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Gold Nanoparticle-Based Enzyme-Linked Antibody-Aptamer Sandwich Assay for Detection of *Salmonella* Typhimurium

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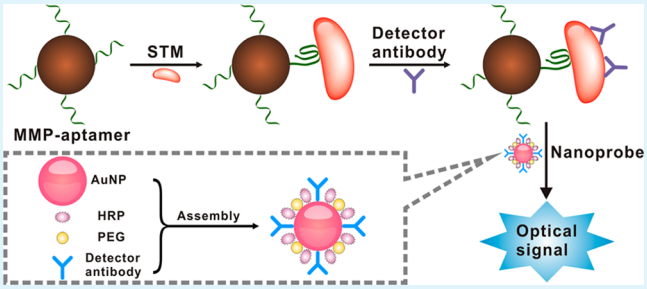
Supporting Information

ABSTRACT: Enzyme-linked immunosorbent assay (ELISA) provides a convenient means for the detection of *Salmonella enterica* serovar Typhimurium (STM), which is important for rapid diagnosis of foodborne pathogens. However, conventional ELISA is limited by antibody−antigen immunoreactions and suffers from poor sensitivity and tedious sample pretreatment. Therefore, development of novel ELISA remains challenging. Herein, we designed a comprehensive strategy for rapid, sensitive, and quantitative detection of STM with high specificity by gold nanoparticle-based enzyme-linked antibody-aptamer sandwich (nano-ELAAS) method. STM was captured and preconcentrated from samples with aptamer-modified magnetic particles, followed by binding with detector antibodies. Then nanoprobes carrying a large amount of reporter antibodies and horseradish peroxidase molecules were used for colorimetric signal amplification. Under the optimized reaction conditions, the nano-ELAAS assay had a quantitative detection range from 1×10^3 to 1×10^8 CFU mL^{-1}, a limit of detection of 1×10^3 CFU mL^{-1}, and a selectivity of >10-fold for STM in samples containing other bacteria at higher concentration with an assay time less than 3 h. In addition, the developed nanoprobes were improved in terms of detection range and/or sensitivity when compared with two commercial enzyme-labeled antibody signal reporters. Finally, the nano-ELAAS method was demonstrated to work well in milk samples, a common source of STM contamination.



KEYWORDS: *Salmonella Typhimurium, aptamer, antibody, nanoprobe, gold nanoparticle, magnetic microparticle*

1. INTRODUCTION

Salmonella enterica serovar Typhimurium (STM) is one of the most common causes of foodborne illnesses, resulting in hundreds of millions of infections and many deaths in humans throughout the world every year.[1,2] It has been a serious burden to public health for many years, and some countries have tried to reduce its morbidity. Recently, due to the increase in consumption of raw or undercooked food[3] and the global spread of multiresistant STM[4] and the like, STM is causing significantly increased concern. According to the Centers for Disease Control and Prevention (CDC) in the United States, between 15 and 20 outbreaks[5] of human STM infections per year associated with the consumption of contaminated fruits,[6] vegetables,[7] milk,[8,9] meat,[10] and so on have been reported and led to massive-scale food recalls.

The gold standard to detect STM is a conventional cultural method that is professional operation limited, labor intensive, and time-consuming (requiring 4−8 days).[11] To prevent outbreaks of STM and reduce the times of food recalls, various types of immunoassay tests have been developed to detect STM rapidly, especially involving enzyme-linked immunosorbent assay (ELISA).[12,13] Since the first report in 1971,[14] ELISA, which was based on antibody−antigen immunoreactions, has become a powerful tool available for biological research and clinical diagnostics because of its convenient operation, ability to test a large number of samples at the same time, and automation. However, despite having good merits, there still remain some problems of ELISA for STM detection. First, the detection sensitivity of conventional ELISA is 10^6 colony forming units (CFU) mL^{-1}, which often could not meet the needs for the detection of STM as the infectious dose for nontyphoidal Salmonellosis is approximately 10^3 CFU.[15,16] Second, antibodies have certain limitations, such as requiring the use of animals or cells, hard labeling at precise locations, limited shelf life, and batch-to-batch variation. Third, the

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conventional ELISA is usually combined with a broth-culture enrichment system or cell lysis to detect STM.

Several attempts were made to solve those problems based on ELISA method, and they provided new opportunities for the high-efficiency detection of pathogens. For example, functionalization of gold nanoparticles (AuNPs) that feature unique physical and chemical properties and biological compatibility[17,18] has resulted in promising candidates in the design of novel ELISA with higher sensitivity.[19] In addition, to rival antibodies in these ways,[20] aptamers are beginning to emerge. Aptamers are short single-stranded DNA or RNA molecules that have been selected through repeated rounds of in vitro selection referred to as systematic evolution of ligands by exponential enrichment to bind specific targets such as small molecules, proteins, nucleic acids, and even whole cells.[21−23] Several studies have demonstrated the use of aptamers as capture probes and/or detection probes in sandwich immunoassays.[24−26] Furthermore, the magnetic bead that is used in combination with ELISA emerges as an attractive tool to separate and concentrate the target pathogens from complex liquid samples.[27,28] However, most published studies are not comprehensive enough and therefore provide little guidance to practical application.[29]

In this work, we designed a comprehensive strategy for rapid, sensitive, and specific quantitative detection of STM by gold nanoparticle-based enzyme-linked antibody-aptamer sandwich (nano-ELAAS) method. Incorporating STM-binding aptamer, magnetic microparticle (MMP), and nanoprobe, the sandwich method could achieve the preconcentration of STM and high-efficiency signal amplification. Also, the developed nanoprobe was compared with horseradish peroxidase (HRP)-labeled reporter antibody and alkaline phosphatase (AP)-labeled reporter antibody. These approaches using these two commercial enzyme-labeled antibody signal reporters were named colorimetric ELAAS and chemiluminescent ELAAS, respectively.

2. EXPERIMENTAL SECTION

2.1. Reagents. DNA oligonucleotides were synthesized and purified by high-performance liquid chromatography (Sangon Biotechnology, Inc., Shanghai, China). The sequence of STM-binding aptamer (5′-ATCCGTCACACCTGCTCTGGAGCAATATGGTG-GAGAAACGTGGTGTTGGCTCCCGTAT-3′) was screened out by Bruno and Chanpong.[30] We selected another DNA oligonucleotide (T20) as blocking DNA. All sequences and their modifications at the 5′-end are listed in Supporting Information, Table S1. BioMag Plus Amine Protein Coupling Kit (MMPs, ∼1.5 μm diameter) was obtained from Bangs Laboratories, Inc. HRP, ExtrAvidin−peroxidase, lipopolysaccharides (LPS) from STM, Tween 20, antimouse IgG (whole molecule)-AP antibody produced in goat, and antimouse IgG (whole molecule)-HRP antibody produced in goat were purchased from Sigma−Aldrich Co. Monoclonal mouse anti-STM was purchased from Hytest Ltd. Goat antimouse IgG was procured from Fitzgerald Industries International, Inc. The chemiluminescent AP ELISA system was purchased from Invitrogen Co. Bovine serum albumin (BSA) was purchased from Amersco, Inc. K-blue substrate (3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2) was obtained from Neogen Co. All other chemical reagents were of analytical grade and purchased from China National Pharmaceutical Group Co. All solutions were prepared by Milli-Q water (18.3 MΩ·cm^{-1}) from a Millipore system.

2.2. Buffers and Solutions. Phosphate buffered saline (PBS), comprising 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 10 mmol L^{-1} Na_2HPO_4, 2 mmol L^{-1} KH_2PO_4, pH 7.4, was prepared by Milli-Q water and autoclaved at 121 °C for 15 min. PBS with 0.05% (v/v) Tween 20 for use as washing buffer. Storing buffer was prepared by dissolving 0.5% (w/v) BSA, 2.5% (w/v) sucrose, and 0.1% (w/v) poly(ethylene glycol) (PEG, average molecular weight 6000) in PBS (as above). Blocking buffer was procured from Thermo Fisher Scientific.

2.3. Bacterial Cultures. STM (CMCC 50115, ATCC 14028, and two clinical isolates), *Salmonella enterica* serovar Typhi (*S.* Typhi, CMCC 50071), *Salmonella enterica* serovar Paratyphi A (*S.* Paratyphi A, CMCC 50433), *Shigella sonnei* (*S. sonnei*, ATCC 25931), *Shigella flexneri* (*S. flexneri*, CMCC 51571), *Escherichia coli* (*E. coli*, ATCC 25922), and *Staphylococcus aureus* (*S. aureus*, ATCC 25923) were donated by the Central Laboratory of Biology and the First Affiliated Hospital of Wenzhou Medical University (Zhejiang, China). *E. coli* O157:H7 (CICC 21530) was purchased from China Center of Industrial Culture Collection (CICC, Beijing, China). *E. coli* O111 (CMCC 44151) was purchased from National Center for Medical Culture Collections (CMCC, Beijing, China). All bacteria were propagated overnight in Luria Broth with shaking at 37 °C. The calculation of CFU mL^{-1} was done according to published protocols.[13] Finally, the cells were resuspended in PBS to the required concentration.

2.4. LPS-Aptamer Plate Binding Assay. LPS from STM was diluted to 10 μg mL^{-1} in PBS and used to coat a 96-well microplate overnight at 4 °C. After being washed three times with washing buffer and passivated with 100 μL blocking buffer at 37 °C for 0.5 h, a concentration series of biotinylated STM-binding aptamers (0, 5, 20, 100, 250, 500, 750, and 1000 nmol L^{-1}) was added and incubated at 37 °C for 1 h. Then 100 μL of diluted ExtrAvidin−peroxidase (1:2000) was added to each well and incubated at 37 °C for 1 h. The color was revealed with 100 μL of TMB substrate. Finally, the absorbance value was measured with a Tecan GENios microplate reader (Switzerland) at 450 nm after blocking the reaction with 50 μL of 0.5 mol L^{-1} H_2SO_4 solution.

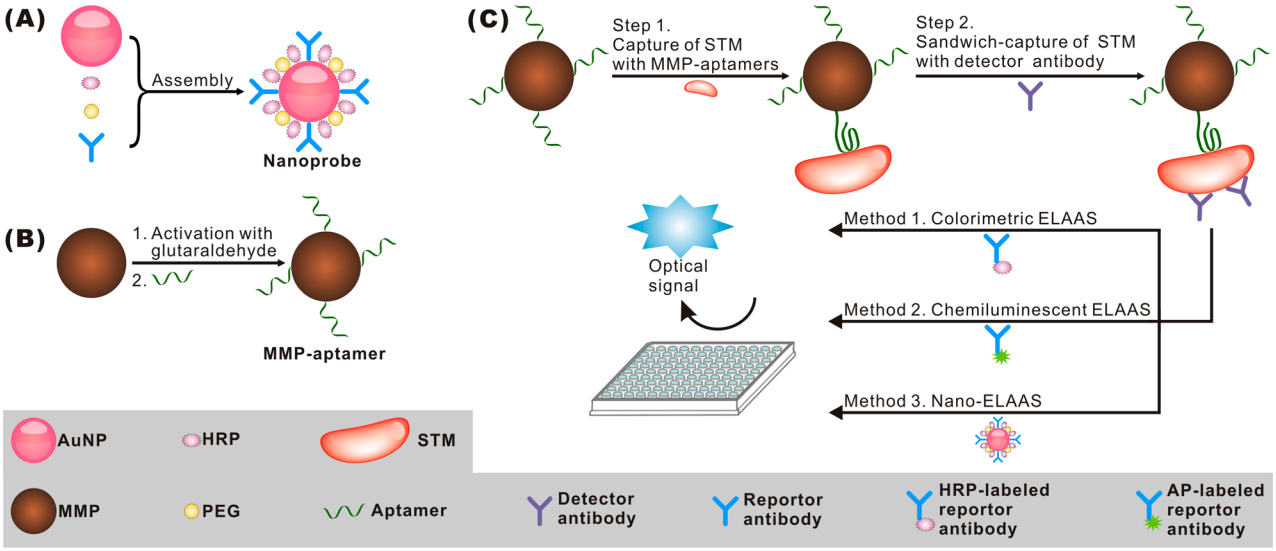
2.5. Confocal Laser Scanning Microscope (CLSM) of Aptamer Targeted to STM. Five μL (0.1 mmol L^{-1}) of fluorescein isothiocyanate (FITC)-labeled STM-binding aptamer and blocking DNA were incubated with bacteria in a dark bottle at room temperature overnight. Then the mixtures were purified with PBS. The mixtures were redispersed finally in PBS and placed onto a glass slide. After complete drying, the slides covered with coverslips were observed under CLSM (Fluoview FV1000, Olympus, Japan) equipped with an excitation wavelength of 488 nm.

2.6. Capture of STM Using MMP-Aptamers. The primary amine groups on the surface of MMPs were activated by glutaraldehyde, allowing amine groups on DNA oligonucleotides to be covalently attached. Briefly, 1 nmol of amino-labeled STM-binding aptamers and blocking DNA were functionalized with MMP solution (6.25 mg mL^{-1}, 100 μL) and were then washed and prepared according to the manufacture's protocol. Finally, the MMP-aptamers were ∼5 mg mL^{-1} as suspension and stored at 4 °C. Of note, on the basis of the consideration to expose the recognition part of the aptamer outside the MMP, we designed a mute spacer (T20) part at the amino end of its sequence.

Two μL MMP-aptamers were transferred into an Eppendorf tube and passivated with 20 μL of blocking buffer at 37 °C with gentle shaking for 0.5 h, followed by washing three times with washing buffer and adding a 100 μL aliquot of STM or other sample solution. After shaking for 45 min and washing three times with washing buffer, 10 μL detector antibodies (monoclonal mouse anti-STM, 2 mg mL^{-1}) were directly injected into each tube and incubated for 45 min at 37 °C with gentle shaking. Next, the MMP-aptamer/STM/detector antibody complexes were magnetically collected and rinsed in washing buffer. These complexes were subsequently used in three methods of signal amplification.

2.7. Signal Amplification Using Colorimetric ELAAS. Ten μL of HRP-labeled reporter antibodies (antimouse IgG-HRP antibodies) were incubated with the above complexes for 45 min at 37 °C with gentle shaking. Then the resulting complexes were magnetically collected and incubated with 100 μL of TMB substrate for 15 min after washing up to five times with washing buffer. Absorbance values

Scheme 1. Schematic Presentation of STM Detection Using Colorimetric ELAAS, Chemiluminescent ELAAS, or Nano-ELAAS: (A) Preparation of Nanoprobes; (B) Preparation of MMP-Aptamers; (C) Comparison of the Three Signal Amplification Methods (Note: Drawing Is Not to Scale)



at 595 nm on 96-well microplate were obtained with the Tecan GENios microplate reader at room temperature.

2.8. Signal Amplification Using Chemiluminescent ELAAS. Ten μL of AP-labeled reporter antibodies (antimouse IgG-AP antibodies) were incubated with MMP-aptamer/STM/detector antibody complexes for 45 min at 37 °C with gentle shaking. Then, Invitrogen chemiluminescent AP ELISA system's procedure was used in these chemiluminescent ELAAS studies. The chemiluminescence was measured in the Tecan GENios microplate reader at 5 min intervals until the light emission reached a plateau.

2.9. Signal Amplification Using Nano-ELAAS. AuNPs (~15 nm diameter) were prepared according to a previously published protocol.[31] The coating process was carried out by adding the minimum reporter antibodies and HRP molecules concentration determined by aggregation test of AuNPs.[32] Briefly, various amounts of reporter antibodies or HRP molecules were added to the 100 μL as-prepared (pH was adjusted to 8.5 by 0.1 mol L^{-1} K_2CO_3 solution) AuNPs. After incubating for 10 min, 20 μL of NaCl (10%, w/v) was introduced to the mixtures, followed by UV–vis characterization (U-3010, Hitachi, Japan).

The schematic of this experimental setup to prepare nanoprobes, which represents reporter antibodies (goat antimouse IgG) and HRP molecule double-codified AuNPs, is shown in Scheme 1 and detailed as follows. The reporter antibodies (1 mg mL^{-1}) and HRP molecules (1 mg mL^{-1}) were mixed at different mass ratios (9:1, 4:1, 1:1, 1:4, and 1:9). Thirty-five μL of mixture was added to 1 mL of the pH-adjusted (pH = 8.5) AuNPs. Then the mixed solution was incubated for 10 min at 25 °C under gentle shaking and stood overnight at 4 °C without mixing. Following addition of 5% (w/v) PEG to a final concentration of 0.5% (w/v) PEG and incubation for 30 min at 25 °C, the mixture was centrifuged (12 000 rpm, 20 min, 4 °C, supernatant decanted) to remove the unbound antibodies, HRP molecules and PEG, and rinsed with storing buffer. The centrifuging/rinsing procedure was repeated 3–4 times. The final deposition was suspended in 200 μL of storing solution and stored at 4 °C for further use. The quality of the particles was monitored with UV–vis spectrophotometer, zeta potential analyzer (NICOMP 380ZLS, Agilent Technologies, CA, USA), and transmission electron microscopy (TEM; JEM-2011, JEOL, Japan).

Ten μL of nanoprobes was incubated with MMP-aptamer/STM/detector antibody complexes for 45 min at 37 °C with gentle shaking. The following experimental setup is the same signal amplification using colorimetric ELAAS.

3. RESULTS AND DISCUSSION

The principle of this nano-ELAAS method was illustrated in Scheme 1. MMPs were modified with STM-binding aptamers to act as the capture probes. STM was then sandwiched by MMP-aptamers and detection antibodies. A magnet was used to collect the sandwich complex, followed by the binding of three signal reporters: HRP-labeled reporter antibody, AP-labeled reporter antibody, and double-codified nanoprobe, developing colorimetric ELAAS, chemiluminescent ELAAS, and nano-ELAAS, respectively. In this study, various parameters such as the titers of nanoprobes and commercial signal reporters had been optimized to improve signal-to-background ratio (S/B).

3.1. Characterization of the Affinity and Specificity of STM-Binding Aptamer. Binding affinity of the aptamer for STM was characterized by dissociation constant (K_d) determination. The K_d of the STM-binding aptamer was determined by carrying out LPS-aptamer plate binding assay in which a concentration series of biotinylated STM-binding aptamers was incubated with a fixed concentration of LPS from STM using biotin–avidin ELISA. A binding curve plotting the absorbance measured against the corresponding inputted aptamer concentration is presented in Supporting Information, Figure S1. Using nonlinear regression analysis, the STM-binding aptamer was found to have a K_d value of 19.59 ± 0.35 nmol L^{-1}, showing it has a high affinity toward LPS from STM. CLSM was used to further prove the success of capture of STM using this aptamer. Shown in Figure 1, green fluorescence was detected on STM (CMCC 50115) with FITC-labeled STM-binding aptamer, and no fluorescence signal was observed from either STM with FITC-labeled blocking DNA or *E. Coli* (ATCC 25922) with FITC-labeled STM-binding aptamer. These results clearly demonstrated the high specificity between STM and this aptamer. Because of the high affinity and specificity, there is a great potential use of STM-binding aptamer as a capture probe for STM analysis.

3.2. Colorimetric ELAAS. After the target bacteria were captured by the MMP-aptamers, detection antibodies were used to improve selectivity in a sandwich configuration. Then

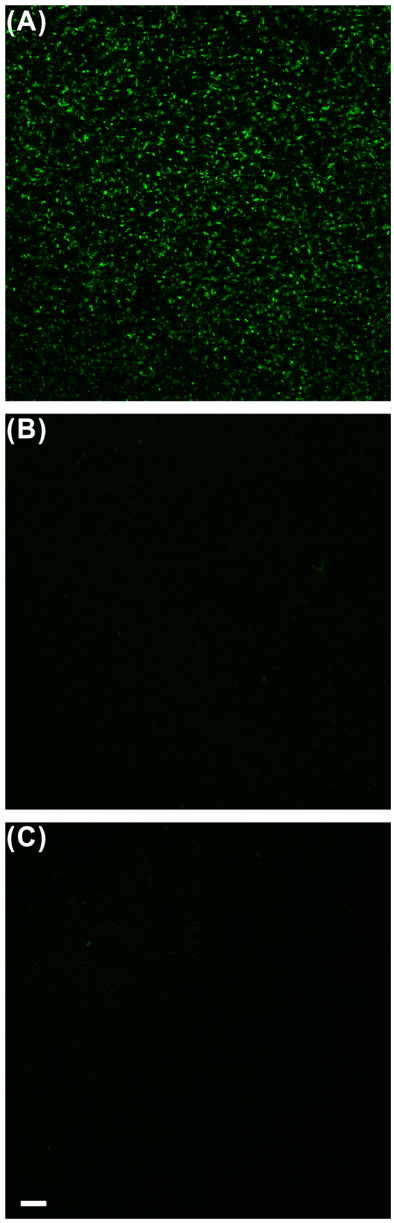


Figure 1. CLSM images of (A) STM treated with FITC-labeled STM-binding aptamer; (B) STM treated with FITC-labeled blocking DNA; and (C) *E. coli* treated with FITC-labeled STM-binding aptamer. Scale bar, 10 μm.

HRP-labeled reporter antibodies were employed to serve as signaling probes for signal amplification. Serially 2-fold diluted HRP-labeled reporter antibodies (1:1250 to 1:20000) were added in the absence and presence of STM (CMCC 50115). Results were evaluated in terms of S/B, defined as S/B = A/A_0. A_0 and A are the absorbance of the colorimetric ELAAS method in the absence and presence of STM, respectively. As shown in Figure 2A, the HRP-labeled reporter antibodies at a titer of 1:5000 were the best ones for the colorimetric ELAAS system. Varying amounts of STM (CMCC 50115) were employed to produce a calibration curve. As shown in Figure 2B, the absorbance was increased along with the STM concentration (from 5×10^3 to 1×10^8 CFU mL^{-1}). Though displaying a relatively low signal, the absorption of 1×10^4 CFU mL^{-1} STM was still obviously higher than average blank response plus

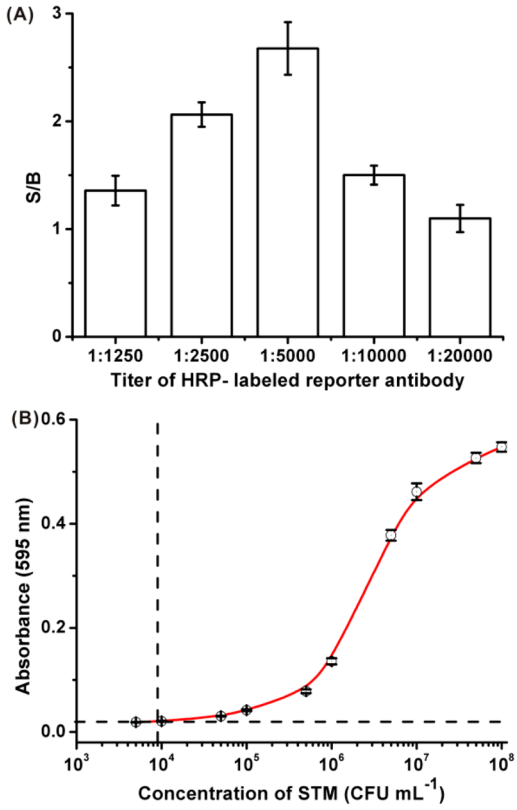


Figure 2. (A) Comparisons of the S/B with the various HRP-labeled reporter antibody titers generated by STM (1×10^5 CFU mL^{-1}). (B) Calibration curve for the absorbance vs concentration of STM by the colorimetric ELAAS method. The horizontal dashed line marks average blank response plus 3 SD, and the vertical dashed line indicates the detection limit. The absorbance signal was recorded at 595 nm. Error bars represent SDs from three independent assays.

three times the standard deviation (3 SD) by the colorimetric ELAAS method.

3.3. Chemiluminescent ELAAS. To meet lower levels of detectable bacteria, the colorimetric ELAAS assay was also configured to a format based upon chemiluminescence. We first compared the effects of different dilution rates of AP-labeled reporter antibody (1:300000 to 1:1500000) by analyzing S/B = CL/CL$_0$, where CL and CL$_0$ are the chemiluminescent signals of this method in the presence and absence of STM (CMCC 50115), respectively. The results showed us that the optimization of AP-labeled reporter antibodies dilution rate was 1:600000 for this chemiluminescent ELAAS assay (Figure 3A). Utilizing the optimal conditions in this system, a calibration curve for STM assay with different concentrations of 1×10^2 to 1×10^8 CFU mL^{-1} was then determined (Figure 3B). We could detect levels as low as 1×10^3 CFU mL^{-1} of STM as estimated from the derived calibration curve (>3 SD, Figure 3B, inset). This detection limit was 10-fold lower than that in the previously described colorimetric ELAAS approach. Moreover, this calibration curve showed a plateau around 5×10^7 CFU mL^{-1} STM, indicating that the chemiluminescent ELAAS method had a wider detection range than the colorimetric one (a plateau around 1×10^7 CFU mL^{-1}, Figure 2B). These results are comparable with other research that compared the colorimetric and chemiluminescent methods, as others have seen greater sensitivity and wider detection range with the use of the chemiluminescent assay.[33,34]

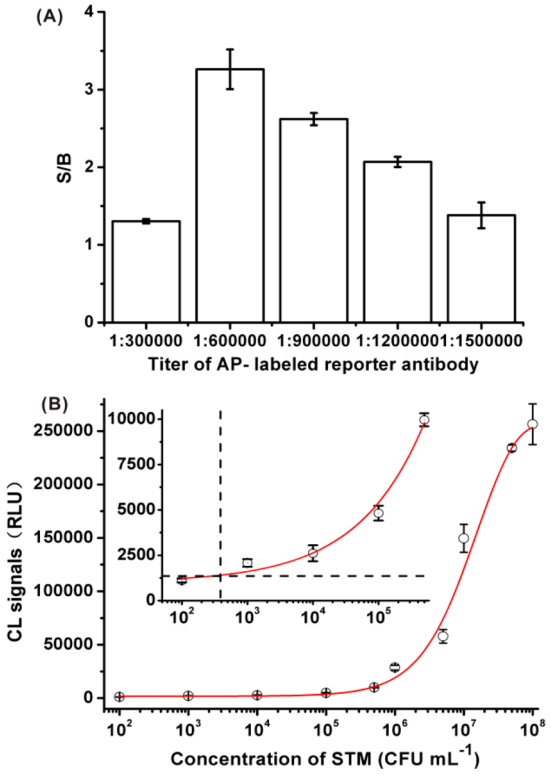


Figure 3. (A) Comparisons of the S/B with the various AP-labeled reporter antibody titers generated by STM (1×10^4 CFU mL^{-1}). (B) Calibration curve for the CL signals vs concentration of STM by the chemiluminescent ELAAS method. The horizontal dashed line marks average blank response plus 3 SD, and the vertical dashed line indicates the detection limit. Error bars show mean ± SD of three determinations.

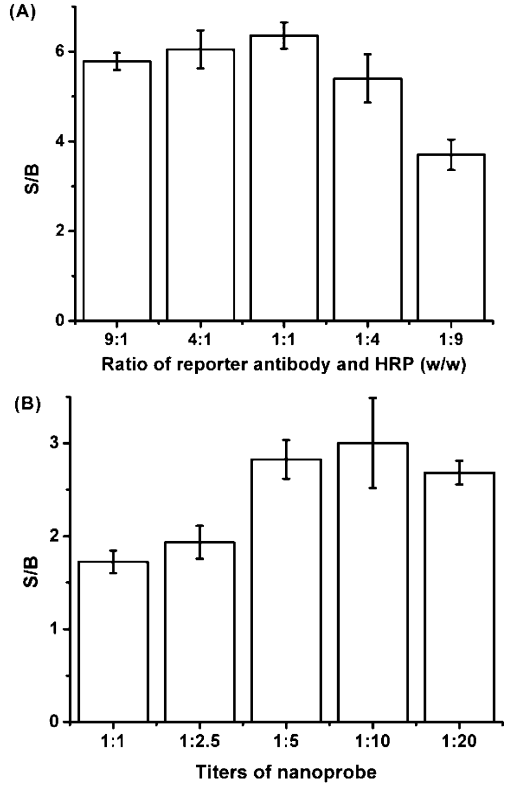


Figure 4. (A) Comparisons of the S/B with the various ratios of reporter antibody and HRP (w/w) generated by detector antibodies (2 μg mL^{-1}). (B) Comparisons of the S/B with the various nanoprobe titers generated by STM (1×10^4 CFU mL^{-1}). Data represent average ± SD (error bars) from at least three independent assays.

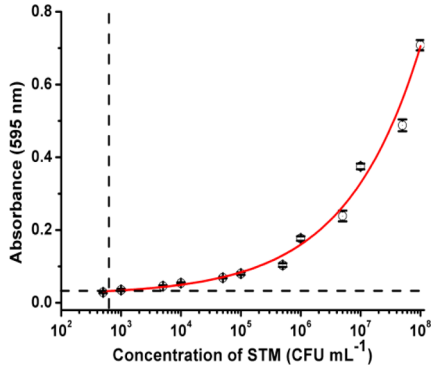


Figure 5. Calibration curve for the absorbance vs concentration of STM by the nano-ELAAS method. The horizontal dashed line marks average blank response plus 3 SD, and the vertical dashed line indicates the detection limit. The absorbance signal was recorded at 595 nm. All data were collected from at least three independent sets of experiments.

3.4. Preparation and Characterization of Nanoprobes.

AuNPs possess a high surface-to-volume ratio that allows the attachment of multiple kinds of biomolecules to the surface and the design of multifunctional nanoprobes.[35,36] In this work, we used reporter antibody rather than detection antibody to modify AuNPs, together with HRP molecules. Such designed nanoprobes could be used directly for other bacteria without changing the antibodies on them. Figure S2 in the Supporting Information shows the results of the aggregation test used to evaluate the loading of reporter antibodies or HRP molecules on AuNPs. NaCl (final concentration = 1.64% (w/v)) was added to AuNP solutions containing different concentrations (0−50 μg mL^{-1}) of reporter antibodies or HRP molecules. The absorbance gradually increased and reached a constant value at >25 μg mL^{-1} of reporter antibodies (Supporting Information, Figure S2A) or 35 mg mL^{-1} of HRP molecules (Supporting Information, Figure S2B), indicating that 35 μg mL^{-1} of reporter antibody/HRP mixture might be an optimized concentration, allowing the modified AuNPs to be stable in the presence of high-concentration salts.

AuNPs before or after coating with reporter antibody and HRP were characterized by TEM, zeta potential measurement, and UV−vis spectrophotometer. Bare AuNPs appeared to be nearly monodispersed, with an average size of 15 nm (see details in Supporting Information, Figure S3A). After modification, the AuNPs kept the original red color with naked eyes and were well-separated with no change in the morphology under TEM (Supporting Information, Figure S3B). Compared with bare AuNPs, there was a blurred shadow around each nanoprobe, which showed that antibody and HRP were adsorbed to the surface of AuNPs.[37,38] This was further confirmed by zeta potential measurement. After modification, the zeta potential value of AuNPs owing to the presence of citrate ions on their surface increased (became less negative) from −33.7 ± 2.7 mV (n = 3) to −26.8 ± 3.1 mV (n = 3). The decrease in surface-exposed negative charges still indicated the formation of nanoprobes. Moreover, according to Beer's law, the concentration of bare AuNPs (15 nm) based on their

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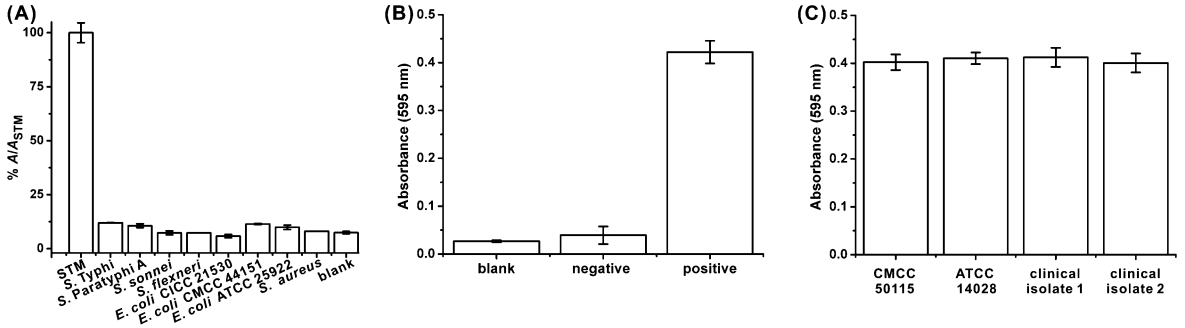

Figure 6. Cross-reactivity studies against (A) other bacteria, (B) a mixture of other bacteria, and (C) four different STM isolates using nano-ELAAS method. Note the high specific signals of STM, low background noise against other bacteria, and no statistically significant difference between the four different STM isolates (one-way ANOVA, $P > 0.05$). Data are expressed as mean ± SD. All samples for the assay were prepared in triplicate.

absorption at 520 nm (see details in Supporting Information, Figure S4) was measured at about 2.2 nmol L^{-1} using an extinction coefficient of 3.64×10^8 L mol^{-1} cm^{-1}.[39] In contrast to the bare AuNPs, the absorption peak of nanoprobes shifted from 520 to 528 nm. The red-shift of surface plasmon resonance band still implied the coating of reporter antibody/HRP at the surface of AuNPs.[32,37]

Although the two kinds of proteins could be assembled onto AuNPs, the molecular ratio between them would greatly influence the performance of the resulting nanoprobes for biorecognition and signal amplification. Reporter antibodies and HRP molecules were mixed at different mass ratios (total protein mass was 35 μg per 1 mL of AuNPs) to modify AuNPs for the preparation of different nanoprobes. These nanoprobes were incubated with a fixed concentration of detection antibodies (2 μg mL^{-1}), which were diluted in carbonate buffer (Na_2CO_3/$NaHCO_3$, pH 9.6) and coated on 384 microplate wells. Results were evaluated in terms of S/B, defined as S/B = A/A_0. A_0 and A are the absorbance (595 nm) in the absence and presence of detection antibodies, respectively. Also, we evaluated the effects of optimized nanoprobes at different dilution rates (1:1 to 1:20) by determining S/B (A/A_0), where A_0 and A are the absorbance of the nano-ELAAS method in the absence and presence of STM (CMCC 50115), respectively. The results indicated that the optimized reporter antibody/HRP (w/w) ratio and optimized nanoprobe titer were about 1:1 (Figure 4A) and 1:10 (Figure 4B), respectively.

3.5. Nano-ELAAS. In a typical nano-ELAAS, nanoprobes were used for signal amplification instead of HRP-labeled reporter antibodies or AP-labeled reporter antibodies. Their sensitivity was calculated by monitoring the absorbance change following the addition of various amounts of STM (CMCC 50115), showing in the calibration curve in Figure 5. This nanobased method had a limit of detection (LOD) of 1×10^3 CFU mL^{-1} STM estimated from the derived calibration curve (>3 SD). Its sensitivity was at least an order of magnitude higher than the colorimetric ELAAS method and comparable to the chemiluminescent ELAAS. Moreover, this calibration curve showed no plateau from 1×10^3 to 1×10^8 CFU mL^{-1} STM, indicating that the nano-ELAAS method had a wider detection range than the other two ELAAS methods; the reason for this could be the higher loading of HRP molecules per reporter antibody in the nano-ELAAS assay. Also of note, the format of the nano-ELAAS assay could meet the robustness requirement because its color response can be tuned for rapid qualitative detection with the naked eye.

The specificity of the nano-ELAAS system was investigated using negative bacteria controls including other serovars of *Salmonella* (*S.* Typhi and *S.* Paratyphi A), some other species of *Enterobacteriaceae* (*S. sonnei*, *S. flexneri*, three strains of *E. coli*), and Gram-positive bacteria (*S. aureus*). STM cells (CMCC 50115) were used at the concentration of 1×10^7 CFU mL^{-1} while the solutions of other bacteria were prepared to the concentration of 1×10^8 CFU mL^{-1} with PBS. As shown in Figure 6A, although the concentrations of other tested bacteria were larger than that of STM, only STM gave the significant signals, demonstrating that STM could be detected while the signals from other bacteria showed no significant difference from a blank control sample (PBS). We further challenged this method with 1×10^7 CFU mL^{-1} of STM in the coexistence of other tested bacteria. As shown in Figure 6B, the absorbance of the mixture of all bacteria (positive, with STM strain CMCC 50115) was >10 times higher than that of the mixture of other bacteria (negative, without STM), indicating that our nano-ELAAS system was highly selective for STM. Furthermore, to quantitatively compare the difference responses of nano-ELAAS to the different STM isolates including two standard strains (CMCC 50115 and ATCC 14028) and two clinical isolates, a one-way ANOVA was conducted. On the basis of Figure 6C, there was no statistically significant difference ($P > 0.05$) for the detection of the four different STM isolates by the nano-ELAAS. It was thus clear that the nano-ELAAS system could equally detect the different STM isolates.

To estimate the reproducibility of the nano-ELAAS method, seven repetitive measurements of STM (5×10^4 CFU mL^{-1}) with the proposed system were made. The obtained signals with relative standard deviation of 3.05% showed the acceptable detection precision. We also performed studies of the storage stability of the nano-ELAAS system by determining S/B of the system in the absence and presence of STM (5×10^4 CFU mL^{-1}). When the MMP-aptamers and nanoprobes were stored at 4 °C for 2 weeks, no obvious change of the S/B was observed, which proved the stability of the nano-ELAAS system. Furthermore, it has been well-documented that highly curved surfaces of AuNPs improve the stability and the activity of enzymes,[40,41] which might also contribute to the long lifetime and high sensitivity of the nano-ELAAS assay.

3.6. Analysis of STM in Real-Life Samples. To estimate the accuracy of the nano-ELAAS method in practical applications, recovery tests were used. The milk was purchased from a local supermarket without any pretreatment and was first analyzed STM by standard culture and colony counting method. Then, the milk was spiked with STM at the desired

concentrations (1×10^3, 1×10^5, and 1×10^6 CFU mL^{-1}), and STM was analyzed by the nano-ELAAS method. Recoveries of STM in the range of 90.2 ± 8.3% and 96.4 ± 5.2% (see Supporting Information, Table S2) were calculated, indicating that the proposed nano-ELAAS method could be employed to detect STM in real-life samples with good accuracy.

4. CONCLUSIONS

In the nano-ELAAS method, MMPs were modified with STM-binding aptamers instead of antibodies to act as the capture probe, while nanoprobes containing a large amount of HRP and reporter antibodies were used as signal amplifiers. By comparing to the colorimetric and chemiluminescent assay, nano-ELAAS achieved a low detection limit of 10^3 CFU mL^{-1} and wide detection range, with low cross-reactivity with other bacteria and high specificity to STM. In addition, this method has a potential to detect other bacteria by changing aptamer and detector antibody and holds a great promise for the determination of multiple pathogens in a short period of time. In conclusion, the nano-ELAAS, with its comprehensive design and high-efficiency analytical performance, could help in escaping the limitation of the ELISA.

■ ASSOCIATED CONTENT

Supporting Information

Relevant experimental results and data not included in the paper associated with binding curves of STM-binding aptamer, aggregation test of AuNPs, TEM images and UV−vis spectra of AuNPs before or after coating, sequences of oligonucleotides employed in this work, and analysis of STM in milk samples by nano-ELAAS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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