Bacteria-template synthesized silver microspheres with hollow and porous structures as excellent SERS substrate

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Template-driven strategy is widely explored for the synthesis of nano/micro materials. Of all the templates studied, naturally occurring biological systems such as proteins, viruses and bacteria have attracted more attention due to the prolific sources and complex structural diversities. Herein, we report a simple bacteria templated synthesis of silver microspheres over a bottom-up controlled route. These as-prepared silver microspheres not only have narrow size distribution but possess hollow and porous structures. Surface enhanced Raman scattering (SERS) experiments using 2-mercaptopyridine (2-Mpy) as probing molecules show that these hollow porous microspheres can act as excellent substrate for ultrasensitive detecting. The detection limit is as low as 10^{-15} M and the enhancement factor reaches to 10^{11}. Compared with other conventional SERS substrates, the reproducible, high sensitive and cost-effective Ag microspheres could become an ideal substrate choice for practical SERS application.

Introduction

With the urgent voices of energy-saving and environment protection, the processing and synthesis of nanomaterials are undergoing a paradigm shift, from traditional acute conditions[1] (for example, high temperature and pressure,[2] organic solvents,[3] even for toxic reagents[4]) to relative "green" approaches[5] (for example, the preferred use of microwave,[6,7] sonochemical,[8] laser ablation[9] and aqueous solvent[10]). Integrating green chemistry principles into the field of nanoscience defined "green nanoscience" is a challenging task faced by scientists.[5] Inspired by nature's strategy to fabricate bio-minerals, some green and sustainable methods have been largely developed for the synthesis of nanomaterials in recent years.[11–16] Plant extracts,[17,18] algal solution[19] and starch solution[20] were successfully explored for the synthesis of Ag and Au nanomaterials. However, the above-mentioned system is farraginous and it is not easy to clarify which ingredient on earth plays the key role. Compared with the crude extracts derived from plants and other organisms, the single-component biological systems are alternative in controlling the size, shape and assembly of nanomaterials. These naturally occurring biological systems such as, proteins, DNA and viruses can act as effective templates to synthesize materials with some interesting features from nanometre to micrometre dimensions.[21] For example, Xie *et al.* prepared Au nanoclusters based on Bovine serum albumin (BSA).[22] Li *et al.* completed DNA-directed assembly of multifunctional nanoparticle networks using metallic and bioinorganic building blocks.[23] Lee *et al.* used genetically engineered M13 bacteriophage to form ordered zinc sulfide (ZnS) nanocrystals;[24] Erik *et al.* organized metallic nanoparticles using tobacco mosaic virus as templates.[25] Such obtained nanomaterials show intriguing properties and promising applications. However, DNA and proteins are usually expensive and viruses need special technicians to deal with it. Bacteria, as another kind of important microorganisms in nature, exhibit a large variety of well-defined stunning morphologies, such as bacillus, coccus, spirillum, fusiform bacilli, star-shaped bacteria.[26] These interesting morphologies afford us natural templates to fabricate nano/micro structures. Importantly, the sources of bacteria are cheap and easily-handled. Currently, bacteria-based controlled assembly of metal chalcogenide hollow nanostructures[26] and ZnO hollow microspheres[27] have been successfully synthesized. These hollow assemblies possess superior photo-catalytic activity compared with the corresponding solid counterparts. It is expected that this bacteria-based template process could be extended to the synthesis of hollow metal microspheres/tubes, such as Au, Ag, Cu, Fe and Pt *etc*. Especially in recent years, noble metal nano/micro structures have been of great interest in many areas, such as catalysts,[28] biomedical imaging,[29,30] optoelectronics,[31] drug carriers,[32] surface-enhanced Raman scattering (SERS),[33] *etc*. To the best of our knowledge, there have been few reports on the preparation of hollow porous noble metal microspheres by using bacteria as templates. Herein, we report a simple and cost-effective approach to synthesize micron-sized hollow silver microspheres with bacteria *cocci* (Gram positive bacteria) as templates. SERS tests of as-prepared Ag microspheres were also carried out using 2-Mpy as probing molecules.

Experimental

Materials

Lactobacillus of *Streptococcus thermophilus* (*Str. Thermophilus*) was kindly provided by Professor Shi Xianming (School of

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agriculture and biology, Shanghai Jiaotong University). Hydrogen peroxide (H_2O_2), Silver nitrate ($AgNO_3$), ascorbic acid (AA) and 2-mercaptopyridine (2-Mpy) were of commercial grade and were used without further purification.

Preparation of hollow silver microspheres

Typically, 0.2 ml *Str. Thermophilus* suspension (OD = 0.02) and $AgNO_3$ (212.5 mg) were dispersed in DI water (100 mL), the mixture was under vigorous magnetic stirring for 2 h at room temperature. Subsequently, AA (220 mg) was added into the above-mentioned solution. The color of the solution rapidly changed from colorless to gray. After 4 h reaction, the mixture was sonicated with an ultrasonic cleaner at room temperature for less than 2 h. The resulting product was collected by centrifugation, washed three times with distilled water and ethanol, respectively, and then dispersed in ethanol for further characterization. To identify the hollow characters, 2 mg Ag microspheres were added into 30% H_2O_2 (1 mL) and sonicated for half an hour. The resulting products were washed using DI water for SEM characterization.

SERS experiments

A sample of 2-mercaptopyridine (500 μL, 1×10^{-9} M) prepared in water was added to silver microspheres (1.0 mg) in a centrifugal tube and allowed to stay for 5 h to reach adsorption equilibrium under ultrasound. Washed 2 times with distilled water and ethanol, respectively, and dispersed in 20 μL ethanol. Then, an aliquot of 10 μL of purified 2-Mpy capped silver micro-spheres ethanol solution was dropped onto a Si wafer. The dropped solution was spread evenly into a circle. After evaporation of ethanol, the sample was measured. All the experiments were carried out at room temperature. The measurement of different concentrations of 2-Mpy (1×10^{-9}, 1×10^{-12} and 1×10^{-15} M) was completed using the same method. 0.1 M 2-Mpy was also used for contrast.

Characterization

The structural characterization was performed with field emission scanning electron microscopy (FESEM: ZEISS) operated at 5.0 kV, a JEM-2100EXII transmission electron microscope (JEOL Co., Ltd) operated at 200 kV, X-ray diffraction measurement using a Bruker-AXS D8 advance instrument operated at a voltage of 40 kV and a current of 40 mA with Cu-Kα radiation (λ = 1.5406 Å). BET surface areas and porosities were determined by nitrogen adsorption and desorption with a Micrometritics ASAP2010 analyzer.

Raman scattering signal was obtained by using a Jobin Yvon micro-Raman spectroscope (Super LabRam). A He–Ne laser at 632.8 nm with a power of *ca.* 5 mW was used as the excitation source for the Raman experiments. Each spectrum was obtained using five accumulations, and the acquisition time in each case was 8 s.

Results and discussion

In a typical synthesis, based on bacteria as templates, we used AA to reduce metal salt precursor (In our case, $AgNO_3$) without adding any catalyst or surfactant. The XRD patterns of as-prepared products are shown in Fig. 1. The diffraction peaks correspond to the (111), (200), (220) and (311) planes, which can be indexed to the face-centered cubic (fcc) structure of silver. Fig. 2 shows the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of the silver microspheres. From the SEM image (Fig. 2.A), one can see that these silver microspheres are in the range of 1.3–1.7 μm with an average diameter of *ca.* 1.6 μm. The magnified SEM image (Fig. 2.B) indicates that these microspheres possess walnut-like morphologies with many trenches on their surfaces. Energy dispersive spectroscopy (EDS) is also performed to assert that no bacterial residual is left in the microspheres after ultrasound treatment (ESI, Fig. S1†). It seems that every large microsphere is made up of many nanometre scaled "Ag islands", indicating the independent nucleation and growth of Ag nanoparticles around the surfaces of bacteria. In order to confirm the hollow structures, we etched the sample using H_2O_2 aqueous solution. The hollow insides are clearly shown for the broken samples (Fig. 2.C). It is estimated that the shell thickness is *ca.* 200 nm which can be controlled by changing the precursor concentration (ESI, Fig. S2†).

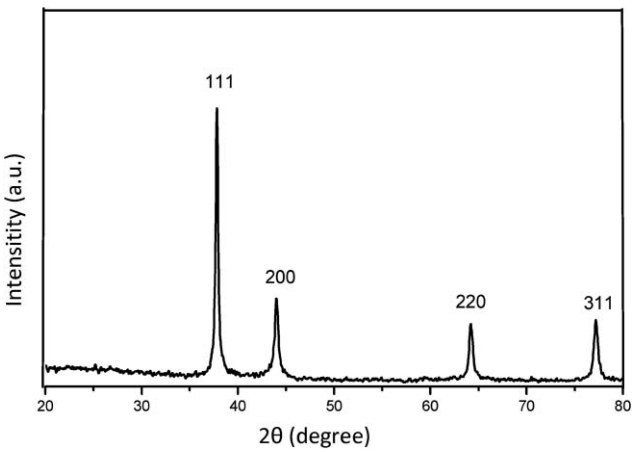

Fig. 1 XRD pattern of the hollow silver microspheres.

Transmission electron microscopy (TEM) is also performed to investigate the morphologies of the as-prepared sliver microspheres (Fig. 2.D). One can see some convex structures on the edges of microspheres, showing their surfaces are very rough, which is consistent with the observation of SEM and AFM (ESI, Fig. S3†). The electron diffraction pattern (Fig. 2.D, inset) shows diffuse rings, indicating that these hollow Ag microspheres are polycrystalline. Attempts to observe the hollow structures though TEM failed, one possible explanation is the walls of as-prepared Ag microspheres are too thick to be penetrated by the electron beam. Therefore, there is no obvious contrast between the edge and middle of each microsphere as observed by others.[34] It should be noted that the walnut-like silver microspheres can also be obtained without involvement of bacteria, but no hollow structures were present (ESI, Fig. S4†). Interestingly, the representative nitrogen adsorption-desorption isotherms show that the silver microspheres are porous with an average diameter of 3.2 nm (Fig. 3). The porous structures might be an ideal substrate for gas adsorption and sensing.

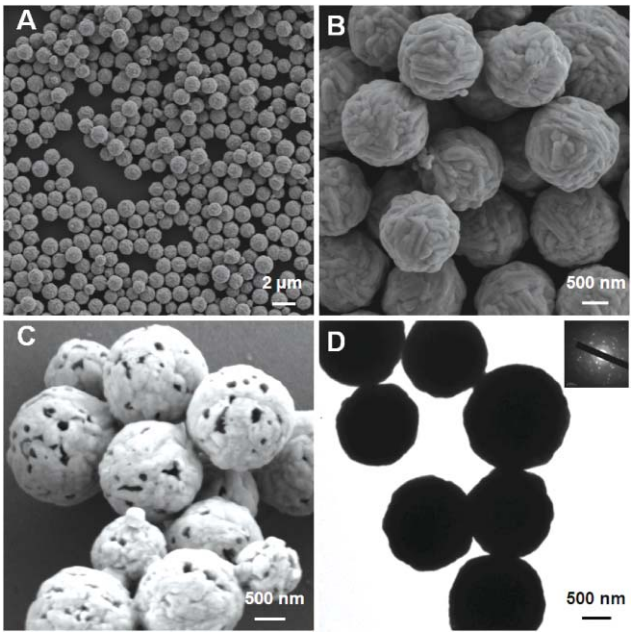

Fig. 2 SEM images of (A) Hollow Ag microspheres observed at low magnification. (B) walnut-like Ag microspheres observed at high magnification. (C) H_2O_2 etched Ag microspheres at higher magnification. (D) TEM image of Ag microspheres. The inset shows the selected area electron diffraction (SAED) pattern of the sample.

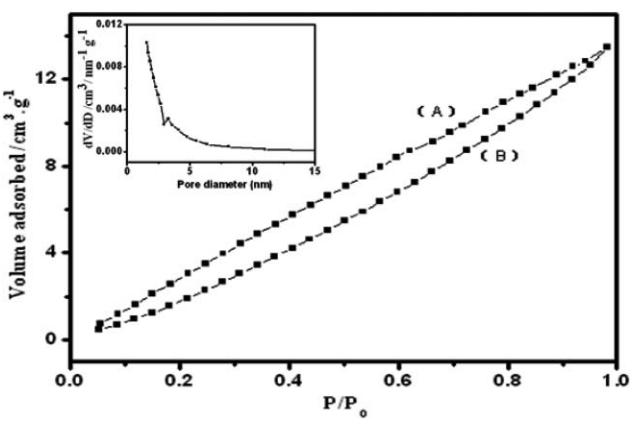

Fig. 3 N_2 adsorption-desorption isotherms for the sample, with corresponding BJH (Barrett–Joyner–Halenda) pore-size distribution (inset). The BET specific surface area of the hollow silver sphere is 25 m^2g^{-1} and the BJH desorption average pore size is 3.2 nm. (A): Desorption; (B) Adsorption.

Actually, Zhou *et al.* employed the same bacteria with the aid of sonication for the *in situ* one-step synthesis of ZnS hollow nanostructures.[34] Compared with their work, several different aspects are elucidated as follows: firstly, the crystal formation mechanism is different in the two systems. The formation of ZnS nanostructures depends on radicals' production and precipitation reaction. Our experiment relies on metal ions adsorption and reduction. Secondly, sonication is used and plays a key role (forming radicals) during the whole experimental process of synthesizing ZnS microspheres. To synthesize Ag hollow microspheres, we only use sonication to disrupt cells and release organic residues. Lastly, Zhou *et al.* also tried to use simple inorganic salts ($ZnCl_2$ and $Zn(NO_3)_2$) to replace $Zn(AC)_2$, but the results failed. They attributed the successful results to acetate ligand which plays an important role in the grafting of zinc onto the cell surfaces during sonication. Therefore, the negatively charged groups as well as ultrasound play significant roles during the mineralization. Fortunately, we successfully used $AgNO_3$ to get Ag microspheres without sonication. As is well known, Ag^+ is an effective antimicrobial agent.[35] Although the antimicrobial mechanism is very complex, a basic fact is that Ag ions interact easily with the cell wall containing many layers of peptidoglycan, teichoic acids and s-layer proteins for Gram-positive bacteria. A large number of negatively charged groups ($R–COO^-$, $R–OH^-$, $R–SH^-$)[36] are located on the cell wall of Gram positive bacteria just like many "hands". When suitable quantities of Ag^+ are added into the solution of bacteria, these "arms" immediately catch them through the interactions of positive-negative charges as well as chelation. Under the powerful magnetic stirring, the Ag ions are evenly attached on the surfaces of the bacteria. Thus, Ag starts to nucleate and grow with the addition of the weak reducer-ascorbic acid. Last, the bacteria coated a layer of silver nano-clusters is disrupted under ultrasound and a hollow microsphere is obtained. The above possible formation process is schematically illustrated in Fig. 4.

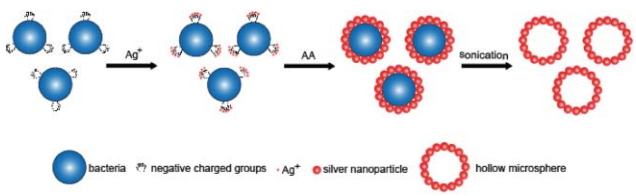

Fig. 4 Schematic illustration of the synthesis strategy of hollow silver microspheres with bacteria as morph-template. There are many functional groups exposed on the surfaces of bacterium. It is easy for Ag^+ to interact with these groups through electrostatic interactions as well as chelation. Once Ag^+ are attached onto the surfaces of bacterium, they can serve as nucleating sites for the subsequent silver nanoparticles reduced by AA to adhere and grow. Last, under the effect of ultrasound, hollow silver microspheres are formed.

Since Ag is an excellent substrate of SERS, the SERS enhancement factor depends strongly on the morphology, *e.g.*, the size, shape, or aggregation of the substrate.[37–40] It is reported that the aggregates of silver or gold nanoparticles are the best suitable structures for optimal SERS substrates.[38] Therefore, to rationally design and assemble such kind of structures has been a hot topic. According to our observation of SEM and TEM, the as-prepared silver microspheres are actually made up of some aggregates of silver nanoparticles. Furthermore, considering their porous and hollow structure natures, rough and large specific surface areas, Raman experiments were carried out using 2-Mpy as a probing molecule to evaluate their SERS ability. Raw Raman and SERS spectra of 2-Mpy (different concentrations) are shown in Fig. 5. The peak assignments of bulk and adsorbed 2-Mpy on Ag microspheres are based on those previously reported.[41,42] The observed vibrational modes are consistent with those reported 2-Mpy on Ag electrodes. The red shift in the models involving ν(C–S) at 732 cm^{-1} to 716 cm^{-1} and of ring breathing (RB)/ν(C–S) band at 1140 cm^{-1} to 1117 cm^{-1} indicates chemisorptions of 2-Mpy to the Ag microspheres surfaces through the sulfur atom. The detection

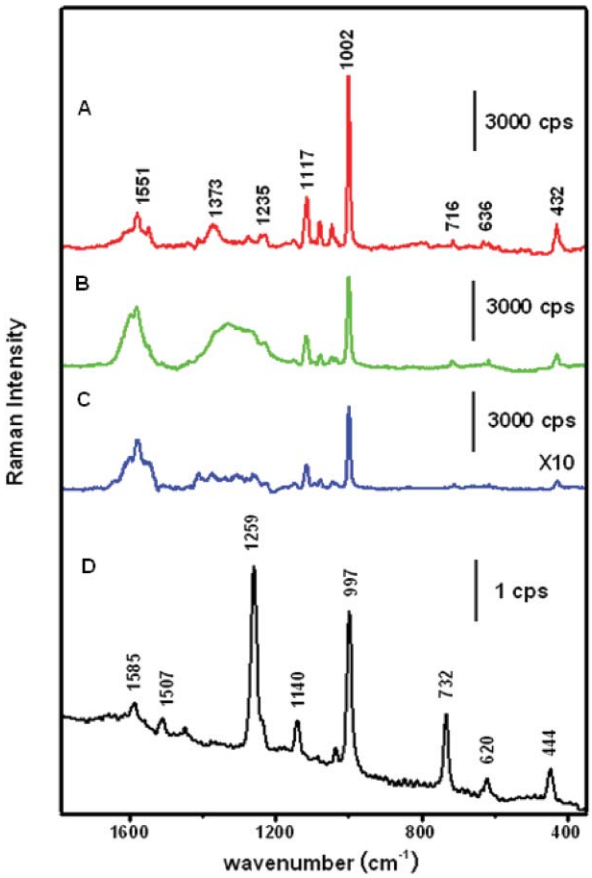

Fig. 5 Representative SERS spectra of 2-Mpy molecules on the hollow silver microspheres substrate at different concentrations (A) 1×10^{-9} M; (B) 1×10^{-12} M; (C) 1×10^{-15} M. (D) Raman spectra of 0.1 M 2-Mpy. It should be noted that the Raman signal is still strong at the concentration of 1×10^{-15} M, showing the silver microspheres are excellent substrate for SERS.

limit is as low as 10^{-15} M and the enhancement factor is calculated as ~10^{11} orders of magnitude (Supporting information, EF calculation) which is much higher than reported other SERS substrats.[42,43] The great enhancement effect is believed to be due to the excitation of the surface plasmon resonance (SPR) on the metal surface which greatly strengthens the local E-field near the surface. Several features could be responsible for the enhanced Raman scattering signal:

1. The huge, roughened surface area can absorb more molecules onto the substrate so that the number of effective excited molecules will increase greatly. It is worth noting that due to the existence of nanoporous structures, both inside and outside of the silver shell might adsorb a large number of 2-Mpy molecules.

2. The trenches and voids formed between "silver islands" afford abundant "hot spots"[44,45] to amplify the local E-fields as well as the Raman signal.

3. The hollow and porous structures enable photos give rise to complex multiple scattering processes. It will be very interesting to study the unique optical features experimentally and theoretically.

4. In some methods, surfactants are usually involved in the synthesis of SERS substrates. However, the existence of surfactants will affect the interaction between Raman molecules and substrate. Our surfactant-free synthesis approach tactfully circumvents the hindrance.

Conclusion

In summary, a facile and environmentally friendly way to synthesize hollow porous silver microspheres has been successfully developed by using bacteria as template, where the individual microsphere is made up of some silver nanoparticle aggregates. The detection limit of 2-Mpy molecules using as-prepared hollow and porous Ag microspheres is as low as 10^{-15} M, and the enhancement factor is calculated as ~10^{11} orders of magnitude. The superior SERS ability to 2-Mpy molecules is attributed to the enlarged E-field which is developed by the complex structures of silver microsphere. We believe that the bacteria-based controlled synthesis of hollow porous silver microspheres may be extended to other metallic materials with great application potentials in catalysts, SERS, photoelectronic devices, antimicrobial agents, gas adsorption, *etc*.

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