solution (see insets of Figs. 4a and 4b). From these measurements, the occurrences of the Raman intensity have been calculated and the related histograms are shown in Figs. 5a and 5b. These plots show, in both the cases, a single-mode distribution that can be well described by a Gaussian shape (see continuous lines in Figs. 5a and 5b). However, the Mb-containing sample displays a much wider distribution (FWHM = 140 c/s) with respect to that of the pure colloidal sample (FWHM = 24 c/s). As was done in the previous section, the Gaussian distributions taking into account the data variability, due to the CCD noise, have been plotted for comparison in Figs. 5a and 5b (dashed curves). It appears that while for the colloidal sample this distribution is almost identical to that derived from the data spread, it markedly underestimates the spread of the data coming from the Mb

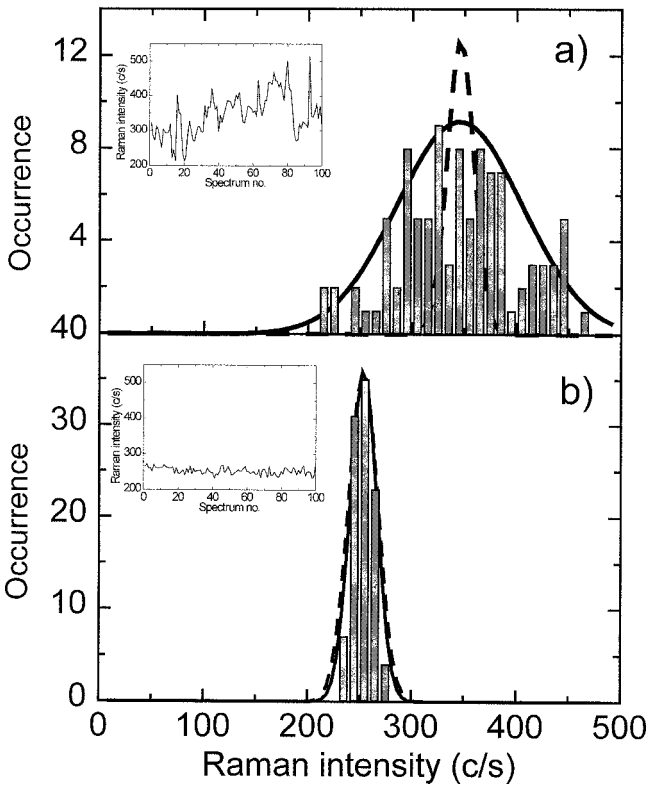

FIG. 5. Statistical analysis of the Raman intensity at 1620 cm^{-1} for 100 SERRS spectra from: (**a**) immobilized Mb silver colloidal particles sample; (**b**) immobilized colloidal. The dashed lines are Gaussian curves centered at the average value and with a standard deviation as derived from the noise expected from the CCD detection apparatus. The continuous dashed lines are Gaussian curves extracted from a fit of the data. The histograms have been performed by using, in both cases, 30 bins whose width is 10 c/s, corresponding to about 5% of the maximum of the Mb colloidal sample. Inset: Raman intensity as extracted at 1620 cm^{-1} for 100 SERRS spectra. The analysis has been performed directly on the raw data.

colloidal sample. This means that besides the noise due to the detection apparatus, additional effects are expected to contribute to the spread of the counts detected in the Mb colloidal sample.

In order to better understand such a result, we have analyzed the Raman intensity detected in correspondence with the 1000, 1340, and 1570 cm^{-1} lines for 100 spectra of the Mb colloidal samples. The corresponding trends are shown in Fig. 6, where the Raman intensity detected at 1620 cm^{-1} is also shown for comparison. First of all, one finds that the variability of the Raman intensity at the other analyzed lines exceeds that expected from the detection apparatus (not shown). Notably, under visual inspection, the trend of the Raman intensity as a function of the spectrum number appears to be very similar between the different lines considered. In other words, increase or decrease of the Raman intensity, from one spectrum to the next, occurs simultaneously for the different lines of the same spectrum. To quantitatively investigate such a phenomenon, we have analyzed the correlation between the Raman intensity for couples of two different lines. In particular, we have calculated the cross-correlation, ρ, at a delay 0, expressed by:

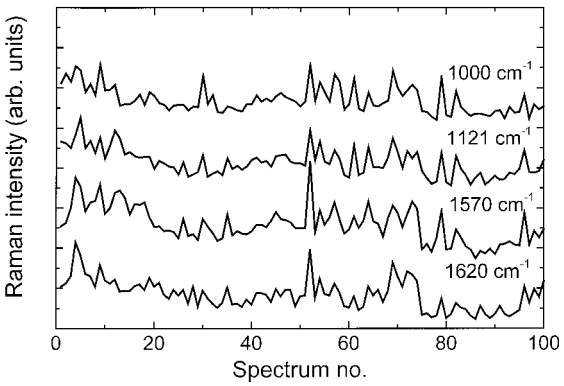

FIG. 6. Raman intensity as extracted at 1000, 1340, 1570, and 1620 cm^{-1}, from a series of 100 SERRS spectra of the immobilized Mb colloidal sample. The values of the cross-correlation, ρ, calculated by Eq. 1 with $I(\nu_2)$ detected at 1620 cm^{-1} and $I(\nu_1)$ at 1000, 1121, and 1570 cm^{-1} are 0.81, 0.85, and 0.86, respectively.

$$\rho = \frac{\sum_i [I_i(\nu_1) - \overline{I(\nu_1)}][I_i(\nu_2) - \overline{I(\nu_2)}]}{\left\{\sum_i [I_i(\nu_1) - \overline{I(\nu_1)}]^2 \sum_i [I_i(\nu_2) - \overline{I(\nu_2)}]^2\right\}^{1/2}} \quad (1)$$

where $I_i(\nu_j)$ are the Raman intensities, detected at the wavelength ν_j for the spectrum i; $\overline{I(\nu_j)}$ is the Raman intensity, detected at the wavelength ν_j, averaged over a series of 100 spectra. By fixing ν_1 as 1620 cm^{-1}, the calculated cross-correlation ρ for $I(\nu_2)$ extracted at 1000, 1340, and 1570 cm^{-1}, respectively, gives values higher than 0.8 for the Mb colloidal sample (see the values reported in the legend of Fig. 6). We note that much lower values (less than 0.3) are, instead, obtained if the same analysis is performed on the pure colloidal sample. Therefore, a strong correlation occurs in the Raman intensity detected in different regions of a Mb colloidal SERRS spectrum; such behavior suggests a common origin of the fluctuations observed in different regions of the SERRS spectra. This provides further evidence that the single molecule limit has been reached. Actually, if the SERRS spectra arose from a collection of molecules, each one characterized by a temporal fluctuation, the various regions of the spectra would have been characterized by a different temporal trend. On the other hand, the presence of the observed correlations could offer an additional element to explain the temporal variability affecting the single molecule SERRS spectra. As already mentioned, the occurrence of fluctuations, or blinking, represents one of the hallmarks of the single molecule. Blinking SERRS, similar to intermittent fluorescence emission, has also been observed when one or a few molecules, possibly adsorbed at a junction site between two particles, contribute to the SERRS signals.[3,22,25] It has been hypothesized that SERS and SERRS effects can be interpreted as due to a concomitant action of both the EM and the CT mechanisms;[20] the largest contribution comes from the EM effect to which a more specific, and smaller, effect related to a chemical interaction between the target molecule and the surface is added.[22] Recently, it has been suggested that a modulation of the CT effect could be at the origin the SERRS spectral fluctuations.[22,23] In particular, fluctuations could be attributed to variations of the local work function at the molecular adsorption site through modification of the extent of the charge transfer mechanism.[23] Such a modulation was tentatively assumed to arise from the diffusion of the target molecule on the metallic site or the diffusion of silver atoms.[23,27] Irrespective of its origin, a variation in time of the enhancement factor experienced by the molecule would affect different regions of the spectrum according to our finding about the correlation in the line intensity. On the other hand, changes in the relative intensity of the different lines from one spectrum to the next suggest the presence of additional effects. It has been speculated that adsorbate motion, by determining variations of the heme-group orientation, could result in changes of the local polarization direction.[27] Alternatively, this effect might also be described in terms of an intrinsic temporal variability of the target molecule itself. In other words, the fluctuations of the Raman signals might also reflect the dynamic behavior of the molecule as well as fluctuations caused by the local environment. In connection to this, we remark that a protein macromolecule samples, during its dynamic evolution, many local minima of the energy landscape, assuming slightly different conformational substates, which then influence the functionality.[8] On such grounds, the Raman signal, monitoring the vibration of the heme groups, might reflect such a variability, which becomes evident when the ensemble average is ruled out. Such a picture finds support from MD simulation results on a single protein molecule.[29] It has been shown that the structural and dynamic fluctuations on single molecule plastocyanin, connected to the CS sampling and detected in the picoseconds time scale, give rise to wide fluctuations in the line intensities and positions in the calculated Raman spectrum. Notably, these fluctuations in the Raman spectrum are expected to be detected in a single molecule Raman experiment when integration times in the second time scale are taken into account.

We finally remark that single molecule detection, in connection with the evidence that the sum of many SERRS spectra can reproduce the ensemble average Raman signal, suggests an ergodic behavior, in agreement with a recent single molecule fluorescence experiment.[13]

CONCLUSION

The SERRS technique provides a valuable tool for investigating the vibrational features of proteins in the single molecule limit. The vibrational modes of a single molecule of Mb, one of the most studied proteins, for which a large amount of data has been collected by several theoretical and experimental approaches, can be followed by adsorbing the macromolecule on silver particles in solution and immobilized on a glass surface. An analysis of the temporal fluctuations in the Raman intensity of the single molecule SERRS spectra may help to elucidate the mechanisms at the origin of the SERRS effect.

ACKNOWLEDGMENTS

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