



A colorimetric assay method for *invA* gene of *Salmonella* using DNAzyme probe self-assembled gold nanoparticles as single tag

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ABSTRACT

A simple colorimetric sensing method was developed for detection of *invA* gene of *Salmonella* by combining DNAzyme probe self-assembled gold nanoparticles and polymerase chain reaction (PCR) technique. Amino-modified capture probe was covalently immobilized on the surface of DNA-BIND plate, and further hybridized with target sequence and DNAzyme probe self-assembled gold nanoparticles to form a sandwich-type hybridization structure. In the presence of hemin, the DNAzyme probe could form a G-quadruplex/hemin complex, which catalyzed the H₂O₂-mediated oxidation of 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB) to cause a dramatic color change. The *invA* gene was isolated from *Salmonella* and amplified using a polymerase chain reaction technique, and signal was further enhanced by DNAzyme probe self-assembled gold nanoparticles. Under optimal conditions, the absorbance value was linear with the logarithm of target sequence concentrations in the range of 0.5–50 nM, with a limit of detection of 0.44 nM for target sequence. In addition, the designed method allowed the quantitative *Salmonella* detection in water from 3×10^3 to 3×10^6 CFU mL⁻¹. This strategy exhibited good stability, sensitivity and simplicity and might be a potential tool for the practical *Salmonella* detection in clinical diagnosis, food safety and environmental monitoring.

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1. Introduction

Salmonella, belongs to enterobacteriaceae, is a gram-negative enteric bacilli. It is one of the most important pathogens in food-borne diseases [1]. The number of cases of *Salmonella* infection is increasing in recent years, which brings about great social and economic problems [2]. Therefore, a simple and sensitive method for the detection of *Salmonella* is urgent demanded.

Different methods have been developed for the detection of *Salmonella*, including traditional culture methods, enzyme-linked immunosorbent assay (ELISA) [3] and polymerase chain reaction (PCR) [4–6]. The traditional culture methods are laborious and time consuming, and take four to seven days to obtain a confirmed

positive result [7]. Another widely used method is ELISA based on the presence of specific antibodies of bacterium and flagellar antigen. The antibodies have the disadvantages such as low affinity, poor chemical stability, complicated preparation and high cost [8,9]. In addition, the new molecular-based detection technique such as PCR is extensively developed. It has distinct advantages in sensitivity, but its successful application has been limited by the lack of a sensitive and convenient method for direct detection of PCR products [10]. Recently, colorimetric sensors have attracted considerable attention for its intrinsic advantages such as low cost, simple, and even only utilizing naked eyes [11–14].

DNAzyme probe is single-stranded DNA oligonucleotide with rich guanine, which exhibits considerable potential as new biocatalyst formed by hemin and G-quadruplexes [15–17]. It is proven that G-quadruplex/hemin complex has peroxidase-like activity and can effectively catalyze the H₂O₂-mediated oxidation of 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB) to cause a dramatic color change [18–21]. Owing to the competitive advantages of DNAzymes probe over traditional protein enzymes

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including high chemical stability, ease synthesis and low price [22,23], nowadays DNAzyme probes have been widely applied in many fields, such as biomedicine and bioanalysis for the detection of DNA, metal ion, small molecules and proteins [24–26]. Meanwhile, to achieve highly sensitive signal, gold nanoparticles (AuNPs) have been widely applied in different biosensing systems for signal amplification [27–29]. The detection assay based on bioactive molecules, including protein enzymes, antibodies functionalized AuNPs has been reported for detection of DNA [30]. To date, there is no report for the simple and sensitive colorimetric assay of DNA by using DNAzyme probes on the AuNPs, which can overcome the inherent disadvantages of protein enzymes.

In this paper, the development of colorimetric sensing assay based on DNAzyme probe self-assembled gold nanoparticles is described for detection of *invA* gene of *Salmonella*. The *invA* gene, which is located on *Salmonella* pathogenicity island 1 (SPI-1), makes the specific detection of *Salmonella* possible because the encoded protein is essential for the invasion of epithelial cells by *Salmonella* [31,32]. The G-quadruplex/hemin complex (DNAzyme) exhibited highly catalytic activity, leading to dual signal amplification for detection of low-concentration target sequence. The designed method is simple, sensitive and specific, and could be a powerful tool for the practical *Salmonella* detection in clinical diagnosis, food safety and environmental monitoring.

2. Experimental

2.1. Reagents

3,3',5,5'-Tetramethylbenzidine dihydrochloride hydrate (TMB), hemin, bovine serum albumin (BSA) and salmon sperm DNA were obtained from Sigma–Aldrich (USA). $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$ was purchased from Sinopharm Chem. Ltd. (Shanghai, China). Premix Taq Version 2.0, DL500 DNA Marker and agarose were purchased from Takara (Dalian, China). All other reagents were of analytical grade, and Millipore-Q water ($\geq 18 \text{ M}\Omega$) was used in all experiment.

2.2. Oligonucleotides

The *invA* target gene sequence and other DNA oligonucleotides were obtained according to the literature [32]. All oligonucleotides were synthesized by Sangon Inc. (Shanghai, China), and the detail base sequences were showed in Table 1.

2.3. Apparatus

DNA-BIND™ plates were purchased from Corning Costar Corporation (New York, USA). The absorbance measurements were performed on an ELIASA (TECAN, Switzerland). UV–vis spectra were carried out on a UV 2550-Vis spectrophotometer (Shimadzu, Japan). Transmission electron microscopic (TEM) image was carried out using an H-7500 transmission electron microscope (Hitachi, Japan). The PCR reaction was taken with a My Cycler thermal cycler (Bio-Rad Laboratories, USA). Gel images were completed on an imaging system (Bio-Rad Laboratories, USA).

2.4. Preparation of AuNPs and DNA-functionalized AuNPs

AuNPs were prepared according to a reported method [27]. Briefly, 3.5 mL of 1% trisodium citrate was added to 100 mL of boiling 0.01% HAuCl_4 solution and was rapidly stirred. The color of solution turned from pale yellow to deep red, indicating the formation of AuNPs. Then the solution was cooled to room temperature with continued stirring and stored at 4 °C before use.

The DNA-functionalized AuNPs were synthesized by adding 80 μL of 10 μM DNAzyme probes and 20 μL of 10 μM detection probes to 400 μL of Au colloidal solution. After incubating for 12 h at 4 °C with slight stirring, the DNA conjugates were “aged” in 0.1 M NaCl for another 12 h. Subsequently, the conjugates were collected by centrifugation at $8000 \times g$ for 30 min and kept at 4 °C.

2.5. Preparation of DNA samples and PCR amplification

The *Salmonella typhimurium* strains were obtained from Chongqing Center for Disease Control and Prevention (Chongqing, China). These strains were grown aerobically at 37 °C for 16 h in sterile liquid Luria–Bertani medium with shaking. For further purification, the bacterial culture was ultracentrifuged at $10,000 \times g$ for 5 min at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 100 μL ultrapure water. The enumeration of viable *Salmonella* was carried out by plating 100 μL of appropriate 10-fold dilutions in sterile ultrapure water onto plate count agar. After incubating the plate for 24 h at 37 °C, the concentration of *Salmonella* was estimated by calculating the average number of CFU. Meanwhile, DNA extraction was performed by incubating the culture in a water bath for 15 min at 100 °C and immediately chilling on ice. Subsequently, the mixture was centrifuged at $10,000 \times g$ for 5 min at 4 °C and the supernatant containing genome DNA was used as template for the PCR.

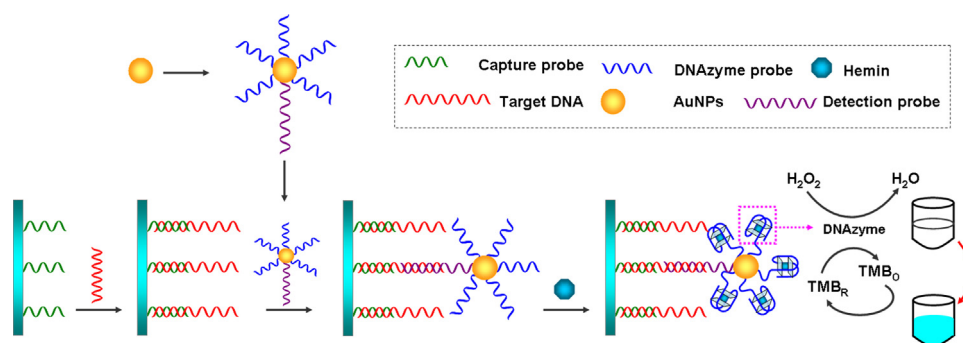
The PCR reactions were performed in 50 μL volumes consisting of 5.0 μL genomic DNA, 1.0 μL of 20 μM forward and reverse primers, 25 μL of Premix Taq (1.25 U of DNA polymerase, $2 \times$ Taq buffer, 0.4 mM of dNTPs) and 18 μL of water. The cycling parameters consisted of 35 cycles of denaturation at 95 °C (30 s), annealing at 64 °C (30 s) and extension at 72 °C (30 s) followed by a final extension at 72 °C for 4 min. PCR products were determined by running 10 μL of PCR mixtures in 2% agarose gel for 20 min and observed under ultraviolet light.

2.6. Preparation of colorimetric sensor

The amino-modified C12 linker-armed capture probe was dissolved in oligo binding buffer (50 mM Na_2HPO_4 , 1 mM EDTA, pH=8.5) at a concentration of 0.25 pmol μL^{-1} , and 100 μL /well was added to the DNA-BIND™ plate to covalently couple and incubated overnight at 4 °C. Then the plate was washed five times with Tris–buffered saline (10 mM Tris, 150 mM NaCl, pH=8.0) to remove uncoupled capture probe. To block the unreacted *N*-oxysuccinimide ester (NOS) groups, 200 μL of 3% BSA and 125 $\mu\text{g mL}^{-1}$ salmon sperm DNA in oligo binding buffer were added to each well and incubated for 30 min at 37 °C. The colorimetric sensor was developed successfully and stored for future use at 4 °C.

Table 1
Oligonucleotides used in the present work.

Oligonucleotide	Sequence (5'–3')
Capture probe	C12-NH ₂ -TTTTTCGCACCGTCAAAGGAA
Detection probe	TAC CGG CCT TCA AAT CGG CAT TTT TTT TTT-(CH ₂) ₃ -SH
Target oligonucleotide	TGCCGATTTGAAGGCCGGTAGCTAGATTCCTTTGACGGTGCC
DNAzyme probe	HS-(CH ₂) ₆ -TTTTTTGGGTAGGGCCGGTTGGG
Single-base-mismatched oligonucleotide	TGCCGATTTGAAGGCCGGTAGCTAGATTCATTGACGGTGCC
Non-complementary oligonucleotide	CTAGCTAAATCCCATGAATCGCTCTAATTAACCTACCGAGA



Scheme 1. Schematic representation of colorimetric sensing assay for *Salmonella* target DNA detection using DNAzyme probe self-assembled gold nanoparticles as single tag.

Then, the mixed solution of PCR product and DNA-functionalized AuNPs in $2 \times$ SSC (30 mM trisodium citrate ($C_6H_5Na_3O_2 \cdot 2H_2O$), 300 mM NaCl, 0.1% SDS, pH = 7.6) was heated for 5 min at $95^\circ C$ in a water bath and immediately chilled in ice. After the plate was washed by washing buffer, the mixed solution was added into the plate, following incubation for 1 h at $37^\circ C$ to form a sandwich-type DNA hybridization. Then, the plate was washed 5 times with $60^\circ C$ -heated $2 \times$ SSC, each for 5 min. Next, $100 \mu L$ of $2 \mu M$ hemin was added and the colorimetric sensor was incubated at room temperature for 1 h, which would allow the formation of G-quadruplex/hemin complex (DNAzyme). Finally, $50 \mu L$ of 0.2 mg mL^{-1} TMB and 0.012% H_2O_2 solution were added as the substrate of above peroxidase-like activity DNAzyme. After 2 h, add an equal volume of $2 M H_2SO_4$ into the solution to stop this reaction. The absorbance value was measured at the wavelength of 450 nm.

3. Results and discussion

3.1. Principle of colorimetric assay for *Salmonella* detection

The principle of the *Salmonella* detection was illustrated in Scheme 1. The capture probe was 5'-end chemically modified with a primary amino group and covalently linked to the plate coated with a layer of reactive *N*-oxysuccinimide ester (NOS groups) in a slightly alkaline environment. In the presence of target sequence, the hybridization reaction, between the capture probe and the detection probe-functionalized AuNPs, occurred to form a sandwich-type hybridization structure. Then in the presence of hemin, the DNAzyme probe, assembled on AuNPs, could form a G-quadruplex/hemin complex, which can catalyze

the H_2O_2 -mediated oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) to cause a dramatic color change. In this method, G-quadruplex/hemin complex (DNAzymes), a new label instead of traditional horseradish peroxidase (HRP), showed inherent advantages of simplicity, low price and stability. In addition, the sensitivity was effectively enhanced by using AuNPs modified by DNAzyme probe as single tag. This sensing platform not only enlarged the target library of the DNAzyme probes based AuNPs colorimetric sensors, but also provided a new simple and economical method to detect *Salmonella*.

3.2. Characteristics of DNA-functionalized AuNPs

Fig. 1A illustrated the size and shape of AuNPs obtained by transmission electron microscopy (TEM) measurement. It can be seen that most of the AuNPs were spherical and homogeneous distributed with a mean size of 24 nm in diameter. The bioconjugation of DNA on the synthesized AuNPs was confirmed by UV-vis absorption spectra (Fig. 1B). The AuNPs exhibited an absorption peak at 520 nm (curve a), and the color of solution was red. After the AuNPs were functionalized with thiolated DNA, the absorption peak at 520 nm was obviously observed and the color of the solution was still red, when 0.1 M NaCl solution was added (curve b). As shown in curve c, AuNPs without SH-DNA after addition of 0.1 M NaCl, the absorption peak declined sharply and the solution color switched from red to gray black. This could be attributed to the fact that the unmodified AuNPs were unstable in high-concentration salt solution and gathered speedily with each other. The above results strongly demonstrated that the AuNPs were successfully labeled with thiol-modified oligonucleotides.

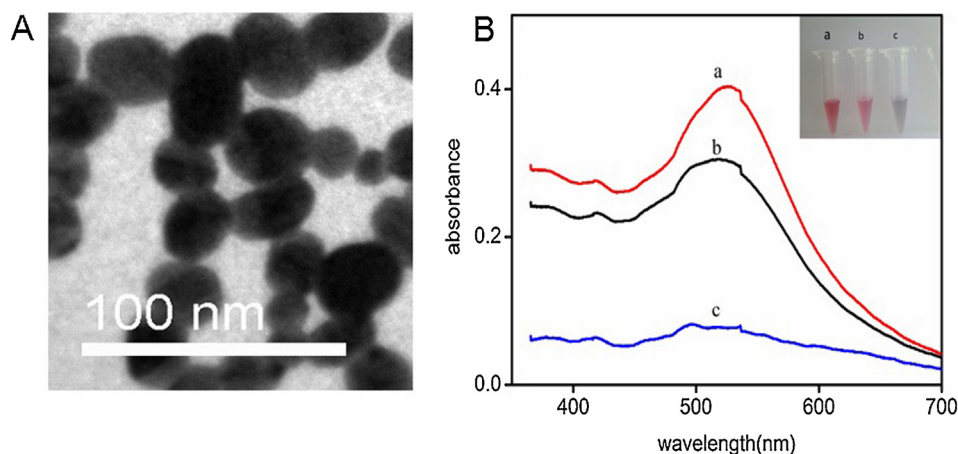


Fig. 1. (A) TEM image of AuNPs. (B) UV-vis absorption spectra of (a) AuNPs, (b) AuNPs with SH-DNA after addition of 0.1 M NaCl, and (c) AuNPs without SH-DNA after addition of 0.1 M NaCl.

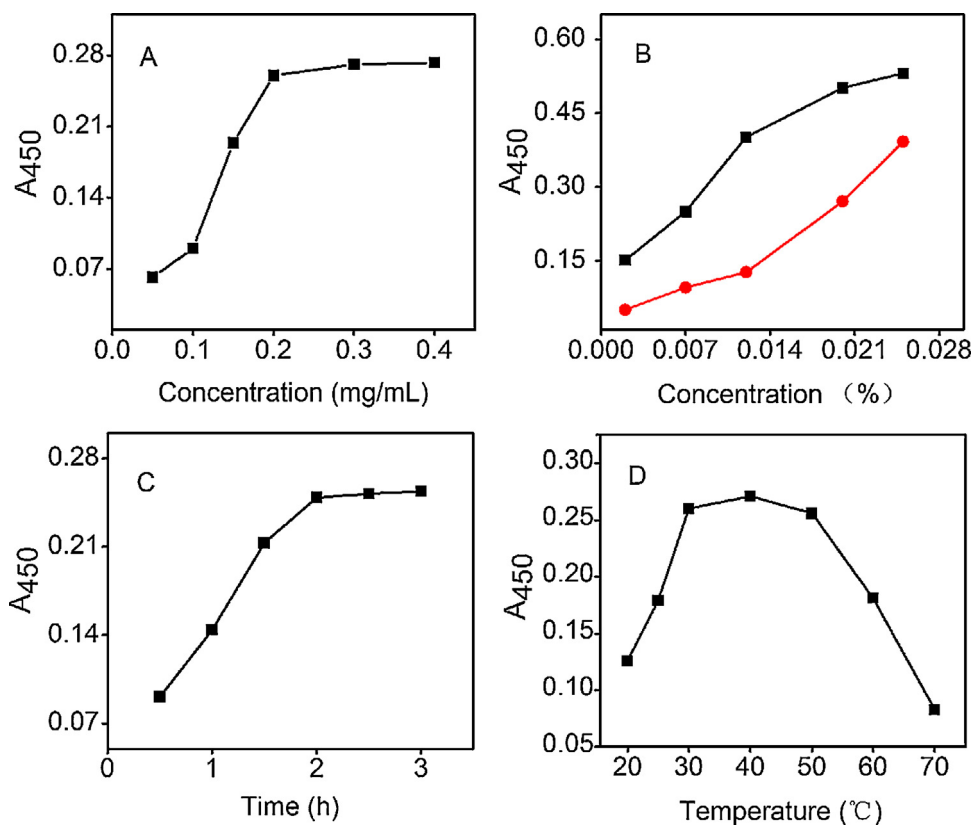


Fig. 2. Dependences of OD value on TMB concentration (A), H₂O₂ concentration (B), Time (C), temperature (D), when one parameter changed and the others were under their optimal conditions.

3.3. Optimization of experimental conditions

The appropriate reaction conditions played an important role in the improvement of sensitivity. So some basic variables were optimized. Fig. 2A showed the absorbance value of different concentration of TMB. With the increasing concentration of TMB, the optical density (OD) sharply increased and tended to a steady value after 0.2 mg mL⁻¹. Therefore, 0.2 mg mL⁻¹ was chosen as the optimum TMB concentration for colorimetric detection. In addition, the concentration of H₂O₂ was also optimized. As shown in Fig. 2B, with the increasing concentration of H₂O₂, the OD value increased rapidly (line a), meanwhile the blank response also increased, and the maximal difference was obtained at 0.012% (line b). Thus, 0.012% was selected as the optimal concentration to obtain high ratio of signal to background.

The incubation time and temperature of substrate were important parameters that influenced the enzyme-catalyzed reaction. The OD values sharply increased with the increasing incubation time up to 2 h and then reached a plateau (Fig. 2C), which suggested that 2 h was enough for the reaction of DNAzyme and substrate. Thus, the incubation time of 2 h was adopted. Fig. 2D showed the OD values of different temperature from 20 °C to 70 °C. It exhibited a plateau and obtained maximum response between 30 °C and 50 °C. In this experiment, 40 °C was chosen as optimal temperature.

Some other important experimental conditions such as the concentration of hemin and the dilution ratio of DNA-functionalized AuNPs also influenced the assay performance. As shown in Fig. 3A, the OD values increased significantly with the increasing concentration of hemin, and the signal exhibited no further remarkable variation after more than 2 μM hemin. So the concentration of hemin was fixed at 2 μM in following experiments. The effect of dilution ratio of the prepared DNA-functionalized AuNPs solution

on the absorbance response was shown in Fig. 3B. The OD values tended to be constant and decreased gradually when the dilution ratio was greater than 1:3. Therefore, the dilution ratio of 1:3 was used for the optimal dilution.

3.4. Sensitivity of colorimetric assay

To explore the sensitivity of the colorimetric sensors for target DNA detection, the absorbance signals of different concentrations of target DNA at 450 nm were measured. As shown in Fig. 4A, the OD value increased remarkably with the increment of target DNA concentration. Fig. 4B showed the good linear relationship between the OD values and the logarithm of target DNA concentration was obtained in the range of 0.5–50 nM, with correlation coefficient of 0.996. The limit of detection (LOD) was calculated to be 0.44 nM, which was lower than the other reported DNAzyme based DNA sensors [33,34]. In addition, the color change of different concentrations of target DNA could be readily differentiated with naked eye, as shown in Fig. 4C.

3.5. Specificity and reproducibility of the assay

In order to investigate the selectivity of the established colorimetric biosensor for the detection of target DNA, three different synthetic oligonucleotides including full complementary oligonucleotides, single-base-mismatched oligonucleotides and non-complementary oligonucleotides were analyzed. Fig. 5 displayed the changes of absorbance value after hybridization with 3 nM and 30 nM of the three different oligonucleotides and the background. The assay gave a very clear response with full complementary oligonucleotides, but the interaction with single-base-mismatched oligonucleotides was significantly weaker

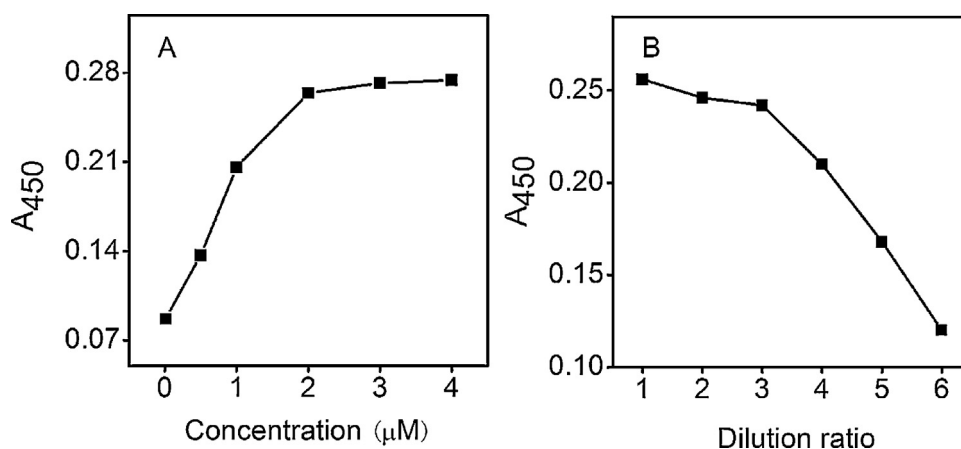


Fig. 3. Dependences of OD value on Hemin concentration (A), AuNPs dilution ratio (B), when one parameter changed while the others were under their optimal conditions.

than that of the complement sequences. The absorbance value with the non-complementary oligonucleotides exhibited significant decrease and were of the same order of magnitude as the signal of the blank background. Therefore, the results demonstrated that the designed colorimetric sensor was highly selective to the sequence of the target DNA.

To estimate the reproducibility of the developed colorimetric sensor, the sensor was investigated by detecting synthetic target DNA at 3 nM and 30 nM with six replicates, respectively. The relative standard deviation (RSD) of six replicates measurements was less than 5%, which suggested a satisfactory precision and acceptable reproducibility of the designed assay.

3.6. Detection of *Salmonella* in water

To evaluate the feasibility of established colorimetric sensor for the detection of actual *Salmonella* in water, the absorbance values of different concentrations *Salmonella* were measured. The cultivate bacteria was processed as described steps by extracting

genomic DNA with a rapid and efficient boiling method and the PCR was performed using the extracted *invA* gene. Then the post-PCR analysis was performed by both colorimetric biosensor and agarose gel electrophoresis. The amplification of a 284 bp fragment of *invA* gene was successfully achieved, which could be verified by 2% agarose gel electrophoresis (Fig. 6A). The colorimetric-PCR assay could be applied to analyze the denatured PCR products for *Salmonella* from 3×10^3 to 3×10^6 CFU mL⁻¹, and the absorbance values to different PCR samples were shown in Fig. 6B. However, no target band could be observed in PCR products when the concentration was below 3×10^5 CFU mL⁻¹, this is attributed to the low EtBr staining efficiency for ssDNA. On the contrary, the established colorimetric-PCR sensor could effectively detect *Salmonella* as low as 3×10^3 CFU mL⁻¹, which was much lower than other methods reported previously for the detection of *Salmonella* [35,36]. Thus, the designed colorimetric-PCR assay is simple, fast and sensitive, which could be regarded as a valid alternative to conventional assays for the detection of *Salmonella*.

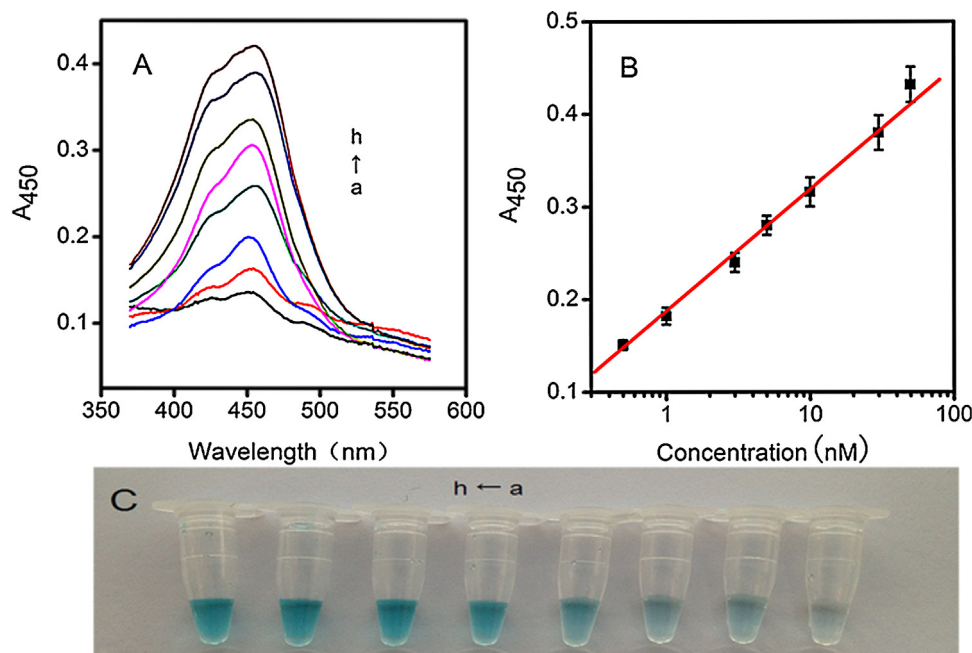


Fig. 4. (A) UV-vis absorption spectra for different target DNA concentrations of 0, 0.5, 1, 3, 5, 10, 30, 50 nM (from a to h). (B) Plot of absorption for target different DNA concentration. The error bars represent average standard errors for three measurements. (C) Colorimetric responses of the sensor to different concentrations of target DNA.

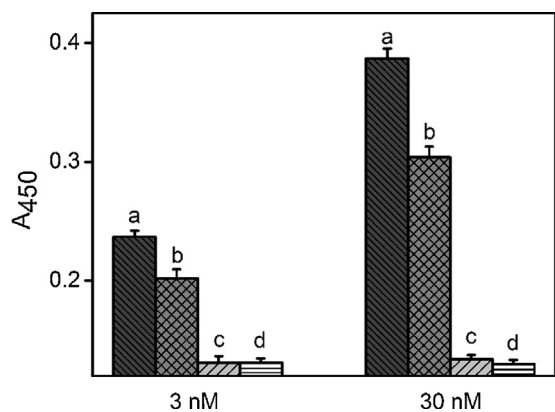


Fig. 5. Comparison of OD values after hybridization with 3 nM and 30 nM of target oligonucleotides (a), single base mismatched oligonucleotides (b), non-complementary oligonucleotides (c), blank (d), respectively.

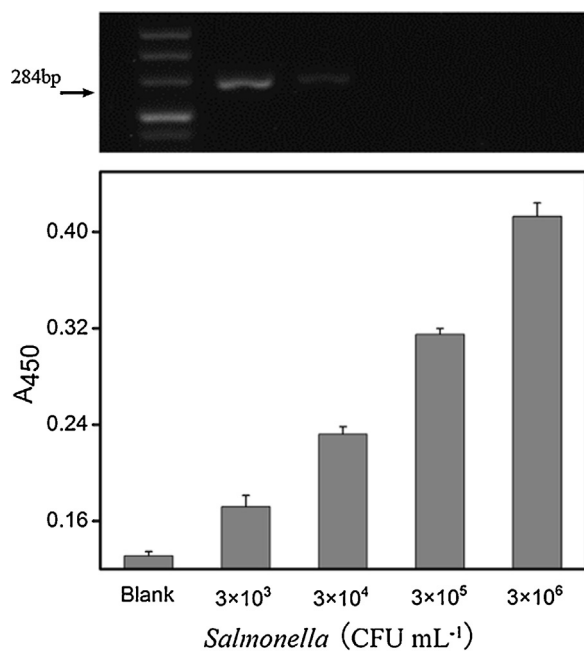


Fig. 6. (A) Gel electrophoresis photos of 500 bp size maker (M) and PCR products of 3×10^6 (a), 3×10^5 (b), 3×10^4 (c), 3×10^3 (d) CFU mL⁻¹ *Salmonella*. (B) Typical OD curves of colorimetric assay with PCR products obtained from serial dilutions of *Salmonella* in the range of 3×10^3 – 3×10^6 CFU mL⁻¹.

4. Conclusions

This work we demonstrated a simple and sensitive colorimetric biosensor assay for the detection of *Salmonella* by using the species-specific *invA* gene and integrating PCR technique and DNAzyme probe self-assembled gold nanoparticles for signal amplification. On the optimal experimental conditions, the amplification greatly improved the sensitivity for detection of target DNA down to 0.44 nM, and the color of 0.5 nM target DNA could be observed directly by naked eyes. In addition, the designed assay had been successfully applied to detect PCR amplified products from *Salmonella* and could detect *Salmonella* as low as 3×10^3 CFU mL⁻¹ in water. In conclusion, this method possessed the advantages of simplicity, sensitivity, low-cost and reliability, which might become a powerful tool for *Salmonella* monitoring in real samples and has a promising prospect for clinical diagnosis, food safety and environmental monitoring.

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