Single-Molecule Surface-Enhanced Raman and Fluorescence Correlation Spectroscopy of Horseradish Peroxidase

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We report on the spectroscopy and enzymatic activity of horseradish peroxidase (HRP) coupled to Ag nanoparticles. We show that vibrational spectra of single HRP molecules can be detected by measuring surface-enhanced Raman scattering (SERS) from isolated and immobilized protein−nanoparticle aggregates. However, the nanoparticle−protein interaction results in decreased enzymatic activity, as shown by an ensemble-averaged HRP activity assay. We demonstrate that H_2O_2, one of the enzyme substrates in the assay, reacts with the Ag surface and that it is necessary to pretreat the Ag particles with H_2O_2 to retain full HRP activity in the Ag sol. However, the concomitant changes in the Ag surface adsorbate layer caused by this treatment prevent strong HRP adsorption and therefore also single-molecule SERS sensitivity. Layers of H_2O_2-treated Ag particles can instead be used to enhance the fluorescence signal from single functional HRP molecules.

1. Introduction

The great interest in single-molecule spectroscopy (SMS) comes from the possibility of obtaining detailed information on molecular heterogeneity, information that is normally averaged out in ensemble measurements.[1,2] A few single-molecule *fluorescence* studies of enzyme activity have been reported. Xue and Yeung measured the time-averaged product formation from single lactate dehydrogenase enzymes and found large variations in catalytic activity of different individual molecules.[3] Such static disorder was also found by Lu et al., who measured the enzymatic turn-over rates of individual cholesterol oxidase molecules, for which the active site toggles between a fluorescent and nonfluorescent state during the enzymatic cycle.[4] These latter measurements also indicated large fluctuations in reaction rates for a given enzyme molecule over time (dynamic disorder). Similar results were reported by Edman et al. in their study on immobilized HRP.[5] In this case, the enzyme catalyzes the conversion of a nonfluorescent substrate molecule to a fluorophore product and the fluctuations in product formation are measured. Single-molecule fluorescence spectroscopy has thus provided important evidence for heterogeneity and fluctuations in enzyme proteins, although the number of studies is, so far, limited.

The single-molecule sensitivity of surface-enhanced Raman scattering (SERS) is remarkable, both because of the enormous enhancement factors involved and because of the possibility, in principle, of identifying and characterizing a single molecule by virtue of its vibrational spectrum. Although the prerequisites for surface-enhanced Raman SMS are only beginning to be understood, it is clear that the degree of sensitivity depends on many parameters, such as excitation wavelength, particle size, and state of aggregation. We have previously shown that visible excitation ($\lambda = 514.5$ nm) of aggregated 90-nm Ag particles is an efficient route to SERS of single hemoglobin molecules.[6] This observation was explained by the enhanced EM fields that exist between particles, which are predicted to be up to 3 orders of magnitude higher than the incident field, yielding an enhancement factor of the order 10^{10}.[6,7] Because of the rapid decay of the EM field out from the surface and potential problems with "spectral crowding" of coadsorbed molecules, the analyte has been adsorbed *directly* on nontreated particles in previous Raman single-molecule studies.[8,9,6] The only adsorbate molecule present in these studies, apart from the analyte, is citrate. Most previous ensemble SERS studies of proteins have also utilized direct adsorption, that is, the proteins have been attached to the metal surface without any spacer or coupling molecules. One popular scheme is to let the proteins stabilize colloidal particles through charge or steric interactions or both and then aggregate the particles by addition of salt.[10−12] However, protein adsorption alone can also induce aggregation, an effect known as *polymer-bridging flocculation.* In this case, the protein is assumed to be positioned between particles, in the region of highest SERS enhancement,[6] thus facilitating single-molecule spectroscopy.

Although metal nanoparticles conjugated to proteins have been extensively used as markers in electron and light microscopy, for example, to locate specific target molecules in cells,[13,10] there is a continuous debate to what extent direct adsorption on a metal surface influences the biological function of the protein. This question is of particular relevance to protein SERS. An additional concern is that free Ag ions are known to seriously affect enzyme activity.[14] The purpose of this study is to further the understanding of the spectroscopy and the biological activity of Ag nanoparticle−enzyme assemblies, focusing on the redox-enzyme horseradish peroxidase (HRP). We report on single-molecule SERS measurements of HRP using polymer-bridging flocculation to aggregate the Ag particles, as well as ensemble

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and single-molecule measurements of enzymatic activity. We show that a hydrogen peroxide treatment of the nanoparticles makes them fully HRP compatible but, unfortunately, also destroys their SERS single-protein sensitivity. The utility of these particles for optical investigations of enzymes is demonstrated by fluorescence correlation spectroscopy (FCS) measurements of single functional HRP molecules.

2. Experimental Section

Chemicals. All used chemicals were of analytical reagent grade. Sodium citrate, sodium chloride, silver nitrate, 3-aminopropyltrimethoxysilane (APTMS), rhodamine 6G (R6G), and glycerol were purchased from Sigma-Aldrich Co. Dihydrorhodamine 6G, from Molecular Probes, was stored at −80 °C. Guaiacol and potassium phosphate were obtained from Merck, and H_2O_2 (puriss.) was obtained from Fluka. Horseradish peroxidase (HRP) from Roche was stored at −20 °C in 50% glycerol and kept on ice in 100 mM phosphate buffer, pH 7.0, when used. All buffers were adjusted to pH 7.0. The water was purified in a Milli-Q Plus system.

Raman Spectroscopy. The 514.5 nm line of an argon-ion laser (Spectra-Physics 2060) was used for excitation. The illumination and collection system consisted of a confocal microscope (Olympus) coupled to a single-grating spectrometer (Dilor XY800) equipped with a liquid-nitrogen-cooled CCD-detector (Wright Instruments). The system has a frequency resolution of ∼6 cm^{-1} at the slit settings used here. A 100× (Olympus, air, NA = 0.95) or a 60× (Olympus, water, NA = 0.9) microscope objective was used to excite and collect the Raman signal in a backscattering configuration. The incident/scattered beam paths were split by a 20/80 beam splitter, and a holographic notch filter (Kaiser) was used for stray-light rejection.

Ag Nanoparticles. The Ag nanoparticles were made by reduction of Ag^+ in aqueous solution using citrate as a reducing agent.[15,16] The particles have a heterogeneous size and shape distribution with an average diameter of ∼90 nm, and citrate is adsorbed on the particles.[6,7] For a controlled particle immobilization, we used an APTMS silane coating,[17] which leads to well-dispersed and isolated single particles on the surface (if the particles are isolated before immobilization).

Single-Molecule SERS—Protocol A. For details of the methodology for single-molecule SERS experiments, see ref 6. The measurement strategy was as follows: the Ag sol was mixed in equal volumes with HRP in sodium phosphate buffer (100 mM) and NaCl (1 mM final concentration). The reaction mix was then stored on ice for 2.5 h. A HRP concentration of 2×10^{-11} M, equivalent to a protein/particle ratio of 1:1, was used. After adsorption, the HRP−particle aggregates were immobilized on a surface so that on average only one protein molecule was in the Raman detection volume. The surface was then immediately scanned for "hot", SERS-active, particle clusters using a manually controlled motorized $x-y$ sample stage (0.1 μm resolution) and the Raman setup described above.

HRP Activity Assay. The state of HRP was determined from absorption spectra (Cary 500) see Figure 1. To measure the enzyme activity, we used an absorbance assay at 436 nm that monitors the turnover of guaiacol for 30 min (see ref 5). The reaction was started by the addition of cold H_2O_2 or by addition of HRP. To measure the activity in the Ag sol, we diluted the sol by 1:7 in buffer to decrease the sol extinction background. Corrections due to the decrease in optical density caused by aggregation induced by buffer and HRP were made.

Fluorescence Correlation Spectroscopy (FCS). The experimental setup for the FCS measurements was the same as used in ref 5. Slides with immobilized HRP were applied in a hanging drop configuration. The focal volume of a 40× (NA = 0.9) objective was centered 2 μm below the surface in the buffer droplet. The excitation wavelength was 542.5 nm. A scan was performed for each substrate until a fluorescence signal was observed. The autocorrelation curve was measured as previously described.[5]

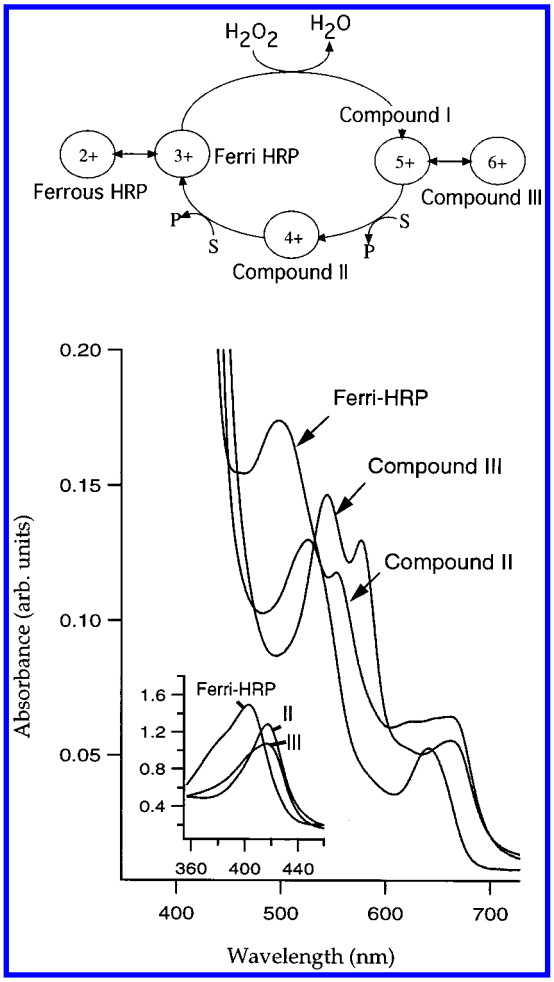

Figure 1. The enzymatic cycle of HRP with H_2O_2 as substrate. Peroxidase forms three compounds with H_2O_2: the green photolabile compound I, which is the precursor of the two red compounds II and III (oxyperoxidase). Compound II forms with as little as one molecule of H_2O_2 per Fe atom, and even in the absence of acceptor, it reverts back to free peroxidase (ferri-HRP). Compound II shows absorption bands at 527 and 555 nm, whereas the addition of 10−20 molecules of H_2O_2 leads to formation of compound III with absorption bands at 546 and 583 nm. Note that compound III can be obtained also from other forms of HRP than compound I.

HRP on Particle−Substrate Slides—Protocol B. Active immobilized HRP was prepared as follows: After coating the glass slide with APTMS, it was washed with methanol and water. The Ag particles were pretreated with H_2O_2 by mixing 867 μL of Ag sol with 133 μL of 7 mM H_2O_2 in a final NaCl concentration of 1 mM. Then, 20 μL of the reaction mixture was applied on the APTMS-coated glass slide. The droplet was left on the slide for 10 min, after which the substrate was washed repeatedly with phosphate buffer (100 mM). A droplet of HRP and buffer was then applied (1 nM for single-molecule experiments) to that part of the slide on which the Ag particles had been immobilized, after which the sample was incubated for 3 h at 8 °C and at controlled vapor pressure. Finally, nonimmobilized HRP was washed away with buffer. The HRP−substrate was kept in buffer for both FCS and SERS measurements.

Protocol B produces well-defined particle layers with an average interparticle distance of ~700 nm, as measured by scanning electron microscopy (SEM).

Single-Molecule FCS. A reaction mix was added to the immobilized HRP, prepared according to Protocol B (see above), consisting of H_2O_2 and the nonfluorescent leukodye dihydrorhodamine 6G.[5] During one enzymatic cycle of HRP, two leukodye molecules turn into the fluorescent product molecule R6G. Neither product formation nor intensity fluctuations were observed in control experiments with a molecule missing in the reaction mix.

3. Results and Discussion

3.1. The Enzymatic Cycle of Horseradish Peroxidase. For the sake of clarity, Figure 1 illustrates the well-known enzymatic cycle of HRP. In the first step, the substrate is hydrogen peroxide and the "resting" ferri-HRP (III) is turned into a species known as compound I. Compound I is a short-lived intermediate, two oxidizing equivalents above the resting enzyme. A one-electron reduction of compound I by a second substrate (S) produces a red species known as compound II. Finally, compound II is reduced back to ferri-HRP by reaction with a second substrate molecule S. Addition of H_2O_2 to HRP, in the absence of substrate S, leads to a slow rate of disappearance of compound I and compound II. In addition to the above-mentioned species, a compound known as oxyperoxidase, or compound III, can form upon addition of hydrogen peroxide. The oxidation state of HRP in the enzymatic cycle can be directly followed by optical absorption spectroscopy,[18] as shown in Figure 1.

3.2. SERS of Single Horseradish Peroxidase Molecules. Both ferri-HRP and compound II have characteristic resonance Raman spectra[19,20] as shown by Figure 2 D and E. The most obvious differences at 514.5 nm excitation are a 10 cm^{-1} shift of the depolarized υ10 mode at 1630 cm^{-1} and a 14 cm^{-1} shift of the anomalously polarized υ2 mode at 1575 cm^{-1}. Normal resonance Raman[19,20] and ensemble-averaged SERS[21] spectra of HRP are stable over time. In contrast, single-molecule SERS spectra have been found to fluctuate with time, both in the intensities and in the positions of different vibrational modes.[6] Figure 2 shows that this is the case also for HRP. The time average of the single-molecule spectra (C) has an overall resemblance to the ensemble resonance Raman spectrum of ferri-HRP (D), but there is a large variation and "spectral diffusion" among individual 1-s spectra (A). These experimental observations, together with the "single-molecule concentration" of the sample, confirm our previous finding that single-protein SERS is possible.

Although we cannot positively determine the cause of fluctuations at this point, it may be worthwhile to indicate some *possible* scenarios. We first note that the spectral series in Figure 2 exhibits a slow variation in *overall* spectral intensity, by as much as 5 times in magnitude. This effect was not reported by Xu et al. in the first single-protein SERS study.[6] Although the intensities of some spectra are very weak, illustrated by spectrum B in Figure 2, they still exhibit HRP marker modes. The intensity variation is reminiscent of the "intermittent" SERS signal reported for R6G by Krug et al.,[22] although the time-scale is longer in the present case. We believe that the most likely explanation for this effect is a slow variation in the enhancement factor experienced by the molecule. This could in turn be due to adsorbate *motion*, either of the molecule relative to the position of highest SERS enhancement or of the heme-group orientation relative to the local polarization direction or particle motion. The latter possibility appears less likely, considering the immobilization strategy used.

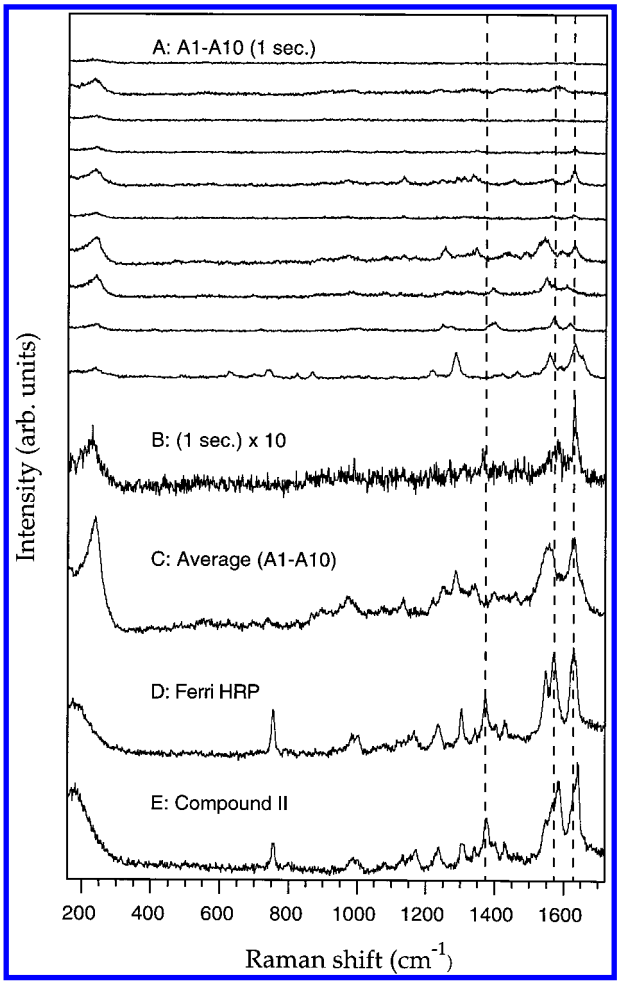

Figure 2. Single-molecule SERS spectra of HRP excited with 514.5 nm light. All spectra (A−C) are from the same time series from *one* HRP molecule adsorbed on an immobilized aggregate of approximately five 90-nm Ag particles (laser power 15 μW). The spectra show large variations in time (A), particularly in overall intensity. For comparison, a weak 1 s spectrum (B) has been multiplied by a factor of 10. The average of the time series (C) is compared to ordinary resonance Raman spectra of ferri-HRP (D) and compound II (E). Single-molecule spectra exhibit large temporal fluctuations in mode positions, as indicated by the marker-mode lines of ferri-HRP at 1375, 1575, and 1630 cm^{-1}, possibly due to fluctuations in oxidation state.

The fluctuations in peak positions in the absence of substrate turnover could, in principle, be caused by a number of different mechanisms.[6] Turning again to Figure 2, we note that the average SERS spectrum (C) exhibits the υ4 mode centered at around 1350 cm^{-1}, which would indicate an average oxidation state of ferrous HRP (2+). On the other hand, many of the most intense modes in the single-molecule spectral series are close to the expected ferri-HRP (3+) spectrum, for example, spectrum B exhibits the polarized oxidation-state marker mode υ4 at 1374 cm^{-1}. Similar fluctuations in oxidation state marker modes were found in all single-molecule SERS experiments. A natural, albeit speculative, interpretation is that the variation in mode positions are in fact due to oxidation-state fluctuations due to dynamic charge transfer between the metal surface and the protein. A report by Zhao et al. [23] is especially interesting in this respect. On the basis of measurements of active HRP adsorbed directly on 30-nm Au particles, the authors propose that adsorption of HRP on Au particles not only preserves but even increases the enzyme activity because the metal can act as an electron-transfer mediator in the enzymatic cycle.[23] It is also interesting to note that recent observations by Zhu and Lu[24] indicate that the

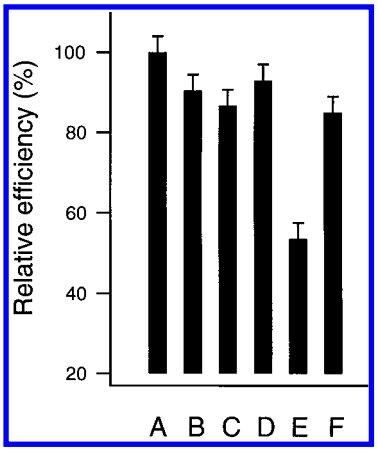

Figure 3. Enzyme activity in the Ag sol. A standard guaiacol ensemble assay was used to determine the enzymatic activity in different buffers and in the Ag sol. The effect of different buffers on HRP activity is shown: potassium (A) and sodium (B) phosphate buffers, and a citrate buffer (C) (error bars are standard deviations, $n = 8$). The HRP activity in the supernatant of the Ag sol (D), and in the Ag sol (E) when HRP was added to the Ag sol before the substrate H_2O_2. However, 85% HRP activity was observed if HRP was added to the Ag sol after H_2O_2 (F).

fluctuation rate in single-protein SERS scales with photon intensity, a result that may indicate photon-driven charge-transfer processes. In any case, the spectral shifts together with the expected extreme environment experienced by the enzyme, situated between metal particles in a high local photon flux, raise questions on the integrity and enzymatic activity of the protein.

3.3. Enzyme Activity in the Ag Sol. Although there are reports of protein denaturation on Ag surfaces,[25] most previous reports claim that citrate-reduced particles[26−28,11] and even Ag electrodes[29] are compatible with proteins. These conclusions were based on the similarities between protein SERS spectra and ordinary Raman spectra measured from solution. As noted above, the dramatic fluctuations in the single-molecule SERS spectra raise questions on the stability of the proteins adsorbed on the particles. We attempted to address this issue by measuring the ensemble enzymatic activity of HRP in the Ag sol, in the supernatant of the Ag sol, and in phosphate and citrate buffers (the assay was started by addition of H_2O_2 after 10 min incubation). The results, summarized in Figure 3, show that the enzyme was fully active in all buffers and in the supernatant. However, when HRP was mixed with Ag sol, the enzymatic activity decreased to 50%. This result shows that the Ag *particles* cause the decrease in activity.

We further found that by simply reversing the order in which H_2O_2 and HRP were added to the Ag sol (so that the Ag sol was now premixed with H_2O_2 for 10 min before the activity assay started with addition of HRP), 85% HRP activity was observed in the Ag sol. We then titrated the Ag sol with H_2O_2 to obtain full HRP activity. This occurred when an extra 18% of the H_2O_2 used to start the assay was added (i.e., an extra 3 μL of 7 mM H_2O_2 was added to the 150 μL of Ag sol in 850 μL of 100 mM phosphate buffer, in a final NaCl concentration of 1 mM). This strongly indicates a side reaction of H_2O_2 with the Ag particles, which cause an apparent loss of enzyme activity in the Ag sol assay.

3.4. H_2O_2 Treatment of the Ag Sol. The assay measurements indicated that H_2O_2 reacts with the Ag particles in the sol. Indeed, two further lines of evidence lead us to conclude that this is the case: (1) When H_2O_2 was mixed with Ag sol (in 1 mM NaCl) the extinction spectrum was red-shifted and the sol

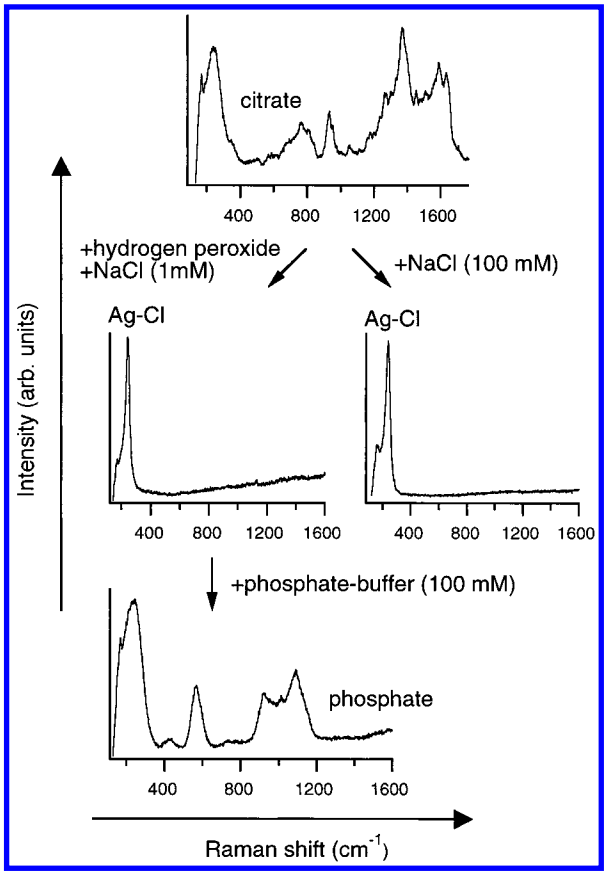

Figure 4. The effect of H_2O_2 on the Ag particles. The SERS spectrum of aggregated nontreated Ag particles shows the vibrations of adsorbed citrate (top). The spectrum from H_2O_2-treated particles (middle left) is completely free of intramolecular vibrations, like citrate vibrations. Instead, it exhibits a peak, which is assigned to a Ag−Cl vibration from comparison to the spectrum from nontreated particles, which also has been "cleaned", in this case by an excess of NaCl salt (middle right). Other adsorbate molecules such as phosphate ions from the protein buffer (bottom) could easily be adsorbed after the H_2O_2 treatment and gave rise to intense SERS spectra.

color turned from milky yellow into a darker brownish color (data not shown). This indicates a change in the dielectric properties of the adsorbate surface layer or a change in the particle morphology or both. (2) Figure 4 shows that the SERS spectrum from Ag particles mixed with H_2O_2 and NaCl (1 mM) was identical to the SERS spectrum of particles washed with excess NaCl (100 mM). The single peak at 235 cm^{-1} is assigned to the Ag−Cl stretching mode, and thus, an ion exchange of citrate and Cl^- had taken place at the surface. Such a complete ion exchange does not occur using only 1 mM of NaCl in the absence of H_2O_2. It is obvious that both the optical properties of the solution and the surface adsorbates change upon mixing the Ag sol with H_2O_2. The most plausible explanation for these observations, in agreement with a recent report,[30] is that the primary reaction of H_2O_2 at the Ag-particle surface is a redox reduction of Ag^+ ions to Ag.

After the H_2O_2 treatment, small analyte molecules such as phosphate ions from the buffer or R6G adsorbed to the particles and exhibited intense SERS spectra as shown in Figure 4. However, despite long incubation times with high concentrations of HRP in phosphate buffer, there was no trace of HRP in the SERS spectrum, which was dominated by phosphate ions from the protein buffer. Given that SERS primarily comes from molecules adsorbed in the first surface layer, this indicates that direct HRP adsorption on the particle is prevented by the phosphate coating. We also consistently failed to aggregate the

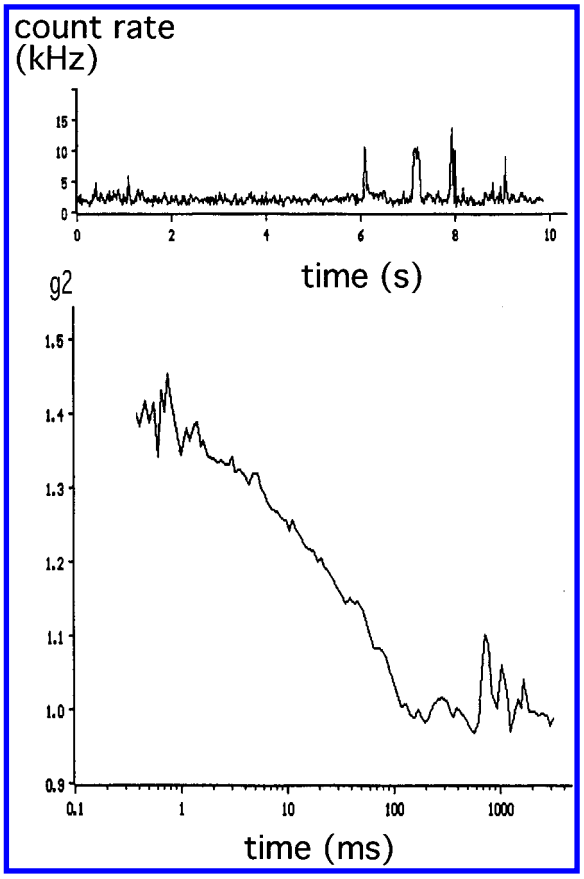

Figure 5. FCS spectra of HRP immobilized on a film consisting of dispersed and isolated Ag particles. In agreement with the activity assays, we found that HRP adsorbed on H_2O_2-treated particle films was enzymatically active. Control measurements were made with a molecule in the reaction mix missing. The lack of activity in this case demonstrated that turnover of substrate to product (R6G) could not be catalyzed by the Ag particles themselves.

H_2O_2-treated particles with HRP in a controlled way and consequently could not perform single-molecule SERS spectroscopy of HRP in buffer with a H_2O_2-treated sol. This was in stark contrast to our single-molecule SERS measurements, which despite a high buffer concentration (50 mM, see Protocol A in Experimental Section) still gave rise to a strong HRP SERS signal from particle aggregates. This result agrees well with our previous reports of protein-bridged particle aggregates being a prerequisite for single-protein sensitivity in SERS. Because of the difference in HRP adsorption between ordinary and H_2O_2-treated, phosphate-coated particles, it is difficult to ascertain from the activity assay whether adsorption in itself affects HRP activity or not.

3.5. FCS Measurements of Single Active HRP. To further study the enzyme activity in the presence of H_2O_2-treated Ag particles, we measured FCS fluctuations due to the turnover of nonfluorescent substrates into fluorescent products catalyzed by single, immobilized HRP. The enzyme was adsorbed to surfaces covered with a dispersed layer of single Ag nanoparticles. Note that the absence of a HRP SERS signal from these phosphate-coated particles prevented SERS studies of HRP and that the stability of the phosphate SERS spectrum, shown in Figure 4, ensured that all fluctuations observed were fluorescence fluctuations and not SERS fluctuations. As noted above, the lack of a HRP SERS signal for H_2O_2-treated Ag particles indicates that the protein does not adsorb directly on the particles. A representative FCS measurement of single HRP enzymatic activity is shown in Figure 5. The fluorescence intensity time traces recorded from the particle-covered surfaces confirmed the principal findings of Edman et al.,[5] namely, that there is a substantial disorder in the catalytic efficiency of HRP, both in the population sense (static) and for a given single-molecule over time (dynamic). The latter effect is seen as a nonperiodic "blinking" in the intensity time trace in Figure 5.

Although the number of single molecules measured (about 10 molecules) is too small to make quantitative conclusions, we found that the intensity levels in the FCS measurements were generally higher, by a factor of 3−4, than what was previously observed for standard surfaces, that is, surfaces without any Ag particles present. This observation is not entirely unexpected. As shown by Wokaun and co-workers, surface-enhanced fluorescence, although modest in magnitude, occurs when the fluorophore is situated some distance away from the metal surface.[31−33] Yokota et al. reported enhancement factors of 2−15 times, depending on the metal used, as a result of plasmon-induced evanescent fields.[34] Interestingly, although Ag exhibited the largest enhancement factor, it also acted as a poison for the motor protein investigated in this study. Nevertheless, the present data show that layers of peroxide-treated and phosphate-coated Ag particles are biocompatible with respect to HRP and that such layers can be used to produce a substantial signal enhancement in single-protein fluorescence investigations.

4. Summary and Concluding Remarks

We have reported on single-molecule Raman and single-molecule fluorescence measurements of HRP using Ag nanoparticles to enhance the optical signal, with an emphasis on biocompatibility of the nanoparticles. For aggregated nontreated protein−particle conjugates, in which HRP is assumed to be located at the region of the greatest SERS enhancement between particles, the SERS spectra are dominated by vibrations of single HRP molecules. Time-dependent spectral fluctuations together with an extremely low HRP concentration indicate that single molecules were observed. An interesting possibility is that the observed fluctuations are HRP oxidation-state fluctuations due to charge transfer between the metal surface and the protein. We also found that HRP retains full enzymatic activity in a Ag sol if the Ag particles have been pretreated with hydrogen peroxide. We demonstrate that the enzyme activity of single HRP copies immobilized on a surface together with pretreated and isolated 90-nm Ag particles can be measured by fluorescence spectroscopy and that the intensity level is enhanced compared to Ag-free surfaces. However, we were not able to measure SERS spectra of HRP using hydrogen peroxide-treated particles in phosphate buffer. Figure 6 shows simplified and schematic views of the two Ag-particle structures used in the SERS and FCS measurements. Note that the exact position of HRP with respect to the particles and the number of particles in the SERS aggregates can only be assumed in this study. In addition to the difference in aggregation state, we also found that ion exchange takes place at the particle surface in the Figure 6B scenario but not in the Figure 6A scenario under the experimental conditions used here. The full implications of such surface reactions for surface-enhanced spectroscopy should be further investigated.

The present study also shows that, depending on the desired application or on the scientific question, the metal particles should be chosen with care. There will always be some tradeoffs, for example, it is better for the Raman surface enhancement to adsorb the analyte directly on the surface, but this will affect, for example, enzyme activity and may cause quenching of fluorescent probes. Similarly, a buffer will keep the enzyme

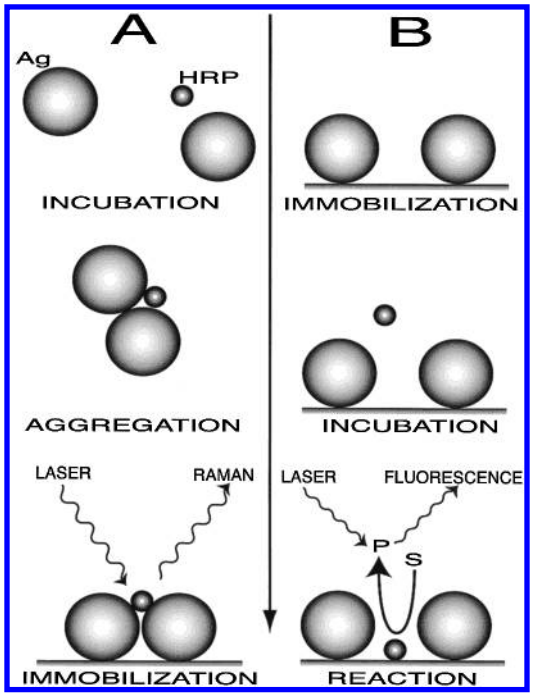

Figure 6. Simplified and schematic views of the two types of Ag-particle structures that, according to the main results reported in this study, are most likely involved in single-molecule Raman and fluorescence spectroscopy of HRP. Panel A shows aggregated colloids with adsorbed citrate and HRP. Such aggregates of 90-nm Ag particles are optimal for single-molecule SERS excited at 514.5 nm. Panel B shows films of immobilized and dispersed pretreated Ag particles. The surface of the particles was redox-titrated ("cleaned") by H_2O_2 and then coated by phosphate molecules from the protein buffer (see Figure 4). The fluorescence signal from substrate turnover due to the activity of a single HRP molecule is enhanced by 3−4 times compared to a particle-free surface.

active in solution but may compete with the protein for adsorption sites. For ensemble measurements, Van Duyne and co-workers recently reported an elegant strategy to overcome these problems using spacer molecules that orient active cytochrome C, either through an electrostatic interaction or specifically via a covalent bond.[35] Yet, for single-molecule studies, one would want to position the protein between particles and still keep it active. For this purpose, small spacer molecules and molecule-controlled particle aggregation, for example, using particles coated with different molecules such as biotin and avidin, appears promising. A controllable distance between the protein and the particles should also allow for the optimization of the fluorescence enhancement.

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