# Single-molecule surface-enhanced Raman scattering of R6G in aqueous environment under non-resonance conditions

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The single-molecule surface-enhanced Raman scattering (SERS) spectra of Rhodamine 6G (R6G) in an aqueous environment under non-resonance conditions are studied. Series of spectra are recorded in time-mapping mode, and intensity fluctuations of SERS signals and spectral diffusion are observed. The correlations between the presence frequency of SERS spectra and number of hot spots as well as the quantity of molecules in scattering volume are examined thoroughly. The results indicate that only molecules located at hot spots produce good signal-to-noise ratio Raman spectra and the origin of fluctuating SERS signals are mainly ascribed to the movement of hot spots.

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Initial reports on single-molecule surface-enhanced Raman scattering (SMSERS) were presented simultaneously by the Nie *et al.* in 1997[1,2]. In their experiments, there was less than one molecule, on average, in scattering volume by preparing ultra-low concentration solution and then recording the SMSERS afterwards. Moreover, they observed spectral fluctuations and considered these as comprising a character of "single-molecule" Raman signals. Since then, there has been increasing attention given to the domain of SMSERS, and various strategies have been provided to improve the experimental methods and explore the nature of SMSERS. For instance, some groups have prepared substrates using the Langmuir–Blodgett technique in order to achieve better distribution of molecules on the surface-enhanced Raman scattering (SERS) substrate, thus obtaining single-molecule Raman signals[3−6]. The technique of tip-enhanced Raman scattering (TERS) with high spatial resolving power obtained Raman signals of a molecule that are localized at the hot spots[7−9].

In recent years, a significant technique, the bi-analyte SERS, has emerged. This method provides a strong proof for the existence of SMSERS based on frequency, rather than intensity[10−15]. Of note, the substrates used in previous SMSERS studies are mostly solid-based, generally with immobilized nanoparticles on smooth film[3−9,12,14]. This is because SERS signals in aqueous solutions are known to be of poor stability due to the Brownian motion of particles[16]. Nevertheless, although the aqueous-based SERS substrates have some drawbacks, they have potential applications in various domains, especially in the field of biology[17]. Since water is the biggest component in the body, and most biochemical processes take place in an aqueous environment, aqueous-based SMSERS studies are crucial in providing a more reliable environment to simulate biological systems than with solid-based substrates.

At present, few studies of SMSERS in aqueous solution have been reported; most of the previous studies reported on acquiring "single-molecule" Raman signals by statistical methods from "high-concentration" probing molecules, signals originating from several molecules[10,11], and under resonance conditions[16]. The previous studies have also shown that SMSERS only originates from the special sites in substrates, which are called hot spots[1,18−20]. Theoretically and experimentally, hot spots have a heterogeneous structure (e.g., aggregated Ag and Au colloids) and only some sites are SMSERS-active in the SERS substrates[18,19,21]. Therefore, gaining better understanding of the relationship between SMSERS and hot spots is significant in SMSERS research.

In this letter, we focused on SERS of Rhodamine 6G (R6G) under non-resonance condition in colloidal silver suspension, from which we obtained the Raman signals with single-molecule characteristic. The spectral fluctuations were found to be correlated to the number of hot spots. The results suggest the significance of applying SMSERS to biological systems in the future.

R6G was purchased from Acros Company and used as received. Deionized water was used throughout the experiments. All other reagents employed were of analytical grade. Raman spectra were obtained with a spectrometer (JY Raman System Model 800, JOBIN YVON, France) with a 50-fold-long working length lens and a grating of 600 lines/mm. The 632.8-nm He-Ne laser was used as the excitation light. The laser power at the sample position was about 3.0 mW.

The silver colloids were prepared according to the procedure described by Lee and Meisel. In brief, 36-mg $AgNO_3$ was dissolved in 200-mL water. The solution was boiled with vigorous stirring. Then, 10 mL of 1% trisodium citrate aqueous solution was added to the boiled $AgNO_3$ solution drop by drop with vigorous stirring. The silver colloids were obtained when the solution was kept boiling with continuous stirring for 30 min. The solution was allowed to cool down naturally. The solution was diluted to 200 mL before use. Aliquots of R6G and KCl aqueous solutions were added into the Ag solution to obtain the sample with the concentration of $1\times10^{-12}$ mol/L R6G and $1\times10^{-2}$ mol/L KCl, respec-

 

tively. SERS measurements were carried out when the sample was kept for more than 1 h for full incubation.

The concentration of colloidal particles was estimated to be no less than $1\times10^{-10}$ mol/L, according to the size of the Ag particles. The concentration of the analyte was $1\times10^{-12}$ mol/L, and the ratio of the number of particles to analyte determinately exceeded 100. This meant that beyond 100 particles, only one molecule was captured, which made it unlikely that there could be more than one R6G molecule per particle. The scattering volume was estimated based on the method reported[22]. In brief, the intensity of Si 520-cm$^{-1}$ band was obtained when Si wafer was immersed into water. The signal intensity varied with the defocus distance in the axial direction ($z$ axis); the relationship profile of intensity–$z$ was then obtained. The microscope detection distance of $z$ axis was ~300 $\mu$m, and the focus length was defined as the full-width at half-maximum (FWHM) of the intensity versus defocusing-distance profile of ~100 $\mu$m (Fig. 1). In the radial direction, the beam diameter was estimated to be ~2 $\mu$m[22]. As a result, the scattering volume was estimated to be ~300 $\mu$m$^3$.

In the SMSERS experiment, the concentration of R6G was $1\times10^{-12}$ mol/L. The quantity of analyte in the scattering volume was estimated to be ~0.2 according to the obtained scattering volume and analyte concentration. Another problem in the test that was taken into account was the Brownian motion of colloidal particles in an aqueous solution. We obtained the resident time of particles in scattering volume according to the formula of Ag nanoparticles diffusion in water: $\tau_{\rm D}=V^{2/3}/(2D)$, where $\tau_{\rm D}$ represented the resident time of particles in scattering volume, $V$ represented the scattering volume, and $D$ represented the diffusion coefficient. The diffusion coefficient of Ag particles in water with diameter of 150 nm was 0.75 $\times10^{-8}$ cm$^2$/s. Considering that the velocity of particles was inversely proportional to their sizes, and the particle average diameter in the experiment was ~50 nm, small aggregated clusters composed of several particles were also presented, and $\tau_{\rm D}$ was calculated to be more than 10 s. As a result, the spectra originated from the molecules adsorbed on the same particle (or cluster) during each measurement since the collection time was only 1 s. We determined that the Raman signal was SMSERS based on a previous analysis.

In the SMSERS experiment, 1,000 SERS spectra were recorded in time-mapping mode with 1-s collection time per spectrum and 1-s interval between two adjacent measurements. As expected, the series of R6G Raman spectra showed obvious dynamics. Intensity fluctuations have been reported by different groups with varied elucidations[16,22,23]. In the present work, we focused on the spectra blinking. Intensities of spectra exhibited quasi periodic variation with the time (Fig. 2). One group of strong enhancement (SE) bands emerged per tens of seconds. We checked all of the 1,000 spectra and found that 142 of these could distinguish the R6G Raman signals from the background. In addition, all of the discernible spectra were divided into 59 groups on the basis of the intensity variation and the time of the spectra recorded in consideration of resident time in scattering volume of the the Ag particles/clusters. Among these, 36 groups contained strong Raman bands.

Recently, Etchegoin *et al.* have reported that SMSERS signals of R6G could not be detected unless the SERS-active substrates give rise to no less than $1\times10^8$ times of enhancement[23]. At the same time, Fang *et al.* have synthesized immobilized Ag particles with diameter of 300 nm on solid wafer and detected the distribution of hot spots. Their results indicated that only about 1% sites could come into being at no less than $1\times10^8$ times of enhancement *et al.*[21]. Of note, SERS-active substrates used in the current work were different from those of Fang *et al.*[21]. We evaluated the number of hot spots in our sol system with a simple method. Being consistent with the SMSERS detection condition, only R6G concentration was changed up to $1\times10^{-7}$ mol/L, and the concentration of the KCl and Ag solutions were kept constant. In the SMSERS test, 100 SERS spectra were recorded in time series with 1-s collection time per spectrum and 1-s interval between two successive measurements. In all, 19 spectra gained stronger enhancement, taking up 19%; then, 4 spectra were mostly enhanced, accounting for 4% (Fig. 3). In contrast to the results of the SMSERS experiment, 59 segments (probability is 5.9%) produced distinguished signals and of these, 36 groups had SE spectra (probability is 3.6%). Moreover, 200 spectra was achieved when every single molecule entering the scattering volume was detected (the product of the presence probability of the molecule in scattering volume was 20%, and the number of measurements was 1,000). In the following, we considered the quantitative relationships of Raman signals, hot spots, and molecules. If only molecules adsorbed at hot

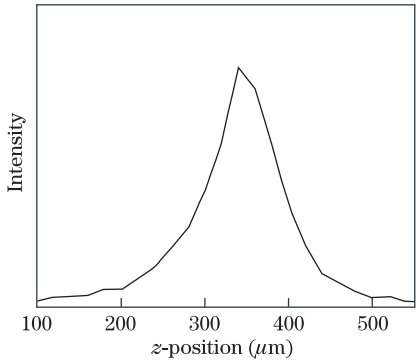


Fig. 1. Intensity of the Si 520-cm$^{-1}$ Raman band varies with the defocusing distance in the axial direction during the confocality test. The FWHM of this curve (~100 $\mu$m) defines the scattering length in the axial direction.

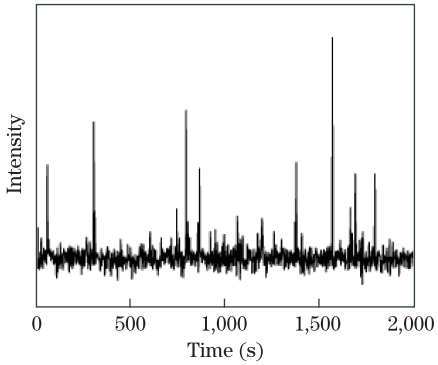


Fig. 2. Intensity of R6G Raman signals around the 613-cm$^{-1}$ band varies with the time in the SMSERS experiment.

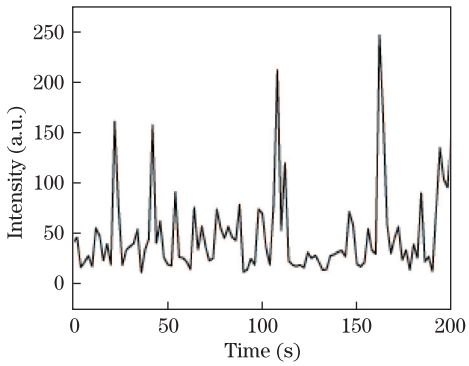


Fig. 3. Intensity of R6G Raman signals around the 613-cm$^{-1}$ band varies with the time in the hot spot detecting experiment.

spots could be detected, 38 spectra would be obtained (200×19%), which were approximately 36. The varying intensity of R6G Raman spectra with time was observed, and there were 8 huge enhancement (HE) signals in all spectra (Fig. 2). The presence probability of HE signals in all measurements was 0.8%, which was equal to the product of the presence ratio of molecules (20%) in scattering volume and the hot spots (4%). The results showed that molecules had similar opportunities to adsorb on every particle, with no special tendency to adsorb on the hot spots.

In addition, in this work, we focused on the intensity variations of spectra in each group. The results indicated that most groups changed in a similar way. Generally, one group persisted for about tens of seconds, as long as the residence time of particles in the scattering volume was the same. Each group usually contained several weak signals and one or two SE lines (Fig. 4 is one typical group). SE signals were recorded when molecule-hot spots system on the position of the focus spot. Raman signals turned weak due to molecule-hot spots system rotating or moving away from the center position. There were no Raman spectra detected when the system went out of the scattering volume. The intensity fluctuations of R6G Raman signal definitely indicated that hot spots were the dominant factord in SMSERS detection in an aqueous environment.

As reported previously, variations of frequency positions, relative intensities and line-widths, and even in no assigned bands were discovered in series spectra (Fig. 5). Bands at 1,033 cm$^{-1}$ of spectra were obtained at 540 and 552 s, and bands at 636 cm$^{-1}$ in the 1,112-s line were not assigned to any vibrations of R6G. The "spurious" SERS signals were considered to have originated from different residual molecules in the analyte-sol system[24−26]. Spectral diffusion was also found (Fig. 6), indicating statistical fluctuations of the spectral bands around an average frequency position, relative intensities, and line-widths. Spectral diffusion is a characteristic phenomenon in SMSERS, which has been widely investigated in previous work. Referring to previous work on single-molecule fluorescence[27,28] and SMSERS[25,29,30], the variations found in the present study were mainly ascribed to three reasons. The first was the different interactions between analyte and Ag particles. The R6G molecules occupied different sites with diverse local environment consisting of metal surface, citrate anions, and chloride anions. These locations had multiplex nanostructures that produced various effects on the residing molecules, including electromagnetism and chemistry influences. The second was the orientation change between laser line and the molecule-particle compound. That molecules stayed on the Ag surface in different orientations was regarded as taking some responsibilities as well.

In conclusion, we investigate the SMSERS spectra of R6G in Ag sol under non-resonance conditions. 1,000 spectra are recorded in time-mapping mode. The fluctuating intensities of the SERS signals and spectral diffusion are also observed. The number of hot spots in Ag sol is checked. In addition, the correlations between the present frequency of SMSERS signals and number of hot spots as well as molecules in the scattering volume are examined thoroughly. The results show that good signal-to-noise ratio Raman spectra originated from molecules

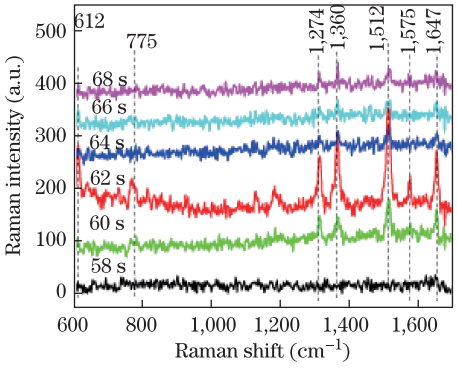


Fig. 4. Group of typical SERS spectra with gradual changes from 58 to 68 s.

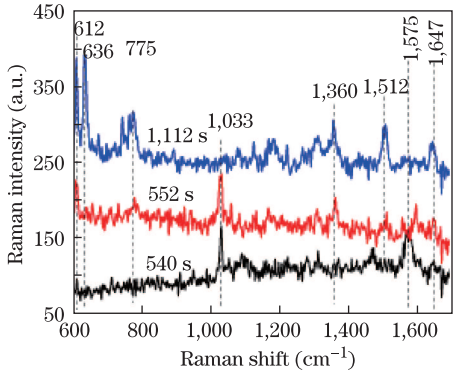


Fig. 5. Selected SERS spectra with "spurious" signals.

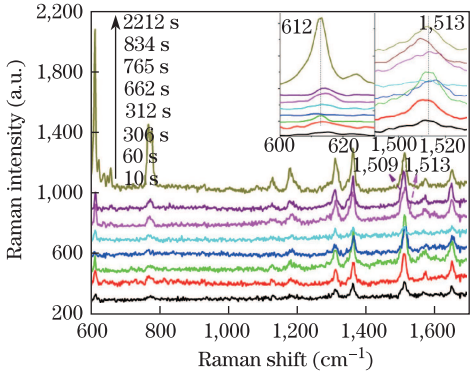


Fig. 6. SE Raman signals of R6G with spectral fluctuations and frequency wandering; the expanded views around the 612 and 1,513 cm$^{-1}$ bands are inserted.

locate at the hot spots, and the phenomenon of fluctuating SERS signals is mainly ascribed to the movement of hot spots. Moreover, spectral diffusion result from the cooperation of the complicated structure of particle surface, varies angles between molecules and metal surface, as well as the different directions of laser lines relative to the analyte-particle complexes. On account of an aqueous environment being similar to actual human body conditions, this study is expected to benefit the application of SMSERS in biological systems in the future.

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