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ORIGINAL PAPER

Separation and detection of multiple pathogens in a food matrix by magnetic SERS nanoprobes

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Abstract A rapid and sensitive method was developed here for separation and detection of multiple pathogens in food matrix by magnetic surface-enhanced Raman scattering (SERS) nanoprobes. Silica-coated magnetic probes (MNPs@SiO_2) of ~100 nm in diameter were first prepared via the reverse microemulsion method using cetyltrimethylammonium bromide as a surfactant and tetraethyl orthosilicate as the silica precursor. The as-prepared MNPs@SiO_2 were functionalized with specific pathogen antibodies to first capture threat agents directly from a food matrix followed by detection using an optical approach enabled by SERS. In this scheme, pathogens were first immunomagnetically captured with MNPs@SiO_2, and pathogen-specific SERS probes (gold nanoparticles integrated with a Raman reporter) were functionalized with corresponding antibodies to allow the formation of a sandwich assay to complete the sensor module for the detection of multiple pathogens in selected food matrices, just changing the kinds of Raman reporters on SERS probes. Here, up to two key pathogens, *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus*, were selected as a model to illustrate the probability of this scheme for multiple pathogens detection. The lowest cell concentration detected in spinach solution was 10^3 CFU/mL. A blind test conducted in peanut butter validated the limit of detection as 10^3 CFU/mL with high specificity, demonstrating the potential of this approach in complex matrices.

Keywords Silica-coated magnetic nanoparticles · Surface-enhanced Raman scattering · Multiplex pathogen detection · Food matrix

Introduction

Rapid and sensitive detection of pathogens is an important step in food safety and public health assurance. Traditional methods for the identification of pathogens involve elaborate procedures including culturing (6–24 h growth) and morphological and biochemical characterization (additional 12–24 h) [1–3]. Technologies comprising of electrochemical biosensors [4], polymerase chain reaction [5, 6], and enzyme-linked immunosorbent assay [7–9] have been developed for the separation and detection of pathogens in a food matrix. Advances in nanotechnology and biotechnology offer new possibilities for the rapid and sensitive identification of harmful pathogens. For instance, biosensors based on magnetic beads and fluorescence have been developed to detect pathogens [10, 11]. However, magnetic beads of size between 1.5 and 3 μm have a strong autofluorescence that interferes with the target [11, 12]. Although quantum dots (QDs) have been used to shift the emission signals away from the autofluorescence range, QDs are smaller, expensive, and their surface modification not trivial [13]. On the contrary, magnetic nanoparticles (MNPs) with its super-paramagnetic property can be fabricated to any desired size and functionality, such as that functionalized with antibodies specific to target pathogens [14–16]. Since the size of MNPs is typically one to two orders of magnitude smaller than the pathogens,

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attachment of multiple NPs onto a bacterial cell permits a robust magnet-mediated separation step [15, 17]. When MNPs are coated with a silica layer (MNPs-SiO_2), surface modification of the composite structure becomes seamless because these could be used in a variety of applications, involving separation and detection, explored in this research, which have great advantages such as easy control and strong magnetic properties because several small MNPs were encapsulated together by silica layer.

Surface-enhanced Raman scattering (SERS) has the potential to function as a highly sensitive bioassay [18, 19] due to the enhancement of the electromagnetic field in the vicinity (<10 nm) of metal nanoparticles [20–22]. An excellent example of a SERS immunoassay reported by Porter et al. uses a sandwich structure to detect proteins [23–25]. Label-free efforts have been proposed, but the SERS substrates require careful optimization to achieve the highest enhancement [26–28]. Other issues constitute the lack of signal reproducibility and multiplex detection capability, which are critical issues that need to be addressed for label-free detection of pathogens [29–32]. However, if SERS nanoprobes can be constructed using appropriate Raman reporters and target-specific antibodies, the potential to identify multiple pathogens indirectly from the spectra of representative Raman reporters is possible. Although this is an indirect approach, highly sensitive multiplex detection platform is possible due to the availability of Raman labels with high cross-section [21, 33].

In this research, we propose a MNPs@SiO_2-based SERS platform for the detection of multiple pathogens in a food matrix. Two types of interactions are involved in the constructed biosensor: (1) magnetic dipole interactions that have a tendency to aggregate the MNPs@SiO_2 under a magnetic field and (2) highly sensitive multiplex SERS immunoassay for the recognition and detection of pathogens with high specificity due to the sandwich structure formation of MNPs@SiO_2 and SERS probes.

Experimental

Chemicals

Oleic acid, iron chloride ($FeCl_3 \cdot 6H_2O$, Aldrich, 98%), cyclohexane, $HAuCl_4$, sodium citrate, mercaptobenzoic acid (MBA), mercaptopyridine (MPY), ethylacetate, cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), glutaraldehyde, NaOH, and ethanol were all purchased from Sigma-Aldrich. Antibodies constituting anti-*Salmonella enterica* serovar Typhimurium and anti-*Staphylococcus aureus* were purchased from Abcam (Cambridge, UK).

Procedure

Preparation of amino-functionalized silica-coated magnetic nanoparticles (MNPs@SiO_2)

Monodisperse MNPs (Fe_3O_4) with the diameter of 15 nm capped with oleic acid were first synthesized according to the method reported by Park et al. [34]. Amino group-functionalized silica-coated MNPs (MNPs@SiO_2) were prepared via the reverse microemulsion method using CTAB as a surfactant and TEOS as the silica precursor according to the protocol reported by Kim et al. with minor modification [35]. Briefly, 4 mg of oleic acid-stabilized monodisperse MNPs dispersed in 1 mL cyclohexane was poured into 5 mL of 0.2 M aqueous CTAB solution, and the resulting solution was sonicated vigorously for 60 min. The formation of an oil-in-water microemulsion resulted in a turbid brown solution. The mixture was then heated to 60 °C and aged at that temperature for 10 min under stirring to evaporate the cyclohexane to form a transparent MNPs/CTAB solution. The resulting solution was then added to a mixture of 22 mL of water and 150 μL of 2 M NaOH solution and heated to 70 °C under stirring. Then, 250 μL of TEOS, 25 μL of APTES solution, and 1.5 mL of ethylacetate were added to the reaction solution in sequence. After 10 min, 25 μL APTES was added, the solution was stirred for another 3 h, and the functionalized MNPs@SiO_2 were washed three times with ethanol to remove the unreacted species and dispersed in 10 mL of ethanol.

Functionalization of monoclonal antibody against pathogens onto amino-functionalized silica-coated magnetic nanoparticles (MNPs@SiO_2)

Functionalization of goat anti-*S. enterica* serovar Typhimurium and *S. aureus* antibodies onto the amino-functionalized MNPs@SiO_2 was accomplished using the well-established glutaraldehyde spacer method [13]; 1 mL of amino group-functionalized MNPs@SiO_2 was dispersed into 0.01 M phosphate buffer solution (PBS; pH 7.4) containing 5% glutaraldehyde for about 1 h with shaking at room temperature. The modified nanoparticles were subjected to a copious wash with PBS and isolated by a permanent magnetic and incubated with pathogen-specific antibodies (10 μL 0.6 mg/mL anti-*S. enterica* serovar Typhimurium and 10 μL 0.9 mg/mL anti-*S. aureus* antibodies) suspended in PBS buffer solution (pH 7.0) for 12 h at 4 °C. The antibody-modified nanoparticles were then washed with PBS to remove excess antibodies and kept at 4 °C at pH 7.4 in PBS overnight. The functionalized probes were found to be very stable for several days. The fabrication of MNPs@SiO_2 and antibody functionalization steps are outlined in Fig. 1a.

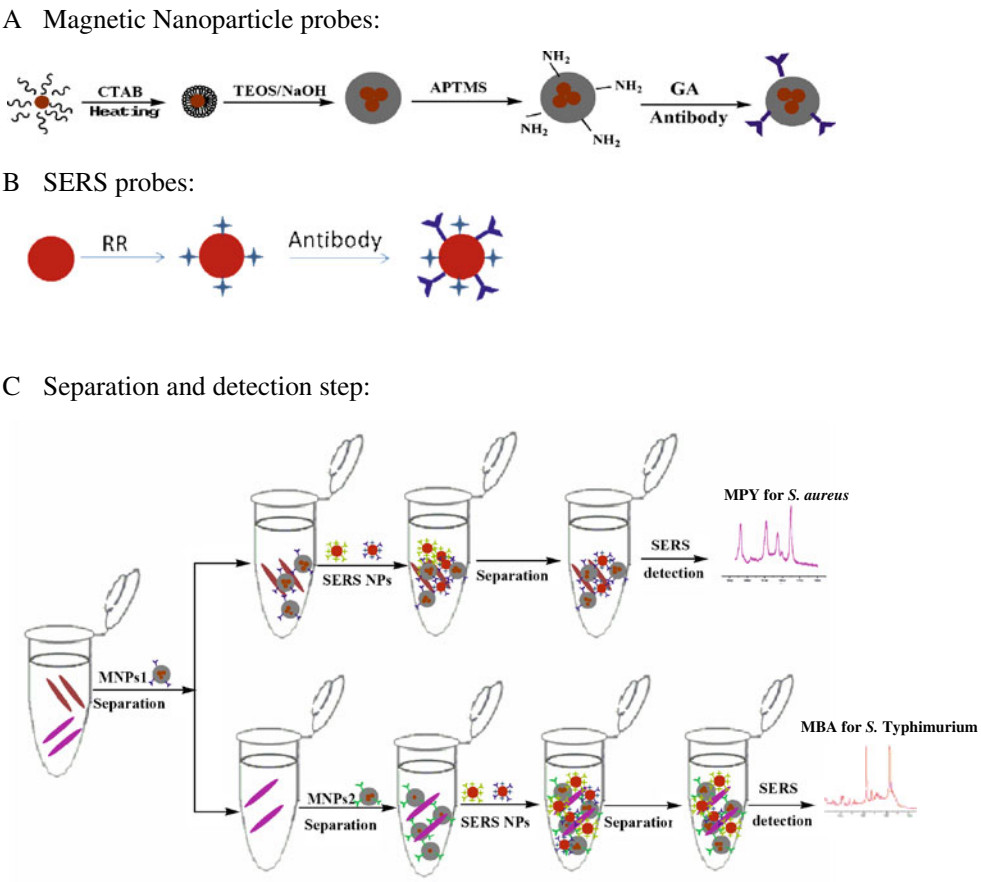

Fig. 1 Scheme for the fabrication of MNPs@SiO_2 (**a**), SERS probes consisting of AuNPs labeled with Raman reporters and antibody (**b**), and scheme for the separation and detection of multiple pathogens in a food matrix (**c**)

Preparation of pathogen-specific SERS probes

SERS probes constitute gold nanoparticles (AuNPs) as enhancer substrate and non-fluorescent molecules, MBA or MPY, as Raman reporters along with pathogen-specific antibody as depicted in the scheme shown in Fig. 1b. The selectivity and specificity of the biosensor are greatly improved by immobilization of antibodies on to the surface of AuNPs. AuNPs were first prepared by aqueous reduction of $HAuCl_4$ with trisodium citrate according to Frens's method [36]. Transmission electron microscopic (TEM) image shows the size of AuNPs to be approximately 40 nm (Fig. S1A), and the UV–Vis spectra show a plasmon resonance peak at 526 nm (curve a in Fig. S1B). It should be noted that AuNPs with 40 nm in diameter were chosen as the enhancer probe because of its stability and enhancement capability [37, 38], although larger AuNPs might provide a higher enhancement. However, the probe conditions and characteristics that provided more stable probes for the sensitivity expected were key factors in developing a practical sensing protocol. Raman reporters were attached onto AuNPs by the addition of 15 μL of 50 μM MPY and 30 μL of 30 μM MBA in 1 mL of AuNPs solutions, respectively, and allowed to react overnight. The conjugates were then centrifuged at 6,000 rpm for 10 min, at which point the excess unbound Raman reporter molecules were discarded and the loose sediment resuspended in borate buffer. To a 1-mL AuNPs–MBA conjugate solution, 5 μL of 0.12 mg/mL anti-*S. enterica* serovar Typhimurium was added to detect a specific pathogen from the Raman signature of MBA. To a 1-mL AuNP–MPY conjugate solution, 5 μL of 0.18 mg/mL anti-*S. aureus* was added and allowed to react overnight at 4 °C. Since the AuNPs were functionalized with antibodies and its corresponding Raman reporter labels, the concentration of antibodies needs to be carefully optimized to avoid aggregation and ensure monodispersity. UV–Vis absorption spectra in Fig. S1B showed the red shift of AuNPs after binding to the Raman reporter–antibody conjugate (the peak of the bare AuNPs is located at 526 nm and then red shifted to 530 and 536 nm, respectively, after attachment of Raman reporters and antibodies), which is most likely due to the surface chemistry changes on the particle surface due to the replacement of the citrate-ligand layer by Raman reporters and antibodies, demonstrating the formation of the probe complex [39, 40]. Next, 10 μL of 2 μM BSA was added to each of the AuNPs conjugate solution to prevent nonspecific adsorption of AuNPs on the pathogen or the MNPs@SiO_2 surface.

Preparation of pathogen samples

S. aureus and *S. enterica* serovar Typhimurium were obtained from the *Escherichia coli* Reference Center at The Pennsylvania State University (University Park, PA, USA). These strains were cultured on LB agar plates for 24 h at 37 °C, and a single colony from each plate was transferred into culture tubes containing sterile LB broth. These liquid cultures were incubated overnight at 37 °C under shaking conditions. Cells were extracted by centrifugation at 5,800 rpm for 10 min and washed with sterile PBS (pH 7.4) three times before resuspending in PBS for detection experiments. Serial dilution plating was used to count live bacteria and determine the number of bacteria in a given population.

To detect pathogens in a food matrix, about 100 g of fresh spinach was obtained from a local grocery store (West Lafayette, IN, USA) and transferred into a sterile 500-mL conical flask. Sterile nanopure water was used to clean the spinach sample three times and was finally suspended in 50 ml of PBS. The food matrix was then artificially spiked with bacteria (500 µl of overnight cultures suspended in PBS) and incubated at 37 °C for 3 h. At the end of the incubation period, the mixture was thoroughly shaken, and 0.5 ml of the spinach extract was used for detection [41].

Detection of bacterial pathogens

The procedure constituting separation and detection of pathogens is shown in Fig. 1c. Briefly, antibody-functionalized MNPs@SiO_2 were incubated in PBS buffer or spinach solution (0.5 mL) containing different concentrations of pathogens for 60 min at room temperature under shaking conditions. After isolation and a PBS wash, the pathogen-bound particles were further reacted (with continuous shaking) with 0.5 mL SERS nanoprobes in PBS solution (pH 7.0) for another 60 min at room temperature. As a final step, the pathogens were isolated from the bulk solution and analyzed by a Raman spectrometer.

The detection scheme was also validated in a different sample through blind studies by artificially spiking 50 g of peanut butter with 5 mL of an unknown pathogen (grown in LB broth and resuspended in PBS buffer, 10^3 CFU/mL) and thoroughly shaken for 10 min to obtain a uniform mixture of peanut butter and buffer solution, and the enumeration was done post-assay, using the same method. Assuming the cells were about 10^8 cells/mL based on OD value, the dilution was done to obtain 10^3 cells/mL. The same protocol was employed for separation and detection of the unknown pathogens as discussed above.

Measurement

The size and morphology of MNPs, MNPs@SiO_2, and AuNPs were determined from TEM images acquired with a Philips CM-100 TEM (Philips, Eindhoven, Netherlands) operating at 100 kV. Absorption spectra of AuNPs and AuNPs labeled by Raman reporters and antibodies were measured with a Jasco V570 UV/visible/NIR spectrophotometer (Jasco Inc., Easton, MD, USA) in the 400- and 900-nm wavelength range. SERS measurements of the probes on AuNPs were conducted using the SENTERRA confocal Raman system (Bruker Optics Inc., Billerica, MA, USA) with a ×20 air objective at 633 nm excitation and a 50-µm pinhole for confocality. The laser power and accumulation time were set at 20 mW and 20 s, respectively.

Results and discussion

Principle of the SERS immunoassay for pathogen detection

A schematic of the SERS immunoassay integrated with the magnetic separation module for detecting multiple pathogens is illustrated in Fig. 1c. The silica-coated MNPs will provide a platform for rapid capture and separation of pathogens from the food matrix. The SERS nanoprobes will bind to the MNPs@SiO_2 particles containing pathogens through the specific antibody–target interaction to form a sandwich assay for detection by SERS. Owing to the enhancement of the signature of the Raman reporters by AuNPs, a sensitive assay could be developed to detect multiple pathogens simply by changing the Raman reporters and the related antibodies on the SERS probes.

To demonstrate the SERS-based multiplex detection, two non-fluorescent molecules, MBA and MPY, were chosen as Raman reporters to detect *S. enterica* serovar Typhimurium and *S. aureus* as test pathogens in this study [42, 43]. The distinct fingerprint of the two labels chosen is shown in Fig. S2, where the main peaks for MBA at 1,078 and 1,589 cm^{-1} can be assigned to the $_{12}$ and $_{8a}$ aromatic ring vibrations, respectively [44, 45], and the fingerprint peaks for MPY at 1,013, 1,094, 1,212, and 1,578 cm^{-1} can be assigned to the C–C, C–N, C–S, and C–C vibration, respectively [46, 47].

Characterization of MNPs and MNPs@SiO_2

MNPs and MNPs@SiO_2 were first characterized by TEM images as shown in Fig. 2a, b. TEM images show the MNPs possess a uniform diameter of ~15 nm with excellent magnetic properties, indicated by the photograph of MNPs dispersed in aqueous solution with and without the external

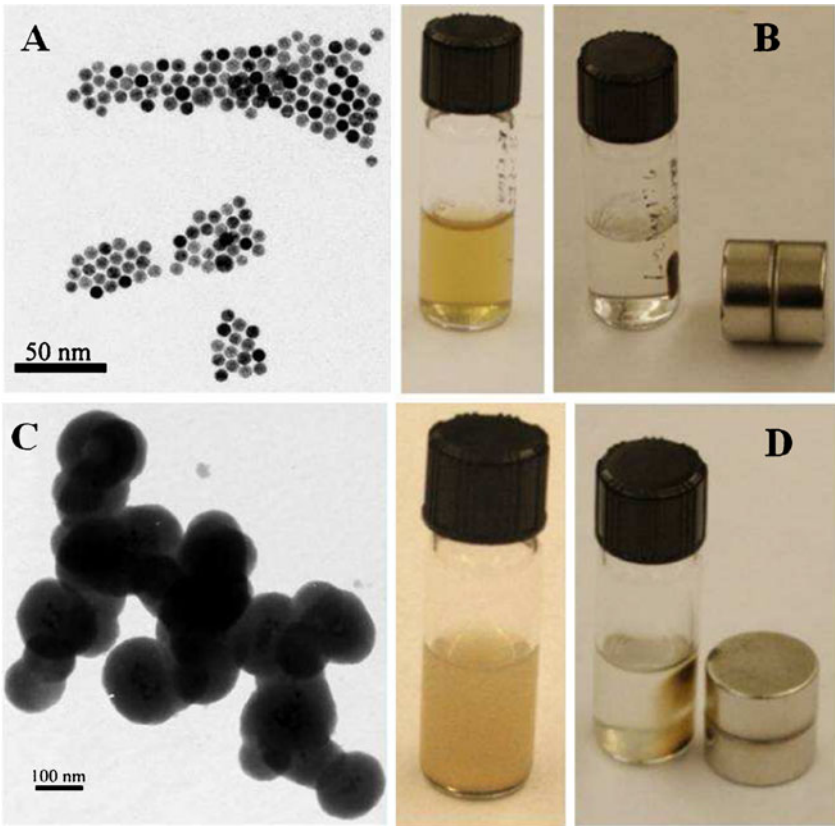

Fig. 2 TEM images of MNPs (**a**) and MNPs@SiO_2 nanostructure (**b**), and optical photographs of MNPs (**c**) and MNPs@SiO_2 nanostructures (**d**) dispersed in an aqueous solution with and without an external magnetic field

magnetic field (Fig. 2c). Under an external magnetic field, MNPs move towards the magnet; however, if the magnet is removed, they re-disperse in solution, almost uniformly. When these MNPs are coated by a silica layer, the size of the nanoparticles increased to ~100 nm to form the MNPs@SiO_2 structure. Figure 2b shows several as-prepared silica-coated MNPs (MNPs@SiO_2) with the desired magnetic property (Fig. 2d) for effective target separation under the magnetic field. Therefore, the dispersity of the MNPs@SiO_2 nanostructures was found to be adequate for the separation of pathogens.

Comparison of pathogen detection scheme with and without Raman reporters

To test for the feasibility of the proposed design, *S. enterica* serovar Typhimurium (~10^4 CFU/mL) was first chosen as a model pathogen to test the separation and detection scheme in PBS buffer. Through the specific interaction of pathogens with the suspended MNPs@SiO_2 and SERS nanoprobes, which were functionalized with the antibody and Raman reporters, *S. enterica* serovar Typhimurium was isolated from the PBS solution and its identity confirmed by the strong and distinct fingerprint of the Raman reporter as shown in curve a in Fig. 3. Independently, the Raman fingerprint of the pathogen was obtained (curve b in Fig. 3), and the vibration modes of carbohydrates at 1,097 cm^{-1}, aromatic amino acids from proteins at 1,206 cm^{-1}, and adenine and guanine (ring stretching) at 1,617 cm^{-1} [48, 49] were found to be enhanced by AuNPs, indicating the feasibility of the sensor for isolating and gathering targeted pathogens for detection using the label-free and label method. However, it can be noted that the signal intensity from the Raman reporters is much stronger than that obtained from the pathogen itself due to the large Raman

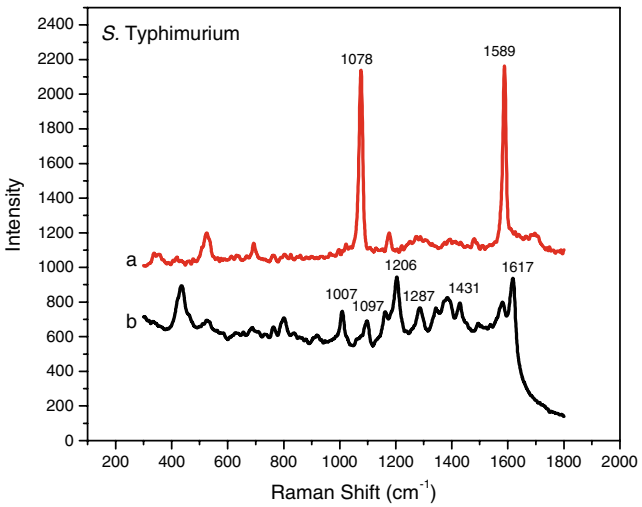

Fig. 3 SERS spectra for *S. enterica* serovar Typhimurium at 10^4 CFU/mL using SERS nanoprobes with (*a*) and without Raman reporters (*b*)

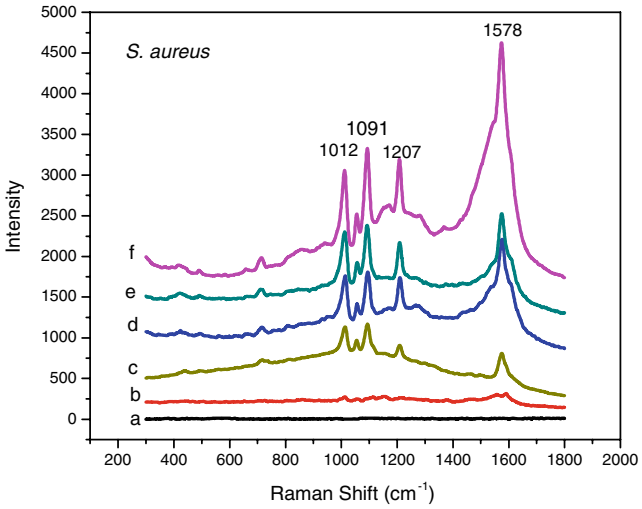

Fig. 4 SERS spectra for *S. aureus* in PBS buffer at different concentrations and with controls ((*a*) 0 CFU/mL; (*b*) non-target pathogen, *S. enterica* serovar Typhimurium; (*c*) 10^2; (*d*) 10^3; (*e*) 10^4; and (*f*) 10^6 CFU/mL)

cross-section of the reporters. Although most of the past work including ours on direct fingerprinting using label-free SERS substrates provides information on the various vibration modes common to organisms, it is often difficult to realize multiplex detection with high specificity due to the similarity of the SERS spectra. Spectroscopic classification of pathogens using Fourier-transform infrared spectroscopy [41, 50, 51] is well established, but often requires post-processing using appropriate chemometrics, and the sensitivity of this approach is at best 10^4 CFU/mL. On the contrary, an advantage of the label-based SERS approach is that multiple pathogens could be detected by just changing the Raman reporter labels. Although labels pose an additional step, the label attachment chemistry is well established [18, 52, 53]. More crucially, the label-based approach can be highly sensitive because of the large cross-section of the Raman reporters. In curve a in Fig. 3, we show that after labeling by one of the Raman reporters, MBA, almost no fingerprint signal of the pathogen could be observed. Although direct fingerprinting may not be highly sensitive, the use of a Raman reporter could provide an assay with excellent signal to noise ratio for direct detection in food matrices.

Detection of selective pathogens in PBS buffer

To further demonstrate the sensitivity and feasibility of the SERS biosensor for pathogens isolation and detection, *S. aureus*, chosen as the second model pathogen, was first magnetically separated from the PBS buffer and then detected by SERS as shown in Fig. 4. Distinct Raman signal from Raman reporters was obtained with respect to the concentrations of the pathogens. The lowest concentration of *S. aureus* detected in PBS buffer was 10^2 CFU/mL (0.5 mL of total sample volume), demonstrating a high sensitivity of the SERS-based biosensor. The reproducibility of the biosensor was demonstrated by monitoring for *S. aureus* in PBS at the same concentration as shown in Fig. S3. Similar spectral properties show excellent reproducibility of the biosensor for the detection of the pathogens. To prove the specificity of the biosensor for *S. aureus* detection, control experiments were conducted using samples without pathogens and a non-targeted pathogen (*S. enterica* serovar Typhimurium) as indicated by curves a and b in Fig. 4, respectively. Almost no signal was observed from the samples without pathogens, and only a very weak signal was noted even at a high concentration of the non-target pathogen (10^4 CFU/mL) due to the negligible

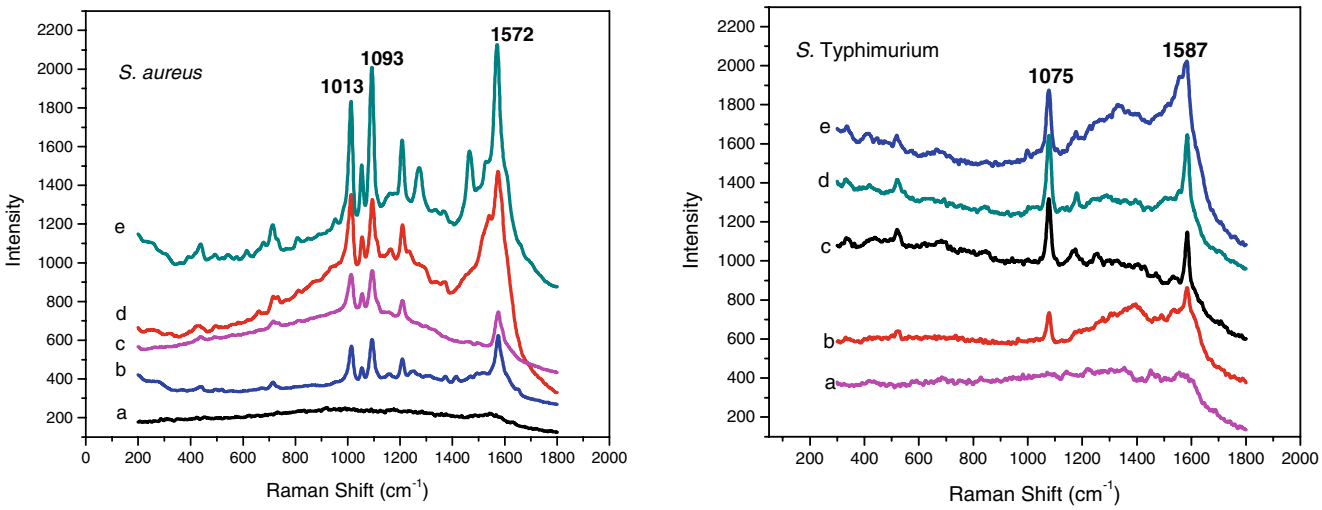

Fig. 5 SERS spectra for *S. aureus* (*left*) and *S. enterica* serovar Typhimurium (*right*) in spinach wash after being separated by a magnet at different concentrations ((*a*) 0, (*b*) 10^3, (*c*) 10^4, (*d*) 10^5, and (*e*) 10^6 CFU/mL)

nonspecific absorption of SERS probes on the cell surface, indicating a high degree of specificity and compatibility of the probes.

Selective detection of multiple pathogens in food matrix

The SERS biosensor platform was then applied to detect multiple pathogens in a food matrix as shown in Fig. 5. Various intensities of the SERS spectra with respect to the concentrations of *S. aureus* (curve a in Fig. 5) and *S. enterica* serovar Typhimurium (curve b in Fig. 5) after isolation from the spinach wash demonstrate the assay sensitivity. Distinct Raman signal could be observed for the two specific pathogens, and no signal was observed when pathogens were absent in the spinach wash, as expected. The organic matter in the spinach suspension gives rise to Raman signal and contributes to some fluorescence, which will diminish the Raman signal from the SERS probes. Thus, the SERS spectra of the Raman reporters from the spinach wash were not as intense as that from a neat buffer, such as PBS. Comparing Fig. 3 and curve b in Fig. 5, the signal intensity for *S. enterica* serovar Typhimurium in PBS was stronger than that in spinach wash for the same concentration, as expected due to interference from the sample matrix. Thus, the lowest detectable concentration was 10^3 CFU/mL (0.5 mL of total sample volume), one order of magnitude lower than the detection in PBS buffer, but higher than the reported limits of detection in the range between 10^4 and 10^5 CFU/mL in food matrices by Fourier-transform infrared [41, 54], indicating a higher sensitivity of the fabricated sensors for detection of multiple pathogens. Using the same procedure, a blind test to detect artificially inoculated pathogen in peanut butter (Fig. S3A) was also demonstrated. Although some background from the peanut butter matrix was observed, the two key peaks at 1,073 and 1,573 cm^{-1} corresponding to the vibration modes of MBA were detectable for the blind samples, indicating the presence of *S. enterica* serovar Typhimurium in the peanut butter emulsion (Fig. S3B). The lowest cell concentration detectable was 10^3 CFU/mL, indicating the sensitivity and specificity of the SERS biosensor for pathogen detection.

Conclusion

A novel immunomagnetic SERS biosensor, centered on a sandwich immunoassay format that combined the respective capture and reporting capability of silica-coated, small MNPs and AuNPs labeled with Raman reporters and antibodies, was fabricated for the separation and detection of *S. enterica* serovar Typhimurium and *S. aureus*. Using SERS detection, the pathogens were separately detected in spinach wash at a limit of 10^3 CFU/mL. A blind test in peanut butter confirmed the validity and robustness of the tools developed to detect at a limit of 10^3 CFU/mL pathogens. With the proper choice of Raman labels and SERS substrate, single pathogen detection SERS platforms could be developed for routine monitoring of raw and processed foods to assure the safety of our food supply chain.

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