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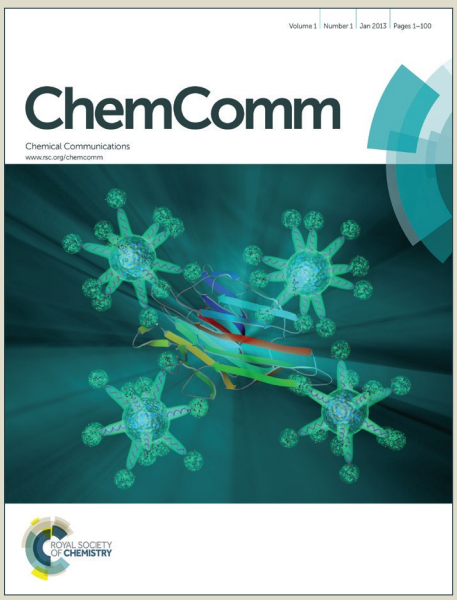

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COMMUNICATION

3D ordered silver nanoshells silica photonic crystal beads for multiplex encoded SERS bioassay

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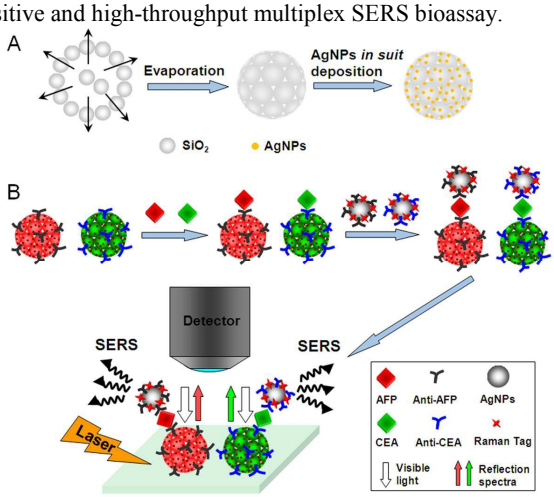
3D ordered silver nanoshells silica photonic crystal beads as novel encoded surface enhanced Raman scattering substrate are proposed for the development of highly efficient multiplex bioassay.

Surface-enhanced Raman scattering (SERS) as a powerful and extremely sensitive analytical technique has been exploited in biochemistry, chemical molecule detection, imaging, environmental monitoring, and sensors applications.[1] It has been demonstrated that the performance of SERS detection strongly depends on the fabrication of the suitable SERS substrates.[2] Especially for the practical applications, an ideal and efficient SERS substrate usually requires not only enormous hot spots to obtain high sensitivity but also uniform distribution of hot spots to ensure good signal reproducibility.[3] Recently, 3D ordered highly SERS-active substrates have attracted increasing attention.[4] Great efforts have been devoted to the design of these types of SERS substrates by self-assembly method,[5] template method,[6] and electron beam lithography.[7] However, it still remains a challenge to fabricate large-scale uniform SERS substrates with high sensitivity and good reproducibility.

Photonic crystals are periodically arranged structured materials, and show attractive advantages for building SERS substrate due to their highly uniform structure and good fabrication reproducibility.[8] To date, 2D photonic crystal film,[9] 3D photonic crystal film[10] and photonic crystal fiber[11] have been reported as SERS substrates. However, photonic crystal films are unstable during SERS detection, and photonic crystal fibers surfer from the complicated fabrication process and high cost. Photonic crystal beads (PCBs), composed with periodically arranged monodisperse nanoparticles show obvious superiority compared with the above-mentioned substrate materials.[12] To the best of our knowledge, PCBs have been not reported for SERS substrates applications so far.

Multiplex bioassays are of great demands for clinical, environmental, and biomedical applications due their unique advantages.[13] In recent years, SERS has been becoming a promising technique for the detection of multiple analytes.[14] The current multiplex SERS technique mainly depends on multiple Raman labels strategy to distinguish the signal from different analytes. However, this mode is heavily restricted by the limited number of Raman labels and complicated resolution of different Raman scattering signals. Moreover, weak intensity of typical Raman labels also limits the sensitivity of multiplex SERS detection.[15] Thus it is highly desired to develop simple, highly sensitive and high-throughput multiplex SERS bioassay.



Scheme 1 The schematic illustration of the fabrication of Ag-SPCBs (A) and multiplex SERS bioassay (B).

In this work, novel silver nanoshells silica photonic crystal beads (Ag-SPCBs) SERS substrate was prepared and further used to develop ultrasensitive multiplex SERS bioassay coupling with Raman signal amplification. The Ag-SPCBs were obtained by the self-assembly of silica nanoparticles to form the monodisperse size-controlled SPCBs using a microfluidic device and then in situ deposition of Ag nanoparticles (AgNPs) onto these SPCBs (Scheme 1A). By changing the diameters of the silica nanoparticles, a series of SPCBs with different reflection peak positions could be obtained. Using reflection peaks of different Ag-SPCBs as the encoding elements, single Raman label-based multiplex SERS bioassay could be achieved for the quantitative detection of multiple analytes (shown in Scheme 1B). Using different encoded Ag-SPCBs to immobilize different antibody molecules, carcinoembryonic antigen (CEA) and alpha fetoprotein (AFP) could be determined in one test tube with a sandwich format. The proposed Ag-SPCBs could be used as

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potential and efficient SERS substrates for high-throughput multiplexed biological assays.

Here, Ag-SPCBs were fabricated by a facile method as efficient SERS substrate. Based on the self-assemble of silica nanoparticles, SPCBs were synthesized by a microfluidic device, and its SEM image was shown in Fig. 1A. The beads are spherically shaped with a smooth edge and the size of about 280 μm. As seen from the enlarged SEM image of SPCBs (Fig. 1B), the beads are composed of hexagonally arranged silica nanoparticles, showing smooth surface and uniform size with the diameter of 240 nm. After the surface of SPCBs was functionalized with SH group, AgNPs were *in situ* deposited onto the SPCBs. It could be clearly observed from SEM image of Ag-SPCBs (Fig. 1C), SPCBs were completely covered with the AgNPs and still retained uniform and ordered structures. This type of nanostructure is very beneficial to significantly induce more hot spots for ultrasensitive SERS detection.[16] After antibody molecules were immobilized onto Ag-SPCBs, its SEM (Fig. 1D) exhibited obviously different surface morphology from that of Ag-SPCBs.

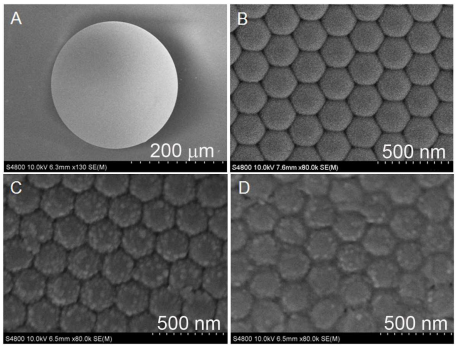

Fig. 1 SEM image of SPCBs (A), enlarged SEM image of SPCBs (B), Ag-SPCBs (C) and antibody immobilized Ag-SPCBs (D).

X-ray photoelectron spectroscopic (XPS) spectra were used to investigate the process for Ag-SPCBs fabrication and antibody immobilization (Fig. S1). As seen from the XPS spectrum of SPCBs, two characteristic O1s and Si2p peaks could be clearly observed. After in-situ growing of AgNPs onto SPCBs, the XPS spectrum of the resultant Ag-SPCBs showed a clear Ag3d peak, indicating the successful synthesis of Ag-SPCBs. The XPS spectrum of antibody immobilized Ag-SPCBs displayed an obvious N1s peak, suggesting the successful immobilization of antibody molecules on the surface of Ag-SPCBs.

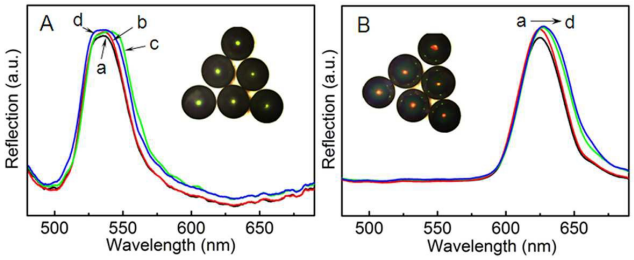

Fig. 2 Reflection spectra of the beads with two kinds of codes (a-d, SPCBs, OH-modified SPCBs, SH-modified SPCBs and Ag-SPCBs), insets: bright field microscope images of the Ag-SPCBs.

In order to obtain a series of encoded Ag-SPCBs, different reflection peaks of SPCBs should be firstly synthesized. By changing the diameter of the silica nanoparticles, SPCBs with different diffraction peak positions could be easily obtained. Here, two kinds of Ag-SPCBs (the size of silica nanoparticles is 240 and 280 nm, respectively) were chosen to illustrate the proposed multiplex SERS bioassay system. As seen from the reflection spectra of Fig. 2, the reflection peaks of two Ag-SPCBs located at 537 and 625 nm, respectively. Their microscope images (insets in Fig. 2) demonstrated that two types of beads were monodisperse, and showed clearly distinguishable structural colors (brilliant green and red). In addition, there are no obvious peak shift during the functionalization of SPCBs and AgNPs deposition process (Fig. 2), indicating the stable and reliable coding of the fabricated Ag-SPCBs substrate.

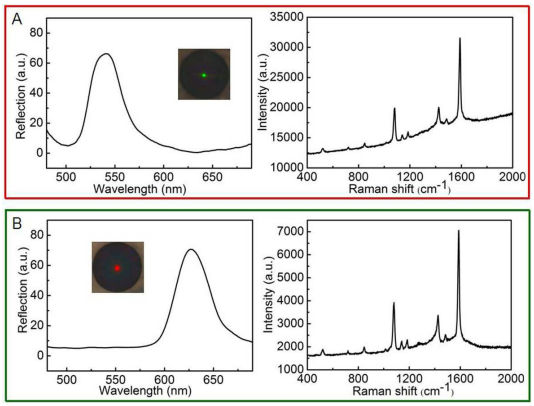

Fig. 3 The refection (left) and SERS spectra (right) of CEA sandwich complex immobilized Ag-SPCBs (A) and AFP sandwich complex immobilized Ag-SPCBs (B) at 10 ng/mL concentrations of two tumor markers, insets: the bright filed microscopy images of CEA and AFP sandwich complex immobilized Ag-SPCBs.

Using CEA and AFP as models, an efficient multiplex SERS bioassay was constructed for detection of two tumor markers using one Raman label. Two kinds of encoded Ag-SPCBs (reflection peaks at 537 and 625 nm) were chosen to immobilize CEA and AFP antibodies, respectively. These two kinds of antibody immobilized beads were mixed with the mixture of CEA and AFP for the first incubation step. SERS signal-amplified probe (4-MBA/antibody/AgNPs) were then added to the system for the second incubation step. The refection and SERS spectra of CEA and AFP sandwich complex/Ag-SPCBs were recorded and shown in Fig. 3. It could be clearly observed that the CEA and AFP sandwich complex immobilized beads showed obviously different structural colors and reflection wavelength. Based on these code information, the Raman signals for CEA and AFP could be distinguished conveniently. Thus the proposed multiplex SERS assay system could be used for the quantitative detection of multiple analytes.

The cross reactivity of the multiplex SERS bioassay was evaluated by comparing the changes of Raman signal at a definite concentration of analytes with the increasing concentration of another interfering analyte. When the concentration of interfering antigens changed in the range of 20-100 ng/mL, the changes in Raman signal (1587 cm^{-1}) for 20 ng/mL of AFP and CEA were less than 5.4% and 4.7%, respectively (Fig. S2), indicating that the cross reactivity between CEA and AFP antibodies and the other noncognate antigens could be negligible. These results suggested that two tumor markers could be determined using the designed multiplex SERS assay system.

Under the selected conditions, the SERS intensities for both CEA and AFP increased with the increasing concentration of two antigens. The calibration curves for the multiplex assay of CEA and AFP were showed in Fig. 4. The very wide linear ranges were obtained for CEA and AFP ranged from 0.01 pg/mL to 1000 ng/mL and from 0.1 pg/mL to 1000 ng/mL, respectively. This can be attributed to the higher surface-to-volume ratio and good biocompatibility of the resulting Ag-SPCBs. The detection limits for CEA and AFP were calculated to be 6.6×10^{-6} and 7.2×10^{-5} ng/mL at a signal-to-noise ratio of 3. The detection limits for CEA and AFP were lower than those of the previous reported SERS assay methods.[17] The ultrahigh sensitivity and very wide linear ranges of the proposed multiplex assay method showed a remarkable superiority in practical clinical diagnosis application. Moreover, the intra- and inter-assay coefficients of variation were 5.3% and 8.7% for 10 ng/mL CEA and 4.9% and 8.2% for 10 ng/mL AFP, respectively, demonstrating acceptable detection and fabrication reproducibility of the multiplex detection system.

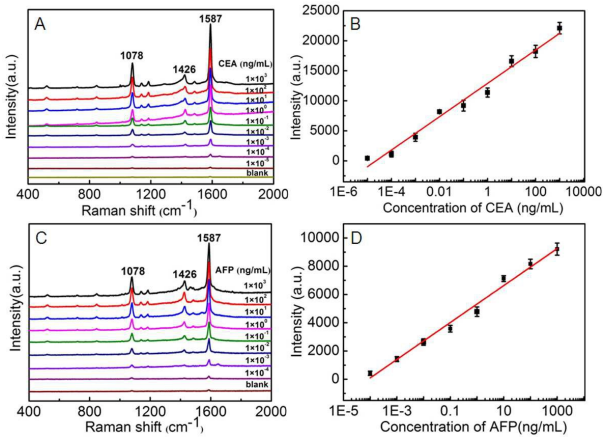

Fig. 4 SERS spectra of Ag-SPCBs substrates for CEA (A) and AFP (C) with different concentrations of analytes, and calibration curves for CEA (B) and AFP (D) produced at 1587 cm^{-1} (n = 5 for each point).

To evaluate the potential application of the Ag-SPCBs based multiplex SERS bioassay, clinical human serum samples were determined using the proposed assay method and the reference method (electrochemiluminescent immunoassay, ECLIA). The ECLIA was carried out by Jiangsu Cancer Hospital. As seen from Table S1, the relative errors between the two methods for the detection of clinical serum samples were not more than 8.18%, indicating the acceptable accuracy of the proposed multiplex SERS bioassay method.

In conclusion, 3D highly ordered Ag-SPCBs were fabricated for efficient SERS substrate by a facile method. The resultant Ag-SPCBs show highly uniform structure and tremendous SERS signal enhancement. Also, the encoded beads keep the characteristics of photonic crystal, and can provide enormous and reliable codes. Using the reflection peak of different types of Ag-SPCBs as codes, a multiplex encoded SERS bioassay was for the first time constructed using only one Raman label. Coupling with a Raman signal-amplified strategy, the Ag-SPCBs based multiplex SERS assay demonstrated ultrahigh sensitivity, very wide linear range, and low cross-reactivity. The detection of human serum samples indicated that the proposed multiplex assay has potential practicality in clinical diagnosis. The designed Ag-SPCBs as a promising SERS substrate provided a novel and efficient platform for developing high-throughput SERS biological assays.

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