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Surface-enhanced resonance Raman scattering spectroscopy of single R6G molecules*

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Surface-enhanced resonance Raman scattering (SERRS) of Rhodamine 6G (R6G) adsorbed on colloidal silver clusters has been studied. Based on the great enhancement of the Raman signal and the quench of the fluorescence, the SERRS spectra of R6G were recorded for the samples of dye colloidal solution with different concentrations. Spectral inhomogeneity behaviours from single molecules in the dried sample films were observed with complementary evidences, such as spectral polarization, spectral diffusion, intensity fluctuation of vibrational lines and even "breathing" of the molecules. Sequential spectra observed from a liquid sample with an average of 0.3 dye molecules in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1 or 2 molecules. Difference between the SERRS spectra of R6G excited by linearly and circularly polarized light were experimentally measured.

Keywords: single-molecule spectroscopy, surface-enhanced resonance Raman scattering, spectral polarization, Poisson distribution

PACC: 3320F, 7830C, 2470, 7730

1. Introduction

Single-molecule detection has been achieved by improvement of experimental methods and sensitivity of detectors.[1−3] The fluorescence indicator is widely used in the biomedical detection of single molecules. However, the fluorescence spectra are so wide that relatively little information about detected molecules can be inferred from that. Raman spectra are determined by the energy of molecular vibrations, which can provide a structural "fingerprint" of the molecules. In the normal state, the cross section of Raman scattering is extremely small, being about 10^{-30}cm^2·molecule^{-1}, which is far beyond the instrument detection limit. In 1974, Fleischman observed unexpectedly high Raman signals from pyridine on a rough silver electrode, which was named surface-enhanced Raman scattering (SERS) now.[4] Raman signal can be enhanced in the order of 10^{14} using SERS or surface-enhanced resonance Raman scattering (SERRS) now, which makes possible the detection of Raman scattering spectra of single molecules.[1,2]

In this paper, R6G was incubated in silver sols with different low concentrations and its SERRS spectra excited by linearly or circularly polarized light were studied. The dye molecules were absorbed on the silver nano-particles because of the opposite charges they hold.[5] From the dried sample films of 10^{-13}M (1M=10^{-3}mol/cm^3) dye colloidal solution, spectral inhomogeneity behaviours from single molecules were observed, such as in spectral polarization and intensity fluctuation of vibrational lines, and even the "breathing" of molecules. From the liquid sample of 5×10^{-14}M dye colloidal solution with an average of 0.3 dye molecules in the probed volume, spectra observed in a time sequence exhibited the expected Poisson distribution for actually measuring 0, 1 or 2 molecules. Difference between SERRS spectra of R6G excited by linearly and circularly polarized light were experimentally measured. A proper method for measuring Raman spectral polarization of single molecules

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was given.

2. Experimental

2.1. Sample preparation

Sliver sols were prepared using the Lee and Meisel method[6] and characterized by UV–VIS absorption spectra (λ_{max}=450nm). 10μL of dye solutions with different concentrations (10^{-7}, 10^{-9} and 10^{-11}M in methanol) were diluted in 1 mL silver sols (100 fold), resulting in a final R6G concentration of 10^{-9}, 10^{-11} and 10^{-13}M, respectively. These solutions were stored for at least 24 hours with frequent shaking to make the dye molecules adsorbed on the silver particles. About 20μL of each solution was spread onto a microscope cover slide respectively. After evaporation of the solvent, which will take tens of minutes to some hours depending on the situation, the dried sample films were mounted onto the scanning stage of the microscope.

Silver nitrate, sodium citrate, and R6G of analytical reagent grade and methanol of HPLC grade and doubly distilled water were used. Polystyrene tubes were used to avoid the absorbing of dye molecules on the wall of vessel.[7] Microscope cover slides were kept more than two days in chromosulfuric acid before use.

2.2. Instrumentation

All optical studies were performed with a custom-made confocal microscope (Fig.1). A microscope objective (40×, NA=0.65) was used to focus the excitation light and collect the scattered light. A single longitudinal mode laser (SUWTECH LDC 2500, at 532nm) served as excitation source and the laser was circularly polarized by a zero order $\lambda/4$ wave plate. A feedback-controlled sample stage (YA07, KOHZU) was used to scan and position the sample with nanometre precision. Raman signal and excitation light were separated by a dichroic filter. A holographic notch filter (Kaiser, λ= 532nm, FWHM=10nm) was used to cut off the remainder of excitation light. Raman spectra were acquired by a spectrograph (SpectroPro 300i, f=300mm, $T = -30^{\circ}$C, Acton). The spectral resolution was 4cm^{-1} and the sample was exposed to air in all experiments.

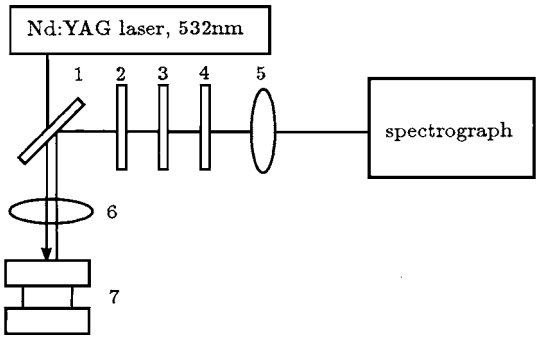


Fig.1. Diagram of experimental system. (1) dichroic filter (2) holographic notch filter (3) analyser (4) $\lambda/2$ wave plate (5) lens (6) objective (7) scanning stage.

The excitation power was about 5mW. In measuring Raman spectral polarization, a zero order $\lambda/2$ wave plate was used to keep the polarizing direction of signal unchanged relative to the slit of spectrograph.

3. The SERRS spectra from the dried sample films

3.1. SERRS spectra of the ensemble and single molecules

Figure 2 shows the spectra recorded from "hot dot" of three samples of R6G with different concentrations (10^{-9}, $10^{-11}$and 10^{-13}M). Both pure fluorescence and the coexistence of fluorescence and Raman spectra can be found from different positions of the first two samples, as shown in Fig.2(a) and 2(b) respectively. The difference between the first two samples is in that the fluorescence dominates if a sample is a little wet, while Raman signal will stand out if the sample is dry enough. Actually Raman signal and faint fluorescence can be detected only from a few positions of the second sample, indicating a greatly inhomogeneous distribution of the silver clusters and the absorbed molecules. With decreasing of the dye concentration (10^{-13}M), fluorescence was quenched[8] and Raman spectra were clearly observed without fluorescent background, as shown in Fig.2(c). This is associated with a further decrease of silver clusters and absorbed molecules in the probed volume. We estimated that only an average of 0.02 R6G molecules

exist in the probe volume in the 10^{-13}M sample.[1)] Furthermore, at this ultra-low dye concentration we found some inhomogeneous spectral behaviour which indicates that surface-enhanced Raman spectra of single dye molecules were being observed.

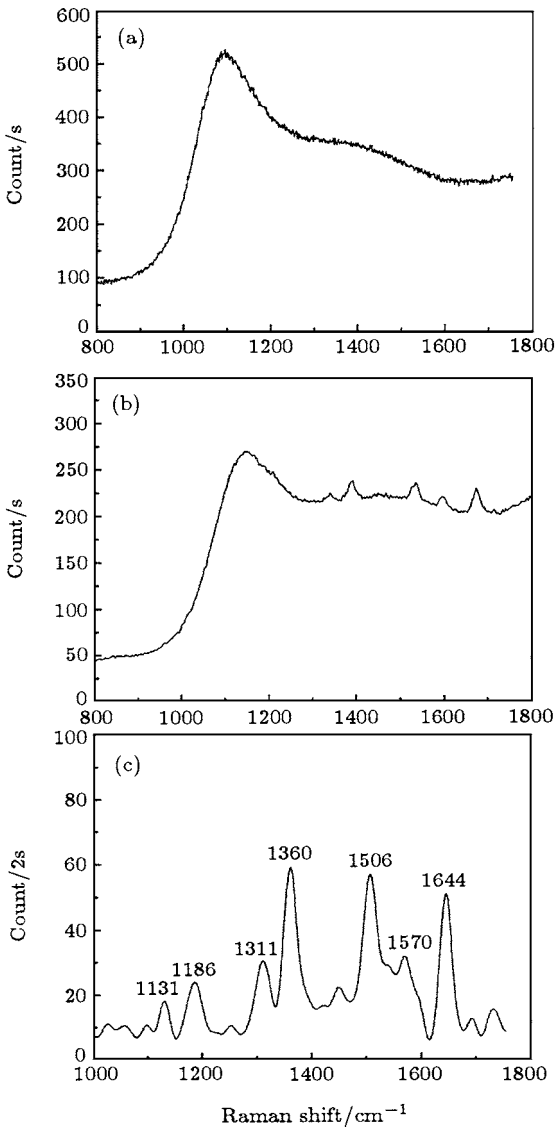


Fig.2. Spectra of R6G colloidal silver sample with concentration of (a) 10^{-9}M, (b) 10^{-11}M and (c) 10^{-13}M. Integration time for spectra in (a) and (b) is 1s and (c) is 2s.

3.2. Confirming the single molecule by the polarized Raman spectra

For more evidence of single molecules, the spectral polarization was examined. An analyser and a $\lambda/2$ wave plate were inserted into the optical path (See Fig.1) and rotated simultaneously. The rotating angle of the $\lambda/2$ wave plate was half that of the analyser to fix the polarizing angle of Raman signal relative to the slit of the spectrograph. Polarized Raman spectra at two mutually perpendicular directions were recorded and shown in Fig.3. Each spectrum was integrated for 5s and circularly polarized light was used for excitation. The vibrational frequencies in the spectra are similar to those of solution resonance Raman and bulk surface-enhanced Raman spectra of R6G although the relative spectral intensities are considerably different.[2] The intensity of the bottom spectrum is much weaker than that of the upper one. Indeed, some vibrational frequencies even disappear in the bottom spectrum. This phenomenon is due to the spatial distribution of the polarization tensor of single molecules which will not appear in the case of many molecules because of the spectral averaging.[9]

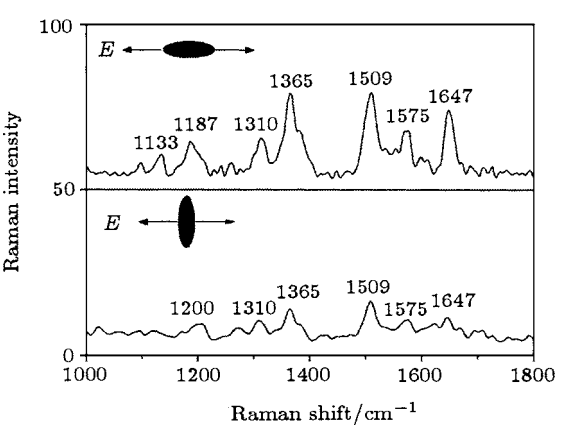


Fig.3. Polarized SERRS spectra of R6G single molecules. Integration time of each spectrum is 5s.

[1)] 20μL (V_{total}) of the sample solution with the concentration of 10^{-13}M was spread onto a microscope cover slide. An area of colloidal film about S_{total}=0.5cm×0.5cm was formed after evaporation of the solvent. Based on the Rayleigh equation, the radius of the focus spot of the excitation light is about $r = 0.61\lambda/\text{NA} = 0.5\mu$m (NA=0.65, λ=0.532μm). The probing area is about $S_{\text{probe}}=3.9\times10^{-13}\text{m}^2$. So we can get that an average of 0.02 R6G molecules exist in the probe area in the 10^{-13}M sample $\left(\frac{S_{\text{probe}}}{S_{\text{total}}} \times V_{\text{total}} \times 10^{-13} \times 6.02 \times 10^{23} = 0.02\right)$. At the ultra-low concentration (10^{-13}M), the collected signal came mainly from the dye molecules adsorbed on the substrate because of the quenching of fluorescence. Therefore the actual average numbers of dye molecules in the area volume may be less than 0.02 but may not be greater than 0.02.

3.3. Observation of spectral diffusion and the "breathing" of single molecules

Figure 4 shows a sequence of 12 consecutive spectra recorded from a "hot dot" of the 10^{-13}M dye sample with an integration time of 1s per spectrum. The vibrational frequencies of Raman signal at about 1365, 1507, 1570 and 1647 cm^{-1} changed at different time (as large as 9cm^{-1} sometimes), which was called spectral diffusion. The total intensity changed gradually from the first to the 12th spectrum in a intense-weak-intense sequence, in which the weakest 6th spectrum became almost flat, which can be due to the "breathing" of molecules. Intensities of Raman signal at about 1647, 1570, 1507, 1365, 1310, 1187 and 1133cm^{-1} changed relatively for each spectrum, and

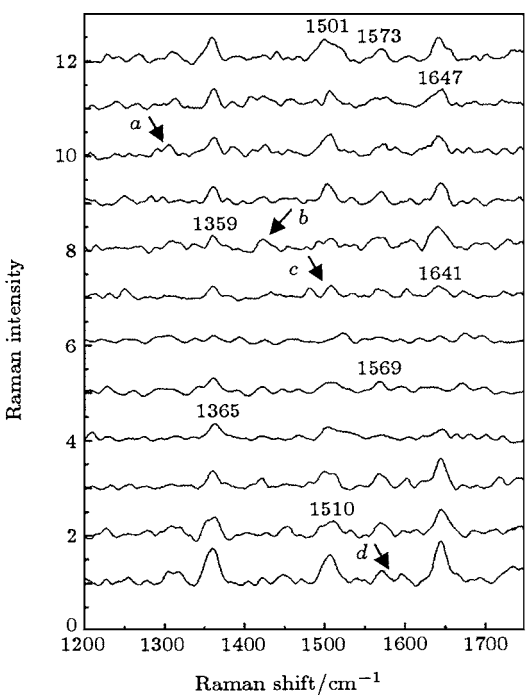


Fig.4. Sequence of 12 consecutive SERRS spectra of R6G with an integration time of 1s per spectrum. peak *a* 1310cm^{-1}, peak *b* 1420cm^{-1}, peak *c* 1507cm^{-1}, peak *d* 1570,1596cm^{-1}.

intensities at 1421cm^{-1} (Fig.4(b)) and 1596cm^{-1} (Fig.4(d)) in some spectra are more intense than their counterpart in the spectrum in Fig.3 (with an integration time of 5s). The spectral inhomogeneity behaviours, such as spectral diffusion and intensity fluctuation, are due to the dynamic interaction of the molecules with local environment. All these evidences are complementary and confirm that the SERRS spectra of single molecules were observed in our experiment.

4. The SERRS spectra of single R6G molecules in liquid

We studied the SERRS spectra of single molecules of R6G in liquid circumstance also. A sample with concentration of 5×10^{-14}M was prepared, which means an average of 0.3 molecules in the probed 10pL volume (the scattering volume in our sample being estimated to be 10pL according to the method described in Refs.[1] and [10]). The same experimental setup as Fig.1 was used, except that the 40× objective was replaced by a 100× microscope objective (100×, NA=1.25 in oil). The sample droplet in a pipette tip was put on the objective upside down to bring the objective into direct contact with the sample solution. Brownian motion of the silver clusters transports the dye molecules in and out of the scattering volume, resulting in a fluctuation of the spectral intensity. The average residence time of a particle in the probed volume can be roughly estimated to be about 10 seconds,[2)] which is ten times longer than the measurement time (1 second per spectrum). We can infer that the detected spectral intensities should fluctuate randomly based on the intensity of single molecules.

A sequence of 100 spectra was recorded consecutively, and the spectral intensity of vibrational line at 1650cm^{-1}, corresponding to the aromatic C–C stretch band,[3] was statistically analysed. The histogram of the intensities was showed in Fig.5. In contrast to the SERRS signal of many molecules, the statistical distribution of the "0.3 molecules SERRS signal" exhibits three relative maxima which can be fit by the superposition of three Gaussian curves conforming to a Poisson distribution. The three maxima reflect the probabilities to find 0, 1 or 2 molecules in the scattering volume during 1 second, respectively. This is the first observation of the SERRS spectra of single R6G molecules in liquid. A more complete analysis about the SERRS spectra of single R6G molecules in liquid will be given in a future paper.

[2)] The estimated residence time of a colloidal particle in the probed volume is $T_D = w^2/2D$, based on the Brownian diffusion, where w is the radius of the volume (approximately 2.5×10^{-6}m), and D is the diffusion coefficient for our colloidal silver particles in water.[15]

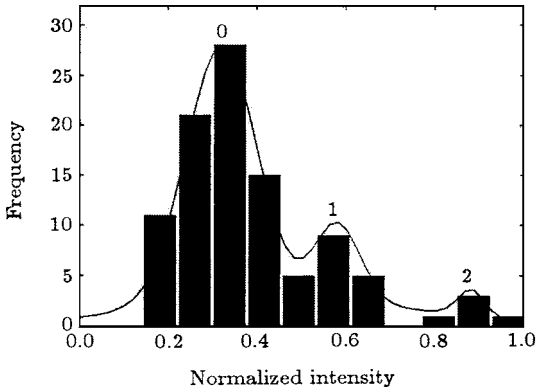


Fig.5. Histogram of the intensities of the 1650cm-1 SERRS signal of R6G molecules. The multiple peaks are associated with the statistical presence of 0, 1 or 2 molecules in the probe volume, where 0.3 molecules are present on average. The curve is a fit with three Gaussian peaks.

5. Discussion

Linearly polarized light is widely used in SERS and SERRS experiments.[2,3] Theoretically, the Raman scattering spectra of the ensemble of molecules should be spatially homogenously polarized in the plane perpendicular to the propagating direction of excitation light irrespective of the polarizing direction of excitation light.[11,12] Meanwhile, polarized Raman scattering spectra should be observed in the case of single molecules. However, polarized Raman signal was observed in our SERRS experiment of R6G excited by linearly polarized laser where there exists much more than one molecule in the probed volume. A new sample was prepared for this measurement. 20 μL of R6G solution in methanol at concentration of 10^{-7}M was dropped on the sample of 10^{-9}M R6G colloidal solution and dried by hot air. At a "hot dot", the SERRS spectra in two perpendicular directions, excited by linearly and circularly polarized light respectively, were recorded after the bleaching of the fluorescence, as shown in Fig.6. The polarizing angle of Raman signal relative to the slit of spectrograph was kept unchanged through rotating the $\lambda/2$ wave plate in the measuring. Polarized spectra were observed despite of the existence of many molecules (about $2\times10^3-2\times10^4$ molecules existing in the probe volume) in the case of linearly polarized excitation (Fig.6(a)) while the intensities of spectra in both polarizing direction are almost the same in the case of circularly polarized excitation (Fig.6(b)). The spectra in Fig.6(a) may be confused with polarized Raman spectra of single-molecules in some circumstances. We attributed it to a great enhancement for molecules with special directions when excited by linearly polarized light.

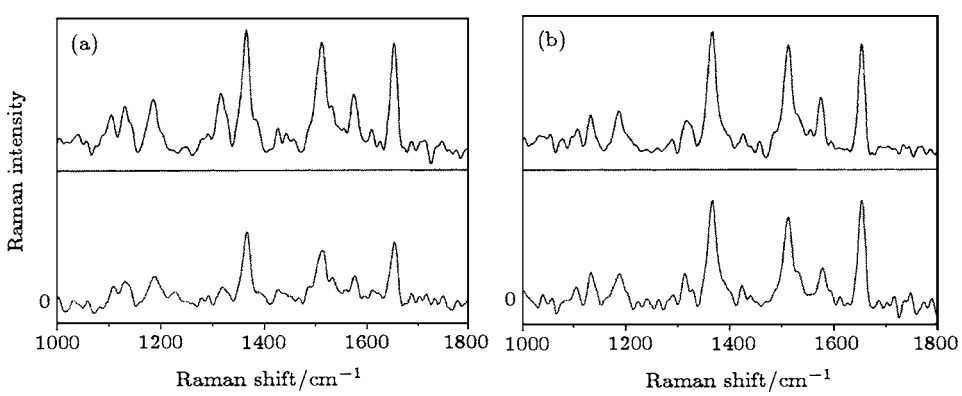


Fig.6. SERRS spectra of R6G excited by linearly (a) and circularly polarized light (b). Integration time of each spectrum is 2s.

6. Conclusion

SERRS spectra of single molecules were recorded in the sample of R6G solution of silver sols at ultra-low concentration. Some spectral inhomogeneity behaviours were observed such as in spectral polarization, spectral diffusion and intensity fluctuation of vibrational lines, and even the "breathing" of single molecules. The SERRS spectra of single R6G molecules in liquid circumstance were observed for the first time, which exhibited the expected Poisson distribution for actually measuring 0, 1 or 2 molecules. In measuring polarizing spectra of Raman signal, the excitation light should be circularly polarized to avoid fake results. Otherwise, the Raman spectra arising from bulk sample and single molecules may be confused. The spectral polarization of single molecules is an important tool in Raman spectroscopy and single-molecule detection, and must be analysed carefully.

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