In Situ Two-Step Photoreduced SERS Materials for On-Chip Single-Molecule Spectroscopy with High Reproducibility

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A method is developed to synthesize surface-enhanced Raman scattering (SERS) materials capable of single-molecule detection, integrated with a microfluidic system. Using a focused laser, silver nanoparticle aggregates as SERS monitors are fabricated in a microfluidic channel through photochemical reduction. After washing out the monitor, the aggregates are irradiated again by the same laser. This key step leads to full reduction of the residual reactants, which generates numerous small silver nanoparticles on the former nanoaggregates. Consequently, the enhancement ability of the SERS monitor is greatly boosted due to the emergence of new “hot spots.” At the same time, the influence of the notorious “memory effect” in microfluidics is substantially suppressed due to the depletion of surface residues. Taking these advantages, two-step photoreduced SERS materials are able to detect different types of molecules with the concentration down to $10^{-13}$ M. Based on a well-accepted bimolecular approach, it is proved that the detection limit reaches the single-molecule level. From a practical point of view, the detection reproducibility at different probing concentrations is also investigated. It is found that the effective single-molecule SERS measurements can be raised up to $\approx 50\%$. This microfluidic SERS with high reproducibility and ultrasensitivity will find promising applications in on-chip single-molecule spectroscopy.

Ultrasensitive detection down to single-molecule level is significant in a wide range of material science, analytical chemistry, and molecular biological diagnostics.[1,–] Among different analytics,[4] surface-enhanced Raman scattering (SERS)[7–13] combining the specificity of molecular fingerprints and sensitivity of plasmonic enhancement demonstrates highly potential applications in single-molecule spectroscopy.[14–20] Benefiting from the quick development of synthetic chemistry and nanolithography, various plasmonic nanomaterials have been designed to facilitate the SERS applications.[21–29] It is found that Raman signals from molecules located in the gap of plasmonic nanoaggregates (or “hot spot”) can be enhanced up to $10^8$–$10^{10}$ times due to the near-field coupling and nanoantenna effect.[30–38] which makes it possible for single-molecule detection (SMD) and imaging.[39–41] However, the practical applications of single-molecule SERS in routine analytics and diagnostics are still hindered by bottlenecks such as the poor reproducibility and low stability.[42,43] One feasible solution is to combine SERS material monitors with the microfluidic technique to enable convenient and controllable on-chip SERS detection.[44–52]

During these years, intensive efforts have been made to incorporate the SERS materials into microfluidic channels.[53–58] and applications on reproducible chemical analysis and biological diagnostics have been demonstrated.[39–62] Among which, the photochemical reduction provides a facile method to directly create SERS monitors into microchannel.[58,63] It has the advantages of in situ, freshly preparing, and real-time detecting for field analysis, thus avoiding the unexpected contamination and oxidation degrading. However, SERS detection in the microfluidic channels has the problem of the “memory effect” caused by the undesired adhesion of analytes/reagents to channel walls/SERS materials, and also encounter the difficulty in increasing the enhancement factor of microfluidic SERS materials to enable SMD.[64–67] These problems inevitably hamper the pursuit of on-chip SERS with high reliability and ultrasensitivity down to the single-molecule level.

Here, we propose a two-step photoreduction method to synthesize SERS materials with the extraordinary performance of highly reproducible single-molecule Raman detection, and incorporate these materials with microfluidic system to realize in situ on-chip single-molecule spectroscopy. Firstly, a spot of rudimentary silver nanoparticle aggregates is created in the microfluidic channel through photochemically reducing the Ag$^+$ ions in a focused laser spot. After washing the microchannel, the aggregates are immediately irradiated for the second time by the same laser. This process has a prominent effect to deplete the residual reactants and analytes owning to the photoreduction and photo-degradation; thus, it effectively eliminates the influence of “memory effect” on subsequent...
SERS measurements. More importantly, the residual reactants absorbing on rudimentary aggregates can be further reduced into new nanoparticles, which greatly improve the quantity and quality of “hot spots.” By this two-step photoreduction method, the “memory effect” in turn helps to extract the full potential of silver aggregates as SERS monitors. It is found that these microfluidic SERS materials have the ability to detect molecules, such as crystal violet (CV), Rhodamine 6G (R6G), methylene blue, hemoglobin, and 5-fluorouracil, at the concentration as low as 10^{-13} M. Standard bianalyte approach proves that it has reached the single-molecule level. Particularly, the reproducibility of SMD is greatly enhanced to ∼50% by this micro-fluidic SERS technique. The time needed for the whole process is only several minutes, which also facilitates fast and reproducible on-chip SMD and monitoring.

A schematic of the microfluidic detection system is shown in Figure S1 (Supporting Information), which is composed of a pump, a microfluidic chip, and a microscope Raman spectroscopy. The designed microchip with three functional modules (injection, mixing, and optical detection) is illustrated in Figure 1a (for details see Experimental Section). Figure 1b demonstrates how to incorporate photoinduced silver nanoaggregates into the microfluidic channel and perform in situ SERS detections of CV. Step ①, AgNO_3 (2 × 10^{-3} M) and sodium citrate (4 × 10^{-3} M) are simultaneously pumped into the channel through inlets 1 and 4 with a flow rate of 200 nL s^{-1} for 24 s. These two solutions meet in downstream of the injection module and then mix in the mixing module, forming the photochemical reagents. Step ②, in the optical detection module, a laser beam (λ = 532 nm, continuous wave) with the power 130 µW is focused on the glass slide through an objective (50×, N.A. = 0.5). After irradiation for 80 s, a spot of silver nanoparticle aggregates with the size ≈ 6 µm is formed in the laser focus due to the photochemical reduction of Ag^+ ions.[68,69] The scanning electron microscope (SEM) image shows that these nanoparticles have the size from 20 to 80 nm. Step ③, deionized water is then pumped into the channel through inlet 2 for 150 s with a flow rate of 1000 nL s^{-1} to wash the whole microchannel and the fresh-prepared SERS material. Step ④, CV (10^{-7} M) is introduced through inlet 3 with a flow rate of 400 nL s^{-1} for 24 s. During the CV injection, the molecules in solution can physically adsorb on the silver nanoparticles surface, thus directly feeling the near-field enhancement from the aggregates. Step ⑤, SERS signals of CV from silver aggregates are in situ recorded by the Raman spectroscopy through the same objective.

The steps ② and ③ are repeated several times. The recorded SERS spectra at each step ⑤ are presented by spectra 1–4 in Figure 1c. In the first spectrum, the Raman fingerprints of CV can be clearly identified at 1174, 1361, 1586, and 1619 cm^{-1}, respectively. Interestingly, the SERS intensity at the second-time measurement suddenly increases, so that more Raman modes (801 and 914 cm^{-1}) can be observed. This feature is clearly demonstrated in Figure 1d, where the peak intensity...
at 1174 cm\(^{-1}\) in each SERS spectrum is summarized in the sequence of measurements. More cases in Figure S2 (Supporting Information) demonstrate that the significant increase of SERS intensity at the second-time measurement is very reproducible. Statistically, the intensity of second SERS measurement is one order stronger than the first measurement. To understand this sudden increase of SERS signals, we first evaluated the intensity fluctuation at different aggregates under the same excitation conditions (Figure S3, Supporting Information). It shows that the relative deviations of SERS intensity for the first and second measurements are 21% and 27%, respectively. Both of them are much less than the magnitude of the significant increase at the second measurement, which indicates the reliability of the observation. We also exclude the influence of immersion time of SERS monitors in CV solution. As shown in Figure S4 (Supporting Information), although the CV injection time is prolonged from 24 to 180 s, apparent sudden increase at the second-time measurement always exists.

Then, we compare the morphology changes of silver aggregates before and after the second-time laser irradiation. As shown in Figure 2, numerous new nanoparticles are formed on the former aggregates (for detailed SEM images of other areas, see Figure S5 in the Supporting Information). It can be understood by the fact that, in experiments, deionized water flushing (Step ③) cannot remove all the photochemical reagents adsorbed on the silver aggregates. During the first SERS measurement, these residual reactants can be reduced into nanoparticles by further laser irradiation. New generated particles would bring numerous nanogaps/hot spots on the former aggregates. Then, these nanogaps are filled with molecules during the CV injection, which gives rise to the prominent raise of SERS at the second measurement. If we prolong the washing time, much less morphology changes can be found after second irradiation due to the great decrease of reactant residues (Figure S6, Supporting Information), which proves the key role of the photochemical reduction of reactant residuals, rather than other effects such as thermal effect. Here, we also note that the relative deviations for the first and second measurements have similar value (Figure S3, Supporting Information), which indicates the well stability of SERS materials before and after the reproduction of silver nanoparticles. After several times of laser exposure, the reactant residues are depleted. Then, the SERS signals decrease due to the photo-degradation of CV molecules on the silver nanoaggregates.\(^{[70]}\)

Based on these understandings, we propose a procedure to enhance the SERS ability of nanoparticle aggregates by introducing a two-step photoreduction process. The whole procedure is shown in Figure 3a. Firstly, after performing steps ①–②, a spot of rudimentary silver aggregates is created at the position shown in Figure 3b (first spot). After the washing step ③, this spot is ripened by a second-time irradiation of 532 nm laser with higher power 2.5 mW for 45 s, which triggers the reduction of reactant residues and depletion of any chemical adsorbing. Then, step ④ (CV injection) and step ⑤ (SERS measurements) are repeated several times and spectra C1–C3 are recorded. The whole procedure is carried out several times at different positions in the microchannel (e.g., the aggregates spots second and third). In the first round, it shows that there is no Raman fingerprint of CV in spectrum 1-A (Raman signals taken from the washed rudimentary aggregates) and spectrum 1-B (Raman signals taken from the ripened one). However, in the second-round procedure, the spectrum 2-A from the second aggregates obviously contains CV signals. This is a clear indication of the “memory effect” introduced by the first-round procedure. This “memory effect” can be largely eliminated by ripening the aggregates using a supplementary irradiation, as shown in the spectrum 2-B. To clearly illustrate this phenomenon, we summarize the Raman intensities at 914 cm\(^{-1}\) for these spectra (Figure 3d). Comparing the red columns with green columns, we find that the supplementary irradiation process can effectively reduce the “memory effect” by about 6 times on average. At the same time, the SERS spectrum C1 always gives the strongest intensity in all group of spectra, which demonstrates that the enhancement potential of silver nanoparticle aggregates has been fully extracted by the two-step reduction method.

In the following experiments, we put an effort to reach the detection limit of these SERS materials and evaluate the correlation between the reproducibility and probing
concentration. To ensure the validity, new microfluidic chip and syringes were used, and the measurements were performed from lowest \((10^{-13} \text{ m})\) to highest CV concentration \((10^{-6} \text{ m})\). For each measured concentration, the procedure presented in Figure 3a was carried out tens of times for statistics. For each monitor, SERS spectrum was acquired every 60 s during the CV injection, until characteristic peaks were detected. The injection time needed for the first successful SERS detection was recorded. The number of active SERS monitors and corresponding injection time needed are summarized in Figure 4.

At the concentration of \(10^{-13} \text{ m}\), 13% SERS monitors show characteristic Raman signals (5 in 38) after 60 s CV injection. By further increasing the injection time to 120 s, six more SERS monitors become active. Finally, 50% SERS monitors are able to work after 240 s CV injection (Figure 4a). The rest inactive monitors are attributed to null cases.

For higher concentrations \(10^{-12}\) and \(10^{-11} \text{ m}\), the performance is greatly improved (Figure 4b,c). It is found that majority of the fabricated monitors are SERS active in the first 60 s CV injection. After 240 s

Figure 4. Reproducibility of SERS detection. a–c) The statistics of successful SERS detection and the CV injection time needed. If there is no effective SERS signals after 240 s injection, it is attributed to the null case. Different CV concentration cases at \(10^{-13}, 10^{-12}, \) and \(10^{-11} \text{ m}\) are presented in (a), (b), and (c), respectively. d) Reproducibility of SERS detection for CV at different concentrations. The injection time is 240 s. The curve is plotted to guide eyes.
injection, only a small portion of monitors are still inactive. It is also noticed that the relation between the counts of the active SERS and the CV injection time is quite different from the case at $10^{-13}$ m. This difference suggests that SERS detection at $10^{-13}$ m has stepped into few/single-molecule level, where the absorption of a molecule in a potential “hot spot” is random. This is consistent to the SMD criteria $0.075 \times 10^{-12}$ m reported before.[71] Here, we define the SERS reproducibility as the ratio between all active counts within 240 s injection and the total number of measured monitors. As shown in Figure 4d, the reproducibility raises logarithmically as the increase of concentration from $10^{-11}$ to $10^{-10}$ m, and finally trends to 100% at $10^{-7}$ m. This can be understood by the fact that the larger the concentration and longer injection time, the higher the possibility of molecules that could be adsorbed in “hot spots,” thus increasing the probability of successful SERS events. Some representative SERS spectra recorded at different CV concentrations are presented in Figure S7 in the Supporting Information. Here, under the consideration of quick detection and acceptable reproducibility, we only explore the detection limit to $10^{-13}$ m/40 ppq for CV molecule. For lower probing concentration, the ripened SERS monitors could be still effective with the compensation of longer injection time. Besides the detection limit and reproducibility, we evaluate the SERS homogeneity of these two-step reduced aggregates under the same excitation conditions. As shown in Figure S8 (Supporting Information), the relative standard deviations of SERS signal are in the range of 18–30% at the CV concentrations of $10^{-11}$–$10^{-13}$ m, which indicates the good performance of these two-step photoreduced aggregates as SERS materials. These data are indispensable for developing quantitative and reproducible microfluidic SERS detection technique. Furthermore, we also verify that these two-step photoreduced SERS materials can universally work for various types of molecules, such as organic dye (methylene blue), biomolecule (hemoglobin), and medicine (5-fluorouracil) with the concentration of $10^{-13}$ m (Figure S9, Supporting Information). The detection reproducibility can also reach 50%, which is consistent to the case of CV.

Statistically, it can be considered that only a few or single molecule contributes to the SERS signals at the $10^{-13}$ m limitation. Here, we verify it by the well-accepted bianalyte SERS approach.[72–74] Different from the conventional SMD criteria such as spectral fluctuations/blinking,[14,15] the bianalyte approach benefits from reliable statistics of large number spectral sampling and provides a direct evidence for single-molecule analysis. Here, we use two closely spaced modes at 590 cm$^{-1}$ in Nile blue (NB) and 611 cm$^{-1}$ in R6G as the effective statistic indicators. These two peaks have comparable cross-section and energy, which makes NB and R6G ideal candidates for bianalyte single-molecule SERS measurements. The mixture of NB and R6G with respective concentration of $10^{-13}$ m is used in the two-step photoreduction microfluidic SERS measurements. Figure 5a shows four probable spectral events obtained in bianalyte measurements. From top to bottom, the spectra are demonstrated for pure NB event, pure R6G event, mixed event with signals from both NB and R6G, and the null case with signals from neither of the molecules, respectively. The histogram of the event distribution for pure NB, R6G, and mixed case. Figure 5b shows the reproducibility raises logarithmically as the increase of concentration from $10^{-11}$ to $10^{-10}$ m, and finally trends to 100% at $10^{-7}$ m. This can be understood by the fact that the larger the concentration and longer injection time, the higher the possibility of molecules that could be adsorbed in “hot spots,” thus increasing the probability of successful SERS events. Some representative SERS spectra recorded at different CV concentrations are presented in Figure S7 in the Supporting Information. Here, under the consideration of quick detection and acceptable reproducibility, we only explore the detection limit to $10^{-13}$ m/40 ppq for CV molecule. For lower probing concentration, the ripened SERS monitors could be still effective with the compensation of longer injection time. Besides the detection limit and reproducibility, we evaluate the SERS homogeneity of these two-step reduced aggregates under the same excitation conditions. As shown in Figure S8 (Supporting Information), the relative standard deviations of SERS signal are in the range of 18–30% at the CV concentrations of $10^{-11}$–$10^{-13}$ m, which indicates the good performance of these two-step photoreduced aggregates as SERS materials. These data are indispensable for developing quantitative and reproducible microfluidic SERS detection technique. Furthermore, we also verify that these two-step photoreduced SERS materials can universally work for various types of molecules, such as organic dye (methylene blue), biomolecule (hemoglobin), and medicine (5-fluorouracil) with the concentration of $10^{-13}$ m (Figure S9, Supporting Information). The detection reproducibility can also reach 50%, which is consistent to the case of CV.

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Experimental Section

**Microfluidic Device**: The microfluidic system was composed of a pump (Longer-Pump TS-2A/LO107-2A), tin-foil-wrapped syringes (500 µL), and a microfluidic chip. The four syringes (1–4) were filled with silver nitrate (AgNO₃), deionized water, solution of probed molecules, and sodium citrate, respectively, and connected to corresponding inlets on the microfluidic chip through poly(tetrafluoroethylene) tubing with the diameter 0.6 mm. The chip was made of a poly(dimethylsiloxane) layer sealed by a glass slide, where microfluidic channels were fabricated by the standard soft lithography method. The PDMS layer was 60 µm in length, 30 mm in width, and 10 mm in height. There were three functional parts in the chip, including an injection module designed into three “Y” pattern to prevent undesired interfering between reagents, a mixing module where reagents were mixed thoroughly through the channel, and an optical detection module where the photoreduction and SERS measurements were carried out. Wastes from the outlet were finally collected by the reservoir. The height of designed channel was 100 µm, and widths in the three modules were 100, 200, and 200 µm, respectively.

**On-Chip SERS Detection**: The SERS measurements were performed on a confocal microscope Raman spectroscopy (Renishaw 2000) at the excitation of 532 nm laser. The excitation power was about 130 µW and integration time was 10 s, if not stated otherwise. The analyte at a certain concentration was injected into the microchannel with freshly prepared silver aggregates, and was kept in a static flow condition during the SERS acquisitions. Here, various molecules such as organic dye (CV, R6G, and methylene blue), biomolecule (hemoglobin), and medicine (5-fluorouracil) were used to evaluate the SERS performance of the fabricated silver aggregates. The SMD was confirmed by the bianalyte (NB and R6G) SERS measurements.

**In Situ Comparison of Silver Aggregates Morphologies Before and After Second-Time Laser Irradiation**: The silver aggregates were first synthesized on the coordinated indium tin oxide glass. The reaction mixture was prepared by mixing aqueous solutions of silver nitrate and sodium citrate with the respective concentrations of 2 and 4 × 10⁻³ m, in a volume ratio of 1:1. The rudimentary aggregates were obtained by photoreduction of the reagent under the irradiation (532 nm) of 130 µW for 80 s. After washing, the SEM characterization was performed on FEI Quanta 650 under 10 kV. Then, the rudimentary aggregates were immersed into the reagent (silver nitrate and sodium citrate) solution again for 2 min to mimic the experimental condition in microchannel. After washing with deionized water, the aggregates were further in situ irradiated at a power 2.5 mW for 45 s to trigger the second-time growth. The morphology of ripened aggregates was characterized by SEM again and compared with the rudimentary one.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

memory effect, microfluidics, single-molecule spectroscopy, surface-enhanced Raman scattering, two-step photoreduction

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