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ORIGINAL PAPER

Development of a fluorescent DNA nanomachine for ultrasensitive detection of *Salmonella enteritidis* without labeling and enzymes

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Abstract

A capture probe complex containing a specific *Salmonella enteritidis* (*S. enteritidis*) aptamer and partly hybridized signal trigger sequence was designed with the ability to directly detect viable *S. enteritidis*. In the presence of the target *S. enteritidis*, single-stranded trigger sequences were liberated and in turn reacted with hairpins I, II, and III to initiate the triple strand migration reaction; this in turn produced numerous hairpin I·II·III complexes with scaffolds of copper nanoparticles (CuNPs) and replaced the trigger sequence which initiated the next cycle of triple migration reaction. Cyclically, the reuse of the trigger sequences and the successive, cascading production of scaffolds of CuNPs achieved the synthesis of highly fluorescent CuNPs, thus providing significantly enhanced fluorescent signals to achieve ultrasensitive detection of live *S. enteritidis* as low as 25 CFU/mL with a linear range of detection from 50 to 10^4 CFU/mL with an emission wavelength at 590 nm. By integrating the triple cascade strand migration amplification with recyclable trigger sequences, aptamer-based target recognition, and self-protection mediated by CuNPs hairpin scaffolds, this is the first report on a non-labeled, non-enzymatic, modification-free, and DNA extraction-free ultrasensitive fluorescent biosensor for the direct detection of live *Salmonella*, which is distinguished from dead *Salmonella*. It also provides a new strategy to detect viable bacteria by applying the CuNPs, thus extending the application of metal nanoparticles.

Keywords Nanomachine · Label-free · *Salmonella enteritidis* · Aptamer · Copper nanoparticle

Peng Zhang, Mengxiao Song and Linqin Dou contributed equally to this work.

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Introduction

The detection of *Salmonella enteritidis* has great significance to public health and the agricultural industry [1–3]. The conventional method applied to the detection of *S. enteritidis*, which relies on bacterial culturing, is laborious and time-consuming; this usually takes up to 5 days to obtain viable results [4]. Thus, quicker methods such as enzyme-linked immunosorbent assay (ELISA) [5], real-time fluorescence qPCR [6], immunochromatographic strip assay [7], and loop-mediated isothermal amplification [8] have been reported. However, the above methods usually suffer from false positive results and the expense materials. Additionally, the application of PCR-based methods is limited due to the inability to detect *S. enteritidis* directly; moreover, PCR-based methods have suffered from restrictions such as complex procedures including bacterial lysis and DNA extraction, and the inability to discern the difference between live and dead *S. enteritidis*, causing false positive results. Recently, some novel strategies including fluorescent biosensors [1, 2, 9], surface plasmon resonance (SPR)–based biosensors [10, 11], electrochemical biosensors [12–14], and lateral flow biosensors [15] have been developed for the accurate detection of *S. enteritidis*. However, an enzyme-free biosensor for this bacterial detection has not been reported. The development of a label-free, DNA extraction-free, and enzyme-free fluorescent biosensor will be desirable, especially for the direct detection of live *S. enteritidis*.

DNA nanomachines are nanodevices designed by utilizing the accurate pairing of complementary nucleic acids, in which the opening and re-arrangement of complementary DNA/RNA can be initiated by certain triggers (e.g., the addition of nucleic acid, enzyme, or pH changes), followed by controllable changes of the DNA conformation along with mechanical motions. Energy transfers are completed during this process as well. DNA nanomachines can capture, store, and release target molecules, enabling "machine" functionality. Four base types A, T, C, and G contained in DNA give DNA nanomachines sequence diversity. Moreover, they have artificial structural diversity due to the variable sequence combinations and flexible design of DNA bases. The unique base-pairing principle of DNA makes it highly programmable and motion controllable. Accordingly, DNA nanomachines have had important roles in many fields, such as bio-therapy [16, 17], bio-imaging [18], and bio-sensing [19–22]. However, many currently reported nanomachines remain limited due to the single function, thus restricting the application of DNA nanomachines. Therefore, it is especially urgent and consequential to look for a novel multi-functional DNA nanomachine.

In this work, a label-free, modification-free, extraction-free, and enzyme-free fluorescent nanomachine is proposed on the basis of the formation of CuNP scaffolds driven by hairpin-assisted, triple isothermal strand migration reaction. The practicability of nanomachine is exploring by developing a biosensor, allowing for the direct detection of viable *S. enteritidis* based on the specific aptamer. This strategy attempts to establish a novel fluorescent nanomachine, which can be applied as an integration platform of diagnosis of live pathogens.

Experimental

Bacterial strains and cultivation conditions

Salmonella enteritidis (the American Type Culture Collection (ATCC) 13076), *Salmonella typhimurium* (ATCC 14028), *Salmonella pullorum* (ATCC 13036), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) were stored in the laboratory. All above bacterial strains were grown in lysogeny broth (LB) for 12 h at 37 °C with shaking and then incubated at 37 °C for 18 h on plate count agar after a serial 10-fold dilution. The cultured colonies were counted as the number of viable bacteria in CFU/mL. A total of 1 mL of *S. enteritidis* at 10^4 CFU/mL was inactivated by heating it to 100 °C for 15 min. To verify the success of the thermal process, heat-inactivated cultures were plated onto LB plates and incubated at 37 °C for 48 h and to ensure that no colony could be observed.

Chemicals and materials

All high-performance liquid chromatography (HPLC)–purified oligonucleotides (listed in Table S1) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Sodium ascorbate and 3-(N-morpholino) propanesulfonic acid (MOPS) were purchased from Sigma-Aldrich (Shanghai, China). Sodium chloride and copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). All reagents were of analytical grade and were used without additional purification. Ultrapure water (18.2 MΩ·cm) obtained from a Millipore filtration system (Billerica, MA, USA) was used throughout the experiments.

Instrumentation

The fluorescence emission spectrum was obtained on a fluorometer F-7100 (Hitachi, Japan). The transmission electron microscope (TEM) image was obtained by Tecnai G2 F20 (FEI, USA).

Detection of *S. enteritidis*

Before detecting the bacteria, the aptamer (3.12 nM) and trigger sequence (2.60 nM) were heated at 95 °C for 10 min and

gradually cooled to room temperature in 40 μL of 10 mM MOPS buffer. Then, the aptamer/trigger sequences were incubated with 1 mL of *S. enteritidis* at 25 °C for 30 min. After centrifugation (5 min, 3000 ×*g*, 4 °C), the supernatant solution was incubated with hairpins I, II, and III (0.19 μM, each) for 90 min at 37 °C.

Then, ascorbate (3.12 mM) was added into the stock solution of the fluorescent biosensor system and incubated for 30 min at room temperature. Finally, $CuSO_4$ (64.94 μM) was added into the solution and the fluorescence spectra of the resulting CuNPs were recorded immediately. The excitation and emission wavelengths of the resulting DNA-CuNPs were performed at 345 nm and 590 nm, respectively, and the fluorescence intensity was measured using a fluorometer F-7100 (Hitachi, Japan).

S. enteritidis selectivity studies

In order to analyze the influence of negative control bacteria, *S. typhimurium*, *S. pullorum*, *E. coli*, *S. aureus*, and heat-inactivated *S. enteritidis* were used with the biosensor. The experimental process was the same as that described for *S. enteritidis* detection.

Preparation of pork meat samples contaminated by *S. enteritidis*

The cultured *S. enteritidis* was inoculated into 25 g pork meat at the concentrations from 2×10^4 to 2×10^7 CFU/mL and minced under the sterile environment. Then, the minced pork meat samples were homogenized in 200 mL of MOPS buffer (10 mM, pH 7.5), following the filtration to remove large debris. The resultant solutions were detected using the developed biosensor.

Results and discussion

Principle of the fluorescent biosensor

The proposed principle of fluorescent nanomachine-based biosensor for detection of *S. enteritidis* is illustrated in Fig. 1. In this sensing system, a capture probe complex containing the *S. enteritidis* aptamer and partly hybridized signal trigger sequence was designed and used for viable *S. enteritidis* recognition (Fig. S1). Each of hairpins I, II, and III was comprised of a half strand of the double-stranded scaffolds required for the synthesis of fluorescent CuNPs. More importantly, these hairpin structures could lock partial strands of the scaffolds used for the synthesis of fluorescent CuNPs and the templates applied to the performance of strand migration reaction. This enabled hairpins to have dual self-protection function for scaffolds of CuNPs and templates of strand migration reaction (Fig. S2). The protruding 3′-end of each hairpin provided a toehold to initiate a branch migration to conditionally open the hairpin given that they hybridized with the trigger sequences or other hairpins. In the presence of the target *S. enteritidis*, the binding between *S. enteritidis* and aptamer structure led to the liberation of single-stranded trigger sequences from the capture probe complex. The free trigger sequences further hybridized to the toehold of hairpin I, initiating a branch migration to open hairpin I with reporter strand 2* on the 5′-end. Then, the released reporter strand 2* could hybridize to hairpin II via the toehold of hairpin II, initiating a branch migration to open hairpin II, and caused the following formation of the complete CuNP scaffolds with reporter strand 3* on the 5′-end. Afterwards, the released reporter strand 3* could hybridize to hairpin III via the toehold of hairpin III, initiating a branch migration to open hairpin III, and led to the subsequent CuNP scaffolds formation with reporter strand 1* on the 5′-end. Next, the released reporter strand 1* could hybridize to hairpin I to initiate the dissociation of the trigger sequences, thus achieving the cyclic reuse of the trigger sequences. Then, the trigger sequences could hybridize to hairpin I via the toehold strand to initiate a new cycle of isothermal strand migration reaction. Those cycles achieved cascading signal amplification and led to the successive release of hairpin I·II·III Y-shaped scaffolds (Fig. S3). Finally, the Y-shaped scaffolds assisted the synthesis of fluorescent CuNPs in the presence of Cu^{2+} via the reduction of sodium ascorbate, creating enhanced fluorescent signals for the sensitive detection of live *S. enteritidis*. This happened even at low CFU levels, without the use of labeling, enzymes, or DNA extraction.

Characterizations of fluorescent CuNPs

The CuNPs generated via triple strand migration reaction in the presence of *S. enteritidis* were used to achieve enhanced fluorescent signals in a non-label, non-enzymatic, and non-modified form. To verify exactly whether the fluorescent signals were produced by CuNPs or not, the final solution was characterized by using TEM in an intuitive way. As shown in Fig. 2b, well-dispersed nanoparticles in the aqueous solution were almost spherical in shape, and the average size was approximately 5.08 nm (Fig. 2b). The HR-TEM image clearly showed the crystal lattice structure of an individual CuNP (inset in Fig. 2a). The above results indicated that CuNPs were synthesized successfully, proving that the Y-shaped scaffolds generated via the triple strand migration reaction in the presence of *S. enteritidis* were exploited as the template for the synthesis of fluorescent CuNPs in the presence of Cu^{2+} by the reduction of sodium ascorbate. This was a successful attempt to develop an enzyme-free and label-free amplification method to generate the DNA scaffolds for forming CuNPs and

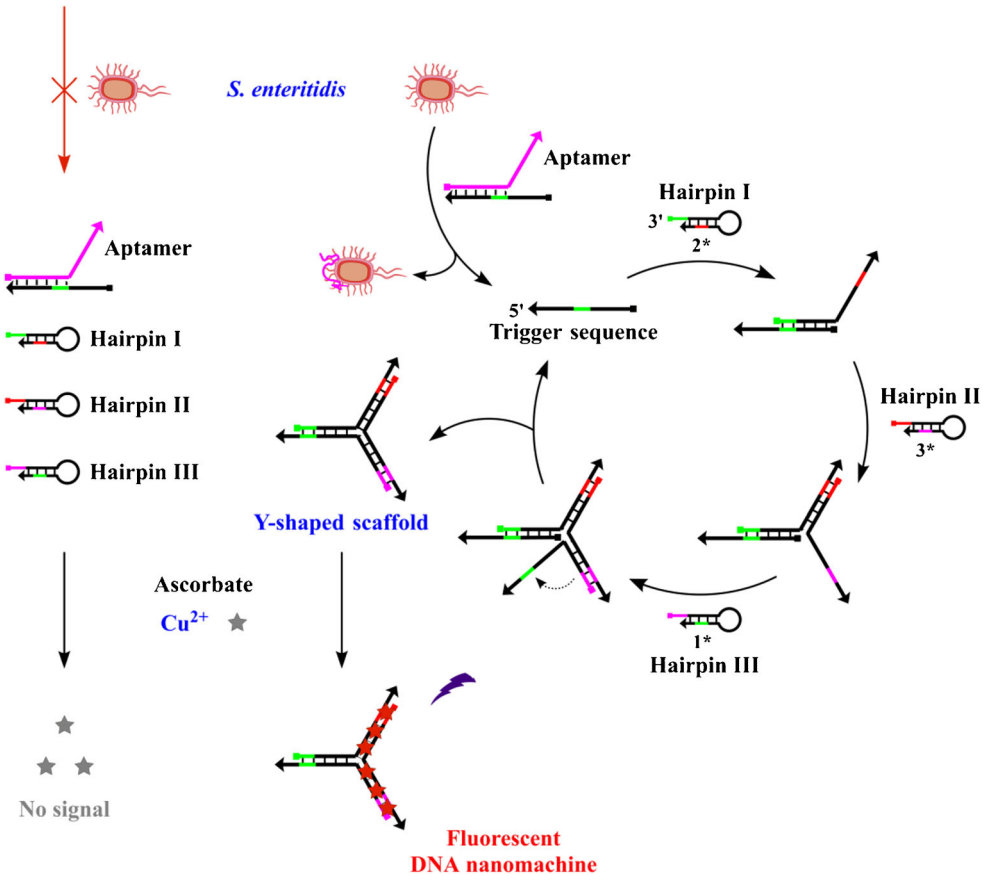

Fig. 1 Schematic illustration of the label-free, enzyme-free, modification-free, and DNA extraction-free direct detection of viable *S. enteritidis* based on a novel fluorescent nanomachine

provide a new approach for producing CuNPs as a functional component of DNA nanomachine.

Verification of the discrimination capability of the biosensor

In order to verify the feasibility of the present method for sensitive *S. enteritidis* detection, the fluorescence emission spectra were recorded under various conditions. As shown in Fig. 3a, there was a negligible fluorescent response from Cu^{2+} and ascorbate (a), revealing that the DNA scaffolds were required for the synthesis of fluorescent CuNPs. A similar low fluorescence intensity (b) was observed when hairpins I, II, and III were exposed to the Cu^{2+} and ascorbate, suggesting that only specific DNA templates had the ability to initiate the synthesis of fluorescent CuNPs, and hairpins I, II, and III could stably coexist. A slightly higher fluorescence intensity (c) was observed when *S. enteritidis* was absent, and this signal was deemed as the background signal. The low background signal may be a result of the stability of the aptamer/trigger sequences, hairpins I, II, and III displayed in the absence of *S. enteritidis*, which thus reduced any nonspecific

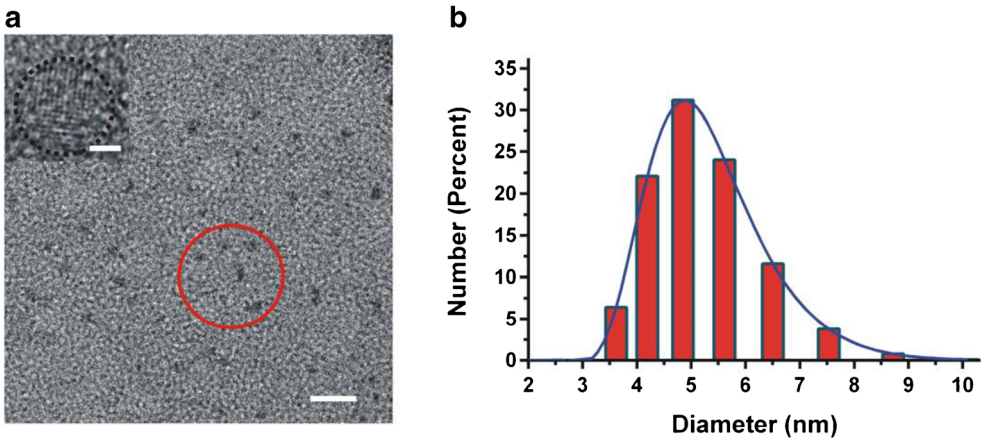

Fig. 2 The fluorescent characterization of the products generated from the reduction reaction. (**a**) The TEM image. Scale bar, 50 nm. Inset: the HR-TEM image of an individual CuNP. Scale bar, 2 nm. (**b**) Size distribution analysis of the reaction products

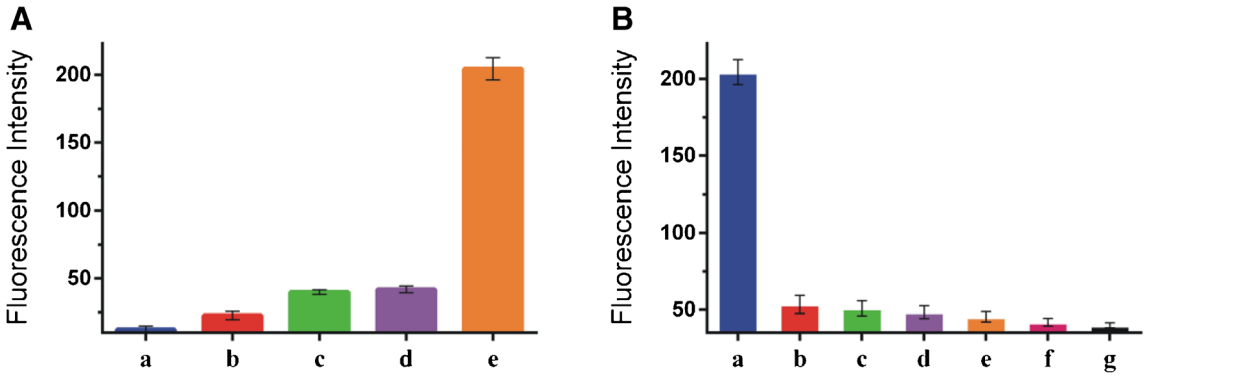

Fig. 3 The analytical characterization of the developed fluorescent nanomachine-basedprotection strategy to achieve high selectivity biosensor. (**A**) Fluorescence responses under different conditions: (a) control experiment in the presence of Cu^{2+} and ascorbate; (b) control experiment in the presence of hairpins I, II, and III; Cu^{2+}; and ascorbate; (c) negative experiment in the absence of *S. enteritidis*; (d) control experiment in the presence of 10^4 CFU/mL *S. typhimurium*; and (e) positive experiment in the presence of 10^4 CFU/mL *S. enteritidis*. (**B**) Selectivity evaluation of the proposed biosensor for the detection of (a) live *S. enteritidis* at 10^4 CFU/mL, (b) *S. pullorum*, (c) *S. aureus*, (d) *E. coli*, (e) heat-inactivated *S. enteritidis*, (f) *S. typhimurium*, and (g) background signal. The concentrations of the control bacteria were all 10^4 CFU/mL. The error bars were standard deviations of three repetitive measurements

strand migration reaction. In the presence of *S. typhimurium*, there was a very weak fluorescence intensity enhancement (d) in comparison with the background signal, which indicated the presence of a tiny amount of CuNPs in the system due to the failure of *S. typhimurium* to open the aptamer/trigger sequences and the subsequent unsuccessful, isothermal strand migration reaction. Conversely, a significant fluorescent signal (e) was obtained in the presence of *S. enteritidis*, indicating that numerous scaffolds had been generated for CuNPs synthesis. This proved that the successful subsequent isothermal strand migration reaction was only initiated by *S. enteritidis*, and the following scaffolds generation and CuNPs synthesis enhanced the fluorescence intensity significantly.

Optimization of methods

The following parameters were optimized: (a) aptamer-to-trigger sequences ratio; (b) concentrations of hairpins I, II, and III; (c) reaction time; (d) concentration of Cu^{2+}. Respective text and figures on optimizations were given in the Electronic Supporting Material. In short, the following experimental conditions were found to give best results: (a) the optimal aptamer-to-trigger sequences ratio was 6:5; (b) the better concentrations of hairpin I, hairpin II, and hairpin III were all 194.81 nM; (c) optimal reaction time was 90 min; (d) the better concentration of Cu^{2+} was 64.94 μM.

Detectability

The detectability of the fluorescent nanomachine-based biosensor was tested by varying the concentration of *S. enteritidis*. It was shown from Fig. 4a that the fluorescence intensity increased as the concentration of *S. enteritidis* increased from 25 to 10^4 CFU/mL, suggesting that the generation of the CuNPs heavily relied on the concentration of *S. enteritidis*. These results validated the working principle that *S. enteritidis* bound to the aptamers and released the trigger sequences, which triggered the isothermal, triple strand migration reaction and generated the scaffolds for the CuNPs. Figure 4b showed the linear correlation between the fluorescence intensity of the CuNPs and the concentration of *S. enteritidis*. Under the optimized conditions, the average fluorescence intensity showed a strong linear relationship with the concentration of *S. enteritidis* varying from 50 to 10^4 CFU/mL. The limit of detection (LOD) of the developed biosensor was determined to be 25 CFU/mL ($n = 3$); this value revealed significantly advanced detection sensitivity compared with some other reported aptamer-based biosensors used in the detection of *S. enteritidis* (Table 1). This high sensitivity and broad detection range may be ascribed to the target-induced, triple cascade strand migration reaction with trigger sequence recycling and the effective fluorescent characteristic of CuNPs. What's more, the biosensor had label-free, enzyme-free, modification-free, DNA extraction-free, and ultrasensitive advantages. These results demonstrated that the developed signal amplification strategy was sensitive, accurate, and cost-efficient for the ultrasensitive detection of *S. enteritidis*.

Selectivity

The specificity of the fluorescent nanomachine-based biosensor was further evaluated by exposing the aptamer/trigger sequences to five kinds of bacteria, including *S. enteritis*, *S. typhimurium*, *S. pullorum*, *E. coli*, *S. aureus*, and heat-inactivated *S. enteritidis* at the same concentration of 10^4 CFU/mL. As shown in Fig. 3b, no significant fluorescence intensity increase was observed upon the addition of the aforementioned bacteria except for *S. enteritidis*. This high specificity may arise from the high affinity and specificity of

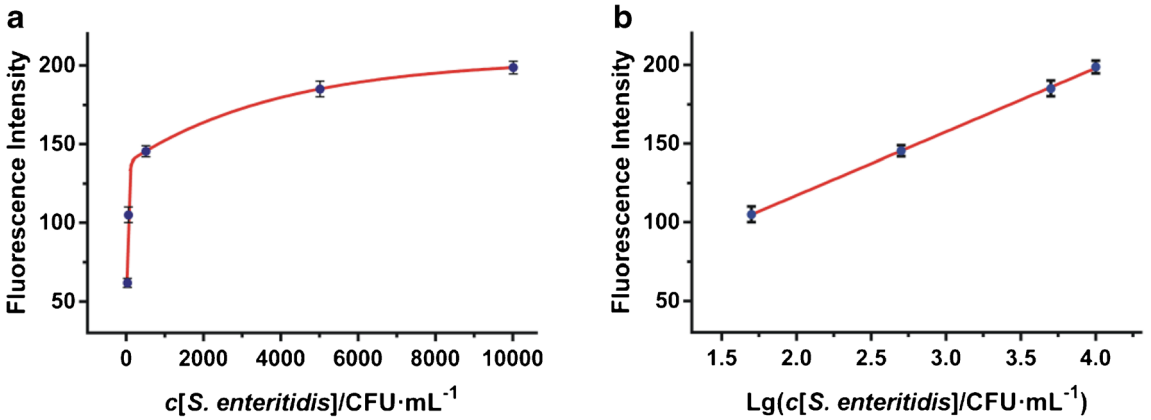

Fig. 4 The sensitivity of the developed fluorescent nanomachine-based biosensor for live *S. enteritidis* detection. (**a**) The relationship between the fluorescence intensity and viable *S. enteritidis* concentration with 25, 50, 5×10^2, 5×10^3, and 10^4 CFU/mL. (**b**) The linear range between the fluorescence intensity and the concentrations of live *S. enteritidis* on a logarithmic scale. The error bars were the standard deviation of three repetitive measurements

the aptamer towards *S. enteritidis*, which was selected via a dozen rounds of the SELEX technique [28]. In addition, precise base pairing, hairpin-assisted dual self-protection, and specific target-induced strand migration reaction could also contribute to the specificity of the biosensor. The ability to differentiate the heat-denatured *S. enteritidis* from viable *S. enteritidis* may be due to the heat inactivation of outer membrane proteins of live *S. enteritidis*, resulting in a weak binding ability with the aptamer and ineffective strand migration reactions. Therefore, these results proved the satisfactory specificity of the developed biosensor in the application of viable *S. enteritidis* detection.

Precision and repeatability

On account of precision and repeatability being significant factors that gauge the practical application of a method, these factors of the developed assay were assessed and verified according to the relative standard deviation (RSD) values of consecutive measurements. The RSD value of the biosensor response to 5×10^3 CFU/mL of *S. enteritidis* was 3.2% (n = 5). The RSD value was found to be 6.2%, at the aforesaid concentration by detecting the same samples after 72 h at the same reaction conditions. The above result revealed that the developed biosensor shared acceptable precision and operational repeatability.

Detection of *S. enteritidis* in pork meat sample

The practicability of the designed biosensor was also evaluated by detecting *S. enteritidis* in pork meat samples. The cultured *S. enteritidis* were inoculated into pork meat using concentrations from 2×10^4 to 2×10^7 CFU/mL; the presented biosensor was then applied to detect the bacteria after the pork meat samples underwent a pretreatment process. The results in Table 2 indicated that the complex components present in the matrix resulting from the pork meat sample did not interfere with the detection of *S. enteritidis*. This outcome could be attributable to the high affinity between *S. enteritidis* and the aptamer and the high efficiency of isothermal triple amplification. These results validated the potential application of this developed biosensor in real samples. Moreover, the proposed

Table 1 The comparisons of the reported nanomaterial-based methods for *S. enteritidis* detection

Detection method	Materials used	LOD (CFU/mL)	Linear range (CFU/mL)	References
Fluorescence	Aptamer, GO	25	10^2–10^7	[23]
SERS-based lateral flow	Au^{MBA}@Ag NPs, antibody	27	27–2.7×10^6	[24]
Fluorescence	Aptamer, GO	40	40–4×10^9	[25]
Fluorescence	Aptamer, enzyme, NMM	60	10^2–10^7	[2]
Lateral flow immunoassay	Nitrogen-rich carbon NPs, antibody	10^2	10^2–10^8	[26]
Colorimetric	Aptamer, streptavidin-poly-HRP	10^3	-	[27]
Fluorescence	Aptamer, CuNPs	25	50–10^4	This work

Table 2 Application of the proposed biosensor for live *S. enteritidis* detection in pork meat samples

Sample	Target *S. enteritidis* added (CFU/mL)	Detected by the biosensor (CFU/mL)	Recovery of the biosensor (%)	RSD (%) ($n = 5$)
1	5.0×10^2	4.49×10^2	89.80	3.22
2	5.0×10^3	5.09×10^3	101.88	4.81
3	1.0×10^4	9.82×10^3	98.20	4.66

biosensor could also be developed as a versatile platform for detecting any bacterium.

Conclusions

By utilizing the aptamer-based target identification and hairpin-mediated dual self-protective strategy, a label-free and enzyme-free DNA nanomachine is established. In the present of target *S. enteritidis*, the DNA nanomachine is triggered specifically, leading to the generation of DNA-templated CuNPs. By innovatively utilizing the aptamer-based target identification and hairpin-mediated CuNP scaffolds protection strategy to achieve high selectivity and reduce the background signal, the nanomachine achieves an ultrasensitive detection of viable *S. enteritidis* with a broad linear range. More importantly, the developed biosensor can distinguish viable *S. enteritidis* from dead *S. enteritidis*. This way is the first study to develop an enzyme-free biosensor to detect *Salmonella*. It is also the first report on the usage of CuNPs to detect bacteria. The developed fluorescent biosensor needs to be further improved to achieve high-throughput, faster, cheaper detection of live pathogens.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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