Single-Molecule Surface Enhanced Resonance Raman Spectroscopy of the Enhanced Green Fluorescent Protein

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Single-Molecule Surface Enhanced Resonance Raman Spectroscopy of the Enhanced Green Fluorescent Protein

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One of the most intriguing findings in single-molecule spectroscopy (SMS) is the observation of Raman spectra of individual molecules,[1,2] despite the small cross section of the transitions involved. To obtain single-molecule Raman spectra, individual molecules have to be adsorbed on metallic nanoparticles, usually silver or gold, at very low concentration. The observation of the spectra can be explained by the surface enhanced resonance Raman scattering (SERRS) effect.[3−5] However, the microscopic picture of the enhancement for single molecules (SM-SERRS) is not yet fully elucidated. There is an ongoing debate about the heterogeneity of active sites on the silver nanoparticles and about the importance of various factors on the enhancement. These factors include enhancement of the electromagnetic fields close to the surface through interactions with the plasmon excitations,[3] chemical bonding and the subsequent formation of a charge transfer state,[6,7] electronic resonance, the particle size,[8] and the presence of anions.[9] Some publications indicate that particle association might help to generate an ultrahigh amplification factor.[6,10,11] At the single-molecule level, the SERRS spectra recorded as a function of time reveal inhomogeneous behavior such as on/off blinking, spectral diffusion, intensity fluctuations of vibrational lines,[6,7,12] and even splitting of some lines within the spectrum of one molecule.[13]

SM-SERS spectroscopy opens up exciting opportunities in the field of biophysical and biomedical spectroscopy,[14−16] where it could provide ultrasensitive detection and characterization of biophysically relevant molecules and processes as well as vibrational spectroscopy with extremely high spatial resolution.[14,16] The first example of SM-SERRS of a heme protein has recently been published.[17] However, some publications argue that the size of the complete protein will not allow for the local huge amplification of the Raman signal. It was suggested that the noncovalently bound porphyrin group might diffuse out of the protein to be adsorbed directly on the Ag nanoparticles.[18]

To avoid this problem, we decided to use green fluorescent proteins (GFPs) for SM-SERRS studies. GFPs are a class of proteins in which the chromophore is formed autocatalytically inside a barrel structure (length 4 nm, height 2 nm). Thus, the chromophore (constituted by three amino acid residues of the protein) is part of the protein and is kept in place by a complex hydrogen-bonding network. GFP and its mutants are widely used as fluorescent probes for cellular and biological studies.[19]

Polydisperse siver particles were produced by the Lee and Meisel citrate reduction method,[20] and the average size was shown to be 70 nm by AFM. Measurements were performed on an inverted microscope used for SM detection.[21] The time evolution of the

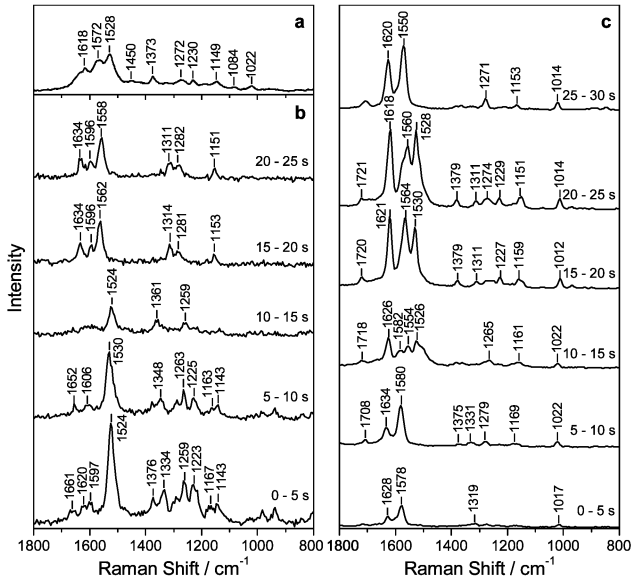

Figure 1. SERRS spectra of a single EGFP molecule (5 s integration time, 500 nW excitation power) adsorbed on a silver colloid. (a) Averaged spectrum built up by 80 different individual molecules. (b,c) The spectral time series from two individual EGFP molecules.

SERRS spectra of a single enhanced green fluorescent protein (EGFP) molecule adsorbed on a silver colloid excited at 488 nm is shown in Figure 1. The wavelength resolution of the system is approximately 10 cm^{-1}. The low ratio of EGFP molecules to colloid particles (1:1) together with the low number of Raman active particles ensures that spectra of individual molecules are measured. The averaged spectrum from 80 molecules showing a SERRS signal is depicted in Figure 1a.

Because the excitation wavelength corresponds to the maximum of the absorption spectra of the deprotonated form of the EGFP chromophore ($\epsilon = 56\,000\ M^{-1}\ cm^{-1}$), this results in a resonance enhanced Raman spectrum which allows to probe the chromophoric site without spectral interference from the surrounding environment. Most of the peaks in the spectrum are in agreement with the ensemble Raman spectrum of EGFP published before[22−24] within an error of $\pm 10\ cm^{-1}$, confirming that the Raman spectrum of EGFP was measured. Furthermore the spectra show all characteristics for Raman spectra of individual molecules mentioned in the introduction.

As can be seen in the time series of the spectra from one EGFP molecule, the signals abruptly changed in both frequency and intensity. The most important feature within this series of the spectra is the sudden frequency jump from 1524 to 1562 cm^{-1} at 15 s. The band peaking at 1524 cm^{-1} can be assigned to the delocalized

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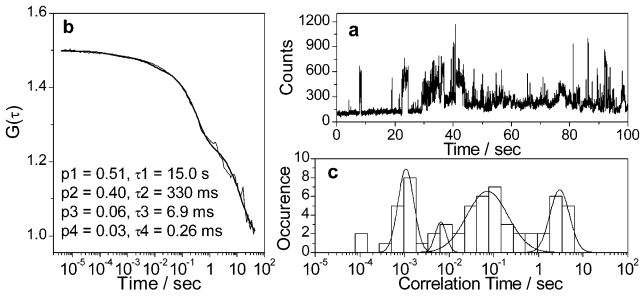

Figure 2. (a) Intensity time trace of the integrated SERRS signal detected from single EGFP molecule. Bin size is 5 ms. (b) Autocorrelation curve obtained from a whole trace of the SERRS signal of EGFP displayed in Figure 2a. (c) Histogram of the correlation times obtained from an integrated SERRS trajectory. The histogram is built up by the traces from 20 different molecules.

imidazolinone/exocyclic C═C mode of the deprotonated form of the chromophore, while the peak at 1562 cm^{-1} can be attributed to the same mode of the protonated form.[25] This frequency jump can be interpreted in terms of a conversion of the chromophore from the deprotonated to the protonated form. Although the conversion of the chromophore between the deprotonated and the protonated form is commenly accepted to occur in EGFP and related proteins, the presence of the protonated form has been assigned on the basis of off-periods in the fluorescence of the protein in SM experiments.[26] This hypothesis is further corroborated here at the single molecule level via the vibrational fingerprints of the protonated/deprotonated forms. Another time series of the spectra (Figure 1c) clearly displays the reversible conversion between the two forms. While only the protonated form is observed in 0−5, 5−10, and 25−30 s, the contribution of the deprotonated form is dominant in 10−15 s. Between 15 and 25 s, the peaks of both form are present. These findings point to the fact that the reversible conversion takes place in less time than the accumulation time (5 s).

In Figure 2a a time trajectory of the integrated Raman signal (including the broad background discussed in a number of publications[6]) of a single EGFP molecule adsorbed on a silver colloid is shown. The contribution of the fluorescence of EGFP to the transient is excluded because no fluorescence was detected in the spectra measured simultaneously (data not shown). This indicates efficient excitation transfer to the metal. The count rate of the SERRS signal is 1−2 orders of magnitude higher than that of the fluorescence signal from single EGFP molecules measured at the same excitation wavelength and same power. From these data one can calculate a SERRS cross section of roughly 6×10^{-15} cm^2, about an order of magnitude smaller than the SERRS crosss section reported for rhodamine 6G (R6G).[6] The difference in cross section between EGFP and R6G could be explained by the fact that the chromophore of EGFP is separated by the β-barrel from the surface of the silver particle, knowing that the electromagnetic enhancement factor decreases dramatically with increasing the distance between the particle and the molecule. Nevertheless, the high cross-section of EGFP is actually surprising as the chromophore cannot directly bind to the silver colloids. Such binding, and subsequent contribution to the enhancement, should in this case occur via the β-barrel amino acid residues. The fact that the enhancement of the SERRS signal for EGFP is the result of a complicated combination of factors is further proven by constructing the autocorrelation function of the intensity fluctuations of SERRS time trajectories and of single bursts in such a trajectory. Similar experiments were performed for R6G on silver colloids, and observed fluctuations in the second range were attributed to electron-induced desorption followed by lateral displacements.[7,8] The autocorrelation trace obtained from an EGFP SERRS intensity trajectory shows multiexponential behavior (Figure 2, b and c). Correlation times from hundreds of microseconds to several tens of seconds were found (Figure 2, b and c). Even autocorrelation analysis of one burst turned out to be multiexponential (data not shown). At this stage we can only speculate on the origin of fluctuations in the SERRS signal of EGFP. Possible actors are desorption followed by small lateral diffusion,[27] rotational diffusion, the photophysical processes taking place in the chromophore, rearrangements in the hydrogen-bonding network, and subsequent coupling to the β-barrel. Nevertheless, we believe this first SM-SERRS study of EGFP shows the potential of the technique to gain structural evidence for photophysical processes thought to occur in fluorescent proteins. We were able to demonstrate dynamic conversion between the protonated and the deprotonated forms of the chromophore in individual EGFPs. Our data show that GFPs, genetic tags recently developed for fluorescence measurements, are also good SERRS probes.

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Supporting Information Available: Experimental details, additional discussion (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Nie, S. M.; Emory, S. R. *Science* **1997**, *275*, 1102−1106.
(2) Kneipp, K.; Wang, Y.; Kneipp, H.; Perelman, L. T.; Itzkan, I.; Dasari, R.; Feld, M. S. *Phys. Rev. Lett.* **1997**, *78*, 1667−1670.
(3) Moskovits, M. *Rev. Mod. Phys.* **1985**, *57*, 783−826.
(4) Otto, A.; Mrozek, I.; Grabhorn, H.; Akemann, W. *J. Phys.: Condens. Matter* **1992**, *4*, 1143−1212.
(5) Otto, A. *Phys. Status Solidi A* **2001**, *188*, 1455−1470.
(6) Michaels, A. M.; Nirmal, M.; Brus, L. E. *J. Am. Chem. Soc.* **1999**, *121*, 9932−9939.
(7) Weiss, A.; Haran, G. *J. Phys. Chem. B* **2001**, *105*, 12348−12354.
(8) Krug, J. T.; Wang, G. D.; Emory, S. R.; Nie, S. *J. Am. Chem. Soc.* **1999**, *121*, 9208−9214.
(9) Doering, W. E.; Nie, S. M. *J. Phys. Chem. B* **2002**, *106*, 311−317.
(10) Michaels, A. M.; Jiang, J.; Brus, L. *J. Phys. Chem. B* **2000**, *104*, 11965−11971.
(11) Xu, H. X.; Aizpurua, J.; Käll, M.; Apell, P. *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.* **2000**, *62*, 4318−4324.
(12) Bosnick, K. A.; Jiang, J.; Brus, L. E. *J. Phys. Chem. B* **2002**, *106*, 8096−8099.
(13) Meixner, A. J.; Vosgrone, T.; Sackrow, M. *J. Lumin.* **2001**, *94*, 147−152.
(14) Kneipp, K.; Kneipp, H.; Itzkan, I.; Dasari, R. R.; Feld, M. S. *J. Phys.: Condens. Matter* **2002**, *14*, R597−R624.
(15) Bjerneld, E. J.; Földes-Papp, Z.; Käll, M.; Rigler, R. *J. Phys. Chem. B.* **2001**, *106*, 1213−1218.
(16) Petry, R.; Schmitt, M.; Popp, J. *ChemPhysChem* **2003**, *4*, 14−30.
(17) Xu, H. X.; Bjerneld, E. J.; Käll, M.; Börjesson, L. *Phys. Rev. Lett.* **1999**, *83*, 4357−4360.
(18) Otto, A. *J. Raman Spectrosc.* **2002**, *33*, 593−598.
(19) Tsien, R. Y. *Annu. Rev. Biochem.* **1998**, *67*, 509−544.
(20) Lee P, C.; Meisel, D. *J. Phys. Chem.* **1982**, *86*, 3391−3395.
(21) Cotlet, M.; Hofkens, J.; Habuchi, S.; Dirix, G.; Van Guyse, M.; Michiels, J.; Vanderleyden, J.; De Schryver, F. C. *Proc. Nat. Acad. Sci. U.S.A.* **2001**, *98*, 14398−14403.
(22) Bell, A. F.; He, X.; Wachter, R. M.; Tonge, P. J. *Biochemistry* **2000**, *39*, 4423−4431.
(23) Schellenberg, P.; Johnson, E.; Esposito, A. P.; Reid, P. J.; Parson, W. W. *J. Phys. Chem. B* **2001**, *105*, 5316−5322.
(24) Kruglik, S. G.; Subramaniam, V.; Greve, J.; Otto, C. *J. Am. Chem. Soc.* **2002**, *124*, 10992−10993.
(25) He, X.; Bell, A. F.; Tonge, P. J. *J. Phys. Chem. B* **2002**, *106*, 6056−6066.
(26) Dickson, R. M.; Cubitt, A. B.; Tsien, R. Y.; Moerner, W. E. *Nature* **1997**, *388*, 355−358.
(27) Eggeling, C.; Schaffer, J.; Seidel, C. A. M.; Korte, J.; Brehm, G.; Schneider, S.; Schrof, W. *J. Phys. Chem. A* **2001**, *105*, 3673−3679.

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