Surface-Enhanced Raman Scattering of Single Adenine Molecules on Silver Colloidal Particles

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To find feasibility of identifying nucleotides at the single-molecule level, we implemented Raman imaging and spectroscopy of adenine molecules adsorbed on Ag colloidal nanoparticles. Surface enhanced Raman scattering (SERS) of adenine molecules showed an intermittent on-and-off behavior called "blinking". The observation of blinking provides substantial evidence for detecting single adenine molecules.

Fluorescence single-molecule detection and spectroscopy are now established as important technologies in condensed matter physics[1] and bioanalytical chemistry,[2] in which biomolecules are labeled with fluorescent dye molecules such as rhodamine and cyanine dyes. The fluorescence efficiency (Φ_f) of all these dye molecules is larger than ~0.1. Incorporating nucleotides labeled with fluorescent dye molecules into DNA is a fundamental requirement for the proposed DNA sequencing based on single-molecule detection.[3] However, the dye molecules labeled raise serious issues in incorporating dye-labeled nucleotides into a template DNA molecules using polymerase and also in digesting dye-labeled nucleotides using exonuclease. Indeed, the dye molecules labeled disturb enzyme activities.[4] On the other hand, SERS can be applied to detecting single nucleotides without dye labeling if the Raman scattering cross section σ_R is enhanced from 10^{-30} cm^2, which is a representative value of σ_R without resonance enhancement, to ~10^{-16} cm^2. This expected value of σ_R ~10^{-16} cm^2 is comparable to the absorption cross section σ_A~10^{-16} cm^2 of representative fluorescent dye molecules amenable to single molecule detection and spectroscopy. In fact, Raman spectroscopy has inherent advantages over fluorescence spectroscopy: no mandatory requirement of excitation in resonance, useful to less fluorescent molecules like nucleotides ($\Phi_f \sim 10^{-4}$), and showing sharp spectrums nicely distinguishing molecular structures. Recently, success in single-molecule SERS imaging and spectroscopy of chromophores has been reported including rhodamine 6G (R6G),[5,6] crystal violet,[7,8] pseudo-isocyanine,[9] and hemoglobin,[10] thus encouraging our goal of detecting and identifying individual nucleotides.[11]

Colloidal Ag nanoparticles were prepared in an aqueous solution following a previous report.[12] An aliquot of a diluted Ag colloidal solution was incubated for 3 h with NaCl (10 mM) and R6G (10^{-7} to 10^{-10} M) or adenine (10^{-6} to 10^{-9} M) as a neutral form. Ag particles covered by the chromophores were prepared by spin-coating one drop of diluted Ag particles onto a glass cover slip coated with 3-aminopropyltrimethoxysilane. The number of chromophores adsorbed on a Ag particle, without distinction between isolated and aggregated, was evaluated by the following method. In this evaluation, all of the chromophores in solution is assumed to be totally adsorbed on Ag particles. In short, the concentration and the surface area of Ag particles were roughly estimated from the amount of Ag ions added, the ionic radius of a Ag ion, and the average radius of a Ag particle (~40 nm) assuming that all particles are in a sphere. SERS images and spectrums were obtained using Raman microscopic spectrophotometer (Ramascope System 2000, Renishaw, UK) composed of a light microscope, a CCD video camera, and a spectrometer. The use of 488-nm excitation was ~40 W/cm^2 for imaging experiments and ~9 kW/cm^2 for spectroscopic experiments. Morphology of Ag particles was evaluated using a tapping-mode AFM (Nanoscope III, Digital Instruments, CA).

Before tackling adenine we implemented SERS experiments involving R6G to find that our experimental methods are valid. The use of R6G is a touchstone for single-molecule SERS imaging and spectroscopy, because R6G is photostable and suitable for standard blue-to-green excitation. The following two observations (data not shown) by ourselves provide substantial evidence for single-molecule SERS imaging and spectroscopy of R6G. First, the observed SERS spectrum was identical to that of R6G previously reported.[5] Second, SERS of R6G showed blinking in intensity and spectral diffusion with time[5,6] when the average number of R6G molecules on a particle was 3 and 0.3.[13] Indeed, blinking and spectral diffusion are key observations not only for single-molecule SERS[5,6,8] but also for fluorescence single-molecule spectroscopy.[1,2] Furthermore, the following three concentration-dependent observations (data not shown) showed that R6G was quantitatively adsorbed on Ag particles. (i)The number of observable SERS-active "hot" particles, (ii) the number of hot particles without blinking, and (iii) the intensity of SERS from hot particles without blinking increased with increasing the average number of R6G molecules on a particle: 0.3, 3, 30, and 300/particle. Note that each of these average numbers corresponds to 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M of the R6G solutions used for preparing samples, respectively. The observation (i) means that Ag particles are not identified as hot ones unless possible hot sites are occupied by R6G molecules. The observations (ii) and (iii) mean that many R6G molecules occupy possible hot sites on a hot particle with increasing R6G molecules. Under the 0.3/particle condition, the number of observed hot particles was smaller than 1% of the total particles; and all the observed hot particles showed blinking. All the observed hot particles were aggregated ones, not isolated single particles. This observation is also consistent with the previous reports.[6,7,9,10]

We are now ready to implement single-molecule SERS imaging and spectroscopy of adenine after the R6G experiment. Note, however, that two decided differences between R6G and adenine make it difficult to follow exactly the R6G experiment using adenine. First, R6G is cation in aqueous solutions, whereas adenine is not always so. Thus, R6G is favorable for adsorption on Ag particles because Ag particles intrinsically load a negative charge.[14] Almost all of adenine was in the neutral form in the solution (pH = 7) we prepared. The p*K* value of N1 in adenine is 4.15;[15] thus, the molar ratio of neutral adenine to cationic

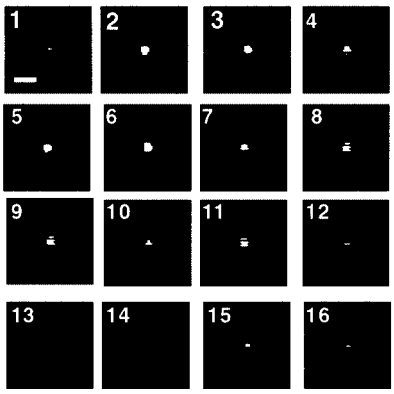

Figure 1. Time-resolved SERS images of adenine adsorbed on a single hot Ag particle prepared by using a 10^{-6} M adenine aqueous solution. Sequential images from 1 to 16 were recorded every 1/30 s. The scale bar in 1 is 2 μm in length. The contrast of images was modified for enhancing visibility.

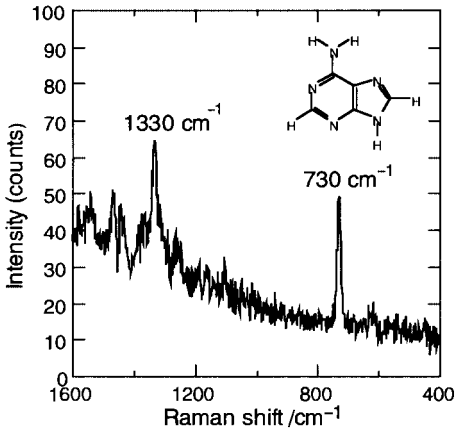

Figure 2. SERS spectrum of adenine adsorbed on a single hot Ag particle prepared by using a 10^{-6} M adenine aqueous solution. Data accumulation time was 1 s. The inset shows the molecular structure of neutral adenine.

adenine is ~1000:1 at pH = 7. Second, the 488-nm excitation is in resonance with R6G but is totally out of resonance with adenine. The enhancement factor under the resonance varies from experiment to experiment (10^2 to 10^5).[16]

Figure 1 shows a temporal change in the intensity of SERS from a hot particle on which adenine molecules were adsorbed. These time-resolved images identify a blinking behavior of SERS. Figure 2 shows a Raman spectrum from a hot particle showing the blinking behavior. This Raman spectrum is identical to that of adenine previously reported; a peak of a breathing mode (730 cm^{-1}) and a peak of a ring-stretching mode (1330 cm^{-1}) were identified. From the blinking and spectrum, we consider that single-molecule SERS imaging and spectroscopy of adenine is achieved. However, the concentration of an adenine solution used for preparing the sample was 10^{-6} M.[17] If all of adenine molecules in a 10^{-6} M solution is adsorbed on Ag particles, the average number of adenine molecule on a particle is ~3000/particle. This estimation sharply contradicts the observation of blinking of adenine SERS; indeed, observation of blinking is the most convincing evidence for single-molecule SERS imaging.[6,8] To decide the contradiction we should remember the two differences between R6G and adenine described in the previous paragraph. Assuming that only cationic chromophores adsorb effectively on a Ag particle[18] and that only extreme hot sites among hot sites compensate lack of a gift of the resonance, the use of a 10^{-6} M adenine solution could be equivalent to that of a 10^{-9} to 10^{-10} M R6G solutions. Anyway, real aspects of this contradiction are unknown at the moment.

In conclusion, with the help of Ag colloidal nanoparticles we have observed blinking of SERS involving adenine molecules, which is the most important evidence for achievement of single-molecule SERS.

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References and Notes

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11 A previous study (K. Kneipp, H. Kneipp, B. Kartha, R. M. Manoharan, G. Deinum, I. Itzkan, R. R. Dasari, and M. S. Feld, *Phys. Rev.*, **E57**, R6281 (1998)) reported single-molecule detection and spectroscopy of adenine. This study, however, like refs 7 and 9 detected SERS intensities of adenine on SERS-active hot particles subject to Brownian movement in a probe volume in solution using burst detection technique. Without imaging technique, no one identifies where are hot particles and what percentages of all particles are hot particles. Moreover, the temporal trajectory of SERS intensities on a single hot particle cannot be obtained by using burst detection technique.
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17 In the SERS of adenine we observed blinking behavior not only showing regular adenine spectrums, but also showing unexpected broad spectrums without peaks like the broad background in Figure 2 and sudden appearance of unidentified peaks not ascribable to adenine. These unexpected phenomena became dominant and the signal-to-noise ratio of regular adenine spectrums was reduced with decreasing concentrations of adenine solutions (10^{-7}, 10^{-8}, and 10^{-9} M). We observed clear adenine spectrums only when using adenine solutions of 10^{-6} M or higher concentrations. These unexpected phenomena were not observed for R6G.
18 To increase the number of cationic adenine molecules, the pH of the solution was changed. Unfortunately under acidic conditions (pH = 3–4) Ag particles formed precipitations, which are much larger than hot particles composed of aggregated particles. Thus, it is impossible to implement SERS experiment using acidic solutions as it is.