

A Novel Colorimetric Detection of *S. typhimurium* Based on Fe₃O₄ Magnetic Nanoparticles and Gold Nanoparticles

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Abstract *Salmonella typhimurium* is one of the most common causes of food-associated disease. A colorimetric nanosensor was developed to detect *S. typhimurium* which was based on the gold nanoparticles' (GNPs) color change effect. Fe₃O₄ magnetic nanoparticles (MNPs) and GNPs were synthesized separately. Two complementary sequences of the *S. typhimurium* target DNA were combined to Fe₃O₄ MNPs and GNPs to fabricate capture probes and signal probes. Fe₃O₄ MNPs could achieve the rapid separation and enrichment of target DNA. With the addition of *S. typhimurium* target DNA sequences, the sandwich-like structures were formed via the DNA hybridization recognition effect. The original good dispersion of GNPs was broken. GNPs showed different degrees of aggregation with different amount of *S. typhimurium* target DNA. The color changed from red, purple to blue which could be characterized by UV-Vis spectrophotometer. The absorbance spectra of GNPs red shifted constantly with the intensity ratio of A700/A521 changed regularly. There was a linear correlation between the ratio of A700/A521 and the amount of *S. typhimurium* target DNA. Thus, this was calculated for the basis of quantitative detection of *S. typhimurium*. This method is simple and rapid with high

sensitivity and specificity which could be used to detect actual samples.

Keywords Colorimetric detection · Gold nanoparticles · Magnetic nanoparticles · *S. typhimurium*

Introduction

Salmonella typhimurium is a common anaerobic gram-negative bacterium which is widely distributed in the environment. Disease caused by *S. typhimurium* is one of the most common foodborne bacterial diseases around the world. It is also the most widespread zoonotic disease which is of important significance in public health (Arnold et al. 2011; Cheung et al. 2007; Magliulo et al. 2007). The traditional culture method for *S. typhimurium* detection is tedious and time-consuming which includes the sequential steps of pre-enrichment, selective enrichment, and selective differential plating (Patel et al. 2006). It is difficult to meet the needs of the current detection of foodborne bacteria. Therefore, researchers all over the world do a lot of researches for the rapid detection of *S. typhimurium*. With the gradually improving detection technology, a variety of detection methods have been reported such as immunofluorescence detection, immunodiffusion method, enzyme-linked immunoassay (ELISA), and latex agglutination test (Falkenhorst et al. 2013; Jain et al. 2012; Ma et al. 2014; Cucchi et al. 2013; Imre et al. 2013; Luo et al. 2014; Altinok et al. 2008). However, each method has its own drawbacks which limit the application.

Nanoparticles have attracted a wide application in biomedical fields, chemical fields, life sciences, and new materials owing to their specific characteristics such as surface effect, small size effect, and quantum size effect. Among which, gold nanoparticles (GNPs) have been extensively concerned for

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their simple preparation and many excellent optical properties (Currivan et al. 2013). Chloroauric acid is usually used as the raw material with the addition of a reducing agent to prepare GNPs. Various reducing agents, capping agents, and experimental conditions will result in the formation of GNPs with different sizes, morphologies, and aggregation degrees (Dai et al. 2002; Ojea-Jime et al. 2010). When the distance of GNPs gets closer to each other, the absorbance spectra red shifted and the color of the solution will be changed (Storhoff et al. 2004). Besides, GNPs also exhibit good biological compatibility with the interaction of biomacromolecules (Afonso et al. 2013; Feng et al. 2013; Wang and Zhang 2013). In addition, GNPs and sulfhydryl group have a strong bond and force. Therefore, biomolecules with thiol labeling could be combined to the surface of GNPs through Au–S bond for the preparation of nanoprobe and application in the detection of biological systems (Liu et al. 2013; Preechakasedkit et al. 2012).

With the increasing development of nanomaterials, magnetic nanomaterials (MNPs) have also attracted extensive attention due to their unique and super paramagnetic properties (Mascaraque et al. 2013; Tan et al. 2014; Wang et al. 2013a; Shokrollahi et al. 2014; Yan et al. 2014). MNPs obtain magnetism and are vulnerable to external magnetic field control. And the small size obtains with larger specific surface area. MNPs have been widely used in biomedical and life science applications with surface modification of various bioactive molecules (such as protein, antigen, and DNA sequence) through different functional groups (such as amino, carboxyl, hydroxyl, and mercapto groups) (Samoilova et al. 2014; Liu et al. 2014; Ma et al. 2009). MNPs could be easily separated from the other substances under the action of magnetic field. After the removal of the external magnetic field, MNPs can be dispersed in solution again. The unique properties of MNPs have important applications in the separation and rapid detection of biological molecules. Combined with the advantages of GNPs and MNPs, there are several related methods for the detection of proteins, bacteria, and so on (Yin et al. 2014; Wang et al. 2013b; Weidemaier et al. 2015).

In this study, a new technique for the colorimetric detection of *S. typhimurium* is discussed. The specific oligonucleotide DNA sequence of *S. typhimurium* was used as the target. The two complementary sequences of target DNA were combined to Fe₃O₄ MNPs and GNPs, respectively, to fabricate capture probes and signal probes. Based on the DNA hybridization recognition technology, sandwich-like structures could be formed by target DNA, capture probes, and signal probes. Based on the optical distance-dependent properties of GNPs, the increased concentration of the target DNA could result in the changes of polymerization degree for GNPs. The absorbance spectra red shifted constantly. The color changed from red, purple to blue which could be observed by naked eyes. By means of the characterization of UV-Vis spectrophotometer, a

rapid and sensitive method was developed for the detection of gram-negative bacterium *S. typhimurium* specific DNA sequences. It is expected to be applied in other biofunctionalized DNA sequence detection.

Materials and Methods

Materials

Sodium acetate trihydrate (CH₃COONa), ferric chloride (FeCl₃), chloroauric acid tetrahydrate (HAuCl₄·4H₂O), trisodium citrate (Na₃C₆H₅O₇), sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na₂HPO₄·12H₂O), potassium biphosphate (KH₂PO₄), potassium carbonate (K₂CO₃), ethylene glycol (C₂H₆O₂), 1,6-hexanediamine (C₆H₁₆N₂), ethanol anhydrous (CH₃CH₂OH), glutaraldehyde (C₅H₈O₂), and sodium dodecyl sulfate (SDS, C₁₂H₂₅NaO₄S) were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Streptavidin was purchased from Sigma-Aldrich. *S. typhimurium* target DNA sequence and the complementary strand were synthesized by Shanghai Sangon Biological Science and Technology Company (Shanghai, China). The capture DNA sequence 1 was 5'-GAG CGT GCC TTA CCG ACG ATA-biotin-3'; the signal DNA sequence 2 was 5'-SH-ATA TCC ACG CAG GAA ATA ACA GGA CTT-3'; the target DNA sequence 3 was 5'-TAT CGT CGG TAA GGC ACG CTC AAT TGT CGT TAA AGT CCT GTT ATT TCC TGC GTG GAT AT-3'. The ultrapure water used in the experiments was prepared using a Millipore Direct-Q® 3 system (Merck Millipore, MA, USA) and had a resistivity of 18.2 MΩ cm.

Preparation of Streptavidin-Functionalized Fe₃O₄ MNPs

The amino-functionalized Fe₃O₄ MNPs were prepared according to the literature (Chen and Gao 2007) with some modifications. A solution of 6.5 g 1,6-hexanediamine, 2.0 g anhydrous sodium acetate, 1.0 g FeCl₃, and 30 mL glycol was stirred vigorously at 50 °C until the formation of a transparent solution. The mixed solution was transferred to teflon-lined autoclave and reacted at 195 °C for 6 h. The lower black liquid was rinsed with water and ethanol using magnetic force for separation and dried at 50 °C to get the amino-functionalized Fe₃O₄ MNPs and stored at 4 °C for further use.

Two milligram amino-functionalized Fe₃O₄ MNP powder was added to 2 mL glutaraldehyde (5%) and reacted at 37 °C for 2 h. The uncombined glutaraldehyde was removed by rinsing with PBS for at least five times using magnetic force. Then, the Fe₃O₄ MNPs was added with 2 mL PBS and 250 μL streptavidin (1 mg/mL) and reacted at 4 °C for 12 h. Similarly, the uncombined streptavidin was removed by rinsing with

PBS using magnetic force. The precipitation (streptavidin-functionalized Fe_3O_4 MNPs) was collected and used for further characterization.

Preparation of GNPs

GNPs were prepared with some modifications as described by Grabar et al. (1996). First, 4.2 mL $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$ (1%, w/w) and 95.8 mL ultrapure water were added to a flask with three necks. The mixture was heated to boil until 10 min under uniform magnetic stirring with oil bath. Then, 10 mL sodium citrate (1%, w/w) was rapidly injected and reacted for another 15 min. The obtained wine red solution was GNPs. The resulting GNPs were purified by three times of centrifugation (10,000 rpm, 25 min) and were redispersed in 40 mL of ultrapure water. The GNPs were stored at 4 °C for further use and characterization.

Preparation of Signal Probes and Capture Probes

Twenty microliter thiolated signal DNA 2 with gradient dilutions was added to 0.5 mL GNPs and reacted at 37 °C for 24 h. Five milligram SDS was added and shaken for a while. Then, a certain amount of NaCl was added and aged for another 24 h at 37 °C. The mixture was purified by three times of centrifugation (10,000 rpm, 10 min) to get thiolated signal DNA 2-modified GNPs (signal probes).

Twenty microliter biotin-labeled capture DNA 1 with gradient dilutions was dissolved in 2 mL PBS. Then, 1 mg of streptavidin-functionalized Fe_3O_4 MNPs was added and reacted at 37 °C for 12 h. The resulting solution was purified using magnetic force, and the precipitation was collected as biotin-labeled capture DNA 1-modified MNPs (capture probes).

Colorimetric Detection Procedure

Twenty microliter target DNA sequence 3 with gradient dilutions was dissolved in 2 mL PBS, and 1 mg of capture probes was added and reacted at 37 °C for 12 h. The mixture was purified using magnetic force, and the precipitation was collected. Then, 2 mL of signal probes was added and reacted at 37 °C for another 12 h. The resulting solution was characterized by UV-Vis spectrophotometer.

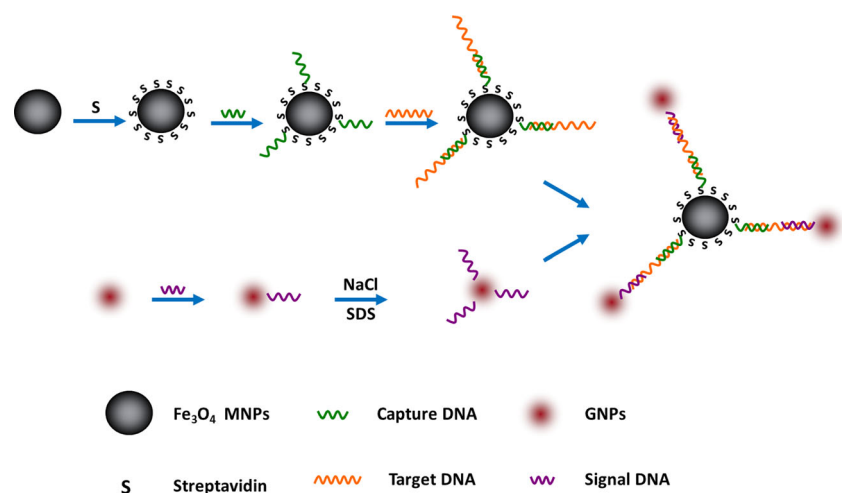
Activation, Cultivation, and Plate Counting of *S. typhimurium*

The *S. typhimurium* were inoculated into the LB liquid medium and cultivated under shaking at 37 °C for 12-h enrichment. The enriched bacterial was centrifuged at 5800 r/min for 10 min (25 °C), and the supernatant was discarded. The precipitate was washed with PBS (0.1 M, pH 7.4) three times and resuspended in PBS. The absorbance was measured at 600 nm. The collection was centrifuged and diluted repeatedly to optical density (OD) value 0.12. This preparation was used as the original *S. typhimurium* sample. Then, the original bacterium was diluted to eight concentrations along a gradient from 10^{-1} to 10^{-8} using physiological saline. One hundred microliter of 10^{-5} , 10^{-6} , and 10^{-7} *S. typhimurium* was coated on the solid LB agar plates. Each plate was coated with three parallel boards. After cultivation at 37 °C for 12 h, the colonies were counted for the calculation of the *S. typhimurium* sample (cfu/mL).

The Extraction of *S. typhimurium* Target DNA and Detection

The DNA extraction kit was used for the target DNA extraction of different dilutions of *S. typhimurium*. Then, after a water bath at 95 °C for 5 min and 0 °C for 5 min, the single-

Fig. 1 Schematic illustration for the colorimetric detection of *S. typhimurium* target DNA based on Fe_3O_4 MNPs and GNPs



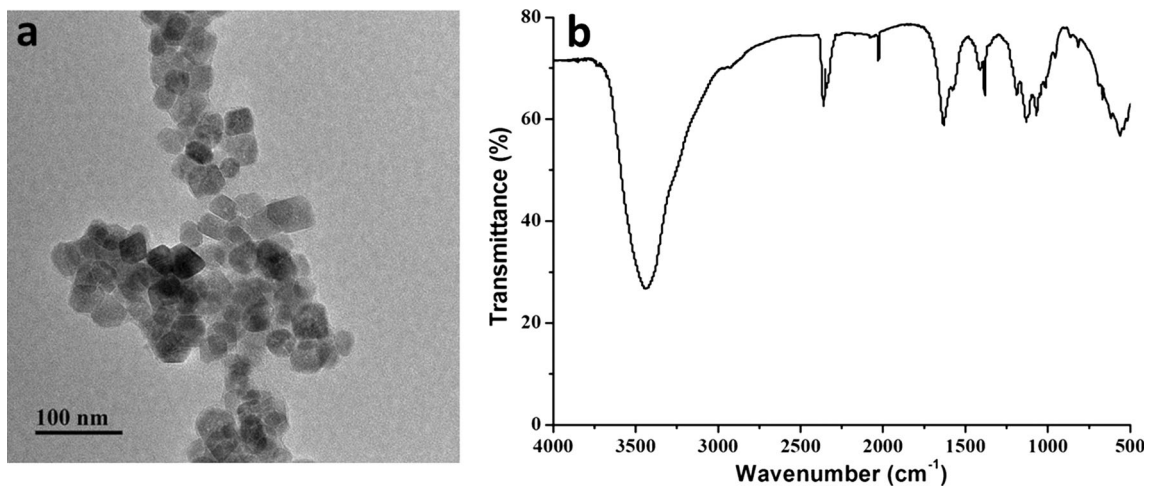


Fig. 2 TEM image (a) of amino-functionalized Fe₃O₄ MNPs and its IR spectra (b)

stranded DNA as the target DNA was produced. This was used to do the colorimetric detection, and the results were compared to the plate counting method.

Recovery Experiments for Milk Sample

In this experiment, the commercial milk was used as realistic samples for recovery experiments in the detection of *S. typhimurium*. Gradient dilutions of *S. typhimurium* were added to the milk sample. Then, the colorimetric detection method was conducted to calculate the detectable amount of *S. typhimurium*. The results were compared with the traditional plate counting method, and the recovery rate was calculated.

Results and Discussion

Principle for Colorimetric Detection

The colorimetric detection process is shown in Fig. 1. Biotinylated capture DNA 1 was modified at the surface of streptavidin-functionalized Fe₃O₄ MNPs. Thiolated signal DNA 2 was decorated to GNPs using covalent attraction between thiol group and GNPs. When the target DNA sequences were added, the unique sandwich structure was formed on account of the base complementation pairing rule. The color of GNPs has a characteristic of light-distance dependence. When the distances between GNPs get closer, the absorbance spectra red shift and the color of GNPs is changed. With the addition of gradient dilutions of *S. typhimurium* target DNA sequences, GNPs show different aggregation states which reflect in color changes. This could be characterized by UV-Vis spectrophotometer.

Preparation of Fe₃O₄ MNPs and Surface Modification

Figure 2 shows the TEM image of the amino-functionalized Fe₃O₄ MNPs and its infrared (IR) spectrum. The particle size was about 40 nm. The strong IR band at 3413.3 and 1627.6 cm⁻¹ from the amino-functionalized nanoparticles matched well with those from free 1,6-hexadiazine, indicating the existence of the free –NH₂ group on the amino-functionalized nanoparticles.

Streptavidin was used for the modification of Fe₃O₄ MNPs for further DNA sequence connection. Results are shown in Fig. 3. In order to get the optimal streptavidin concentration, different volumes (50, 100, 150, 200, 250, 300, 350, 400 μL) of streptavidin (1 mg/mL) were added to react with 2 mL Fe₃O₄ MNPs (1 mg/mL). By calculating the differences of absorbance intensity before and after modification, we can see that the differences almost achieve maximum when the

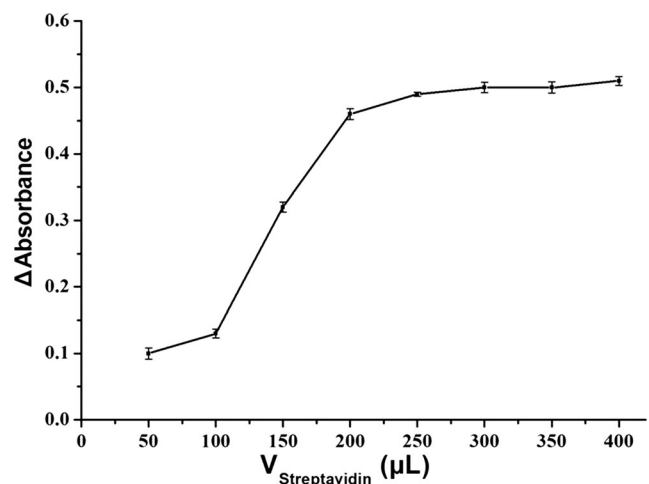


Fig. 3 Absorbance intensity changes of 1 mg/mL streptavidin before and after modification to Fe₃O₄ MNPs (λ = 280 nm)

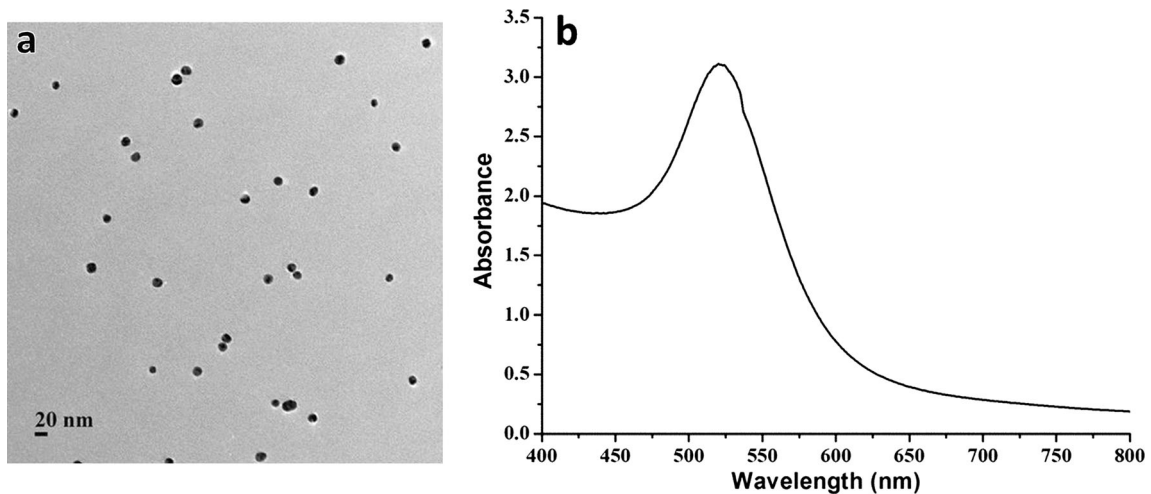


Fig. 4 **a** TEM image and **b** UV-visible absorption spectra of GNPs

volume was 250 μL . So, the optimal streptavidin (1 mg/mL) addition amount was 250 μL .

Preparation of GNPs

Figure 4 is the TEM image and UV-visible absorption spectrum of GNPs. It shows that the prepared GNPs have an absorption peak at approximately 521 nm and the size is ~ 15 nm.

Optimization of Experimental Conditions

In this study, thiol group-labeled signal DNA 2 was connected to the GNPs via the strong affinity of the thiol group and gold to construct signal probes. When the amount of DNA sequence increased, some basic group of oligonucleotide could adsorb to the surface of GNPs which might influence the formation of Au–S chemical bond. The addition of electrolyte

NaCl solution could do the desorption treatment of basic group. It is reported by Demers et al. (2000) that saline ions could weaken the electrostatic interaction of DNA sequences adsorbed on the surface of GNPs. Then, the DNA sequence may stand up on the GNP surface which increases the connection density. Therefore, the fixing amount of signal DNA 2 increases. However, the excessive addition of saline ions may result in the aggregation of GNPs accompanied by the change of color. This will influence the colorimetric detection result. So, the optimal concentration of NaCl is a key factor for this experiment.

When the concentration of NaCl increased from 50 to 200 mM, the absorption intensity decreased slightly and the color of GNPs remained wine red. While the concentration further increased to 250 mM, the color of GNPs changed to purple which could be observed by naked eyes. And the absorption intensity decreased obviously along with the red shift

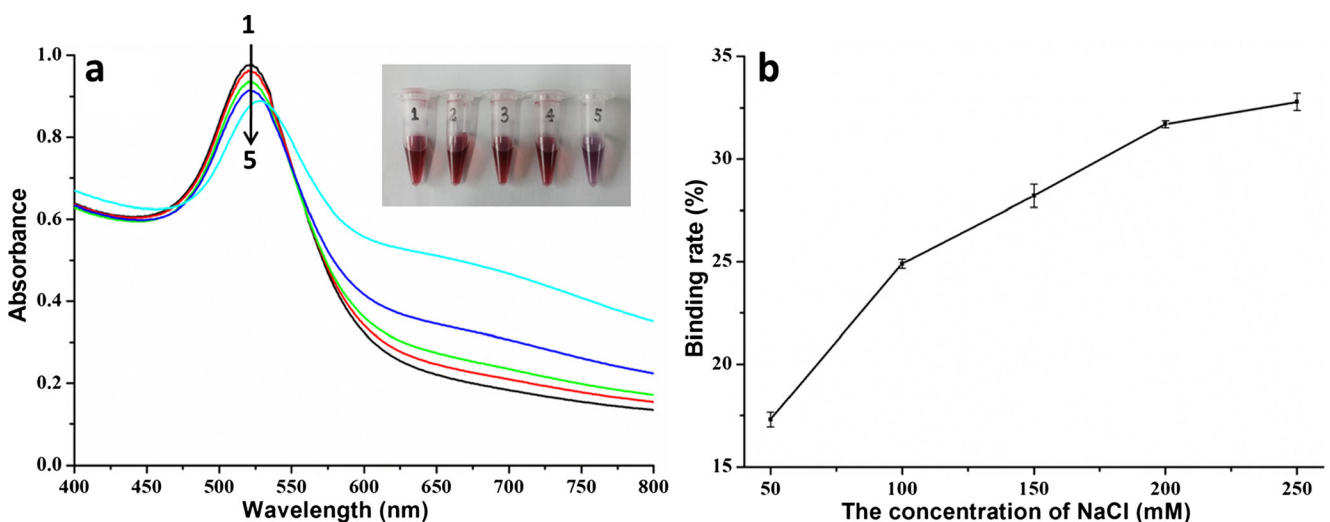


Fig. 5 **a** UV-visible absorption spectra of GNPs with the addition of different concentrations of NaCl solution (0.05, 0.10, 0.15, 0.20, 0.25 M (1–5)). The inset is the photograph of the

corresponding GNP solution. **b** The binding rate of signal DNA linked to GNPs with the treatment of different concentrations of NaCl

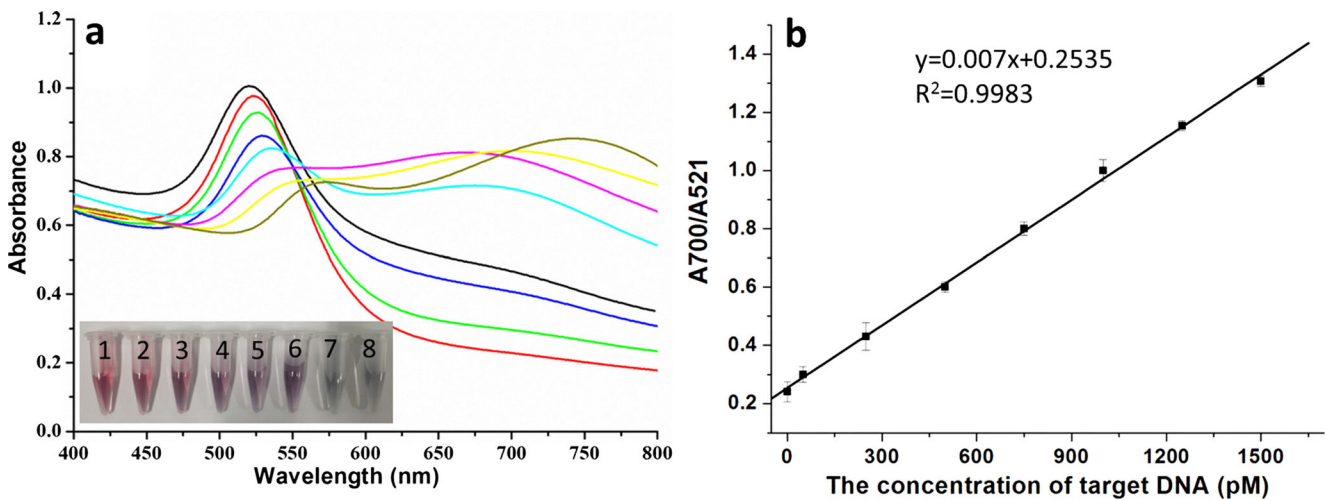


Fig. 6 **a** The colorimetric detection of *S. typhimurium* target DNA (1, 50, 250, 500, 750, 1000, 1250, 1500 pM (1–8)). **b** The linear relationship between different concentrations of *S. typhimurium* target DNA and the ratio of A700/A521

of the absorption peak position which is depicted in Fig. 5a. This indicated that the GNPs were aggregated. Figure 5b shows the binding rate of DNA sequences to GNPs according to the different amount addition of NaCl. The binding rate was calculated by the absorption intensity of DNA at 260 nm measured before and after modification to GNPs. With the amount of NaCl increased from 50 to 200 mM, the binding rate also increased significantly. After that, the binding rate changed slowly. Combined with the results of Fig. 5a, b, the optimal concentration of NaCl was 200 mM.

Analytical Performance

A series of concentrations of target DNA sequence 3 were detected under the optimal experimental conditions. Results are shown in Fig. 6. With the increased amount of DNA 3, the absorbance spectra red shifted constantly. The absorbance intensity at 521 nm gradually decreased, while the absorbance intensity at 700 nm increased (Fig. 6a). The ratio of

A700/A521 was calculated as the basis for quantitative analysis. As depicted in Fig. 6b, there was a good linear correlation between the ratio of A700/A521 and the amount of DNA 3 ranged from 1 to 1500 pM. The linear correlation equation obtained was $y = 0.007x + 0.2535$ ($R^2 = 0.9983$). And the statistical analysis revealed that the detection limit of target DNA 3 was 0.8 pM. 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).

Specificity

Some other DNA sequences were used to do the specificity detection. These included the single base mutation chain, five-base mutation chain, random control chain, *Staphylococcus aureus* target DNA sequences, *Vibrio parahaemolyticus* target DNA sequences, *Bacillus cereus* target DNA sequences, and *Shigella dysenteriae* target DNA sequences. The concentration of the DNA sequences was maintained at 1 nM.

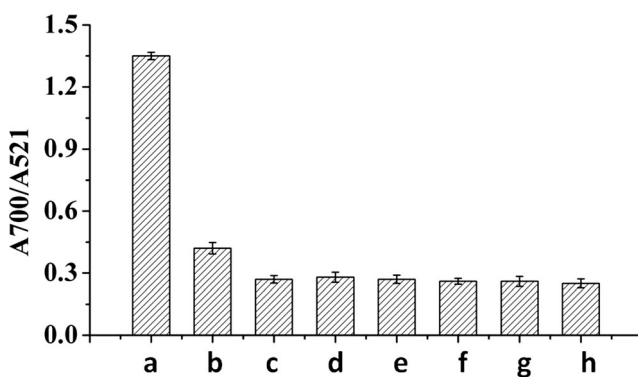


Fig. 7 Specificity result for the detection of **a** *S. typhimurium* target DNA, **b** single base mutation chain, **c** five-base mutation chain, **d** random control chain, **e** *S. aureus* target DNA sequences, **f** *V. parahaemolyticus* target DNA sequences, **g** *B. cereus* target DNA sequences, and **h** *S. dysenteriae* target DNA sequences

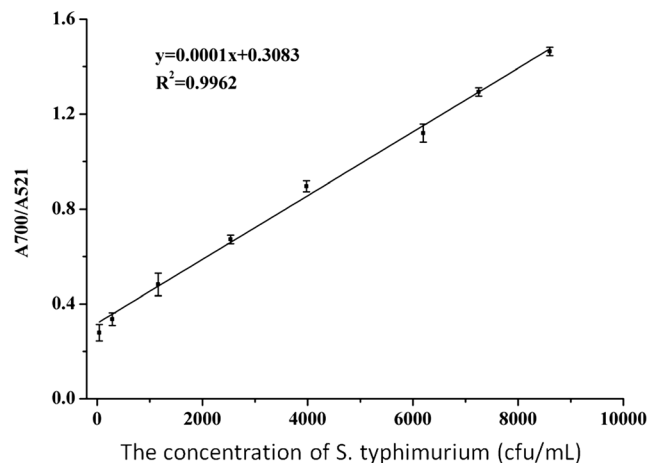


Fig. 8 The linear relationship between different concentrations of *S. typhimurium* and the ratio of A700/A521

Table 1 Comparison of the milk sample results obtained from the colorimetric detection and classical plate counting method

Milk sample	Plate counting (cfu/mL)	Colorimetric detection (cfu/mL)	Recovery ratio (%)
1	16 ± 1	ND	ND
2	54 ± 3	49 ± 3	90.7%
3	197 ± 5	183 ± 6	92.3%
4	642 ± 10	656 ± 9	102.2%
5	1318 ± 12	1301 ± 13	98.7%

All results were repeated three times and shown as average ± SD

Experimental results shown in Fig. 7 clearly showed that the ratio of A700/A521 for the other DNA sequences was much lower than that of the *S. typhimurium* target DNA sequence.

The Extraction and Detection of *S. typhimurium* Target DNA

In addition to the known sequence of *S. typhimurium* target DNA, the DNA extraction kit was used for the target DNA extraction of *S. typhimurium*. The obtained sequence was used as the target DNA to do the colorimetric detection. Results were compared with the plate counting method. As depicted in Fig. 8, the ratio of A700/A521 showed good linear correlation to the amount of *S. typhimurium* obtained by plate counting method ranging from 30 to 8600 cfu/mL. The linear equation is $y = 0.0001x + 0.3083$ ($R^2 = 0.9962$). And the statistical analysis revealed that the detection limit of *S. typhimurium* was 23 cfu/mL. 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).

Compared with the traditional culture method for *S. typhimurium*, this method is fast and free from pre-enrichment treatment. This method combined with the advantages of both MNPs and GNPs. The MNPs have the fast separation and enrichment properties. And the optical properties of signal GNPs will be gradually changed according to the aggregation extent. The color changes could be used as a qualitative and quantitative detection basis which is simple for the analysis of pathogenic bacterium.

Milk Sample Detection

The utility of the colorimetric detection for *S. typhimurium* was examined using milk sample 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 colorimetric detection method were similar to those obtained using the plate counting method. The recoveries were between 90.7 and 102.2%, indicating good accuracy of the proposed test for *S. typhimurium* detection. There is no complicated step for the pretreatment of milk sample. This colorimetric method is expected for the field test of *S. typhimurium*.

Conclusion

The colorimetric detection of *S. typhimurium* using Fe₃O₄ MNPs and GNPs was investigated. Fe₃O₄ MNPs were immobilized with capture DNA to fabricate capture probe. GNPs were modified with signal DNA to fabricate signal probe. The capture probe and signal probe could be connected to *S. typhimurium* target DNA via the DNA base complementary matching principle. MNPs possess good magnetic characteristics, which make it easier to separate the product from the mixture during the experiment. The quantitative detection of *S. typhimurium* target DNA was achieved by the characterization of UV-Vis spectra of GNPs. In addition, this developed method was successfully used to analyze milk samples, and there was no significant difference between a classical plate counting method and the developed method. So, it has the potential for wide use in the detection of other foodborne pathogenic bacteria in food samples.

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Compliance with Ethical Standards

Conflict of Interest Xiaoyuan Ma declares that she has no conflict of interest. Liangjing Song declares that she has no conflict of interest. Yu Xia declares that he has no conflict of interest. Caiyun Jiang declares that she has no conflict of interest. Zhouping Wang declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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