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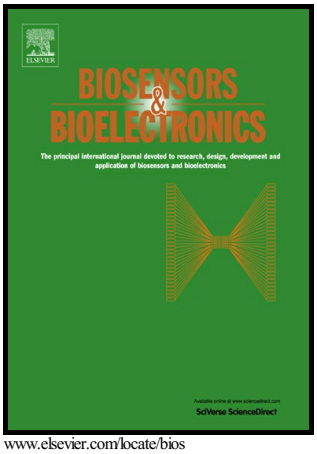

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PII: S0956-5663(16)30914-9
DOI: http://dx.doi.org/10.1016/j.bios.2016.09.037
Reference: BIOS9149

To appear in: *Biosensors and Bioelectronic*

Received date: 19 May 2016
Revised date: 8 September 2016
Accepted date: 10 September 2016

Cite this article as: Peng Zhang, Hui Liu, Xiaocheng Li, Suzhen Ma, Shuai Mer Heng Wei, Jingjing Cui and Hongning Wang, Label-free fluorescent direct detection of live *Salmonella typhimurium* using cascade triple trigger sequences regenerated strand displacement amplification and hairpin template-generated scaffolded silver nanoclusters, *Biosensors and Bioelectronic* http://dx.doi.org/10.1016/j.bios.2016.09.037

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Label-free fluorescent direct detection of live *Salmonella typhimurium* using cascade triple trigger sequences-regenerated strand displacement amplification and hairpin template-generated-scaffolded silver nanoclusters

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Abstract

The harm of *Salmonella typhimurium* (*S. typhimurium*) to public health mainly by the consumption of contaminated agricultural products or water stresses an urgent need for rapid detection methods to help control the spread of *S. typhimurium*. In this work, an intelligently

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designed sensor system took creative advantage of triple trigger sequences-regenerated strand displacement amplification and self-protective hairpin template-generated-scaffolded silver nanoclusters (AgNCs) for the first time. In the presence of live *S. typhimurium*, single-stranded trigger sequences were released from aptamer-trigger sequences complex, initiating a branch migration to open the hairpin template I containing complementary scaffolds of AgNCs. Then the first strand displacement amplification was induced to produce numerous scaffolds of AgNCs and reporter strands which initiated a branch migration to open the hairpin template II containing complementary scaffolds of AgNCs. Then the second strand displacement amplification was induced to generate numerous scaffolds of AgNCs and trigger sequences which initiated the third branch migration and strand displacement amplification to produce numerous scaffolds of AgNCs and reporter strands in succession. Cyclically, the reproduction of the trigger sequences and cascade successive production of scaffolds were achieved successfully, forming highly fluorescent AgNCs, thus providing significantly enhanced fluorescent signals to achieve ultrasensitive detection of live *S. typhimurium* down to 50 CFU/mL with a linear range from 10^2 to 10^7 CFU/mL. It is the first report on a fluorescent biosensor for detecting viable *S. typhimurium* directly, which can distinguish from heat denatured *S. typhimurium*. And it develops a new strategy to generate the DNA-scaffolds for forming AgNCs.

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Keywords: Fluorescence biosensor; Label-free; *Salmonella typhimurium*; Aptamer; Strand displacement amplification; Silver nanocluster

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1. Introduction

Salmonella is one of the most major pathogens and the main cause of foodborne illnesses worldwidely. Among more than 2, 500 serovars of *Salmonella enterica*, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) has emerged as one of the most common zoonotic pathogens worldwidely, mainly by the consumption of animal-related products (Nandakumar et al., 2008). Thus, the detection of *S. typhimurium* has become a more and more important issue. The conventional culture-based approach used for detecting *S. typhimurium* is tedious and time-consuming, usually taking up to 5 days to obtain determined results (Sheikhzadeh et al., 2016). Therefore, rapid methods based on enzyme-linked immunosorbent assay (ELISA) (Lee et al., 1990), real-time multiplex PCR (Maurischat et al., 2015), immunochromatographic strip (Wang et al., 2015) and microfluidic chip (Patterson et al., 2013) have been developed. However, these methods always suffer from false positive, sophisticated, expensive instruments and highly skilled personnel. In addition, the DNA-based methods can not detect *S. typhimurium* directly, which suffer from the complicated procedures, such as cell lysis and DNA extraction, and have an inability to discriminate between live and heat denatured *S. typhimurium*, which may cause meaningless results. Up to present, some novel strategies including fluorescence biosensors (Cho et al., 2014; Kim et al., 2015), FRET-based optical fiber biosensor (Ko and Grant, 2006), electrochemical biosensors (Afonso et al., 2013; Bagheryan et al., 2016; Dong et al., 2013; Labib et al., 2012; Nandakumar et al., 2008; Salam and Tothill, 2009; Sheikhzadeh et al., 2016), microfluidic biosensor (Guo et al., 2015) and Raman based biosensor (Zhang et al., 2015) for rapid detection of *S. typhimurium* have been developed. Among them, the fluorescent biosensor is especially attractive because of its high sensitivity, easy readout, small reaction volume,

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easy-to-manipulation and low background signal. But the existing biosensors are restricted from utilizing immobilized or fluorophores-labeled antibodies as the specific detection probes of *S. typhimurium*. Additionally, the antibody faces the challenge of low stability, expensive modification and is easy to lose activities to bind to target antigens. All these drawbacks limit the application of antibody in the detection. Therefore, it looks particularly important and urgent to look for an alternative to antibody.

Aptamer is a single-stranded oligonucleotide (RNA or DNA) that is obtained *in vitro* using systematic evolution of ligands by exponential enrichment (SELEX) from a large random oligonucleotide library (Ellington and Szostak, 1990; Tuerk and Gold, 1990) and can combine directly and selectively with a wide range of targets, particularly including the *S. typhimurium* (Duan et al., 2013; Kolovskaya et al., 2013; Labib et al., 2012; Wu et al., 2014). Although the antibody-based method can also detect *S. typhimurium* directly, aptamers show many obvious advantages compared with antibodies, including cheap and facile synthesis, easy and multiple-selective labeling, high target affinity and specificity, good stability and design flexibility. Hence, aptamers are increasingly utilized as alternative recognition elements to antibodies for the improvement of the biosensor. However, the fluorescent biosensor with the use of aptamers for the detection of live *Salmonella* reported in recent years is still rare (Zhang et al., 2016). Up to now, the fluorescence biosensor designed for detecting live *S. typhimurium* has not been reported. Thus, the development of ultrasensitive, label-free, modification-free and DNA extraction-free fluorescent biosensor is imperative and meaningful for the direct detection of live *S. typhimurium*.

Trigger sequence-induced strand displacement amplification (T-SDA) is one of isothermal reactions in which one strand of a DNA duplex or a terminal of the stem-loop structure is

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displaced by the trigger sequence at a short overhanging single-stranded domain, forming a new double-stranded structure with more thermodynamic and kinetic stability. So far, T-SDA has attracted ever-increasing attention, especially in fluorescent detection (Du et al., 2016; Liu et al., 2016). However, the reported T-SDA needs to be improved due to the the limitation of the number of trigger sequences which is not of recycled utilization.

In this research, a non-label, non-modification, non-DNA extraction, isothermal and ultrasensitive fluorescent biosensor based on the specific aptamer in combination with triple self-protective hairpin template-assisted strand displacement amplification-driven formation of scaffolds of AgNCs has been developed for detecting viable *S. typhimurium* directly. The biosensor can avoid high background, tedious fluorescent labeling, preconcentration of samples and DNA extraction and can distinguish live *S. typhimurium* directly from other bacteria, particularly including heat denatured *S. typhimurium* with high target affinity and specificity for live *S. typhimurium*. So far as we know, this is the first case of a fluorescent biosensor for the direct detection of live *S. typhimurium* in a label-free, modification-free and DNA extraction-free form, with innovative application of triple strand displacement amplification which takes advantage of hairpin template-mediated self-protection and trigger sequences cyclic generation, introducing innovations to generate the DNA-scaffolds for forming AgNCs, leading to cascade increment of the fluorescence intensity of AgNCs, thereby achieving ultrasensitive detection of viable *S. typhimurium* down to 50 CFU/mL with a linear range of 10^2 to 10^7 CFU/mL. And it is the first report on synthesizing metal nanoclusters to detect live bacteria, revealing the application of metal nanoclusters is extended to the detection of viable bacteria. Furthermore, the proposed strategy can be developed into a versatile platform for detecting various targets, thus it can be

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regarded as a potential improvement direction towards simple, sensitive, rapid, accurate, cost-effective and direct detection for viable pathogens.

2. Materials and methods

2.1. Bacterial Strains and Cultivation Conditions

The *S. typhimurium* (the American Type Culture Collection (ATCC) 14028), *Salmonella enteritidis* (*S. enteritidis*, ATCC 13076), *Salmonella pullorum* (*S. pullorum*, ATCC 13036), *Escherichia coli* (*E. coli*, ATCC 25922) and *Staphylococcus aureus* (*S. aureus*, ATCC 25923) were preserved in our lab. All above bacterial strains were grown in Lysogeny broth (LB) for 12 h at 37 °C with shaking, and then incubated onto plate count agar by a serial 10-fold dilution at 37 °C for 18 h. The culture colonies on the plates were measured as the number of live bacteria in CFU/mL. 1 mL of *S. typhimurium* at 10^6 CFU/mL was used for heat assisted inactivation at 100 °C for 15 min. To check the heat-inactivation treatment, deactivated cultures were plated onto LB plates and incubated at 37 °C for 48 h to confirm that no colony was observed.

2.2. Chemicals and materials

The DNA sequences (listed in Table S1) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The Bsm DNA polymerase and Nb.Bpu10I nicking endonuclease were purchased from Thermo Scientific, USA. Silver nitrate ($AgNO_3$) and sodium borohydride ($NaBH_4$) were purchased from Frontier Scientific, Inc (Logan, Utah, USA). All chemicals were of analytical reagent and were used without further purification. Ultrapure water obtained from a Millipore filtration system was used throughout the experiments.

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2.3. Instrumentation

The fluorescence emission spectra was obtained on a fluorometer F-7000 (Hitachi, Japan). The transmission electron microscope (TEM) image was obtained by Tecnai G^2 F20 (FEI, USA). The 10 % Native PAGE results were scanned by a digital camera.

2.4. Detection of live S. typhimurium

Before the detection, the trigger sequences and aptamer (100 nM) were heated at 95 °C for 10 min and gradually cooled to room temperature in 40 μL of 1× PBS buffer. Then the aptamer-trigger sequences complex was incubated with a certain concentration of *S. typhimurium* at 37 °C for 30 min. After centrifugation at 3000 rpm for 5 min at 4 °C, the supernatant solution was added into 30 μL of the 0.25 μM template I, 250 μM dNTPs, 0.25 U/μL Nb.Bpu10I, 0.15 U/μL Bsm DNA polymerase, 10 mM Tris-HNO_3 (pH 8.0), 10 mM $Mg(NO_3)_2$, 100 mM KNO_3 and 0.1 mg/ml BSA, and then added 0.25 μM of template II and incubated at 37 °C for 90 min.

Then, 500 μM of $AgNO_3$ was added into the reaction mixture in the sodium citrate buffer (10 mM, pH 7.0). After vigorous shaking for 30 s and centrifugation at 12 000 rpm for 5 min at room temperature, the supernatant solution was incubated at room temperature, in the dark, for 15 min. Following with the addition of 18 μM of freshly prepared $NaBH_4$ and vigorous shaking for 30 s, the solution was incubated at room temperature, in the dark, for 20 min, to conduct reduction of Ag^+ ions by $NaBH_4$. The fluorescence intensity of produced DNA-AgNCs was obtained with emission wavelengths at 643 nm with excitation at 573 nm.

2.5. S. typhimurium selectivity studies

In order to investigate the effects of negative bacteria, the *S. enteritidis*, *S. pullorum*, *E. coli*, *S. aureus* and heat-inactivated *S. typhimurium* were added into the sensing system, respectively. The

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experiment procedure was the same as those described in the preceding experiment for live *S. typhimurium* detection.

2.6. Preparation of chicken meat samples contaminated by S. typhimurium

The cultured *S. typhimurium* was inoculated into 25 g chicken meat at the concentrations from 2×10^5 to 2×10^9 CFU/mL and minced under the sterile environment. Then the minced chicken meat samples were homogenized in 200 mL of sodium citrate buffer (10 mM, pH 7.0), following the filtration to remove large debris. The resultant solutions were detected using the presented biosensor.

3. Results and discussion

3.1. Principle of the fluorescent biosensor

The proposed principle for label-free, modification-free and DNA extraction-free fluorescent detection of live *S. typhimurium* based on the target-induced assembly of AgNCs is illustrated in Scheme 1. In the sensing system, an ingeniously designed capture probe complex that contained *S. typhimurium*-aptamer and partially hybridized trigger sequences is used for viable *S. typhimurium* recognition. Template I and II are comprised of the antisense strands of scaffolds for the synthesis of fluorescent AgNCs and two half-recognition sites of the Nb.Bpu10I nicking endonuclease in middle bound by antisense strands of trigger sequences or reporter strands on either side, respectively. More importantly, they are newly designed to form hairpin structures to lock partial complementary strands of scaffolds for the synthesis of fluorescent AgNCs, with protruding the 3′ end which provides as a toehold, initiating a branch migration to open the hairpins only when the latter hybridize to the trigger sequences or reporter strands. In the presence of live *S. typhimurium*,

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it binds to *S. typhimurium*-aptamer, leading to the liberation of single-stranded trigger sequences from the capture probe complex. The liberated trigger sequences further hybridize to the toehold of template I, initiating a branch migration to open template I, and the first cycle of strand displacement amplification is triggered in the presence of dNTPs/Bsm DNA polymerase to generate the scaffolds of AgNCs and reporter strands. And then, the released reporter strands can hybridize to the toehold of template II, initiating a branch migration to open template II, and following the second cycle of strand displacement amplification to generate the scaffolds of AgNCs and trigger sequences, achieving cyclic generation of the trigger sequences. Then, the trigger sequences can hybridize to the toehold of template I, initiating a branch migration to open template I, and the third cycle of strand displacement amplification is induced to produce lots of scaffolds of AgNCs and reporter strands which trigger the next new cycle of branch migration and strand displacement amplification as stated above, achieving cyclic reproduction of the trigger sequences and leading to the successive release of scaffolds of AgNCs. Finally, the generated scaffolds are used for the synthesis of fluorescent AgNCs in the presence of Ag^{+} by the reduction of $NaBH_4$ to achieve enhanced fluorescence signals for sensitive detection of live *S. typhimurium* down to a low CFU level in a non-label and non-modification form.

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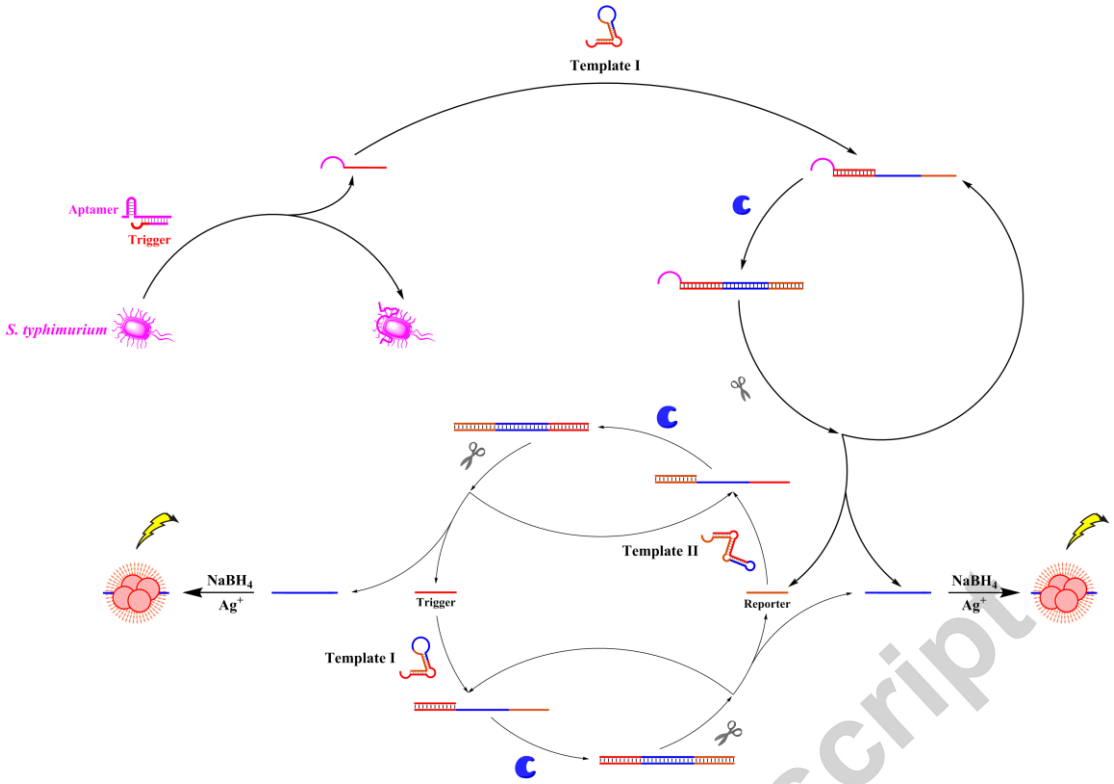

Scheme 1. Schematic illustration of the label-free, modification-free and DNA extraction-free fluorescent direct detection of viable *S. typhimurium* based on the triple trigger sequences-regenerated strand displacement amplification-driven assembly of the fluorescent AgNCs.

3.2. Characterizations of fluorescent AgNCs

In this work, the formation of AgNCs was realized by reducing $AgNO_3$ with $NaBH_4$ stabilized by the DNA template generated *via* the triple strand displacement amplification in the presence of live *S. typhimurium*. And the synthesis of the AgNCs was characterized by using TEM. As shown in Fig. 1, the TEM image clearly showed that AgNCs were synthesized exploiting DNA as the template produced by the triple trigger sequences-regenerated strand displacement amplification successfully. The AgNCs were approximately spherical in shape with the average size of about 2 nm and had well-dispersion in aqueous solution. In addition, the obvious crystal lattice structure of

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an individual AgNC was presented in the high-resolution transmission electron microscopy (HR-TEM) image, indicating the fluorescent AgNC had high crystallinity (Fig. 1 inset). Up to now, it was the first time to develop a target-triggered triple strand displacement amplification to generate the DNA-scaffolds for forming AgNCs.

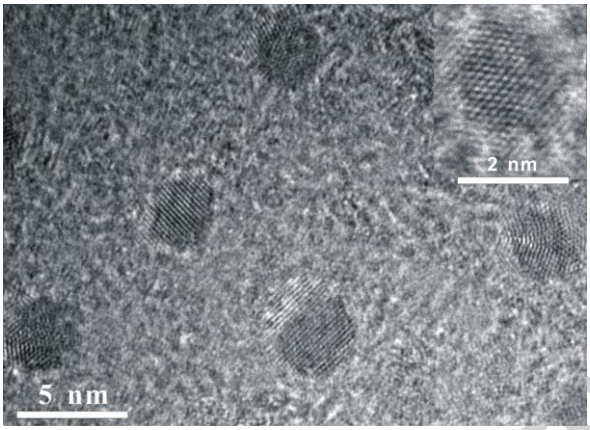

Fig. 1. TEM image of triple trigger sequences-regenerated strand displacement amplification product-templated AgNCs and HR-TEM image of an individual AgNC (inset).

3.3. Verification of the discrimination capability of the biosensor

In order to verify the feasibility of the present method for viable *S. typhimurium* detection directly, the fluorescent emission spectra under various conditions were recorded. As shown in Fig. 2A, a small fluorescent intensity (curve b) was observed when live *S. typhimurium* was absent, which was considered as the background signal. The low background signal might be benefited from the stable hairpin structures of template I and II in the absent of viable *S. typhimurium* to reduce the nonspecific amplification and the following scaffolds of AgNCs. A fluorescent intensity (curve a) as low as the background signal was measured when the template I was removed from the system, suggesting that the first strand displacement amplification failed to be performed efficiently and the subsequent two strand displacement amplifications could not be initiated

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successfully, with almost no generation of scaffolds to synthesize AgNCs. When the template II was taken away from the system, a higher fluorescence intensity (curve c) was observed comparable to the background signal, indicating that the first-step strand displacement amplification was performed to produce a small number of scaffolds for forming AgNCs, but large numbers of scaffolds of AgNCs were generated owing to the cyclic triple strand displacement amplification mediated by the synergies between template I and II. A litter higher fluorescence change (curve d) compared with the background signal was obtained in the presence of *S. pullorum*, which indicated the presence of a tiny amount of AgNCs in the system since *S. pullorum* failed to open the aptamer-trigger sequences complex and then performed an unsuccessful strand displacement amplification. On the contrary, a significantly enhanced fluorescent signal (curve e) was recorded in the presence of live *S. typhimurium*, proving triple strand displacement amplification induced by viable *S. typhimurium* was performed successfully to generate numerous scaffolds of AgNCs, and the fluorescent AgNCs were synthesized successfully, presenting a significantly amplified fluorescent intensity.

The native polyacrylamide gel electrophoresis of different reaction solutions was further used to verify the present method for sensitive *S. typhimurium* detection (shown in Fig. 2B). When the template I was removed from the system, only a template II band was observed (lane a), demonstrating that template I must be needed to initiate the triple strand displacement amplification. When there was no template II, a template I band and a weaker scaffolds of AgNCs band were observed (lane c), revealing that the first cycle of strand displacement amplification was performed successfully, but only producing a small number of scaffolds of AgNCs. When live *S. typhimurium* was absence in the system, hardly any scaffolds of AgNCs band appeared (lane b),

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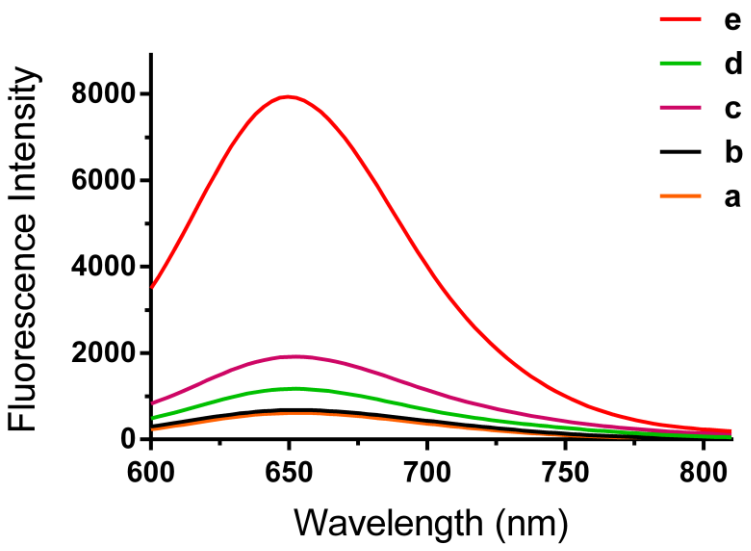
but the new aptamer-trigger sequences complex band was observed, and the template I template II band were as strong as that in lane a and c, respectively, revealing that the triple strand displacement amplification failed to be performed efficiently in the absence of viable *S. typhimurium*. A weak DNA band appeared when *S. pullorum* was presence in the system (lane d), suggesting *S. pullorum* non-specifically bound to the aptamer of capture probe complex to initiate an unspecific amplification, generating some DNA with the same mobility of scaffolds of AgNCs. When live *S. typhimurium* was presence in the system (lane e), a bright scaffolds of AgNCs band and a weaker aptamer-trigger sequences complex band were observed compared with lane b and d, proving the triple strand displacement amplification was initiated successfully due to the presence of viable *S. typhimurium*, and the numerous scaffolds of AgNCs were generated successfully. These results were in agreement with those in Fig. 2A, indicating the feasibility of our sensing protocol.



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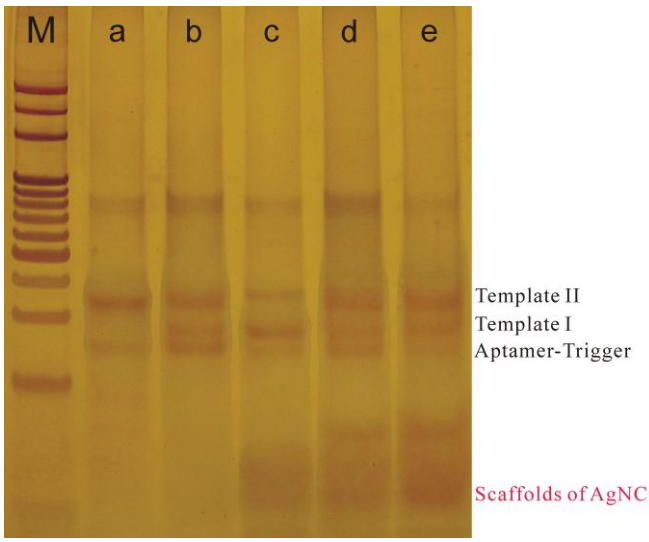

Fig. 2. Fluorescence responses (A) and native polyacrylamide gel electrophoresis image (B) under different conditions: (a) control experiment in the absence of template I, (b) negative experiment in the absence of live *S. typhimurium*, (c) control experiment in the absence of template II, (d) negative experiment in the presence of *S. pullorum* at 10^7 CFU/mL, and (e) positive experiment in the presence of viable *S. typhimurium* at 10^6 CFU/mL.

3.4. Optimization of reaction conditions

The analysis parameters were optimized by selecting F/F_0 or fluorescence intensity as standard to reach the best sensing performance, where F and F_0 were the fluorescent intensities of the reaction solutions in the presence and absence of live *S. typhimurium*, respectively. The concentrations of aptamer-trigger sequences complex, template I, template II, Bsm DNA polymerase, Nb.Bpu10I nicking enzyme and reaction time were optimized (depicted in Fig. S1-S6), respectively. The concentration of aptamer-trigger sequences complex was firstly analyzed on the basis of the F/F_0 due to it would have a key effect on the detection range of the fluorescent biosensor. For one thing, the lower concentration of aptamer-trigger sequences complex could limit the detection range. For another, the higher concentration of aptamer-trigger sequences

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complex would likely lead to higher background signal. The Fig. S1 showed that the concentration of aptamer-trigger sequences complex of 100 nM could obtain the better F/F_0 than that at other concentrations. Therefore, the concentration of 100 nM aptamer-trigger sequences complex was selected as the optimized concentration for the following live *S. typhimurium* detection.

The concentrations of template I and template II played a vital role in the efficiency of the triple strand displacement amplification. On the one hand, the lower concentrations of template I and II might restrict the amplification efficiency because a large number of the trigger sequences or product sequences generated in the triple strand displacement amplification were needed to hybridize to the enough template I or template II, then initiating the strand displacement amplification to generate the scaffolds of fluorescent AgNCs. On the other hand, the higher concentrations of template I and II might cause higher background signal due to the generation of dimmers resulted from potential nonspecific amplification involving in the template I and II. Accordingly, the concentration of template I was assayed from 100 nM to 220 nM, and following the concentration of the template II was varied from 120 nM to 250 nM. As shown in Fig. S2 and S3, the values of F/F_0 reached the maximum when the concentrations of template I and II were 180 nM and 200 nM, respectively. Therefore, these concentrations were applied in the subsequent experiments.

The concentrations of the Bsm polymerase and Nb.Bpu10I nicking enzyme were optimized because they were the core elements in the triple strand displacement amplification. It could be observed from Fig. S4 that, the maximum F/F_0 was observed with a Bsm polymerase concentration of 0.15 U/μL. Similarly, a peak-shape dependence of F/F_0 on nicking enzyme concentration was also recorded (Fig. S5). The maximum F/F_0 was obtained at the point of 0.25

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U/μL. Thus, the 0.15 U/μL and 0.25 U/μL were selected as the optimum concentrations of the Bsm polymerase and Nb.Bpu10I nicking enzyme, respectively, in the following detection.

As the reaction time had an important effect on increasing fluorescence intensity, the reaction time was assayed by recording the fluorescence intensity at a time interval of 15 min from 15 to 120 min. It was observed that the fluorescent intensity was no longer increasing after the reaction time reached 90 min (shown in Fig. S6) because the enzymes were inactivated, achieving the saturation of the reaction. To minimize time delays and increase the production of scaffolds for the synthesis of fluorescent AgNCs, 90 min was chosen as the optimal reaction time.

3.5. Sensitivity

The sensitivity of the fluorescent biosensor based on aptamer-induced cascade triple strand displacement amplification was verified by varying concentrations of viable *S. typhimurium*. It was seen from Fig. 3A that, the fluorescence intensity increased as the concentration of live *S. typhimurium* ranged from 50 to 10^7 CFU/mL, suggesting that the generation of the AgNCs highly relied on the concentration of viable *S. typhimurium*. It validated the working principle that live *S. typhimurium* bound with the aptamer and released the trigger sequences, initiating the triple strand displacement amplification, and finally generating the AgNCs. The Fig. 3B indicated the linear correlation between fluorescence intensity of AgNCs and the concentration of viable *S. typhimurium*. Under the optimum condition, the average fluorescence intensity exhibited a good linear relationship with the concentration of live *S. typhimurium* varying from 10^2 to 10^7 CFU/mL. The detection limit of the proposed biosensor was measured to be 50 CFU/mL on the basis of the rule of the blank measurements plus 3 times the standard deviation (3 σ), achieving a significantly improved detection sensitivity compared with the reported biosensors that involved in the

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detection of *S. typhimurium* (Guo et al., 2015; Kim et al., 2015; Sheikhzadeh et al., 2016). The ultra-sensitivity and the wide detection range might be ascribed to the low background signal, efficient signal amplification strategy and effective fluorescence characteristic of AgNCs.

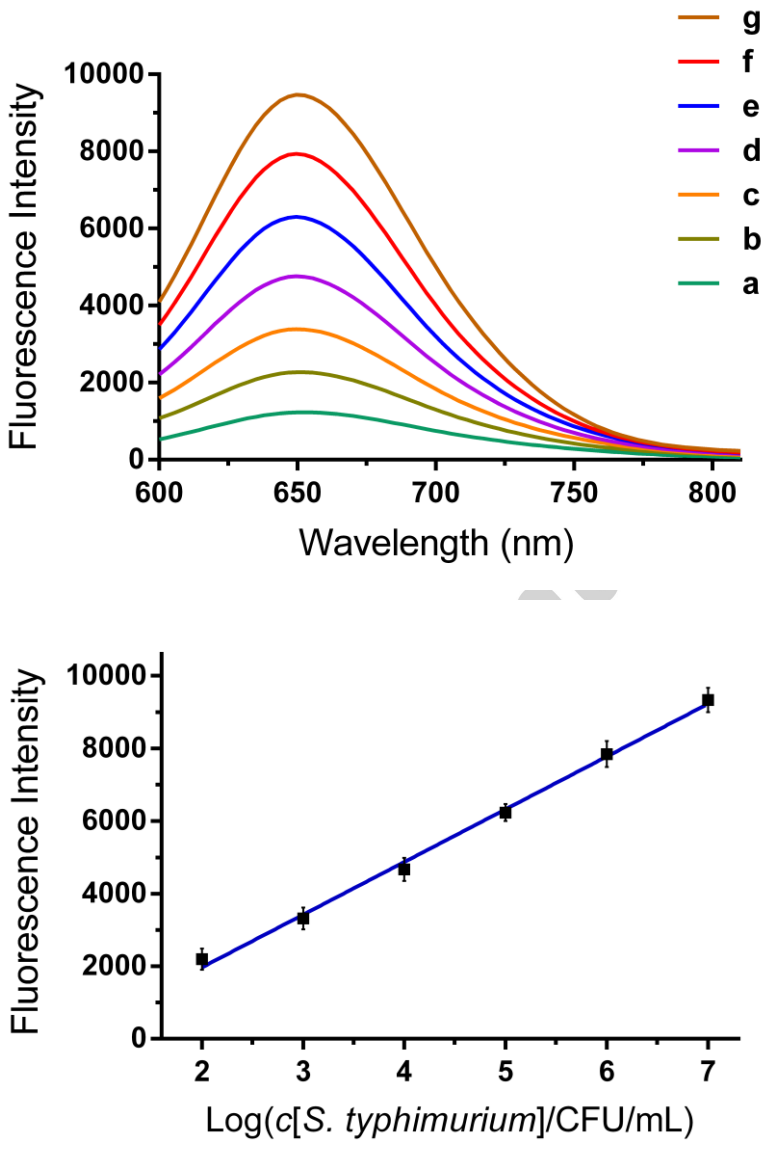

Fig. 3. (A) Effect of different concentrations of live *S. typhimurium* on the fluorescent response: (a) 50 CFU/mL, (b) 10^2 CFU/mL, (c) 10^3 CFU/mL, (d) 10^4 CFU/mL, (e) 10^5 CFU/mL, (f) 10^6 CFU/mL and (g) 10^7 CFU/mL. (B) Linear relationship between the fluorescence intensity and the concentration of viable *S. typhimurium* on logarithmic scales. Each data point represented an average of 3 measurements (each error bar indicated the standard deviation).

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3.6. Specificity

The specificity of the fluorescent biosensor was further tested by adding five kinds of control bacteria, including *S. pullorum*, *S. aureus*, *E. coli*, *S. enteritis* and heat-inactivated *S. typhimurium* at the same concentration of 10^6 CFU/mL and live *S. typhimurium* at 10^5 CFU/mL, respectively. As demonstrated in Fig. 4, there was no significant fluorescence intensity increment upon the addition of aforesaid bacteria except viable *S. typhimurium*. This high specificity could be owed to the good selectivity and high affinity of the aptamer against live *S. typhimurium*, which was selected by a modified Cell-SELEX technology (Kolovskaya et al., 2013). Specifically, the ability to distinguish from the heat denatured *S. typhimurium* might be because of the heat induced degradation of outer membrane proteins of viable *S. typhimurium*, thus resulting in its weak binding ability to the aptamer and unsuccessful strand displacement amplifications. Thus, these results confirmed the good specificity of the proposed biosensor was suitable for the direct detection of live *S. typhimurium*.

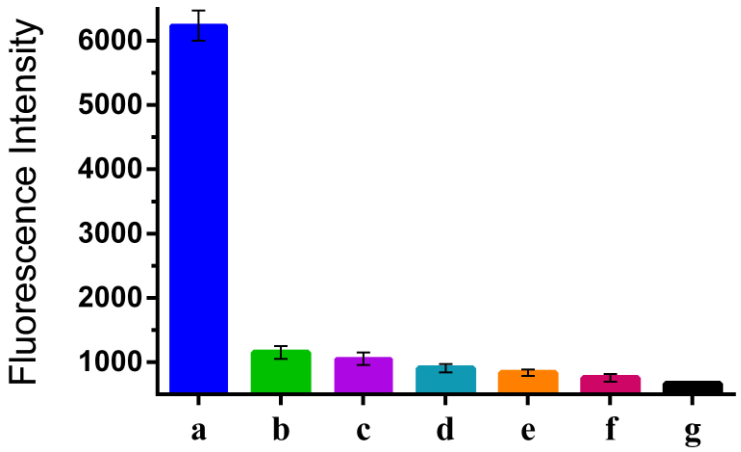

Fig. 4. Selectivity evaluation of the proposed biosensor for the detection of (a) live *S. typhimurium* at 10^5 CFU/mL, (b) *S. pullorum*, (c) *S. aureus*, (d) *E.coli*, (e) heat-inactivated *S. typhimurium*, (f) *S.*

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enteritidis and (g) background. The concentrations of control bacteria were all 10^6 CFU/mL. The error bars were standard deviations of three repetitive measurements.

3.7. Precision and reproducibility

As significant factors to estimate a method used for practical application, the precision and reproducibility of the designed biosensor were evaluated according to the relative standard deviation (RSD). A same sample of viable *S. typhimurium* at 10^3 CFU/mL with five repeated measurements was conducted to measure the precision of the proposed method and obtained a RSD of 3.0 %. The result revealed that the proposed strategy shared acceptable precision and reproducibility.

3.8. Detection of live S. typhimurium in chicken meat samples

The practicability of the developed biosensor was also considered by detecting viable *S. typhimurium* in chicken meat samples. The cultured live *S. typhimurium* was inoculated into chicken meat at the concentrations from 10^3 to 10^7 CFU/mL, the presented biosensor was then used for detecting viable *S. typhimurium* after a pretreatment process for the chicken meat samples. The results in Table S2 indicated that the biosensor for detecting live *S. typhimurium* was not interfered in these complex components. This could be owing to the high affinity between viable *S. typhimurium* and the aptamer, and high efficiency of triple strand displacement amplification. These results validated the potential application of the developed biosensor in real samples.

4. Conclusions

In summary, we have developed a novel fluorescent biosensor for the direct detection of live *S.*

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typhimurium. By creatively utilizing the aptamer-based target recognition with triple self-protective hairpin template-mediated strand displacement amplification to reduce the background signal and achieve trigger sequences regeneration to improve the sensitivity, the biosensor has an ultrasensitive detection limit of 50 CFU/mL with a broad linear range from 10^2 to 10^7 CFU/mL. The merits are that the biosensor is label-free, modification-free, DNA extraction-free, isothermal and ultrasensitive. More importantly, this biosensor can distinguish viable *S. typhimurium* from heat denatured *S. typhimurium*. In addition, the research provides a new approach for generating the DNA-scaffolds for forming AgNCs and it is the first study to apply metal nanoclusters to detect live bacteria, extending the application of metal nanoclusters in the detection of viable bacteria. This fluorescent biosensor can be developed into a potentially versatile system for the direct detection of a large number of viable pathogenic bacteria and other objects of interest by simply altering corresponding aptamers as the objects recognition probes.

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Acknowledgements

This project was supported by the “973” National Basic Research Program of China (No. 2013CB127200), Earmarked Fund for Modern Agro-industry Technology Research System (No. CARS-41) and the Special Fund for Agro-scientific Research in the Public Interest of China (No. 201303044).

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Highlights

- It is the **first** report on a fluorescent biosensor for the direct detection of live *S. typhimurium.*
- An innovative triple self-protective hairpin template-mediated strand displacement amplification is **firstly** established to achieve trigger sequences recycling generation.
- It is the **first time** to develop a target-triggered triple strand displacement amplification to generate the DNA-scaffolds for forming silver nanoclusters.
- The **first** study to apply metal nanoclusters to detect live bacteria.

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