Rhodamine B Isothiocyanate-Modified Ag Nanoaggregates on Dielectric Beads: A Novel Surface-Enhanced-Raman-Scattering and Fluorescent Imaging Material

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ABSTRACT
Rhodamine B isothiocyanate (RhBITC) is a prototype dye molecule that is widely used as a fluorescent tag in a variety of biological applications. We report in this work that once RhBITC adsorbs on Ag on silica beads, it exhibits not only a strong surface-enhanced-Raman-scattering (SERS) signal but also a fairly intense fluorescence, contrary to the usual expectation that the fluorescence will be quenched by metal nanoaggregates. The RhBITC-modified silica beads are well dispersed in ethanol, and they are also readily coated in water with polyelectrolytes for their further derivatization with biological molecules of interest that can bind to target molecules. The application prospects of these materials are thus expected to be very high especially in the areas of biological sensing and recognition that rely heavily on optical and spectroscopic means.

Keywords: surface-enhanced Raman scattering, SERS, fluorescence, silver, silica bead, rhodamine B isothiocyanate, molecular sensing/recognition, biotin-streptavidin interaction

1 INTRODUCTION
Noble metallic nanostructures exhibit a phenomenon known as surface-enhanced Raman scattering (SERS) in which the Raman scattering cross-sections are dramatically enhanced for the molecules adsorbed onto them [1]. When the adsorbed molecules are subjected to resonance Raman scattering, it is called a surface-enhanced resonance Raman scattering (SERRS) by which even single molecule detection is known to be possible, suggesting that the enhancement factor can reach as much as $10^{14}$-$10^{15}$ [2,3]; the effective Raman cross sections are then comparable to the usual fluorescence cross sections. In recent years, there has also been considerable interest in the application of SERS/SERRS in biomolecular detection [4,5]. Although fluorescence is currently the principal detection method in bioassays, it has inherent drawbacks such as photobleaching, narrow excitation with broad emission profiles, and peak overlapping in multiplexed experiments. The latter limitations can be overcome by means of SERS/SERRS. Several groups have thus developed various types of SERS-active tagging materials that can be used in diagnostic bioassays [4-7].

Noble metals such as Au and Ag can support nanoparticle plasmon resonances in the ultraviolet, visible, and near-infrared regions of the spectrum that can be modified by varying the nanoparticle size and shape. The usual, solid metallic nanospheres and nanorods exhibit relatively weak plasmonic tunability compared with metallic nanoshells composed of a dielectric core and a concentric metal shell. This is because nanoshells exhibit plasmon resonances that are critically dependent on the inner and outer shell dimensions due to the hybridization of the two fixed-frequency plasmon modes supported by the inner cavity and outer surface of the nanoshell [8]. Moreover, the plasmon resonance of solid silver nanoparticles appears at shorter wavelength than that of gold, along with stronger and sharper resonance strength [9]. The plasmon tunability of a silver nanoshell is thus greater than that of a gold nanoshell. However, it has not been routine to fabricate a silver nanoshell on dielectric beads.

We demonstrate in this work that Ag can be deposited onto the silica beads simply by soaking them in ethanolic solutions of AgNO$_3$ and butylamine. The extent of silvering could be adjusted by varying the relative concentrations of butylamine and AgNO$_3$. Upon the deposition of silver, the UV/vis absorption peak at ~420 nm gradually red-shifts, finally showing a very broad feature extending from near-UV to near-infrared regions. In accordance with the electromagnetic enhancement mechanism in SERS, the Ag-deposited silica beads are efficient SERS substrates that can be used as core materials of SERS/SERRS-based biosensors. Specifically, we report that once rhodamine B isothiocyanate (RhBITC) adsors on Ag on silica beads, it exhibits not only a strong SERS/SERRS signal but also a fairly intense fluorescence, contrary to the usual expectation that the fluorescence will be quenched by metal nanoaggregates. To our knowledge, this is the first report informing the simultaneous observation of fluorescence and SERS/SERRS for dye molecules assembled on metal nanoaggregates.

2 EXPERIMENTAL
Tetraethyl orthosilicate (TEOS; 99%), silver nitrate (99%), butylamine (99%), poly(allylamine hydrochloride) (PAH, MW~70,000), poly(acrylic acid) (PAA, MW~450,000), and RhBITC (97%) were purchased from Aldrich and used as received. Other chemicals, unless
specified, were of regent grade. Highly pure water (Millipore Milli-Q system), of resistivity greater than 18.0 MΩ·cm, was used throughout. The synthesis of PLL-g-poly(ethylene glycol) (PEG) and biotinylated-PLL-g-PEG was based on the protocols described by Huang et al. [10] and the detailed processes were reported in our recent publication [11].

Monodisperse silica particles were prepared using the Stöber-Fink-Bohn method [12], comprising the base-catalyzed hydrolysis of TEOS in water-ethanol mixtures. The silica particles with a mean diameter of 250 nm thus prepared were cleaned by centrifugation (10000 rpm for 15 min) and redispersion in absolute ethanol repeated 10 times. When silver was deposited onto silica particles, a polypyrrolene container was used as the reaction vessel to avoid nonspecific silvering of the reaction vessel. Specifically, the cleaned silica in ethanol was added into the silvering medium to a final concentration of 0.11 mg/mL (w/v, dried silica mass/ethanol) and then incubated for 50 min at 50 ± 1°C with vigorous shaking. As a silvering mixture, the concentrations of AgNO₃ and butylamine were maintained at 1 mM. The silver-coated silica particles were finally rinsed and redispersed in ethanol.

For the self-assembly of RhBITC on silver, 0.1 mg of silver-coated silica beads were placed in a small vial into which 2 mL of 0.1 mM ethanolic RhBITC solution was subsequently added. After 12 h, the solution phase was decanted and then rinsed with highly pure water. The remaining solid particles were left to dry in a vacuum for 2 h. Subsequently, polyelectrolyte layers were formed by the sequential dipping of the RhBITC-modified silver-coated silica beads into the PAA and PAH solutions (0.1 mg/mL) for 10 min at room temperature. In the interim, to change the polyelectrolyte solution, silver-coated silica beads were intensively rinsed with water. At the final stage, the PAA-derivatized silver-coated silica beads were electrostatically reacted with biotinylated-PLL-g-PEG.

To construct a dose–response curve for streptavidin, a glass slide was initially soaked in a piranha solution to assume negatively charged surfaces. The glass slide was subsequently dipped in PLL-g-PEG (20 µg mL⁻¹) solutions for 10 min. After washing the slide with water and then drying it in a nitrogen atmosphere, 1.5 µL of biotinylated-PLL-g-PEG (100 µg mL⁻¹) was pipetted onto it to obtain a 5-mm domain. The glass slides coated with biotinylated-PLL-g-PEG were soaked in a streptavidin solution at various concentrations for 10 min, followed by extensive rinsing with a phosphate-buffered saline (PBS) solution and drying by a nitrogen stream. The streptavidin-attached slides were subsequently immersed in a solution containing silver-coated silica beads (1 mg mL⁻¹) derivatized consecutively with RhBITC, PAA–PAH, and biotinylated-PLL-g-PEG. After 10 min, the glass slides were washed with water, and the dried slides were finally subjected to Raman spectroscopy measurements.

UV/vis absorption spectra were obtained using a SCINCO S-2130 spectrometer. Field emission scanning electron microscopy (FESEM) images were obtained with a JSM-6700F field emission scanning electron microscope operated at 5.0 kV. Transmission electron microscopy (TEM) images were obtained on a JEM-200CX transmission electron microscope at 200 kV. Confocal Laser Scanning Microscope images were obtained with a MRC-1024 Confocal Laser Scanning Microscope. The 568-nm line from an Ar-Kr laser was used at the excitation source. X-ray diffraction (XRD) patterns were obtained on a Bruker D5005 powder diffractometer for a 2θ range of 30° to 80° at an angular resolution of 0.05° using CuKα (1.5406 Å) radiation. IR spectra were measured using a Bruker IFS 113v Fourier transform IR spectrometer equipped with a globar light source and a liquid nitrogen cooled wide-band mercury cadmium telluride detector. Raman spectra were obtained using a Renishaw Raman spectrometer (model 2000) equipped with an integral microscope (Olympus BH2-UMA). The 514.5-nm line from a 20-mW Ar⁺ laser (Melles-Griot model 351MA520) or the 632.8-nm line from a 17-mW He–Ne laser (Spectra Physics model 127) was used as the excitation source. The Raman peak intensities of RhBITC were normalized with respect to that of a silicon wafer at 520 cm⁻¹.

3 RESULTS AND DISCUSSION

Monodisperse silica particles can be readily prepared by the well-known Stöber-Fink-Bohn method [12] since the particle size depends largely on the relative concentration of reactants. The silica particles synthesized were maintained stable without agglomeration in ethanol. The dried silica powder exhibited a very broad O-H stretching band in the region of 3200-3600 cm⁻¹ in the transmission infrared spectrum. This indicates that the surfaces of silica particles are terminated with OH groups; these OH groups must be in a H-bonded state. Owing to these characteristics, the silica powder can be dispersed in water and ethanol. To deposit silver onto the silica particles, the colloidal silica was dispersed in a reaction mixture consisting of ethanolic AgNO₃ and butylamine. As described in the Experimental Section, the reaction mixture was incubated for 50 min at 50 ± 1°C. The concentration of AgNO₃ and butylamine were maintained at 1 mM. Figure 1 shows a typical FE-SEM image of silica particles taken after the deposition of silver.

Figure 1: FE-SEM image of Ag-coated 250-nm silica beads.
The deposition of silver can also be confirmed from the XRD data. As Ag is deposited onto the silica particles, four distinct XRD peaks are clearly observed at 2θ values of 38.1°, 44.3°, 64.4°, and 77.3° (data not shown), corresponding to the reflections of (111), (200), (220), and (311) crystalline planes of cubic Ag, respectively. It has to be mentioned that we could not identify any colloidal silver formed in the bulk. This indicates that no nucleation center existed in the solution phase. The reduction of silver must have occurred only on the surfaces of silica particles. Butylamine is a very weak reductant, so nucleation centers hardly seem to form in the solution. However, once silver ions are bound to the anionic oxygen sites of the silica particles, silver nitrate will be reduced by butylamine, anchoring onto silica surfaces. The weak reductant characteristics of butylamine can also be confirmed from the observation that the silverying of silica particles hardly takes place when the reaction vessel is maintained at room temperature. The reduction of silver nitrate is facilitated upon increasing the temperature. The formation of a silver shell can be readily controlled at 50°C. However, silver particles may form even in the bulk at temperatures much higher than 50°C.

Figure 2: UV/vis spectra of (a) RhBITC and (b) Ag-coated silica beads.

Figure 2a shows the UV/vis absorption spectrum of 0.1 mM RhBITC in ethanol. The absorption maximum is located at 548 nm, so the resonance Raman scattering would be expected to occur when the excitation wavelength is below 548 nm. Figure 2b shows the UV/vis extinction spectra of the Ag coated silica particles. The spectrum measured for bare silica powder was featureless. As the particles become coalesced into a network-like structure, a distinct peak is no longer observed. Instead, a very broad band appears, extending from near-UV to near-infrared regions (see Figure 2b). Much the same UV/vis extinction spectrum was observed after the adsorption of RhBITC. Since the absorption band of RhBITC extends between 450-600 nm, we may well expect to observe a distinct Raman spectrum for RhBITC adsorbed on silver-coated silica beads not only by 514.5-nm radiation but also by 632.8-nm radiation. As shown in Fig. 3, distinct Raman spectra are indeed observed by using both the 514.5- and 632.8-nm radiation. The former must then be a SERRS spectrum, while the latter is largely a SERS spectrum. Due to the resonance Raman scattering effect, the Raman signal of RhBITC in Fig. 3a is about one and half times more intense than that in Fig. 3b.

Figure 3: SERS/SERRS spectra of RhBITC on Ag/250-nm silica taken at (a) 514.5 nm and (b) 632.8-nm excitation.

As mentioned in the Introduction, we hope to use the RhBITC-modified silver-coated silica beads as a core material of SERS-based biosensors (see Scheme 1). The SERS-marker molecules like RhBITC on Ag have then to be stabilized in one way or the other for the modified beads to be used in buffer solutions. We have reported recently that the layer-by-layer (LbL) deposition of cationic and anionic polyelectrolytes is a useful strategy to protect SERS marker molecules assembled on micrometer-sized Ag particles [13]. We confirmed that PAA and PAH were consecutively deposited onto the RhBITC-modified silver-coated silica beads.

Scheme 1: Fabrication of Ag-coated silica beads usable as a template of biosensor operating via SERS/SERRS.

Positively charged poly(L-lysine) may then adsorb fairly well onto the PAA layer. Accordingly, we have subsequently deposited biotinylated PLL-g-PEG onto the outermost PAA layer of RhBITC-adsorbed Ag on silica beads and then confirmed their interaction with streptavidin molecules by monitoring the SERS/SERRS peaks of the RhBITC. We evaluated further the sensitivity of the biotinylated RhBITC Ag/silica beads in recognizing streptavidin molecules by constructing a dose–response
curve. After the fabrication of biotinylated glass slides, SERS/SERRS spectra were obtained as a function of the streptavidin concentration, ranging from $10^{-6}$ to $10^{-13}$ g/mL, as shown in Fig. 4a. Fig. 4b shows the normalized SERS/SERRS intensity of the characteristic band of RhBITC at 1647 cm$^{-1}$; all the SERS/SERRS peaks were normalized with respect to the peak intensity of a silicon wafer at 520 cm$^{-1}$. A very intense SERS/SERRS spectrum is obtained as long as the concentration of streptavidin is above $10^{-9}$ g/mL. At $10^{-10}$ g/mL, the number of polystyrene particles adsorbed on the biotinylated glass substrates decreases, resulting in a lowering of the SERS/SERRS intensity. The SERS/SERRS peak becomes very weak at $10^{-11}$ g/mL and is hardly detected at $10^{-12}$ g/mL, however. Considering the simplicity of the present method, the detection sensitivity indicated by these results is indeed remarkable.

Figure 4: (a) A typical SERS/SERRS spectrum of RhBITC measured after biotinylated RhBITC/Ag/silica particles were allowed to interact via streptavidin with other biotinylated layers on glass. (b) SERS/SERRS intensity of the characteristic band of RhBITC at 1647 cm$^{-1}$ measured as a function of streptavidin concentration; the SERS/SERRS intensities were the average of 10 different measurements with the error bars denoting their standard deviation.

Considering the appearance of SERS/SERRS peaks, the fluorescence of RhBITC is expected to be quenched. Surprisingly, however, the remaining fluorescence is strong enough to be detected by fluorescence microscopy. Figure 5 shows a typical confocal laser scanning microscope image of RhBITC-modified Ag on 250-nm SiO$_2$ taken simply after spreading them on a glass slide. To our knowledge, this is the first report informing the simultaneous observation of fluorescence and SERS for dye molecules assembled on metal nanoaggregates. The RhBITC-modified silica beads are thus expected to be invaluable in the multiple analyses of biomolecules.

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