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Gold nanoparticles enhanced SERS aptasensor for the simultaneous detection of *Salmonella typhimurium* and *Staphylococcus aureus*

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ABSTRACT

Salmonella typhimurium and *Staphylococcus aureus* are most common causes of food-associated disease. A Raman based biosensor was developed for *S. typhimurium* and *S. aureus* detection simultaneously. The biosensor was based on nanoparticles enhanced Raman intensity and the specific recognition of aptamer. The Raman signal probe and the capture probe are built. Gold nanoparticles (GNPs) modified with Raman molecules (Mercaptobenzoic acid and 5,5′-Dithiobis(2-nitrobenzoic acid)) and aptamer are used as the signal probe for *S. typhimurium* and *S. aureus*, respectively. Fe_3O_4 magnetic gold nanoparticles (MGNPs) immobilized with both aptamer of *S. typhimurium* and *S. aureus* are used as the capture probe. When *S. typhimurium* and *S. aureus* are added in the reaction system, the capture probe will capture the target bacteria through the specific binding effect of aptamer. And then the signal probe will be connected to the bacteria also by the effect of aptamer to form the sandwich like detection structure. The Raman intensified spectrum was measured to quantify *S. typhimurium* and *S. aureus*. Under optimal conditions, the SERS intensity of MBA at 1582 cm^{-1} are used to measure *S. typhimurium* ($y=186.4762+704.8571x$, $R^2=0.9921$) and the SERS intensity of DNTB at 1333 cm^{-1} are used to measure *S. aureus* ($y=135.2381+211.4286x$, $R^2=0.9946$) in the range of 10^2–10^7 cfu mL^{-1}. The LOD is 35 cfu mL^{-1} for *S. aureus* and 15 cfu mL^{-1} for *S. typhimurium*. This method is simple and rapid, results in high sensitivity and specificity, and can be used to detect actual samples.

1. Introduction

Salmonella typhimurium and *Staphylococcus aureus* are most reported food-borne pathogenic bacteria which will cause food poisoning, intestinal infectious diseases and other health problems (Arnold et al., 2011; Francois et al., 2010; Can and Celik, 2012). The conventional methods for *S. typhimurium* and *S. aureus* detection are usually classical culture methods that include the sequential steps of pre-enrichment, selective enrichment and selective differential plating. These methods are time-consuming, labor-intensive and impractical for real-time applications (Patel et al., 2006). The development of new techniques with faster response time, better sensitivity and selectivity, especially the simultaneous detection for different food-borne pathogenic bacteria are of great importance.

Nanoparticles are attracting research interest because of their distinguished properties, such as the small scale effect, surface effect and good biocompatibility properties (Mascaraque et al., 2013; Tan et al., 2014; Shokrollahi et al., 2014; Yan et al., 2014). The synthesis of gold-coated iron nanoparticles (Fe_3O_4@GNPs) is of special attention. The gold coating produces air-stable nanoparticles, which are protected from oxidation. Surface derivatization with gold also helps to reduce particle agglomeration by stericor electronic repulsion and improves the biocompatibility (Liu et al., 2014; Ma et al., 2009). Many nanosensors based on Fe_3O_4/Au core–shell structures and GNPs have been built in the detection of chemistry, biology and food fields, such as protein, antibiotics, food borne pathogens, toxins, and metal ions.

Aptamers are single-stranded DNA or RNA molecules that form sequence-defined unique structural forms with binding affinities for specific targets. Several characteristics of aptamers make them

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attractive for pre-analytical sample processing and biodiagnostic assay development, including their small size, ease of synthesis and labeling, lack of immunogenicity, low cost of production and target binding affinity and specificity (Ahmadi et al., 2014; Jo et al., 2011). Biosensors that use an aptamer as a specific recognition part known as an aptasensor have become an area of research interest (Tombelli et al., 2005; Suh et al., 2014; Kim et al., 2014; Guo et al., 2014; Lee et al., 2014).

Surface-enhanced Raman spectroscopy (SERS) has been used in various biological applications because of its high sensitivity and specificity. Therefore, the SERS technique is an attractive tool for sensing molecules in trace amounts within chemical and biochemical analytics (Cialla et al., 2012). Common SERS-enhanced substrates are gold nanoparticles, silver nanoparticles and other heavy metal nanomaterials, as well as various core–shell nanoparticles (Cai et al., 2014; Huan et al., 2014; Quyen et al., 2014).

Here, we report a SERS-based biosensor for the quantitative detection of *S. typhimurium* and *S. aureus* simultaneously using aptamers and nanoparticles. GNPs combined with Raman signal molecule and aptamers were used as the signal probe. And MGNPs immobilized with aptamers were used as the capture probe. The experimental conditions are optimized. Raman signal intensities are recorded for quantitative detection. In the range of 10^2–10^7 cfu mL^{-1}, *S. typhimurium* and *S. aureus* concentration exhibited a good linear relationship, and the detection limit was 35 cfu mL^{-1} for *S. aureus* and 15 cfu mL^{-1} for *S. typhimurium*. This method showed high sensitivity, good selectivity and short detection time. It has the potential to be improved for daily detection work.

2. Materials and methods

2.1. Materials

Ferric chloride ($FeCl_3 \cdot 6H_2O$), ferrous chloride ($FeCl_2 \cdot 4H_2O$), ammonia solution, hydrochloric acid, ethanol, phosphate buffer solution (10 mM PBS, pH 7.4), trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), chloroauric acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$), and sodium chloride were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Mercaptobenzoic acid (MBA), 5,5′-Dithiobis(2-nitrobenzoic acid) (DNTB) used as Raman signal molecules were purchased from Sigma-Aldrich (Shanghai, China). The *S. typhimurium* aptamer sequence (Joshi et al., 2009) was 5′-SH-TAT GGC GGC GTC ACC CGA CGG GGA CTT GAC ATT ATG ACA G-3′. The *S. aureus* aptamer sequence (Cao et al., 2009) was 5′-SH-GCA ATG GTA CGG TAC TTC CTC GGC ACG TTC TCA GTA GCG CTC GCT GGT CAT CCC ACA GCT ACG TCA AAA GTG CAC GCT ACT TTG CTA A-3′. Thiolated aptamers were synthesized by the Shanghai Sangon Biological Science & Technology Company (Shanghai, China).

2.2. Preparation of the GNPs

The synthesis of ~15 nm GNPs was performed as reported previously (Liu et al., 2014). First, 2 mL of $HAuCl_4 \cdot 4H_2O$ (1%, w/w) was dissolved in 200 mL deionized water and heated to boiling for 10 min. Then, 8 mL sodium citrate (1%, w/w) was rapidly injected with vigorous magnetic stirring. The mixture reacted for another 15 min until the color turned to wine-red. The resulting GNPs were purified by centrifugation (12,000 rpm, 25 min) for three times and stored in a 4 °C for further use.

2.3. Preparation of the signal probe for S. typhimurium

For the preparation of the signal probe for *S. typhimurium*, 0.1 mL of 1 mM MBA (Raman signal molecule) was added into 1 mL of the prepared GNPs and reacted at 37 °C for 24 h. The solution was centrifuged and the precipitate was washed twice and redispersed in 10 mL 0.01 M PBS (pH 7.4). Then, 2 μL 10^{-5} mol/L of the thiolated *S. typhimurium* aptamer solution was added and incubated at 37 °C for 24 h. A trace of 1 M NaCl solution was added for further reaction. The reaction product was centrifuged at 12,000 rpm for 25 min to remove the excess aptamer. Then, the nanoparticles were then blocked with a blocking buffer (1% BSA in PBS) for 1 h at room temperature to occupy the uncoated places and avoid the nonspecific absorption. Finally, the prepared signal probe was resuspended in 10 mL 10 mM PBS (pH 7.4) and stored at 4 °C for further use.

2.4. Preparation of the signal probe for S. aureus

The preparation method of the signal probe for *S. aureus* was similar as described in Section 2.3 while the Raman signal molecule was changed to 0.1 mL of 1 mM DNTB and the aptamer was changed to 2 μL 10^{-4} mol/L thiolated *S. aureus* aptamer.

2.5. Preparation of MGNPs

Fe_3O_4 magnetic gold nanoparticles were prepared as described in previous work (Rawal et al., 2012). First, 8.5 g $FeCl_3 \cdot 6H_2O$ and 3.0 g $FeCl_2 \cdot 4H_2O$ were dissolved in 38 mL hydrochloric acid (0.4 M) and 375 mL ammonia solution (0.7 M) was rapidly added under vigorous stirring for 30 min. The resulting precipitates were isolated under magnetic force and washed thoroughly three times. Then, 5 mL of the prepared magnetic nanoparticles was dissolved in 100 mL of 1 mM $HAuCl_4$ and mixed with 10 mL ethanol (10 mg mL^{-1}) for reaction at room temperature for 15 min. The prepared MGNPs were separated under magnetic force. Finally, the MGNPs were washed three times, dried in the oven, and stored at 4 °C for further use.

2.6. Preparation of the capture probe

First, 1 mg of the prepared MGNPs was dissolved in 1 mL PBS (10 mM, pH 7.4). Then, 2 μL of 10^{-5} mol/L thiolated *S. typhimurium* aptamer and 2 μL 10^{-4} mol/L of thiolated *S. aureus* aptamer were added simultaneously. The mixture was incubated at 37 °C for 24 h. The thiolated aptamer were connected at the surface of the GNPs through Au–S chemical bonds. The resulting capture probes were isolated under magnetic force. And the blocking step was conducted as described in Section 2.3. The product was washed with PBS (10 mM, pH 7.4) and stored at 4 °C for further use.

2.7. Analytical procedure

100 μL of the prepared capture probe and 50 μL of the sample solution containing different concentrations of *S. typhimurium* and *S. aureus* (10^2 cfu mL^{-1}, 10^3 cfu mL^{-1}, 10^4 cfu mL^{-1}, 10^5 cfu mL^{-1}, 10^6 cfu mL^{-1}, and 10^7 cfu mL^{-1}) were mixed and incubated at 37 °C for 70 min. The mixture was isolated under magnetic force and washed with 10 mL PBS (10 mM, pH 7.4) three times. Then, 100 μL of the prepared *S. typhimurium* signal probe and *S. aureus* probe were added to the reaction system for incubation at 37 °C for 70 min. The solution was isolated under magnetic force and redispersed in 50 μL PBS (10 mM, pH 7.4) for Raman test.

2.8. Recovery experiments for pork sample

In this experiment, the commercial pork was used as realistic samples for recovery experiments in the detection of *S. typhimurium* and *S. aureus*. 5 g pork paste was added into 10 mL PBS (10 mM, pH 7.4) and sterilized. And the product was centrifuged at

10,000 rpm for 15 min to remove the solid impurities in the sample. Then gradient dilutions of *S. typhimurium* and *S. aureus* were added to the pork sample. Then the Raman detection method was conducted to calculate the detectable amount of *S. typhimurium* and *S. aureus*. The results were compared with the traditional plate counting method and the recovery rate was calculated.

3. Results and discussion

3.1. Detection method for Raman test

The Raman based detection process is shown in Fig. 1. The MGNPs modified with thiolated *S. typhimurium* aptamer and thiolated *S. aureus* aptamer were used as capture probe. The GNPs immobilized with MBA and thiolated *S. typhimurium* aptamer were used as *S. typhimurium* signal probe. The GNPs immobilized with DNTB and thiolated *S. aureus* aptamer were used as *S. aureus* signal probe. When *S. typhimurium* and *S. aureus* exist both in sample solution, they will be captured by the MGNPs capture probe through the aptamer specificity effect. Then the two pathogenic bacteria could be connected to the signal probe also by the aptamer specificity effect to form the sandwich structure. MBA and DNTB have their own specific Raman spectra. At the same time, the distance between GNPs was closer accompanied by the formation of sandwich structure. Therefore, the Raman intensity was enhanced which was related to the generation of a high density of hot spots. So the Raman signal could be detected to measure the amount of *S. typhimurium* and *S. aureus*.

3.2. Preparation and characterization of GNPs and MGNPs

Fig. S1 shows the UV–visible absorption spectrum and TEM image of GNPs. It shows that the prepared GNPs have an absorption peak at approximately 520 nm and the size is ~15 nm.

Fig. S2 shows the TEM image of the MGNPs and its element analysis by energy dispersive spectroscope. The particle size is ~20 nm (Fig. S2A). Fig. S2B presents the EDX spectrum of the MGNPs, which indicates the presence of oxygen, iron, and gold, which demonstrating the successful coating of the Au shell.

3.3. Preparation and characterization of signal probe

Fig. S3 depicts the absorption peak for GNPs immobilized with MBA and thiolated *S. typhimurium* aptamer. There contains three absorption peaks at 225 nm, 260 nm, and 520 nm. Benzoic acid group in MBA exhibits a special absorption peak at 225 nm. And the aptamer has an absorption peak at 260 nm. However, the absorption peak for GNPs at 520 is relatively much lower than the other two peaks for the relatively low amount of GNPs. The results of spectral characterization demonstrate the successful functionalization of the *S. typhimurium* signal probe.

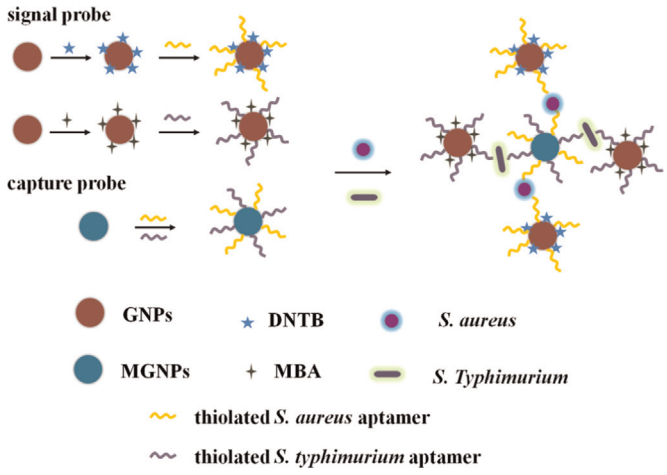

Fig. 1. Schematic illustration of the aptasensor for simultaneous detection of *S. aureus* and *S. typhimurium* based on GNPs enhanced Raman intensity.

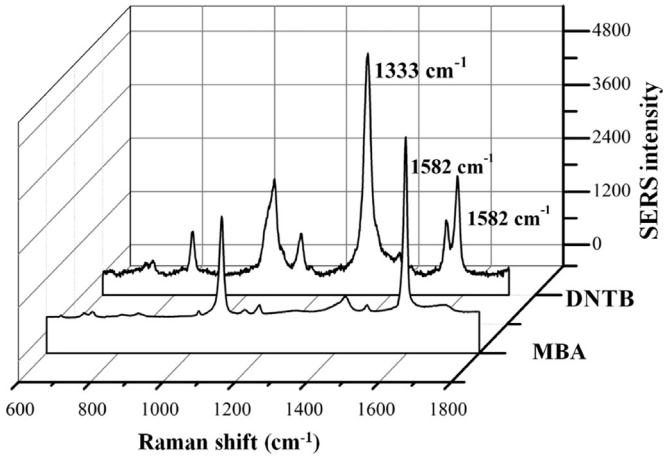

Fig. 2. The Raman scattering spectra of DNTB and MBA.

For the preparation of signal probe for *S. aureus*, the Raman signal probe DNTB and thiolated *S. aureus* aptamer were immobilized in GNPs. First, Fig. S4A shows the Raman spectrum for the compound of DNTB and GNPs. The characteristic absorption peak for DNTB indicated the successful connection of DNTB to GNPs. Then, Fig. S4B shows the UV–visible absorbance of thiolated *S. aureus* aptamer before and after modification to GNPs which also indicate the successful connection.

Fig. 2 depicts the Raman spectra for DNTB and MBA. The Raman signal intensity from DNTB at 1333 cm^{-1} is used to calculate the amount of *S. aureus*. And the Raman signal intensity at 1582 cm^{-1} is used to calculate the amount of *S. typhimurium*. Here, the intensity of 1582 cm^{-1} from DNTB owing to the existence of *S. aureus* is subtracted.

3.4. Optimization of aptamer concentrations

The sandwich detection method is formed through the specific binding effect of aptamer. The amount of aptamer will directly influence the detection results. So the amount of aptamer is optimized for both capture probe and signal probe. Results are shown in Fig. 3.

To determine the optimal concentration, various concentrations of aptamer were added to react with GNPs. As observed in Fig. 3a, which depict the Raman signal for the connection between *S. aureus* and GNP, the signal intensity grew rapidly in the Raman spectrum as the concentration of the aptamer increased from 1×10^{-8} M to 1×10^{-4} M, but remained steady when the concentration increased from 1×10^{-4} M to 1×10^{-3} M. Therefore, 1×10^{-4} M was selected as the optimal concentration for further experiments. Similarly, the optimal concentration for *S. typhimurium* aptamer connected in GNP signal probe is 1×10^{-5} M as shown in Fig. 3b.

3.5. Analytical performance

Gradient concentrations of *S. aureus* and *S. typhimurium* were added in the detection system under the optimal conditions. Results are shown in Fig. 4. Fig. 4A shows the Raman spectrascope upon different concentrations of *S. aureus* and *S. typhimurium*. There was a strong linear correlation ($y=186.4762+704.8571x$,

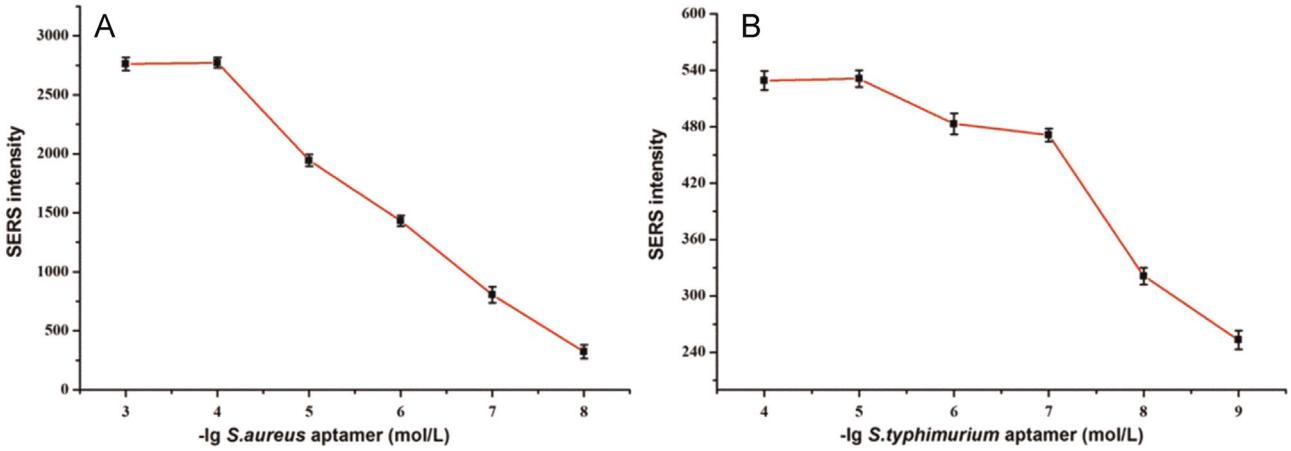

Fig. 3. Optimization for the amount of aptamer. (A) Results for *S. aureus* and (B) results for *S. typhimurium*.

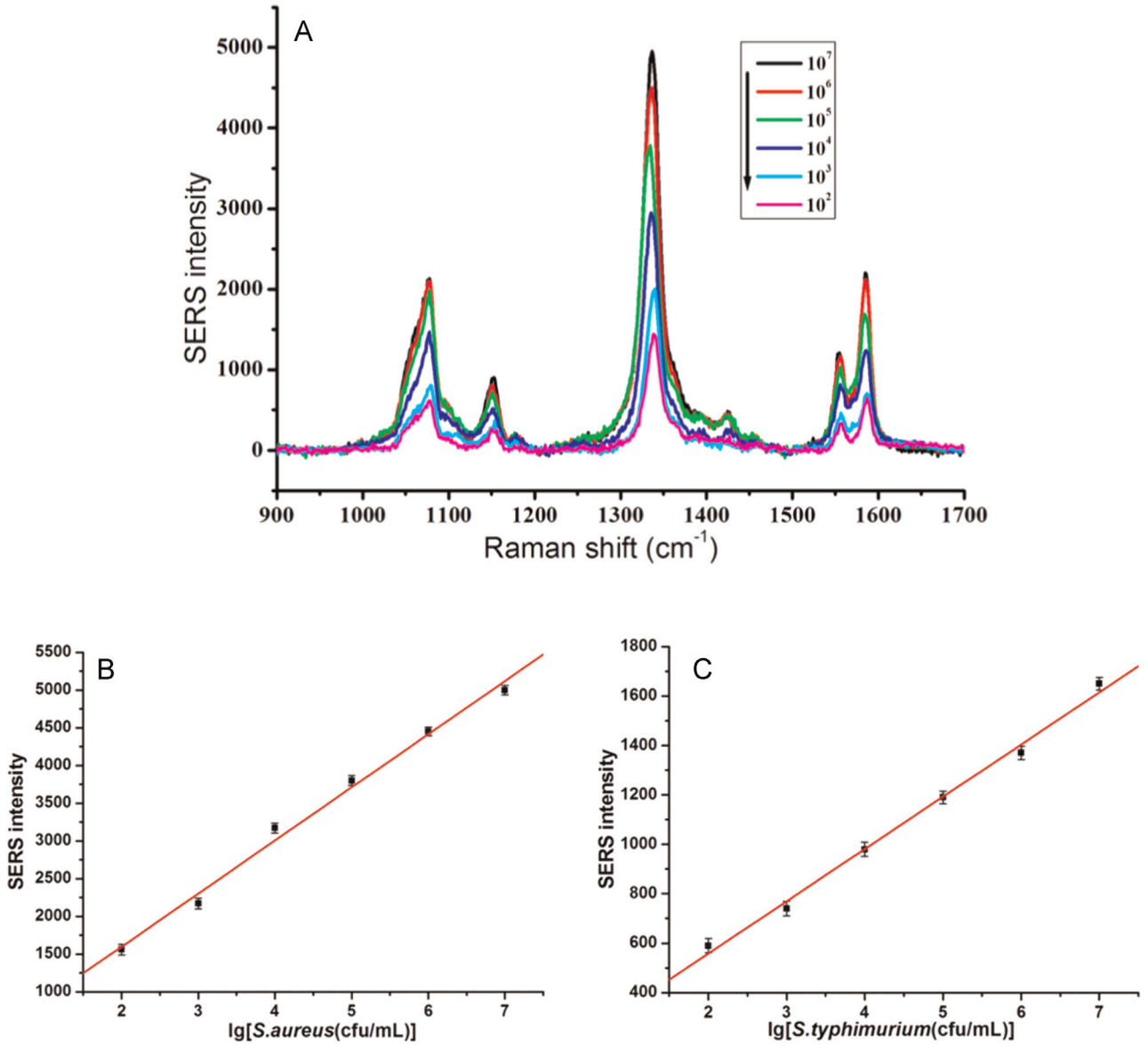

Fig. 4. (A) Raman scattering spectra of different concentration of *S. aureus* and *S. typhimurium* (10^2 cfu mL^{-1}, 10^3 cfu mL^{-1}, 10^4 cfu mL^{-1}, 10^5 cfu mL^{-1}, 10^6 cfu mL^{-1}, and 10^7 cfu mL^{-1}). (B) Correlation between Raman intensity and concentrations of *S. aureus*. (C) Correlation between Raman intensity and concentrations of *S. typhimurium*.

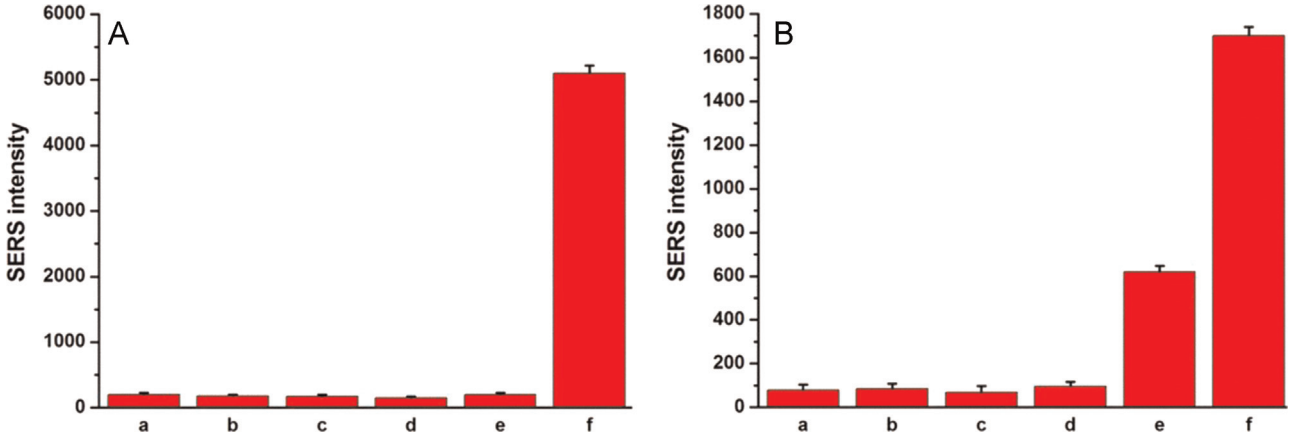

Fig. 5. (A) Specificity result for the detection of *S. aureus*, a: *E. coli*, b: *V. parahemolyticus*, c: *B. cereus*, d: *S. dysenteriae*, e: *S. typhimurium*, and f: *S. aureus*; (B) specificity result for the detection of *S. typhimurium*, a: *E. coli*, b: *V. parahemolyticus*, c: *B. cereus*, d: *S. dysenteriae*, e: *S. aureus*, and f: *S. typhimurium*.

Table 1
Comparison of the pork sample results obtained from the Raman detection and classical plate counting method (all results were repeated three times and shown as average ± SD).

Pork sample	Plate counting (cfu mL^{-1})		Raman method (cfu mL^{-1})		Recovery ratio (%)	
	S. typhimurium	*S. aureus*	*S. typhimurium*	*S. aureus*	*S. typhimurium*	*S. aureus*
1	2676 ± 20	3214 ± 12	2698 ± 24	3210 ± 34	100.82	99.88
2	324 ± 8	423 ± 3	336 ± 10	425 ± 6	103.70	100.47
3	135 ± 6	109 ± 2	129 ± 6	112 ± 9	95.56	102.75
4	35 ± 2	34 ± 1	39 ± 3	32 ± 3	108.33	94.12

R^2=0.9921) between the intensity of the signal and the logarithm of the concentration of *S. aureus* (Fig. 4B) determined using the plate count method in the range of 10^2–10^7 cfu mL^{-1}, with a detection limit of 35 cfu mL^{-1}. The detection limit is based on the calculation formula $D=3N/S$ (N is the standard deviation of blank sample signal. S is the slope of standard curve). And there was a strong linear correlation ($y=135.2381+211.4286x$, $R^2=0.9946$) between the intensity of the signal and the logarithm of the concentration of *S. typhimurium* (Fig. 4C) determined using the plate count method in the range of 10^2–10^7 cfu mL^{-1}, with a detection limit of 15 cfu mL^{-1}. The calculation method is the same as *S. aureus*.

3.6. Specificity

Some other bacterial samples, *Escherichia coli*, *Vibrio parahaemolyticus*, *Bacillus cereus* and *Shigella dysenteriae*, were used to evaluate the specificity of the method for the detection of *S. typhimurium* and *S. aureus*. The concentration of the bacteria was maintained at 10^7 cfu mL^{-1}. Experimental results shown in Fig. 5 clearly show that the signal intensities of the other bacteria are much lower than that of the *S. typhimurium* and *S. aureus*. That is because of the high affinity between the aptamer and its target. As the result in Fig. 5B for the specificity of *S. typhimurium* detection, there is a relative high SERS intensity for *S. aureus*. That is because the DNTB for *S. aureus* detection also has a scattering peak at 1582 cm^{-1}. Actually, in the analytical performance, the intensity of 1582 cm^{-1} from DNTB owing to the existence of *S. aureus* is subtracted for *S. typhimurium* detection.

3.7. Pork sample detection

The utility of the sandwich detection for *S. typhimurium* and *S. aureus* was examined using pork samples obtained from a supermarket. The sample was tested using the new method and the classical plate counting methods. The analytical results are shown in Table 1. The results obtained using the sandwich detection method are similar to those obtained using the plate counting method. As shown in Table 1, the recoveries were between 94.12% and 108.33%, indicating good accuracy of the proposed aptamer-based Raman test for *S. typhimurium* and *S. aureus* detection.

4. Conclusions

The detection of *S. typhimurium* and *S. aureus* simultaneously using a SERS-based sandwich structure is investigated. Gold nanoparticles modified with Raman molecules (MBA and DNTB) and aptamer are used as the signal probe. Fe_3O_4 magnetic gold nanoparticles immobilized with both aptamer of *S. typhimurium* and *S. aureus* are used as the capture probe. The aptamer is of high affinity and specificity, enabling the success of the sandwich detection method. GNPs can enhance the Raman intensity. MGNPs possess good magnetic characteristics, which make it easier to separate the product from the mixture during the experiment. This method can detect the target in approximately three hours and can also be applied to other targets with the transformation of other aptamers.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.07.033.

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