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Short communication

Nicking enzyme-assisted biosensor for *Salmonella enteritidis* detection based on fluorescence resonance energy transfer

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ABSTRACT

Salmonella enteritidis (*S. enteritidis*) outbreaks continue to occur, and have increased public awareness of this pathogen. Nicking endonuclease Nb.BbvC I is widely used for the detection of biomolecules and displays activity for specific double-stranded DNA (dsDNA). In this study, we developed a biosensor to detect *S. enteritidis* based on fluorescence resonance energy transfer (FRET) using nicking enzyme and carbon nanoparticles (CNPs). Because of the quenching effect of black hole quencher 1 (BHQ 1), the CNPs do not fluoresce in the reaction system. When the target bacteria are added, the nicking enzyme recognizes and cleaves the dsDNA fabricated by the interaction between probe and target. As a result, the CNPs dissociate from BHQ 1and emit strong fluorescence. Using the nicking enzyme, the fluorescence signals of the biosensor are greatly amplified. The biosensor exhibited a linear relationship with the concentration of *S. enteritidis* ranging from 10^2 to 3×10^3 CFU/mL in water and from 1.5×10^2 to 3×10^3 CFU/mL in milk. The present results indicate that our FRET-based detection system can be widely employed for the effective detection of pathogens.

1. Introduction

Foodborne pathogens can contaminate food at every step of food production, storage, and distribution. *Salmonella enteritidis* (*S. enteritidis*) is a gram-negative food pathogen that affects the abdomen of exposed individuals and leads to infection, diarrhea and pain. Infection with *S. enteritidis* is an important problem facing humans (Rodrigue et al., 1990), so it is desirable to develop rapid, sensitive, selective and real-time detection technologies to identify this pathogen.

Various monitoring techniques have been developed to detect *S. enteritidis*, such as electrical measurement and the enzyme-linked immunosorbent assay (ELISA). Electrical measurement is based on the impedance characteristics of electrodes and media where bacteria exist (Labib et al., 2012, 2013). ELISA is based on affinity between antibody and antigen. But they all require time-consuming pre-enrichment steps and a relatively costly procedure. The polymerase chain reaction (PCR) has become the gold standard for the detection of *S. enteritidis* (Sokolov and Sokolov, 2013). However, PCR is complicated for diagnosis, and requires sophisticated and expensive equipment and skilled operators. Moreover, PCR can give false positives because of nonspecific amplification during the complicated thermal cycling steps (Zhi et al., 2013).

Existing biosensor technologies use a combination of biological receptor compounds and transducer directing. Examples include surface plasmon resonance (SPR) devices and physical or physicochemical transducers based on specific DNA sequences or aptamers (Hyeon et al., 2012; Jongerius-Gortemaker et al., 2002). These sensors can detect a broad spectrum of analytes in complex sample matrices with high performance. Nevertheless, problems associated with the high cost and poor stability of materials used in assays remain an obstacle. For instance, Mao et al. (2014) employed a method which based on hybridization between oligonucleotides probe and specific DNA sequence (Mao et al., 2014). They have to amplify the target by asymmetric PCR, due to DNA was double strand and it cannot hybridize with probe in nature. Yang et al. (2013) used aptasensor and carbon nanotubes (CNTs) to detect pathogen. Considering the affinity of aptamers that specifically adhere around the target is not as specific as antibodies, this strategy is not impeccable (Jayasena, 1999; Yang et al., 2013).

Ribosomal ribonucleic acid (rRNA) molecules have been used to improve the detecting ability of sensors because they allow the design of taxon-specific oligonucleotides (Harvey et al., 2001; Salazar et al., 2000). Hybridization using oligonucleotides directly as sensors for 16S rRNA could be an interesting alternative to identify *S. enteritidis* much more rapidly than current methods. One prominent hybridization format in microbiology is fluorescence *in situ* hybridization (FISH) with nucleic acid probes (Almeida et al., 2013). FISH is based on the specific binding of a nucleic acid probe, which is a synthetic single-stranded nucleic

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acid capable of hybridizing with complementary nucleic acid targets.

We have developed a biosensor based on oligonucleotide-specific hybridization and fluorescence resonance energy transfer (FRET). FRET involves energy transfer from a carbon nanoparticle (CNP) to a black hole quencher 1 (BHQ 1) when these two species are in close proximity to each other (Tyagi and Kramer, 1996). DNA nicking endonuclease was to construct the FRET-based biosensor because it exhibits high sensitivity for dsDNA, and allows the sensor to operate at room temperature without specialized instruments (Bi et al., 2010; Higgins et al., 2001; Lin et al., 2011). Traditional assays are usually based on a recognition interaction between a target and detection probe with a 1:1 stoichiometric ratio, which means that each target can only bind to one fluorescent probe to generate a signal. In our method, use of DNA nicking endonuclease means that each target strand can go through many cycles, leading to cleavage of numerous molecular beacons (MB), which provides a lower limit of detection (LOD) than other methods (Kim et al., 2007, 2008; Li et al., 2010; Ohk and Bhunia, 2013). Compared with existing technologies like SPR, our method is simpler and more convenient for the rapid detection of *S. enteritidis* (Bokken et al., 2003).

2. Material and methods

2.1. Chemicals and materials

CNTs were obtained from Shenzhen Nanotech Port Co. Ltd. (Shenzhen, China). A specific sequence was chosen from the V3–V6 region of 16S rRNA, and updated from the NCBI gene bank. Oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), as shown in Fig. 1S. The MB contained a probe sequence consisting of nine nucleotides, CNPs and BHQ 1. Four sequences of single-stranded DNA (ssDNA) were fixed on four groups of CNTs respectively.

Nicking endonuclease Nb.BbvC I and NEBuffer 2 were purchased from New England BioLabs (Ipswich, MA, USA). NEBuffer 2 contained 50 mM potassium chloride, 10 mM tris–acetate (pH 7.4), 10 mM magnesium chloride and 1 mM dithiothreitol. Hydrochloric acid and lactose were purchased from Generay Biotech Co., Ltd. (Shanghai, China). Lysozyme egg white, N, N-dimethylformamide (DMF), N-hydroxy-sulfosuccinimide (NHS) and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDAC-HCl) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). SYBR Green I was purchased from Fanbo Biochemical Co., Ltd. (Beijing, China). Water was purified before use by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Apparatus

Fluorescence signal measurements were performed using a fluorometer (Perkin-Elmer Ltd., Beaconsfield, England) connected to a personal computer. Excitation and emission slits were both set with a band pass of 10.0 nm. The CNPs were excited at 490 nm, and emission spectra were collected from 500 to 700 nm. The fluorescence intensity at 515 nm was used to evaluate the performance of the developed assay. All measurements were carried out at room temperature unless stated otherwise.

2.3. Preparation of MBs

CNPs were fabricated by a one-step alkali- or the acid-assisted ultrasonic chemical method (Li et al. 2011). Lactose (5 g) was added to hydrochloric acid (100 mL, 37% w/v). The mixture was treated at 80 °C and 300 W in an ultrasonic bath (Yijing ultrasonic instrument Co., Ltd., Shanghai, China). The pH of the homogenized solution was adjusted to 7.4. After the sediment was separated, the remaining solution was centrifuged at 21,255*g* for 40 min. The supernatant (0.5 μL) was added to water (469.5 μL) to obtain 10^3 times dilution. EDAC-HCl (10 g/L, 10 μL) was added to the diluted solution, which was then treated with NHS (5 g/L, 10 μL) and BHQ-DNA sequence solution (10 μL) after 20 min. The final volume of the solution was 500 μL. The solution was stored at 5 °C overnight.

2.4. Preparation of CNT-DNA

CNTs (40 mg) were dissolved in DMF (100 mL) under ultrasonic conditions (200 W). This solution was then diluted to give a concentration of CNTs of 40 mg/L. EDAC-HCL (10 g/L, 10 μL) was added at room temperature, and then after 20 min, NHS (5 g/L, 10 μL) and BHQ-DNA sequence solution (100 μM, 10 μL) were added. The volume of the final solution was 500 μL, and it was stored at 5 °C until use.

2.5. Analysis of target sequence in water

The model analyte of 16 S rRNA (10^{-7} mol) was dissolved in water (1 mL). CNT-DNA (2.5 μL) was added to water (29 μL), followed by NEbuffer 2 (10 ×, 5 μL), MB (10 μL), and target sequence (2.5 μL). The mixture was incubated at 27 °C for 40 min. Nb.BbvC I (1 μL) was added, and then the fluorescence signal amplification reaction was carried out at 37 °C for 45 min. The same reaction mixture without target sequence was used as a control. The volume of the reaction mixture was 50 μL, and it contained 100 nM CNT-DNA, 400 nM MB, 1 × NEbuffer 2, and different concentrations of target sequence. Finally, the reaction mixture was moved to a suitable vessel for fluorescence detection.

2.6. Analysis of S. enteritidis in milk

S. enteritidis were separately mixed with milk (1.0 mL) in different concentrations. After equilibration of the mixtures at room temperature for 10 min, they were subjected to centrifugation (1735*g*) for 10 min and then the sediments were transferred into different tubes. Lysozyme (200 ppm) was added to each sediment, and then the resulting mixture was aged at room temperature for 2 h to obtain lysate of *S. enteritidis*. CNT-DNA (2.5 μL) was added to water (29 μL), followed by NEbuffer 2 (10 ×, 5 μL), MB (10 μL), and lysate of *S. enteritidis* (2.5 μL) (final concentration from 30 to 5×10^3 CFU/mL). The reaction mixture was incubated at 27 °C for 40 min. Nb.BbvC I (1 μL) was added, and then the fluorescence signal amplification reaction was carried out at 37 °C for 45 min. The same reaction mixture without lysate was used as a control.

2.7. Exploration of the hybridization of 16S rRNA with CNT-DNA

S. enteritidis was diluted into 10^5 CFU/mL (sample A) and then added into reaction mixture (containing CNT-DNA modified by a magnetic bead at 5′ end). The same reaction mixtures with synthesized target sequence (20 bp) (5 μM, sample B) and lysate of *S. typhimurium* (10^5 CFU/mL, sample C) were also prepared. Reaction mixture without target sequence of bacteria was used as a control. And then they were washed out impurity using magnetic grate and added SYBR Green I (300 nM). After 1 h incubation at 37 °C, the fluorescence signal of SYBR Green I was measured (reaction solutions were excited at 485 nm. Excitation and emission slits were both set with a band pass of 10.0 nm.).

3. Results and discussion

3.1. Design of the sensor

This study is the first time that 16S rRNA and nicking enzyme-assisted fluorescence signal-assisted amplification have been simultaneously used to detect bacteria. CNPs were used instead of conventional organic dyes to construct MB because they possess superior chemical inertness and lower toxicity (Li et al., 2011; Ray et al., 2009). Fig. 1 displays a schematic diagram of the sensor. The ssDNA (CNT-DNA sequence) used to modify the surface of the CNTs contains two sections: a complementary DNA sequence of 16S rRNA (blue) and complementary DNA sequence to recognize the nicking enzyme (red). The MB (green) possess a cleavage site, so they can completely hybridize with CNT-DNA to form a double-stranded DNA (ds-DNA) (Ding et al., 2011). The binding of the complementary DNA sequence of 16S rRNA and target unzipped the connection between the CNT-DNA and CNTs, because the CNT-DNA was energetically more stable when hybridized with 16S rRNA than when entwined around CNTs. Meanwhile, MB easily hybridizes with CNT-DNA to form dsDNA, which is a substrate of Nb.BbvC I. Then, the nicking enzyme quickly binds to and cleaves the MB. The MB is then too short to maintain its conformation, so when the CNPs and BHQ 1 disconnect, a fluorescence signal appears. In the absence of the target, MB will not be hybridized and cleaved because the 3′ end of the CNT-DNA sequences is anchored onto the CNTs through a covalent bond, which prevents the release of the sequences from the CNTs.

3.2. Preparation of MB

The effects of different concentrations of BHQ-DNA and CNPs on the behavior of the sensor are presented in Fig. 2S. The concentrations of BHQ-DNA sequence in the mixture (500 μL) were 0.5, 1, 1.5, 2, and 2.5 nM. As the concentration of BHQ-DNA sequence increased, the fluorescence intensity of the CNPs decreased, which was caused by energy being transferred from the CNPs to BHQ 1. It was also found that 0.2 mg CNPs was completely quenched by 10 μL BHQ-DNA sequence (100 μM).

3.3. Optimization of the CNT-DNA sequence

The efficiency of target detection of the MB is measured by its signal-to-background (S/B) ratio, which is the ratio of the fluorescence signal in the presence of the target to the fluorescence signal before the addition of the target. In this work, MB could hybridize with the CNT-DNA sequence only when the probe was released. Based on this strategy, we compared the fluorescence characteristics of three different CNT-DNA sequences by changing the length between the complementary sequence of the target and the cleavage site of the nicking enzyme, as shown in Fig. 3S. In this experiment, the sample contained 5×10^3 CFU/mL lysate of *S. enteritidis* and the control contained all of the same components except lysate. The highest S/B ratio was observed for sensor 2, in which the length between the complementary sequence of the target and the cleavage site of the nicking enzyme was only three bases. This is because the hybridization efficiency of MBs and the CNT-DNA sequence decreased when the length of the CNT-DNA sequence was too long or short. Hence, sensor 2 was used in subsequent experiments.

3.4. Signal generation and optimization

It is well known that CNTs can interact with ssDNA non-covalently through π–π stacking interactions between the nucleotide bases and CNTs (Zhen et al., 2010). Moreover, ssDNA was

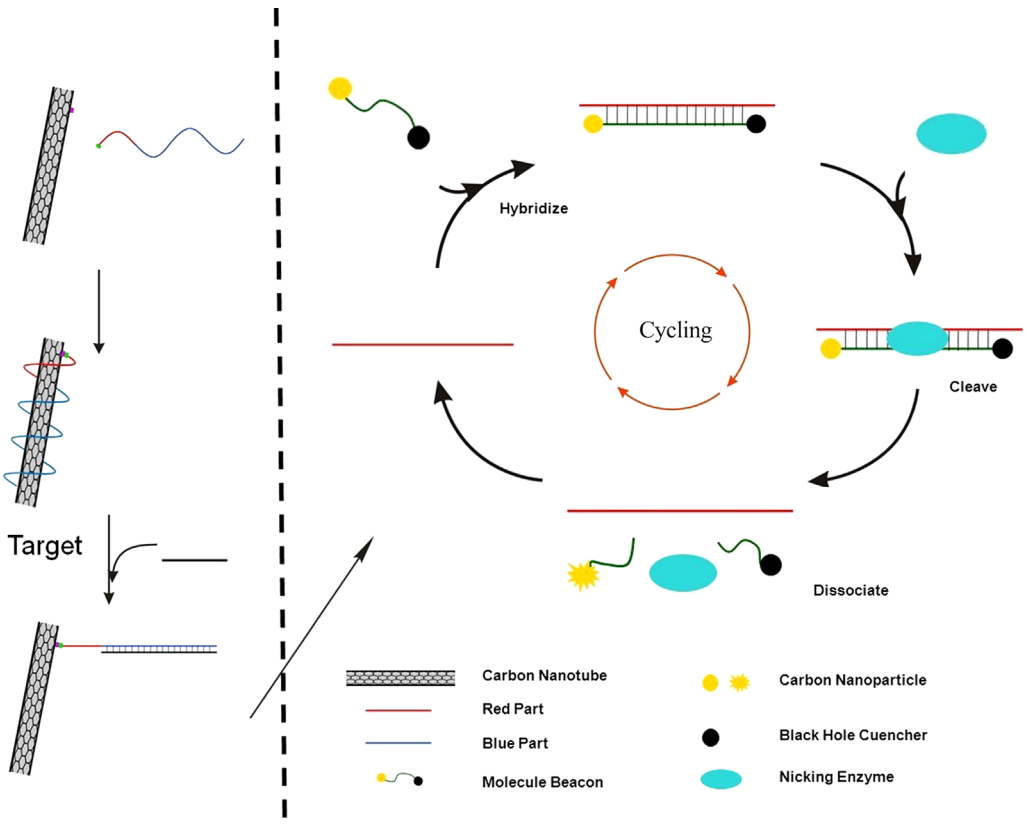

Fig. 1. Schematic diagram of detection based on nicking enzyme-assisted fluorescence signal amplification. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

anchored onto the CNTs to prevent dissociation from the 3′ end, which would cause a false positive. The bacteria medium can interfere with the fluorescence signals of CNPs; this effect was minimized by adjusting the excitation wavelength of the CNPs to 490 nm.

Following cleavage, MB was too short to maintain its conformation, resulting in disconnection of the CNPs and BHQ 1. Then, the CNT-DNA sequence matched with other uncleaved MBs. This means that one target can induce many quenched fluorescent probes to fluoresce to facilitate detection and improve the sensitivity of our method. As shown in Fig. 4S, with the prolongation of the cleavage process, a linear increase of fluorescence intensity was observed from 50 to 250 (a.u.). After 50 min, the fluorescence intensity did not increase over time. Taking both efficiency and rate into consideration, 45 min was chosen as the nicking enzyme reaction time.

3.5. Performing and analyzing detection

To obtain the LOD of this biosensor, a series of target sequences of different concentrations were prepared. Fig. 2 shows the relationship between fluorescence intensity and target concentrations from 0 to 100 nM. Fluorescence intensity increased gradually with the concentration of the target, which implies that the MBs were increasingly cleaved by nicking enzyme. In addition, the fluorescence of the control sample was relatively low because the MB can only be cleaved in the presence of target sequence. The inset in Fig. 2 depicts the linear relativity observed from 0 to 1 nM during target sequence determination. The LOD of the sensor was up to 70 pM ($n=5$, mean $\pm$ SD). Fig. 5S shows the fluorescence intensities of various phases. The sensitivity of this method is higher than that of other existing homogeneous assays for bacteria (Ding et al., 2011; Wu et al., 2013).

3.6. Detection specificity

Specificity is equally important as sensitivity in detection. In our sensor, the intensity of the fluorescence signal depends mainly on the cleavage reaction. When the target is captured, the CNT-DNA sequence can be released and the MB can be cleaved. Thus, the specificity of this biosensor is determined by the hybridization between the target sequence and CNT-DNA sequence. Two types of bacteria, *S. typhimurium* and *E. coli* K88 (5×10^3 CFU/mL) were used to determine the specificity of the biosensor, and compared with the results obtained for *S. enteritidis* (5×10^3 CFU/mL) (Fig. 6S). Averages of fluorescence intensity (a.u.) in water were 38 ± 2.79 (control), 39.01 ± 2.88 (*S. typhimurium*), 37.55 ± 2.93 (*E. coli* K88), and 258.88 ± 2.92 (*S. enteritidis*). Fig. 7S was results of specificity tests when using milk samples. Averages of fluorescence intensity (a.u.) in milk were 32.98 ± 2.88 (control), 34.27 ± 2.87 (*S. typhimurium*), 33.2 ± 2.94 (*E. coli* K88), and 234.44 ± 3.22 (*S. enteritidis*). Group comparisons were performed using Mann–Whitney *U* test ($n=5$). The biosensor could precisely detect 16S rRNA and provide high specificity.

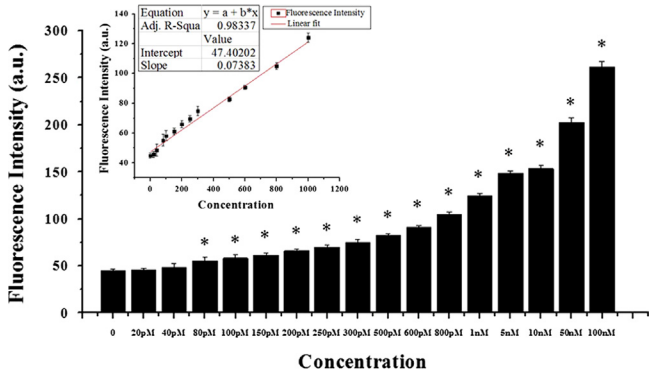

Fig. 2. Fluorescence intensities of target sequences with concentrations of 20 pM, 40 pM, 80 pM, 100 pM, 150 pM, 200 pM, 250 pM, 300 nM, 500 nM, and 600 nM, 800 pM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM and of a control group. The inset shows the linear fitting of fluorescence intensities from 0 to 1 nM. An asterisk indicates a significant difference between control and experimental groups ($P<0.01$, Mann–Whitney *U* test, $n=5$, mean $\pm$ SD).

3.7. Sample test

We then used the biosensor to detect *S. enteritidis* in real samples. The bacterium suspension was diluted, and lysozyme was added. Lysozymes can lyse the cell wall of *S. enteritidis* without damaging its 16S rRNA (Brasca et al., 2013). As illustrated in Fig. 3, the LOD of this assay was 10^2 CFU/mL in water. However, LOD obtained using milk was slightly higher (1.5×10^2 CFU/mL). This increase may be because some components in milk interfere with the specific hybridization of the CNT-DNA sequence to the target, or affected the light transmittance of the reaction mixture.

3.8. Conformation of CNT-DNA/16S rRNA

In order to explore the conformation of CNT-DNA/16S rRNA complex, we developed a method and confirmed that the CNT was entwined by other part of oligonucleotide when CNT-DNA probe was hybridized with a fragment of it.

As shown in Fig. 8S, sample B gave the highest S/B ration, sample A was middle, and sample C was the lowest. Sample A and B illustrated that the other part of 16S rRNA entwined CNT. That is, the SYBR Green I (inserted into probe/target complex) was in close proximity to CNT, and eventually caused the fluorescence intensity of SYBR Green I decreased (Fig. 9S).

When MB was added into these three samples, it was found that fluorescence intensities of CNPs were different, in which sample B was higher than sample A. Therefore, we think other part of 16S rRNA can impact the process of hybridization of target or cleavage of MB.

4. Conclusions

A biosensor based on FRET has been developed for the rapid, sensitive, and convenient detection of *S. enteritidis*. The assay was performed within 2 h (including incubation time) with detection limits of 10^2 and 1.5×10^2 CFU/mL in water and milk, respectively. Compared with existing methods, the sensitivity of this method

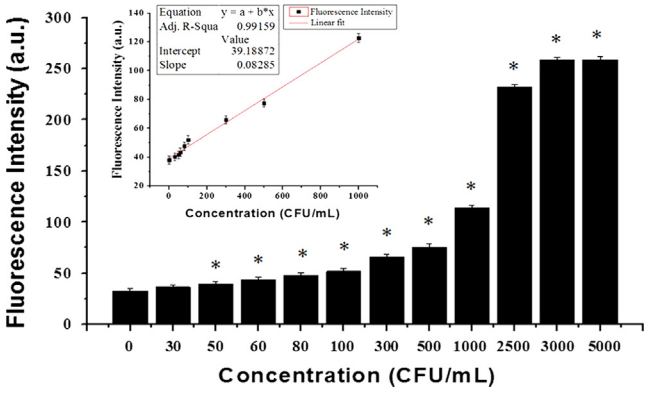

Fig. 3. Fluorescence intensities obtained from different concentrations of bacteria lysate. The inset shows the linear fitting of fluorescence intensities from 0 to 10^3 CFU/mL. An asterisk indicates a significant difference between control and experimental groups ($P<0.01$, Mann–Whitney *U* test, $n=5$, mean $\pm$ SD).

was improved; its LOD was 10 times lower than that of other methods because fluorescence intensity was amplified by the nicking enzyme. The CNPs used in the sensor exhibited more favorable properties than traditional organic dyes. In addition, our method has the potential to detect other protein and biological molecules simply by changing the specific sequence of CNT-DNA. Our results indicate that this method is an effective technology for the detection of *S. enteritidis*, and can be expanded to a rapid, simple strategy to detect many other pathogens in food or the environment. Even though this method is convenient and more sensitive than existing methods based on FRET, further research is needed to decrease the detection time.

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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.2013.12.053.

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