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A label-free ultrasensitive fluorescence detection of viable *Salmonella enteritidis* using enzyme-induced cascade two-stage toehold strand-displacement-driven assembly of G-quadruplex DNA

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ABSTRACT

The harm of *Salmonella enteritidis* (*S. enteritidis*) to public health mainly by contaminating fresh food and water emphasizes the urgent need for rapid detection techniques to help control the spread of the pathogen. In this assay, an newly designed capture probe complex that contained specific *S. enteritidis*-aptamer and hybridized signal target sequence was used for viable *S. enteritidis* recognition directly. In the presence of the target *S. enteritidis*, single-stranded target sequences were liberated and initiated the replication-cleavage reaction, producing numerous G-quadruplex structures with a linker on the 3′-end. And then, the sensing system took innovative advantage of quadratic linker-induced strand-displacement for the first time to release target sequence in succession, leading to the cyclic reuse of the target sequences and cascade signal amplification, thereby achieving the successive production of G-quadruplex structures. The fluorescent dye, N-Methyl mesoporphyrin IX, binded to these G-quadruplex structures and generated significantly enhanced fluorescent signals to achieve highly sensitive detection of *S. enteritidis* down to 60 CFU/mL with a linear range from 10^2 to 10^7 CFU/mL. By coupling the cascade two-stage target sequences-recyclable toehold strand-displacement with aptamer-based target recognition successfully, it is the first report on a novel non-label, modification-free and DNA extraction-free ultrasensitive fluorescence biosensor for detecting viable *S. enteritidis* directly, which can discriminate from dead *S. enteritidis*.

1. Introduction

Salmonella enterica serovar Enteritidis (*S. enteritidis*) has emerged as one of most common zoonotic agents and leading causes of foodborne illnesses worldwidely (Retamal et al., 2015). Thus, as a target of interest, *S. enteritidis* has attracted lots of attention from scientific community because of its great value in public health, medical diagnosis, drug research and pathogenesis. The conventional microbiological culture method used for the detection of *S. enteritidis* is labor intensive and time consuming, usually taking up 72 h to obtain confirmed results (Dziadkowiec et al., 1995). Therefore, rapid methods such as enzyme-linked immunosorbent assay (ELISA) (Holt et al., 1995), real-time fluorescence qPCR (Li et al., 2010), immunochromatographic assay (Moongkarndi et al., 2011) and loop-mediated isothermal amplification methods (Ravan and Yazdanparast, 2012) have been developed. However, these methods usually suffer from complicated DNA extraction, false positive, expensive, sophisticated equipments and highly skilled personnel. Up to now, some novel strategies including fluorescence biosensor (Song et al., 2014; Zhang et al., 2009), surface plasmon resonance (SPR)-based biosensor (Bouguelia et al., 2013; Mazumdar et al., 2010), electrochemical biosensor (Delibato et al., 2009; Labib et al., 2012; Sun et al., 2015) and lateral flow biosensor (Fang et al., 2014) for convenient determination of *S. enteritidis* have been developed. Among them, fluorescence biosensor are particularly attractive due to their high sensitivity, easy readout, small sample volume, easy-to-operation and feasibility of quantification. But these biosensors utilize modified or fluorescence dye-labeled DNA as the specific detection probes of *S. enteritidis*. Besides, the inability of DNA-based methods to detect *S. enteritidis* directly and discriminate between live and dead bacteria also limits their application.

Aptamers are single-stranded oligonucleotides (DNAs or RNAs)

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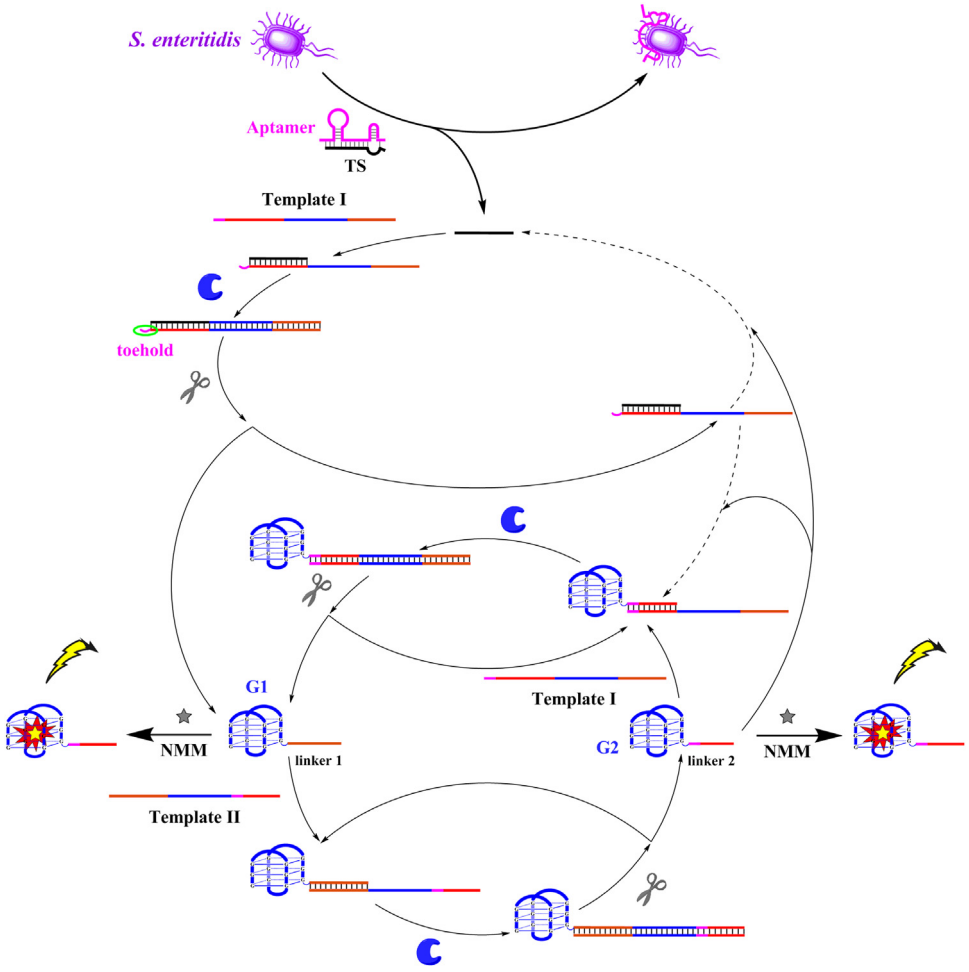
that are obtained in vitro by SELEX (systematic evolution of ligands by exponential enrichment) from large random oligonucleotide libraries (Ellington and Szostak, 1990; Tuerk and Gold, 1990) and can bind directly to a wide range of target molecules, especially including the *S. enteritidis* (Hyeon et al., 2012; Kolovskaya et al., 2013; Labib et al., 2012). Compared with antibodies, aptamers hold many superior characteristics, including simple and cheap synthesis, easy and controllable labeling, high target specificity and affinity, long-term stability and design flexibility. Thus, aptamers have been increasingly utilized as potential probes alternative to antibodies for the development of biosensors. However, the reports on the use of aptamers for the detection of *S. enteritidis* published in recent years are rare (Fang et al., 2014; Labib et al., 2012; Wu et al., 2014). Wu et al. (2014) developed a fluorescence-switch biosensor with low limit of 40 CFU/mL in 30 min and Fang et al. (2014) developed a lateral flow biosensor with lower limit of 10 CFU/mL. However, these methods usually required magnetic bead enrichments, fluorescent labeling and expensive materials. Labib et al. (2012) developed a impedimetric sensor with limit of 600 CFU/mL in 10 min, nevertheless, this strategy usually suffered from poor sensitivity and expensive equipments. Up to now, label-free aptamers that are used in fluorescence biosensor for viable *S. enteritidis* detection have not been reported. Therefore, the development of simple, label-free, modification-free and ultrasensitive fluorescence biosensor is desirable and meaningful for detecting the live *S. enteritidis* directly.

The toehold strand-displacement reaction (T-SDR) is triggered by the stretching toehold, to which the target sequences attach and then force the original stem-loop structures migrate away, forming a new DNA duplex with more thermodynamical stability (Zhang and Winfree, 2009). So far, T-SDR has attracted ever-increasing interest, especially in fluorometric detection (Chen et al., 2016; Xu and Zheng, 2016; Zhang et al., 2016). However, the T-SDR requires improvement due to the the limited number of target sequences which can not be used cyclically.

In the present study, we developed a label-free, modification-free, DNA extraction-free, isothermal and ultrasensitive fluorescence aptamer cascade sensing system based on the combination of the specific aptamer and enzyme-assisted target sequences-recyclable quadratic T-SDR-driven formation of G-quadruplex molecular for detecting viable *S. enteritidis* directly. The sensing system can avoid the tedious process of fluorescence labeling, enrichments of samples and DNA extraction and can discriminate viable *S. enteritidis* directly from dead *S. enteritidis* with the high target specificity and affinity against the viable *S. enteritidis*. To the best of our knowledge, this is the first report on a label-free, modification-free and DNA extraction-free fluorescence biosensor for a direct detection assay of viable *S. enteritidis* with creative application of quadratic T-SDR for the first time, leading to the



Scheme 1. Schematic illustration of the label-free, modification-free and DNA extraction-free sensitive fluorescent detection of *S. enteritidis* based on the cascade two-stage toehold strand-displacement-driven assembly of the G-quadruplex structures. The dashed arrows represent the two different products including the target sequences (TS) in the same reaction.

cyclic reuse of the target sequences and cascade strand-displacement and achieving highly sensitive detection of *S. enteritidis* down to 60 CFU/mL with a linear range from 10^2 to 10^7 CFU/mL. This novel strategy is considered as a potential development direction toward sensitive, simple, rapid, accurate and economic detection of viable pathogens directly.

2. Results and discussion

2.1. Principle of the fluorescence biosensor

The proposed principle for label-free, modification-free and DNA extraction-free fluorescent detection of *S. enteritidis* based on the target-induced assembly of G-quadruplex DNA is illustrated in Scheme 1. The chemically synthetic target sequence (TS) is designed to partially hybridized with the viable *S. enteritidis*-aptamer. Template I and II consist of the antisense sequence of G-quadruplex and half recognition site of the Nb.Bpu10I nicking endonuclease in middle bound by complementary sequence of TS (red) or reporter strand on either sides, respectively. In the presence of the target *S. enteritidis*, it binds to the aptamer sequence due to the high binding affinity between *S. enteritidis* and the specific aptamer and the TS is released. The liberated TS further hybridizes with template I and triggers a strand-displacement in the presence of dNTPs/Bsm DNA polymerase. The replication of the template I finally yields the duplex strand with a full recognition site for Nb.Bpu10I endonuclease, generating numerous complementary sequences that contain the G-quadruplex sequences (G1) with linker strands on the 3′-end (linker 1). Then the released strands can hybridize to the template II by the linker strands to initiate a cycle of replication-cleavage reaction to produce enormous amount complementary sequences that contains the G-quadruplex sequences (G2) with linker strands on the 3′-end (linker 2). And then, a part of the G2 sequences can hybridize to the template I by the linker strands to initiate a new cycle of replication-cleavage reaction to produce lots of G1 sequences and triggered above T-SDR cycles. Most importantly, another part of the G2 sequences can bind to the template I of template I-TS complex competitively and release the TS. The liberation of TS then initiates a new cycle of replication-cleavage reaction and strand-displacement as above, achieving cascade signal amplification and leading to the successive release of G-quadruplex structures. The fluorescent dye NMM binds to these G-quadruplex structures and generates significantly intensive fluorescent signals for sensitive detection of viable *S. enteritidis* down to a low CFU level without labeling and modification.

2.2. Verification of the discrimination capability by fluorescence spectral characteristics

In order to verify the feasibility of the proposed method for sensitive *S. enteritidis* detection, the fluorescent emission spectra under different conditions were analysed. As shown in Fig. 1, comparing with a small fluorescent response of NMM (curve a), which is owing to the tiny fluorescent emission of NMM, a slightly increased fluorescent intensity is obtained when the *S. enteritidis* was absent (curve c), which was identified as background signal. The result might be attributed to nonspecific amplification which involved template I and II. A significant fluorescence enhancement (curve f) resulting from the presence of *S. enteritidis* clearly indicated the successful release of TS and subsequent strand-displacement followed with the formation of numerous G-quadruplex which greatly enhanced fluorescence signal of NMM. On the contrary, in the presence of *S. typhimurium*, a much weak fluorescence change (curve e) compared with that in the presence of target *S. enteritidis* was recorded, which signified the presence of a very small number of G-quadruplex in the system since *S. typhimurium* failed to open the aptamer/TS with an unsuccessful strand-displacement.

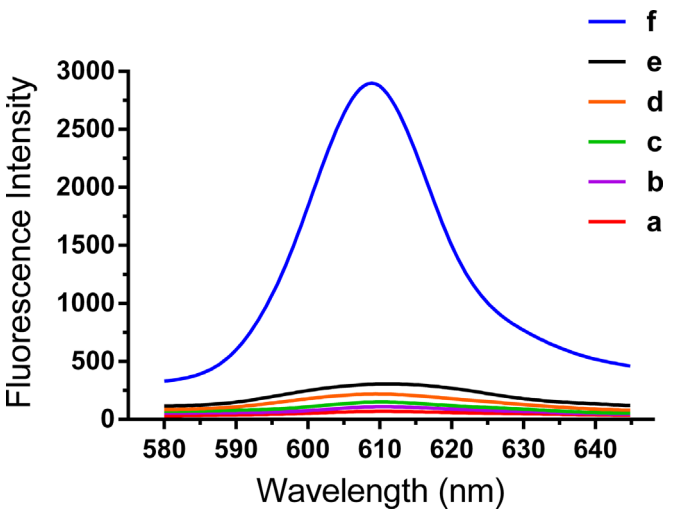


Fig. 1. Fluorescence responses under different conditions: (a) NMM, (b) control experiment in the absence of template I, (c) control experiment in the absence of aptamer/TS, (d) negative experiment in the absence of *S. enteritidis*, (e) negative experiment in the presence of *S. typhimurium*, and (f) positive experiment in the presence of *S. enteritidis*.

In a further control assay, the template I was taken away from the system, a fluorescent response as low as the background signal was observed (curve d), indicating that the 2nd strand-displacement reaction was initiated by the production generated in the 1nd strand-displacement reaction. Additional control experiment was further conducted to remove the template II from the system. In this case, a higher fluorescence response (curve b) was observed comparable to NMM itself, revealing that G-quadruplex was generated mainly due to the cascade amplification mediated by the synergistic effects between template 1 and 2.

2.3. Sensitivity

The sensitivity of the fluorescence biosensor based on enzyme-induced cascade two-stage recycling amplification strategy was examined by varying concentrations of *S. enteritidis*. As shown in Fig. 2A, the fluorescence intensity increased as the concentration of *S. enteritidis* ranged from 0 to 10^7 CFU/mL, indicating that the generation of the G-quadruplex structure highly relied on the concentration of *S. enteritidis*. This verified the working principle that the *S. enteritidis* binded with the aptamer and released the TS which triggered the isothermal amplification, and finally generating the G-quadruplex structures. Fig. 2B shows the linear relationship between the fluorescence intensity of NMM/G-quadruplex and the concentration of *S. enteritidis*. Under the optimal conditions, the average fluorescence intensity presented a good linear correlation with the concentrations of the *S. enteritidis* varying from 10^2 to 10^7 CFU/mL. The detection limit of this biosensor was measured to be 60 CFU/mL according to the rule of the blank measurements plus 3 times the standard deviation (3 σ), providing a significantly improved detection sensitivity compared with those reported fluorescence approaches that involved in the detection of *S. enteritidis* (Labib et al., 2012; Song et al., 2014). The ultrahigh sensitivity and the broad dynamic range revealed that this signal amplification strategy was efficient for ultrasensitive fluorescence detection of *S. enteritidis.*

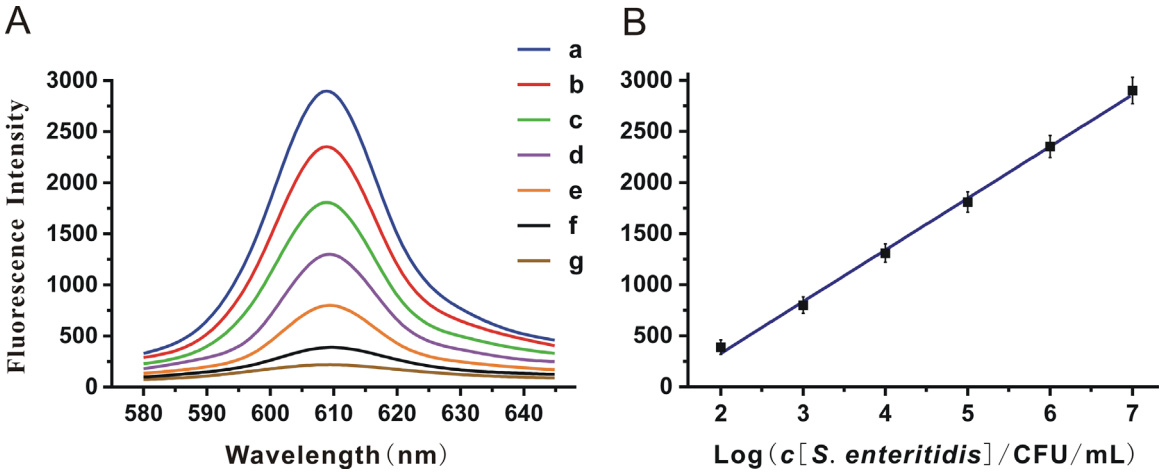


Fig. 2. (A) Effect of different *S. enteritidis* concentrations on fluorescence emission spectra: (a) 10^7 CFU/mL, (b) 10^6 CFU/mL, (c) 10^5 CFU/mL, (d) 10^4 CFU/mL, (e) 10^3 CFU/mL, (f) 10^2 CFU/mL and (g) background. (B) Linear relationship between the fluorescence intensity and the concentration of *S. enteritidis* on logarithmic scales. Each data point represents an average of 3 measurements (each error bar indicates the standard deviation).

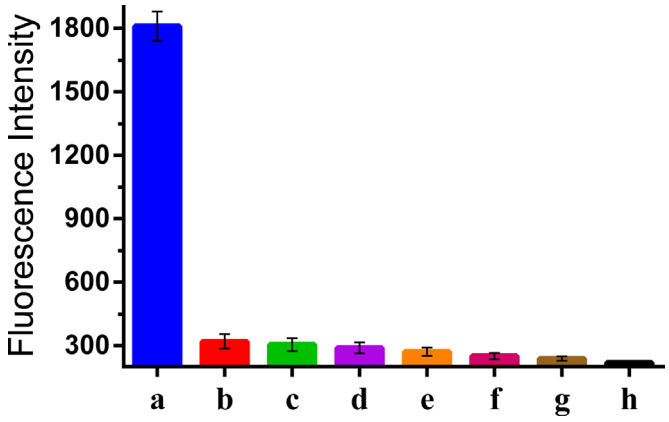


Fig. 3. Selectivity evaluation of the proposed biosensor for the detection of (a) *S. enteritidis* at 10^5 CFU/mL, (b) heat-inactivated *S. enteritidis*, (c) *S. typhimurium*, (d) *S. pullorum*, (e) *E. coli*, (f) *S. aureus*, (g) *L. monocytogenes* and (h) background. The concentrations of control were all 10^6 CFU/mL. The error bars are standard deviations of three repetitive measurements.

2.4. Specificity

The specificity of the fluorescent sensor was further inspected by exposing the aptamer/TS to six kinds of bacteria, including *S. enteritidis*, *S. typhimurium*, *S. pullorum*, *L. monocytogenes*, *E. coli*, *S. aureus* and heat-inactivated *S. enteritidis* at the same concentration of 10^6 CFU/mL. As shown in Fig. 3, there was no significant fluorescence increase upon the addition of above bacteria except viable *S. enteritidis*. This high specificity to target *S. enteritidis* might be attributed to the high affinity and good selectivity of the aptamer against viable *S. enteritidis*, which was selected via twelve rounds of SELEX techniques (Kolovskaya et al., 2013). In particular, the ability to discriminate from the dead *S. enteritidis* might be owed to the heat induced degradation of outer membrane proteins of viable *S. enteritidis*, thus leading to unsuccessful combination between dead *S. enteritidis* and aptamer, consistent with previous research (Fang et al., 2014; Wu et al., 2014). So, these results indicated the good specificity of the biosensor is suitable for detecting the viable *S. enteritidis* directly.

2.5. Precision and reproducibility

As important factors to evaluate a method in practical application, the precision and reproducibility of the proposed strategy were estimated by using the relative standard deviation (RSD) of intra- and interassay ($n=5$). The RSD of the inter-assay were 2.6%, 3.1%, and 4.0% at 10^3 CFU/mL, 10^4 CFU/mL and 10^5 CFU/mL of *S. enteritidis*, respectively. And the RSD of intra-assay were 3.6%, 4.7%, 6.8%, at the aforementioned concentrations by measuring the same samples after 72 h at the same experimental conditions. These results demonstrated that the designed assay shared acceptable precision and reproducibility.

3. Conclusions

In summary, we developed a novel fluorescence biosensor that combined the cascade two-stage target sequences-recyclable toehold strand-displacement with aptamer-based target recognition for the first time, and assessed the method using live *S. enteritidis* as an example. The biosensor had a low detection limit of 60 CFU/mL with a linear range from 10^2 to 10^7 CFU/mL. The merits of the assay are that it is label-free, modification-free and DNA extraction-free. Importantly, this biosensor can discriminate viable *S. enteritidis* from dead *S. enteritidis*. This ultrasensitive fluorescence biosensor is a potentially versatile and modular sensing platform for the detection of numerous viable pathogens directly and other targets of interest by simply changing other aptamers as the molecular recognition probes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.02.031.

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