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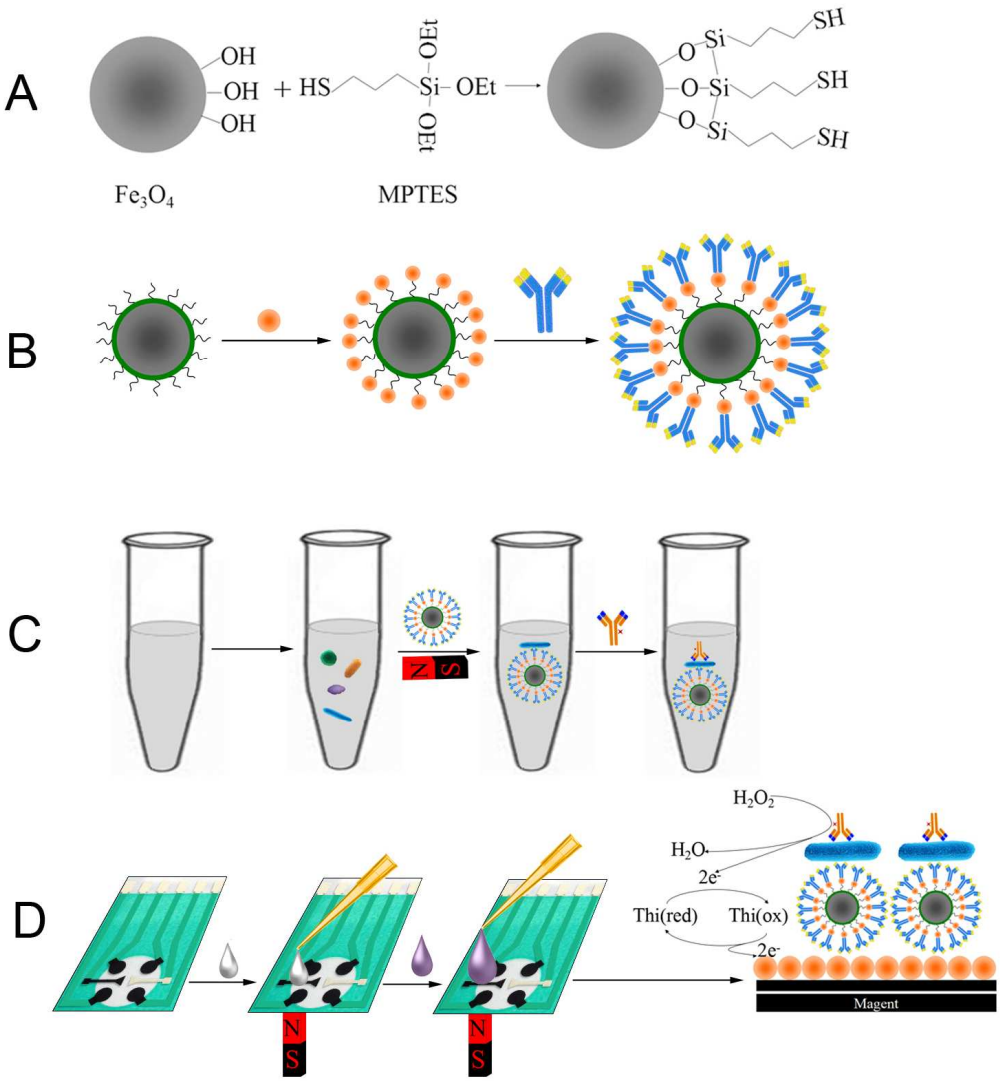
Graphical Abstract: The preparation of immunoassay method has been showed in Schematic illustrations: (A) The synthesis of Fe_3O_4/SiO_2–SH; (B) AuNPs and Ab_1 link with Fe_3O_4/SiO_2–SH based on the self-assembly in order; (C) Functions of IMB for antigen (Ag) enrichment and separation from sample, and HRP-antibody (HRP-Ab_2) for immunoreaction; (D) HRP-Ab_2/Ag/Ab_1/AuMNPs dropped on the AuNPs/4-SPCE, the principle of electrochemical detection.



: Fe_3O_4; : MPTES; ; AuNPs; : anti- *S. pullorum* and *S. gallinmaru*; , : other Bacteria; : *S. pullorum* and *S. gallinmaru*; : HRP-anti- *S. pullorum* and *S. gallinmaru*; : Magnet; : Ag/Ab_1/AuMNPs; : Thionine solution.

A sandwich electrochemical immunoassay for *Salmonella pullorum* and *Salmonella gallinarum* based on $AuNPs/SiO_2/Fe_3O_4$ adsorpting antibody and 4 channels screen printed carbon electrode electrodeposited gold nanoparticles

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ABSTRACT A rapid and high-sensitive sandwich electrochemical immunoassay method was constructed for *Salmonella pullorum* and *Salmonella gallinarum* (*S. pullorum* and *S. gallinarum*) determination based on immune magnetic beads (MB) and enzyme labeled antibody. An abundance of gold nanoparticles (AuNPs) were attached to SiO_2 coated Fe_3O_4 nanoparticles (Fe_3O_4/SiO_2) via the covalent binding between the -SH groups of the Fe_3O_4/SiO_2 and AuNPs. Antibodies against *S. pullorum* and *S. gallinarum* were immobilized on Fe_3O_4/SiO_2/AuNPs nanocomposites (AuMNPs) by automatic adsorption between thiol and AuNPs. *S. pullorum* and *S. gallinarum* in sample were captured by AuMNPs and separated from samples by applying an external magnetic field. The AuMNPs–*Salmonella* complexes (Ag/Ab_1/AuMNPs) were re-dispersed a buffer solution then exposed to Horseradish Peroxidase-labeled anti- *S. pullorum* and *S. gallinarum* ($HRP\text{-}Ab_2$) solution, forming a sandwich-type immune complex ($HRP\text{-}Ab_2/Ag/Ab_1$/AuMNPs). 4 channels screen printed carbon electrode (4-SPCE) was modified by gold nanoparticles (AuNPs) through electrodeposition method to prepare AuNPs/4-SPCE. After magnetically separating the sandwich immune complexes from solution, the $HRP\text{-}Ab_2/Ag/Ab_1$/AuMNPs was anchored on AuNPs/4-SPCE by magnet. A linear response to *S. pullorum* and *S. gallinarum* was obtained in concentration range from 10^2 to 10^6 $CFU \cdot mL^{-1}$, with a limit of detection of 3.2×10^1 $CFU \cdot mL^{-1}$ (at an SNR of 3). This nanoparticle-based immunoassay method offers a way of sensitive, highly specific, and reproducible detection of *S. pullorum* and *S. gallinarum*. Given its low detection limit, it represents a promising potential in detection for other food-borne pathogens by exchanging the antibody.

Keywords: Electrochemical sensor; Gold nanoparticles; Fe_3O_4/SiO_2/AuNPs; Sandwich assay; *Salmonella*.

1. Introduction

Research on electrochemical immunoassay has attracted more attention from scientists in recent years. Such research can be easily conducted using simple electrochemical instruments that have the potential of miniaturization and automation [1, 2]. Electrochemical immunoassays that capitalize on the selectivity of antigen-antibody reactions have excellent detection limits and selectivity. Moreover, it is not affected by the sample components that might interfere with spectroscopic detection, such as particles, chromophores, and fluorophores [3]. Thus, the electrochemical immunosensor is very suitable to detect food-borne pathogens in complex sample without interference from matrix with excellent selectivity, reproducibility and usability [4, 5].

Antibody immobilization is vital in successful development of an electrochemical immunosensor, and the present immobilization methods, such as chemical modification, self-assembly, or physical absorption, are usually quite complex and liable to make the antibody deactivate in real application [6-8]. So if the antibody modification procedure can be excluded, there will be a good prospect for the method. The Horseradish Peroxidase (HRP) can biocatalyze H_2O_2 in the presence of thionine, resulting in an obviously increase of the redox and reduction peak of Cyclic Voltammetry (CV) [9]. The above detection principle has been widely used in the development of novel electrochemical immunosensor. If enriching the amount of above sandwich complexes accumulated on working electrode, the sensitivity of the immunoassay would be greatly improved [10].

More recently, magnetic nanoparticles (MNPs) have received increasing attention due to their high surface-to-volume ratios, allowing for the direct capture, easy separation and concentration of targets in complex samples in an external magnetic field. MNPs are superparamagnetic, which means that they are attracted to a magnetic field but retain no residual magnetism after the field is removed [11]. Therefore, MNPs tagged to the biomaterial of interest can be removed from a matrix using a magnetic field, but they do not agglomerate after removal of the field. These advantages make MNPs desirable candidates for electrochemical immunoassay, as they can function as both an amplifier to increase the sensitivity of the electrochemical immunoassay and simultaneously as a concentration purification agent to reduce background interference [12, 13]. Although MNPs are excellent agents for a low-interference and sensitive electrochemical immunoassay,

they suffer from several drawbacks such as a lack of surface tunability for biocompatible applications, which makes them difficult to couple with bimolecular directly[14]. However, if the proteins were immobilized directly on the surface of MNPs, pure magnetic particles may undergo rapid biodegradation when they are directly exposed to complex environmental and biological systems [10]. Therefore, a suitable coating is essential to prevent such limitations from occurring. Silica has been reported to be one of the best candidate shell materials for the fabrication of novel magnetic core–shell MNPs/ SiO_2, exhibiting the desirable intrinsic properties of the magnetic core and silica shell [15].

It is well known that gold nanoparticles (AuNPs) possess the property of high stability and good biocompatibility [16]. The surface of gold nanoparticles (AuNPs) can be coated with antibody based on the automatic adsorption between antibody and AuNPs, and AuNPs can retain high bioactivity of the adsorbed biomolecules [17, 18]. Thus, combining MNPs with silicon dioxide and AuNPs shell (Fe_3O_4/SiO_2/AuNPs) will have great potential application in biotechnology.

Fowl typhoid (FT) and Pullorum disease (PD), were caused by *Salmonella gallinarum* (*S. gallinarum*) and *Salmonella pullorum* (*S. pullorum*) respectively. FT, as an acute or chronic septicemia infectious disease, primarily transmits by oral or respiratory routes, and affects adult poultries or grower groups. PD is an acute systemic disease and usually found in young birds.[19] The disease can be transmitted by vertically and horizontally to others with contaminated poultries that usually results in a high mortality rate. A huge economic loss and serious threat for the development of intensive poultry industry can be caused by *S. gallinarum* and *S. pullorum*. Therefore, establishing an effective and fast detection measure for these two pathogens are required. Multilocus enzyme electrophoresis and sequence analysis clearly stated that *S. pullorum* and *S. gallinarum* both possess antigen O_1, O_9 and O_{12}, and exhibit high cross-reactivity with each other, they are generally regarded as biotypes of the same serovar, resulting in that they can be simultaneously detected. [20-23] Barrow et al. deemed that it was difficult and unnecessary to differentiate *S. pullorum* and *S. gallinarum* strictly.[24] And Oliveira et al. used ELISA to assess serological response of chickens to *S. pullorum* and *S. Gallinarum*.[25]

The purpose of this study is to establish a sensitive and rapid amperometric

immunoassay method for food-borne pathogens detection. *S. gallinarum* and *S. pullorum* was used as the analysis target models. MNPs were applied to increase the sensitivity of our developed method, which takes advantages of magnetic particles as pre-concentrators. In this study, The Fe_3O_4/SiO_2/AuNPs core-shell magnetic nanoparticles were synthesized by anchoring AuNPs on Fe_3O_4 magnetic composites particles by strong bonding force between –SH and AuNPs. AuNPs acted as the intermediary materials to link Fe_3O_4/ SiO_2–SH and antibody and get the immunomagnetic nanocomposites (Ab_1/AuMNPs). HRP labeled antibody against *S. pullorum* and *S. gallinarum* (HRP-Ab_2) was used as the signal tag. *S. pullorum* and *S. gallinarum* bacteria in sample were captured by Ab_1/AuMNPs and separated from analyte samples by applying an external magnetic field. The MNP–Salmonella complexes were re-dispersed in a buffer solution then exposed to HRP-anti-*S. pullorum* and *S. gallinarum.* The final sandwich immunocomplexes were then attached on the surface of the working electrodes of 4 channels screen printed carbon electrode (4-SPCE) by an external magnetic field. Moreover, 4-SPCE was used to shorten the detection time and improve the reproducibility. In addition, AuNPs were electrodeposited on the working electrodes of 4-SPCE due to its signal amplification function. CV was employed to determine *S. pullorum* and *S. gallinarum* via changes of reduction peak current in the substrate solution of H_2O_2 and the electron mediator of thionine included.

2. Experimental section

2.1. Reagents and solutions

S. pullorum and *S. gallinarum* (CMCC 50770) was employed as the target bacterium, and *Escherichia coli* (*E. coli*, ATCC 8739), *Staphylococcus aureus* (*S. aureus*, ATCC 27217), *Enterobacter Sakazakii* (*E. sakazakii,* ATCC 29544), *Bacillus subtilis* (*B. subtilis*, ACCC 11060), *Bacillus cereus* (*B. cereus*, ATCC 10987) and *Bacillus stearothermophilus* (*B. stearothermophilus*, CICC 20137) were purchased from China Center of Industrial Culture Collection (Beijing, China) and conserved in the laboratory of the authors. Phosphate buffered saline (PBS, 0.01 M, pH 7.4) was used as control group. Anti-*S. pullorum* and *S. gallinarum* were obtained from the China Institute of Veterinary Drug Control (Beijing, China). HRP-labeled anti-*S. pullorum* and *S. gallinarum*

(HRP-Ab_2) were obtained from Shanghai Youke Biotechnology Co., Ltd. Chloroauric acid was obtained from Hangzhou Chemical Reagent Co., Ltd. Thionine (Thi) was obtained from Shanghai Zhongtai Chemical Reagent Co., Ltd. (Shanghai, China). Tetraethyl orthosilicate (TEOS) was obtained from Aladdin Industrial Inc. (Shanghai, China). 3-Mercaptopropyltriethoxysilane (MPTES) was obtained from Nanjing Chengong Organic Silicone material Co., Ltd. (Nanjing, China). And other reagents were all of analytical grade and the water used was doubly distilled.

2.2. Apparatus

CHI 1030 and CHI 760C electrochemical workstation were provided by Shanghai ChenHua Instruments, Inc. (Shanghai, China). 4-Screen-printed carbon electrode (4-SPCE) was developed by Rong Bin Biotechnology Co., Ltd. (Nanjing, China). The diameter of disk-shaped working electrode was 0.25 cm, and the working electrode and counter electrode were made of a carbon ink whereas the reference electrode was made of Silver/Silver chloride (Ag/AgCl), they were all printed on a plastic support. The nanostructures of electrode were characterized by SU-8010 field emission scanning electron microscope (Tokyo, Japan). All electrochemical experiments were performed at 25 ± 2 °C.

2.3. Synthesis of Fe_3O_4 magnetic nanoparticles

The Fe_3O_4 nanoparticles were prepared according to the method of Ziyang Lu [26]. 1.35 mM $FeSO_4 \cdot 7H_2O$ was added to 70 mL double-distilled water, which had been removed oxygen by continuously blowing with nitrogen for 30 min. Under vigorous mechanical stirring and nitrogen protecting, 2.7 mM $FeCl_3 \cdot 6H_2O$ and 5 mL ammonia solution were added to the above double-distilled water. After reacting 80 min at 80 °C, Fe_3O_4 magnetic beads were isolated from the solution by a magnet and rinsed five times by double-distilled water and diluted with water to a total volume of 60 mL.

2.4. Synthesis of Fe_3O_4/SiO_2–SH nanoparticles

The synthesis mechanism of Fe_3O_4/SiO_2–SH nanoparticles was displayed in Fig. 1A and the details as follows: 60 mL ethanol solution and 9 mL ammonia solution were

mixed with 30 mL Fe_3O_4 magnetic beads solution in 100 mL flask. 1 mL TEOS was dropped to the mixture slowly. With the help of stirring, the reaction was carried out for 2 h at 18 °C, then 0.5 mL MPTES was added, and reacted for 12 h. Fe_3O_4/SiO_2–SH nanoparticles were isolated from the solution by a magnet and rinsed five times by double-distilled water and diluted with water to a total volume of 6 mL.

2.5. Synthesis of $Fe_3O_4/SiO_2/AuNPs/Ab_1$ nanocomposites

Gold nanoparticles (AuNPs) were obtained according to the Frens method [27]. In brief, 1 mL of 1% $HAuCl_4$ and 100 mL ultra-pure water were mixed in a 250 mL flask. 5 mL of 1% sodium citrate solution was added quickly to the mixture after boiling, and the boiling of the mixture was kept for another 15 min. As a result, the color of the solution turned to wine red, implying the diameter of gold nanoparticles was between 5 nm and 20 nm. And colloidal gold solution was stored at 4 °C to prevent agglomerate.

Fe_3O_4/SiO_2/AuNPs nanocomposites (AuMNPs) were prepared by automatic adsorption between AuNPs and Fe_3O_4/SiO_2–SH nanoparticles[28]. Fig. 1B shows the procedure of preparation. 20 μL Fe_3O_4/SiO_2–SH suspension (0.15 $mg \cdot mL^{-1}$) dropped into 4 mL centrifuge tube with 1.5 mL colloidal gold solution, and incubated for 12-24 h at room temperature. In order to make each Fe_3O_4/SiO_2–SH homogeneously combine with AuNPs which can improve the stability of experiment results, the centrifuge tube was shook slowly every four hours. AuMNPs were separated by a magnet, and rinsed three times with PBS, then resuspended with 1 mL PBS.

Ab_1/AuMNPs was obtained as follows: 40 μL Anti-*S. pullorum* and *S. gallinarum* (100 $\mu g \cdot mL^{-1}$) and 1 mL AuMNPs suspension was mixed and stirred at 4 °C for 12 h. Ab_1/AuMNPs was blocked by 1 mL 0.2% BSA at 4 °C for 1 h, then rinsed three times with PBS, dispersed in 1 mL PBS, and stored at 4 °C for use.

2.6. Preparation of electrochemical immunoassay method and measurements

The AuNPs (25 nm) deposited 4-SPCE was prepared according to previous report [29]. The electrochemical reduction was performed with 4-SPCE by CV in a dispersion containing 25 $mg \cdot L^{-1}$ $HAuCl_4$ with a magnetic stirring and N_2 bubbling. The scan potential was performed between -1.5 and 0.5 V at a scan rate of 25 $mV \cdot s^{-1}$. Then the

electrode was rinsed with double-distilled water and dried with blowing N_2 at room temperature (25 ± 2 °C).

The preparation of the immunoassay method and mechanism of rapid detection of *S. pullorum* and *S. gallinarum* were displayed in Fig. 1C and D. The *S. pullorum* and *S. gallinarum* was detected according to the following procedure: *S. pullorum* and *S. gallinarum* was captured by 20 μL Ab_1/AuMNPs in 1 mL sample solution, then separated with a magnet and rinsed carefully three times. 20 μL HRP-Ab_2 (7.8 $\mu g \cdot mL^{-1}$) was dropped into the above isolates and incubated for 30 min, rinsed carefully three times and resuspended with 20 μL PBS. Then 5 μL HRP-Ab_2/Ag/Ab_1/AuMNPs was dropped on the AuNPs/4-SPCE and adsorbed by magnet. 300 μL Hac-NaAc (pH=6.5, 0.1 $mol \cdot L^{-1}$) containing 1.0 $mmol \cdot L^{-1}$ Thi and 0.7 $mmol \cdot L^{-1}$ H_2O_2 was dropped on the above modified electrode. CV was conducted with a CHI 1030 at a scan rate of 25 $mV \cdot s^{-1}$ between -0.6 V and -0.1 V. The detection of *S. pullorum* and *S. gallinarum* was performed by measuring the reduction peak current change (ΔIpc) of CV before and after the immune reaction. Before the immunoreaction, the current response was recorded as I_1. Due to the accelerated decomposition of hydrogen peroxide by HRP, the current response of the immunoassay method increased after the immunoreactions and was recorded as I_2. Therefore, changes of immunesensor current value (ΔIpc) was expressed as $\Delta Ipc = I_2 - I_1$. All experimental solutions were desecrated by nitrogen for at least 10 min before measurement, and a nitrogen atmosphere was kept during the whole electrochemical measurements. Three successive CV scans were performed for each measurement, the last cycle was recorded.

3. Results and discussion

3.1. Comparison of 4-SPCE and SPCE

The reproducibility of 4-SPCE and SPCE were compared by using CV. As shown in Fig. S.1, The RSD of 4-SPCE is 5.05% (n=6) and RSD of SPCE is 8.54%, indicating that 4-SPCE owns a better reproducibility than SPCE. The reason maybe that there are four working electrodes on one 4-SPCE, meanwhile the four working electrodes of 4-SPCE use the same auxiliary electrode and reference electrode, which avoid effects of the external factors change. And it can simultaneously examine four samples under the same

test conditions. Conversely, different SPCEs can't be operated at the exact same condition and don't have the completely consistent auxiliary electrode and reference electrode, that is to say external factors can't be exactly the same. And for the high sensitivity of sensor slight change will affect the reproducibility. Therefore, 4-SPCE is more stable and owns a better reproducibility, it was chosen to use in this work.

3.2. Optimizate the dosage of Fe_3O_4/SiO_2–SH

During the preparation of AuMNPs, two kinds of nanoparticles, Fe_3O_4/SiO_2–SH and AuNPs, were linked by coupling agent to form a strong chemical bond. The composites are stable by employing this method because the magnetic particles were coated with a large amount of free thiol group (-SH) on the SiO_2 shell with 3-Mercaptopropyltriethoxysilane (MPTES) which has been found to exhibit a strong binding force to AuNPs. In order to make each AuNMPs combine with sufficient anti-*S. pullorum* and *S. gallinarum*, getting the best effect of enrichment for *S. pullorum* and *S. gallinarum*. An experiment of different dosage of Fe_3O_4/SiO_2–SH from 0.075 to 0.375 $mg \cdot mL^{-1}$ mixed with 1.5 mL AuNPs was carried out. Meanwhile Fe_3O_4/SiO_2 and PBS were used as control groups (tube 1 and tube 2). As Fig. 2 shows that there is no obviously difference between tube 1 (Fe_3O_4/SiO_2) and tube 2 (PBS), suggesting AuNPs cannot react with Fe_3O_4/SiO_2. The solutions from tube 6 to 7 become transparent, and the absorbance almost no longer changes, indicating all AuNPs have linked with Fe_3O_4/SiO_2–SH. Therefore, 0.225 $mg \cdot mL^{-1}$ Fe_3O_4/SiO_2–SH (tube 5) was selected as the optimal condition.

3.3. Characterization of Ab_1/AuMNPs

Agglutination test was utilized to verify whether anti- *S. pullorum* and *S. Gallinarum* had successfully linked with AuMNPs, 10 μL Ab_1/AuMNPs and 10 μL *S. pullorum* and *S. Gallinarum* (10^9 $CFU \cdot mL^{-1}$) were dropped on glass slides, the results were recorded after reacting for 1 min. Meanwhile *E. Coli* and PBS were used as control groups. Ab_1/AuMNPs uniformly disperse in the solution of *E. Coli* and PBS as shown in Fig. 3B and Fig. 3C, but agglomerate appears when *S. pullorum* and *S. Gallinarum* is added (Fig. 3A), indicating antibody has successfully linked with AuMNPs and Ab_1/AuMNPs have a

good dispersibility.

3.4. Characterization of AuMNPs nanocomposite and AuNPs layer

The morphology of bare 4-SPCE, AuNPs/4-SPCE and AuMNPs was characterized using SEM. As shown in Fig. 4A, bare 4-SPCE is covered by smooth and uniform nanoparticles with diameter of about 50 nm. Fig. 4B shows AuNPs with diameter of about 25 nm are successfully electrodeposited on the working electrode. AuNPs were introduced into the fabrication of the immunoassay method to enhance the electrochemical signals and ensure the sensitivity of the test results. Fig. 4C shows AuNPs successfully loaded on the surface of Fe_3O_4/SiO_2–SH, the size of which is about 250 nm. Fig. 4D displays the UV–Vis spectra of Fe_3O_4/SiO_2–SH, AuNPs and AuMNPs. AuNPs show the absorption peak at about 520 nm (curve b). And there is no obvious absorption peak from 400 to 700 nm (curve a). But an absorption peak appears at about 560 nm (curve c) after AuNPs immobilizing with Fe_3O_4/SiO_2–SH, suggesting that AuNPs are successfully loaded on Fe_3O_4/SiO_2–SH.

3.5. Electrochemical characteristics of the stepwise modified electrodes

To investigate the effect of each component on the electrode, the redox behavior of a reversible redox couple was recorded by CV after each modified step. Curves were recorded in 1.0 mM Thi. Fig. 5 displays a pair of reversible redox peaks of Thi at the bare 4-SPCE (curve A). After electrodepositing in $HAuCl_4$, the peak currents of the redox peaks of 4-SPCE (curve B) significantly increases. But the redox current (curve C) gradually decreases when Ab_1/AuMNPs are dropped on the AuNPs/4-SPCE. Compared with curve c, the redox current of Ag/Ab_1/AuMNPs (curve D) significantly decreases. This result indicates that *S. pullorum* and *S. gallinarum* is firmly captured to Ab_1/AuMNPs through the specific binding affinity between antigen and antibody. And the formed electronic barriers hindered electron transfer toward the electrode surface, result in the decreasing of peak current. After the addition of HRP-anti- *S. pullorum* and *S. gallinarum*, the reduction peak current value (curve E) greatly increases, implying the enzyme-labeled antibody is bound onto Ag/Ab_1/AuMNPs through the immune interaction, and the HRP catalyzes reduction of H_2O_2 with the assistance of an electron mediator,

which promotes electron transfer between the enzyme and the electrode. The immunoassay method response is based on the following redox process:

$$HRP\ (red) + H_2O_2 \rightarrow HRP\ (ox) + H_2O \quad (1)$$

$$Thionine\ (red) + HRP\ (ox) \rightarrow HRP\ (red) + Thionine\ (ox) \quad (2)$$

$$Thionine\ (ox) + 2e^- \rightarrow Thionine\ (red) \quad (3)$$

3.6. EIS characterization

Electrochemical impedance spectroscopy (EIS) was employed to monitor the interface properties of the carbon electrode surface during stepwise modifications [30, 31]. Different stages of the modified electrode were characterized in the test base solution containing 0.1 mM KCl and 5.0 mM $[Fe(CN_6)]^{3-/4-}$. As seen from Fig. S.2, the R_{et} of AuNPs/4-SPCE (curve B) significantly decreases compared with bare electrode (curve A), due to the gold nanoparticles not only have a large specific surface area, but also own a highly efficient electron transport property and electro-catalytic activity. The gold nanoparticles greatly reduced the resistance and accelerated the rate of electron transfer. When Ab_1/AuMNPs was dropped onto the AuNPs/4-SPCE, a larger semicircle (curve C) was observed, indicating the R_{et} greatly increased. After *S. pullorum* and *S. gallinarum* was incubated with Ab_1/AuMNPs and dropped onto the AuNPs/4-SPCE, the semicircle (curve D) became larger, the antigen and Ab_1/AuMNPs formed a barrier which impeded electron transfer. Similar situations occurred when HRP-Ab_2/Ag/Ab_1/AuMNPs was dropped onto the AuNPs/4-SPCE (curve E). This result suggested that every step of the modification were successful.

3.7. Optimization of the experimental conditions

To achieve the best performance, experimental conditions were optimized. The results are given in Fig. S.3. the following experimental conditions were found to give best results: (A) A concentration of H_2O_2 is 0.7 $mmol \cdot L^{-1}$; (B) A sample pH value of 6.5; (C) Incubation time between anti-*S. pullorum* and *S. gallinarum* and *S. pullorum* and *S. gallinarum* is 30 min; (D) Incubation temperature between anti-*S. pullorum* and *S. gallinarum* and *S. pullorum* and *S. gallinarum* is 32 °C; (E) Incubation time between *S. pullorum* and *S. gallinarum* and HRP-anti-*S. pullorum* and *S. gallinarum* is 30 min; (F)

Incubation temperature between *S. pullorum* and *S. gallinarum* and HRP-anti-*S. pullorum* and *S. gallinarum* is 30 °C.

3.8. Calibration curve of the immunoassay method

Under these optimal conditions different concentrations of *S. pullorum* and *S. gallinarum* (from 10^0 to 10^8 CFU·mL^{-1}) were detected. As Fig. 6A shows, with increasing concentration of *S. pullorum* and *S. gallinarum*, the amount of HRP-labeled antibody reacted with the immobilized *S. pullorum* and *S. gallinarum* increased, therefore, the ΔIpc increased. The plot of ΔIpc versus the logarithm of *S. pullorum* and *S. gallinarum* concentration shows a linear relationship in the concentration range from 10^2 to 10^6 CFU·mL^{-1}, and the linear regression equations is ΔIpc (μA) = 0.3418x + 0.4698, R^2 = 0.9953. The limit of detection (*LOD*), which was defined as three times the standard deviation of the blank sample measurements, was estimated to be 3.2 × 10^1 CFU·mL^{-1} (Fig. 6A inset). As Table 1 shows, this sensor performance shows a potential in reducing detection limit and more convenient as compared to other systems for bacteria detection. In many of past reports, sample solution was dropped on the surface of SPCE to detect pathogenic bacteria, and the volume of sample solution was always less than 30 μL[32], resulting in the detection limit can never be lower than 10^2 CFU·mL^{-1}. Because 30 μL 10^2 CFU·mL^{-1} pathogen suspension only contains three bacteria in theory. And this problem is well solved in this developed method.

3.9. Specificity, reproducibility and stability of the immunoassay method

The specificity and interference are very important for immunoassay method to distinguish the target bacteria from other foodborne pathogens in samples. To prove the specificity of the constructed immunoassay method, experiments were conducted using *E. sakazakii*, *E. coli*, *B. subtilis*, *B. cereus*, *B. stearothermophilus* and *S. pullorum* and *S. gallinarum*, and all of the bacteria solution concentrations were 10^6 CFU·mL^{-1}, PBS was used as blank control. And *E. sakazakii* and *B. subtilis* were mixed with *S. pullorum* and *S. gallinarum*, respectively. The results are displayed in Fig. 6B, the current increase induced by *S. pullorum* and *S. gallinarum* (ΔIpc = 2.3273 ± 0.1393 μA) is significantly larger than the current increase induced by other bacteria and PBS, the largest

mean value and standard deviation was 0.7823 ± 0.0241 μA, suggesting the immunoassay method had a high specificity for *S. pullorum* and *S. gallinarum*. And the ΔIpc caused by mixed bacteria solution contaminating *E. sakazakii* and *B. subtilis* just had inconspicuous change, indicating the immunoassay method had a high anti-interference ability. The specificity of immunoassay method was attributed to the highly specific antigen-antibody immunoreactions.

A long-term storage stability of the prepared immunoassay method was also measured. Ab_1/AuMNPs were stored at 4 °C when they were not in use, and intermittently measured every five days with three modified electrodes, they retained 93.95% of their initial signal after a storage period of 30 days. Therefore, the modified sensors towards *S. pullorum* and *S. gallinarum* owned good stability.

The reproducibility of the immunoassay method was investigated by independently monitoring the reduction peak current values of five modified electrodes under same experimental conditions. And the relative standard deviation (RSD) obtained at the concentration of 10^6 CFU·mL^{-1} was 5.33%. Therefore, the modified sensors towards *S. pullorum* and *S. gallinarum* owned satisfying reproducibility. Different modified electrodes for determination of *salmonella* were compared, and the dates are displayed in Table 1. The performance of this sensor performance shows a potential in reducing detection limit and more stable as compared to others for bacteria detection.

3.10. Detection of *S. pullorum* and *S. gallinarum* in Real Samples

In order to better verify the application of the newly developed immunoassay method in practical sample detection, a series of food samples: chickens were bought from market, the real sample were tested for *S. pullorum* and *S. gallinarum* by the China national food safety standard (GB/T 17999.8-2008) for the detection of *S. pullorum* and *S. gallinarum*. And we found that all of them were not affected by *S. pullorum* and *S. gallinarum*. A blind method was used and performed by two teams. The detail steps were as follows: one team randomly added a proper dose of *S. pullorum* and *S. gallinarum* into the negative samples and mixed with other samples. Another team used the newly sensors and the China national food safety standard (GB/T 17999.8-2008) in the assays. The two teams were not allowed to interact during the whole process. The results were showed in

Table 2, the number in Table 2 is the number of true positive or negative results detected by the corresponding methods. Accuracy is defined as the compliance between results got by the developed method and the reference standard method for identical samples. By comparing the results of electrochemical immunoassay method with standard culture method, accuracy was 93.3%，the true positive rate was 94.2% and true negative rate was 87.5%. We find this sensor reveals a good agreement with standard method, indicating that there was an acceptable accuracy and reliability of the immunoassay method. This result revealed that the immunoassay method held great promise as a reliable tool for the detection of *S. pullorum* and *S. gallinarumin* real samples.

4. Conclusions

A rapid and high-sensitive electrochemical immunoassay method based on Fe_3O_4/SiO_2/AuNPs and 4-SPCE has been successfully constructed for *S. pullorum* and *S. gallinarumin* detection in this work. AuNPs were used as bridging materials between biomolecules and Fe_3O_4/SiO_2–SH; AuNPs can easily immobilize the antibody onto the Fe_3O_4/SiO_2/AuNPs and retain high bioactivity of the adsorbed biomolecules. Electrodeposited AuNPs on the working electrodes increased the current signal of this method. This biosensor showed wide linear range, low detection limit and high specificity. It can also be used for detection of *S. pullorum* and *S. gallinarumin* in real samples. Importantly, this assay strategy remarkably improved the detect limit of immunoassay method, provided a sensing platform for detection of *S. pullorum* and *S. gallinarumin*, and the whole analytical process was shortened and simplified by using AuMNPs and 4-SPCE. This immunoassay method can be used to develop other biosensors for pathogenic bacteria and would become a useful tool for pathogenic microorganism screening in clinical diagnostics, food safety and environmental monitoring.

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Caption

Fig. 1. Schematic diagram of the modification process of electrochemical immunoassay method and measure mechanism: (A) The synthesis process of Fe_3O_4/SiO_2–SH nanoparticles; (B) The synthesis process of Ab_1/AuMNPs; (C) The process of *S. pullorum* and *S. gallinmaru* being captured from samples by Ab_1/AuMNPs and the formation of HRP-Ab_2/Ag/Ab_1/AuMNPs; (D) HRP-Ab_2/Ag/Ab_1/AuMNPs dropped on the AuNPs/4-SPCE, the principle of electrochemical detection.

Fig. 2. Optimization of the Fe_3O_4/SiO_2–SH dosage: (1) 0.150 $mg \cdot mL^{-1}$ Fe_3O_4/SiO_2; (2) PBS; (3) 0.075 $mg \cdot mL^{-1}$ Fe_3O_4/SiO_2–SH; (4) 0.150 $mg \cdot mL^{-1}$ Fe_3O_4/SiO_2–SH; (5) 0.225 $mg \cdot mL^{-1}$ Fe_3O_4/SiO_2–SH; (6) 0.300 $mg \cdot mL^{-1}$ Fe_3O_4/SiO_2–SH; (7) 0.375 $mg \cdot mL^{-1}$ Fe_3O_4/SiO_2–SH was dropped into 1.5 mL colloidal gold solution.

Fig. 3. The agglutination test results of (A) 10 μL *S. pullorum* and *S. Gallinmaru*, (B) 10 μL *E. Coli*, (C) 10 μL PBS was respectively mixed with 10 μL IMB.

Fig. 4. FE-SEM images of (A) bare SPCE; (B) AuNPs/SPCE; (C) TEM images of AuMNPs; (D) UV-vis absorption spectrum of Fe_3O_4/SiO_2 (a), AuNPs (b) and AuMNPs (c).

Fig. 5. Current curve of different modified electrode (A) Bare 4-SPCE, (B) AuNPs/4-SPCE, (C) Anti-*S. pullorum* and *S. gallinarum*/AuMNPs/4-SPCE, (D) *S. pullorum* and *S. gallinarum*/Anti-*S. pullorum* and *S. gallinarum*/AuMNPs/4-SPCE, (E) HRP-anti-*S. pullorum* and *S. gallinarum*/*S. pullorum* and *S. gallinarum*/Anti-*S. pullorum* and *S. gallinarum*/AuMNPs/4-SPCE

Fig. 6. The performances of immunoassay method: (A) The ΔIpc of different concentrations of the logarithm *S. pullorum* and *S. gallinarum*(Inset: Linear relation between the reduction peak current change (ΔIpc) and of *S. pullorum* and *S. gallinarum* concentration.); The specificity of immunoassay method for *S. pullorum* and *S. gallinarum*: (B) The modified electrodes incubated with *S. pullorum* and *S. gallinarum*, *E.sakazakii, B. cereus*, *B. subtilis*, *E. coli*, and *B. stearothermophilus*, PBS (0.01 M, pH 7.4) under the best reaction conditions, and mixed bacteria liquid A and B (*S. pullorum* and *S. gallinarum* mixed with *E.sakazakii* and *B. subtilis*), respectively.

Table 1 Comparison of recently reported methods for determination of *salmonella.*

Table 2 Accuracy experimental results of a group modified electrodes (n=60).

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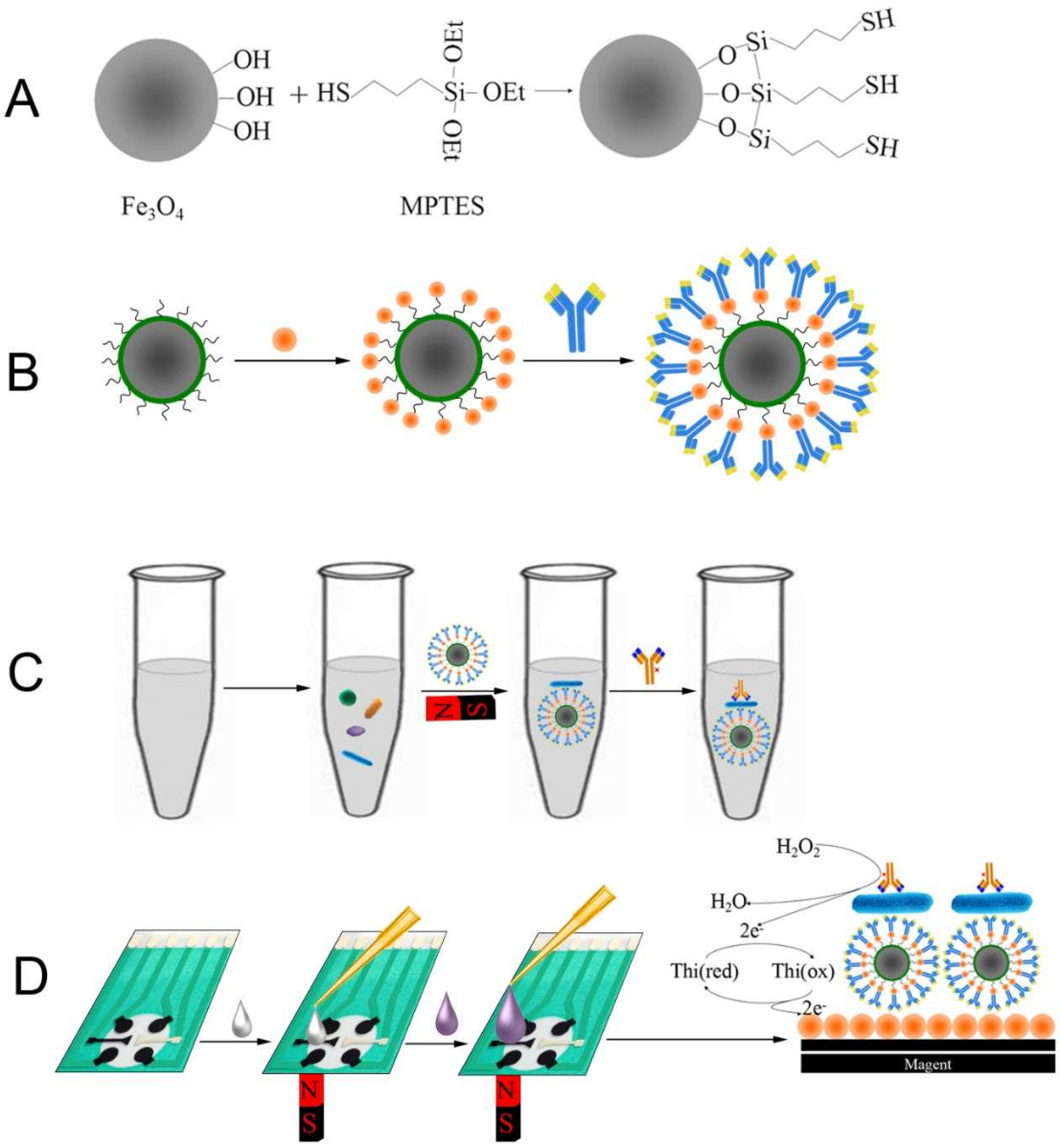

555

556 : Fe_3O_4; : MPTES; ; AuNPs; : anti- *S. pullorum* and *S. gallinmaru*; ,

557 , : other Bacteria; : *S. pullorum* and *S. gallinmaru*; Magnet: Magnet; :

558 $Ag/Ab_1/AuMNPs$; : Thionine solution.

559

560 Fig .1

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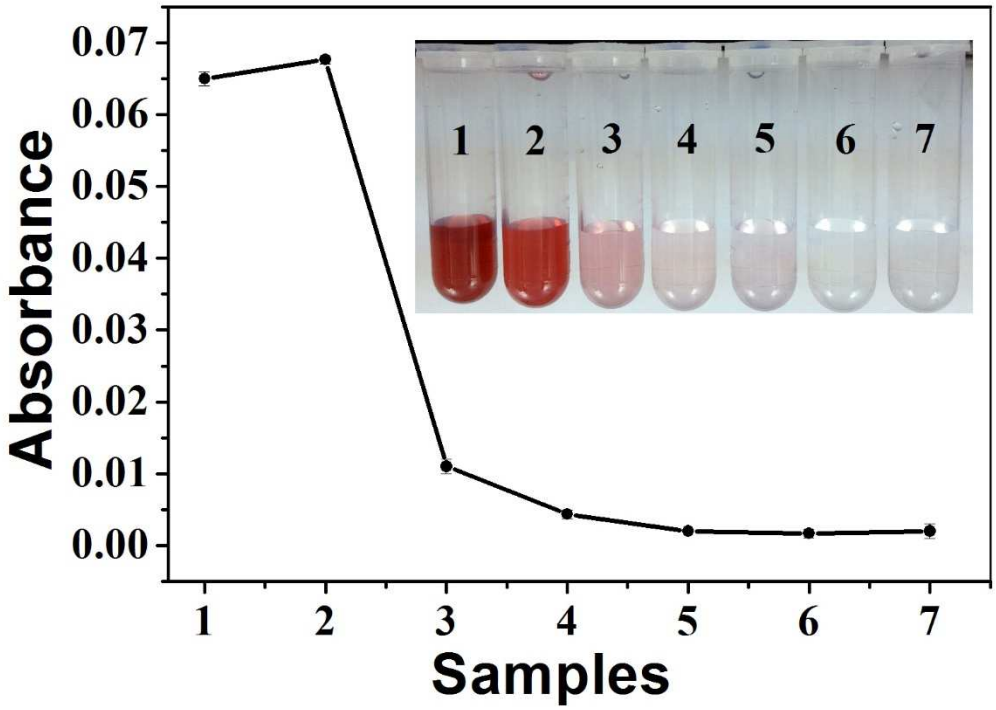

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563 Fig. 2

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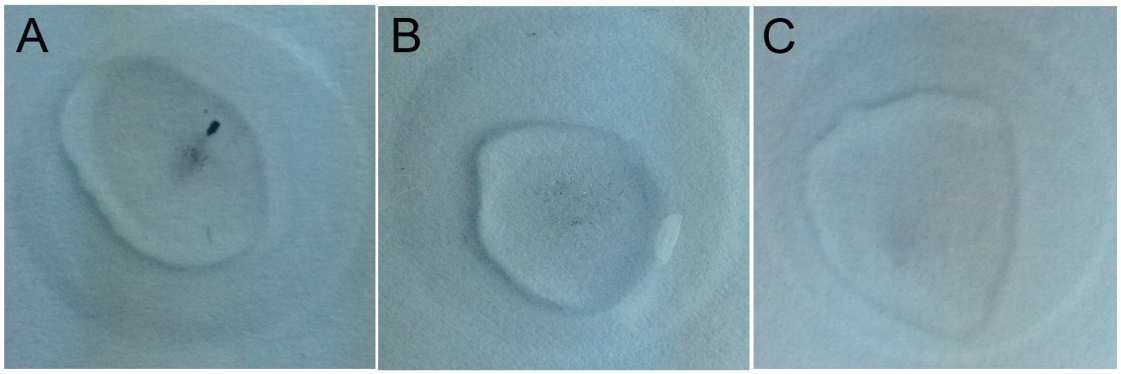

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568 Fig. 3
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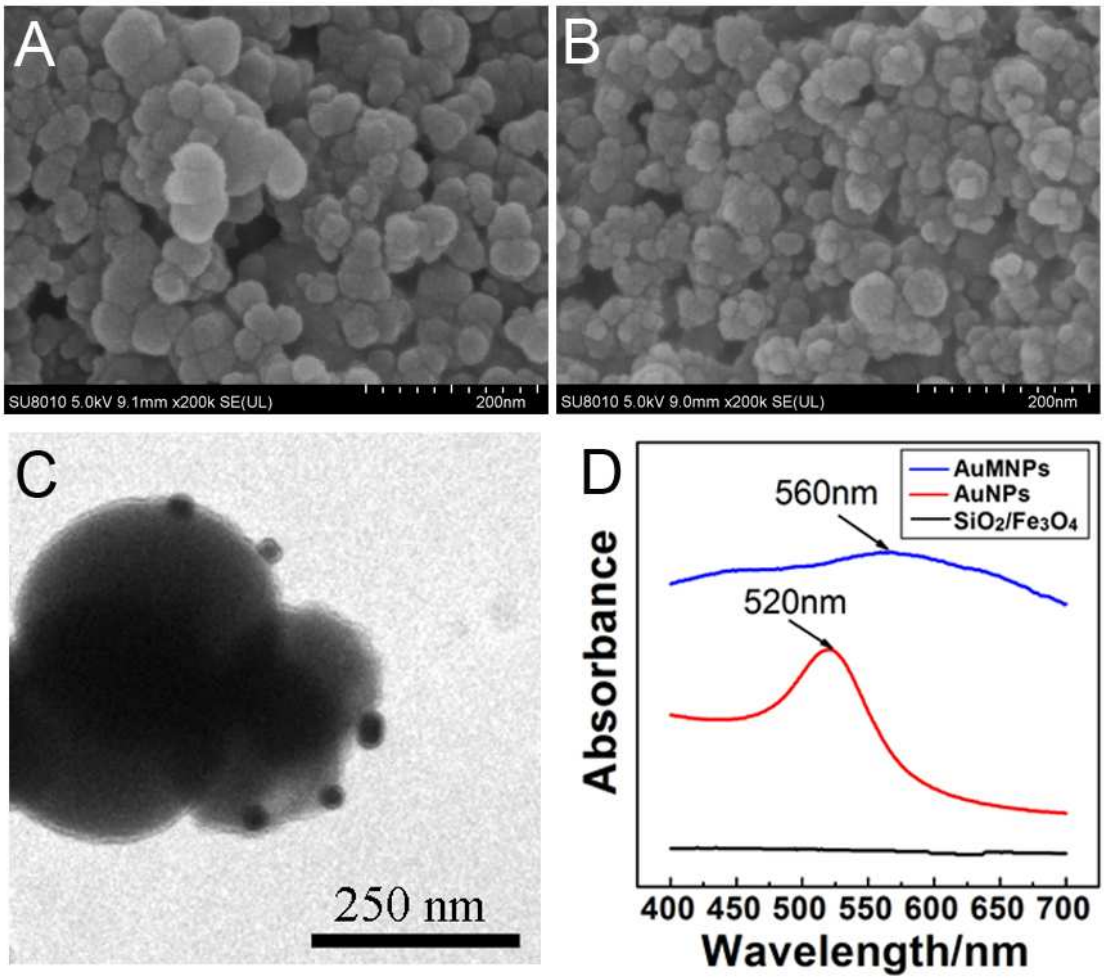

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573 Fig. 4

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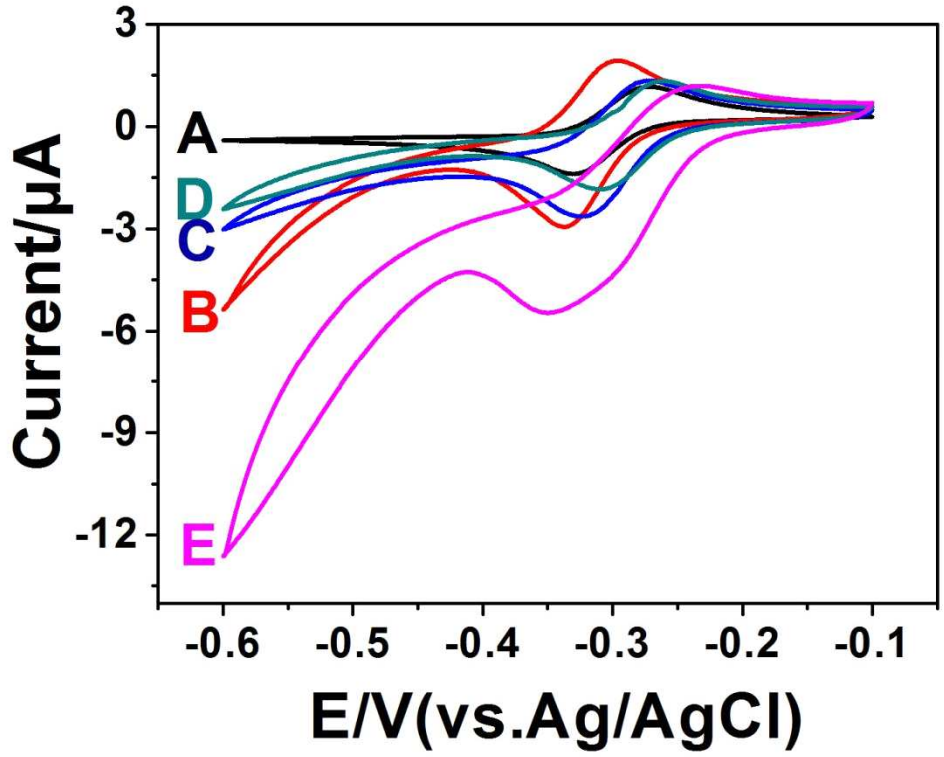

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577 Fig. 5

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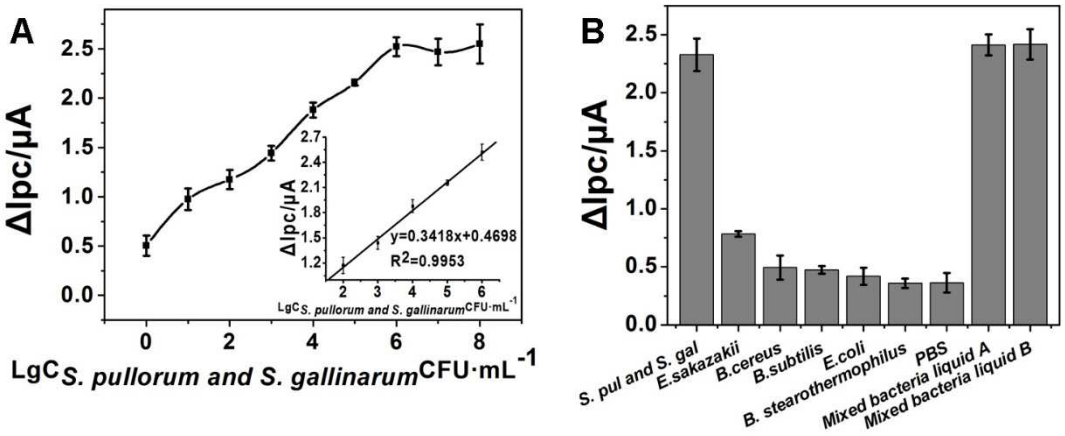

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582 Fig. 6

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Material/method used	Analytical ranges ($CFU \cdot mL^{-1}$)	LODs ($CFU \cdot mL^{-1}$)	Reproducibility	Interferences
OCMCS/Fe_3O_4/GCE (EIS)	$10^3 – 10^7$	1.0×10^3	6.3%	33
MBs-pSAb/S/sSAb-AuNPs/SPCE(DPV)	$10^3 – 10^6$	1.43×10^2	2.4%	1
MSNTs/IDAM(EIS)	$10^3 – 10^7$	10^3	—	34
MNPs/QDs(—)	$2.5 \times 10^3 – 1.95 \times 10^8$	5.0×10^2	—	35
AuNPs/PAMAM/MWCNT/Chi/GCE (EIS)	10^3–10^6	5.0×10^2	3.8%	36
Fe_3O_4/SiO_2/AuNPs/AuNPs/4-SPCE(CV)	$10^2 – 10^6$	3.2×10^1	5.3%	This work

586

587 *AuNPs* gold nanoparticles, *PAMAM* Poly(amidoamine), *MWCNT* Multi wall carbon
588 nanotubes, *Chi* Chitosan, *GCE* glassy carbon electrodes, *OCMCS*
589 O-Carboxymethylchitosan surface, *MBs-pSAb* magnetic beads modified with primary
590 antibodies, *S Salmonella*, *sSAb-AuNPs* AuNPs modified with secondary antibodies,
591 *MSNTs* magnetic silica nanotubes, *IDAM* interdigitated array microelectrodes, *MNPs*
592 magnetic nanoparticles, *QDs* quantum dots.

593 Table 1

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595

		Immunoassay method		
		Positive	Negative	Total
GB	Positive	49	3	52
	Negative	1	7	8
	Total	50	10	60

596 Table 2