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RESEARCH PAPER

Ultrasensitive cloth-based microfluidic chemiluminescence detection of *Listeria monocytogenes hlyA* gene by hemin/G-quadruplex DNAzyme and hybridization chain reaction signal amplification

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Abstract

In this work, a cloth-based chemiluminescence (CL) biosensor has been firstly presented for highly sensitive determination of long PCR amplicons. Under the action of a hybridization chain reaction, a good deal of hemin/G-quadruplex DNAzyme molecules are produced, which can effectively enhance the CL signal. Moreover, effective cloth-based DNA biosensors can be fabricated by sequential wax screen-printing and surface-modification processes. Especially, the integration of a desirable hydrophobic barrier and gravity/capillary flow onto the flow channel of the cloth-based device makes the biosensor easy to be fabricated and to be associated with a flow CL. For the luminol/H_2O_2-based CL system, the signals are triggered by the hemin/G-quadruplex DNAzyme and are recorded by a low-cost CCD. Under optimized conditions, the determination range of target DNA is 0.002–20,000 pM and its limit of detection is calculated to be 1.1 fM. The results show that the proposed CL biosensor has a good analytical performance, such as high detectability and specificity, wide linear range, and receivable reproducibility and stability. Finally, the proposed biosensor is proven by the fact that this method can successfully detect the target DNA prepared from the *Listeria monocytogenes*–spiked milk samples. Therefore, it is believed to have the potential application prospects in food safety and environmental monitoring.

Keywords Cloth-based microfluidics · Chemiluminescence · DNA biosensor · Hybridization chain reaction · Hemin/G-quadruplex DNAzyme · Detection of pathogenic bacteria

Introduction

As is known, some public health issues, like food-borne diseases caused by pathogenic bacteria, are becoming worse increasingly. It is reported that about 20 species of food-borne pathogens have been recognized as the main causes of food-borne diseases, like *Salmonella enterica*, *Listeria bacteria*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Escherichia coli*, which can trigger the outbreak of food-borne diseases [1, 2]. These pathogenic bacteria can be rapidly spread by the ingestion of contaminated water or food, causing the outbreak of food-borne illnesses and leading to human diseases and even death [3]. Millions of people all over the world are subjected to the disease caused by pathogenic bacteria every year, and most of them can be attributed to the ingestion of the pathogenic bacteria–contaminated food or water [4]. To ensure human health and food safety, it is necessary to rapidly and effectively detect these pathogenic bacteria at all stages in the aspects of food safety [5]. Thus, it is of vital role to detect the food-borne pathogens for food safety and environmental monitoring.

Up to now, some traditional methods like the bacterial culture counting, immunology, and polymerase chain reaction (PCR) are often applied for the detection of pathogenic bacteria. In general, the bacterial culture counting is of accuracy and

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reliability for detecting pathogenic bacteria [6, 7]. However, this method possesses the shortcomings of being time-consuming and complicated, which is unsuited to rapid detection of pathogenic bacteria. Recently, the antigen/antibody-based immunology has been developed as an effective avenue for determining food-borne pathogens [8, 9]. It usually has an enough short assay time, but often suffers from a low detectability and poor affinity. Alternatively, the PCR amplification–based methods have been widely utilized to determine the bacteria related to the food safety, and they have a distinct advantage of detecting the target nucleic acid at low concentration in real sample [10, 11]. However, the gel electrophoresis for analysis of PCR products has some disadvantages such as lower resolution, high complication, and use of hazardous materials, which may limit its wide application for bacterial detection. All in all, these abovementioned methods are of accuracy and reliability, while they are greatly restricted by being time-consuming and having low detectability and high complexity. Thus, exploring a speedy but efficient analytical approach is becoming the hot research subject for the detection of pathogenic bacteria.

Recently, the DNA biosensor–based methods have been recognized as popular techniques for detecting pathogenic bacteria, which often possesses some excellent properties such as simplicity, speediness, and sensitivity [5]. In previously reported DNA biosensors, several different methods have been applied to determine the pathogenic bacteria in recent years, including colorimetric [12], fluorescence [13], electrochemical [14], electrochemiluminescence [15], and chemiluminescence (CL) [16, 17] methods. Among these methods, the CL method, as a powerful and significant analytical approach, has been proven to be well-suited to simply and rapidly detect the DNA sequence, associated with low detection limit, low background, and simple instrumentation. Most importantly, it shows the great potential to develop the miniaturized device [16, 17]. Recently, several CL-based biosensors have been well-used to effectively determine the bacterial DNA samples [18–20]. However, the detectability of these biosensors still needs to be improved. Therefore, it is necessary to explore a simple, inexpensive, and sensitive CL biosensor for bacterial DNA detection.

In the past few years, several signal amplification methods, including nanomaterial-based methods [21] and enzyme-based DNA amplification methods (like strand displacement amplification [22], rolling circle amplification [23], and loop-mediated isothermal amplification [24]), have been successfully applied in the design of DNA biosensors, which can efficiently amplify the signal response and bring down the detection limit. For these amplification methods, a high detectability can be achieved, but they still have some drawbacks of complexity and susceptibility to contamination. During the last decade, hybridization chain reaction (HCR), as an alternative technique to signal amplification, was firstly introduced in 2004 [25] and has been recognized as the most popular method for the enzyme-free signal amplification. In short, HCR can form a mass of long double-stranded DNA polymers owing to the self-assembly of nucleic acid, which can be bound with a great deal of luminescent material. Thus, this amplification method can provide stronger signal response and higher detectability. The DNA biosensors based on HCR signal amplification have been widely reported [26–28]. For further improving the detectability, HCR can be integrated with the oxidizing-enzyme amplification [29]. However, the modification and conjugation of the conventional protein enzymes (such as horseradish peroxidase (HRP) [29]) are often complex and expensive. In addition, the conventional protein enzymes are generally poor in stability and inactivated easily. Alternatively, increasing attention has been paid on the hemin/G-quadruplex DNAzyme owing to its higher stability and easier functionalization, which can overcome the abovementioned limitations [30, 31]. What's more, the hemin/G-quadruplex DNAzyme with excellent catalytic performance can catalyze the oxidation of luminol by H_2O_2 to yield the CL signal [32]. Unfortunately, however, the HCR and hemin/G-quadruplex DNAzyme so far have not been reported to be associated with the CL biosensor for bacterial DNA detection.

In recent years, several fibrous materials have gained increasing concerns in the field of the fabrication of the microfluidic paper or cloth-based analytical devices (μPADs or μCADs), since they have excellent properties such as simplicity, low price, and disposability. Especially, the cloth is chosen as a desired substrate, since it has better durability and flexibility than paper. The last years have witnessed a rapid progress in μCADs since the first hydrophilic-hydrophobic patterns were woven on the cloth [33]. Up to date, several detection approaches based on μCADs have been well-explored, including colorimetric [33], electrochemical [34], electrophoretic [35], CL [36], three-electrode electrochemiluminescence [37], and bipolar electrochemiluminescence [38, 39] methods. Within these detection methods, however, the cloth-based CL has not been reported for detection of DNA sequences.

Keeping these considerations in mind, the aim of this work is to exploit a cloth-based CL DNA biosensor for a simple, low-cost, and sensitive detection of *Listeria monocytogenes hlyA* gene. In our designed biosensor, several attractive properties are described as follows. First of all, the processing steps of the cloth-based device are simple, rapid, and cheap and do not require any well-trained personnel. Furthermore, the desirable hydrophobic barrier and gravity/capillary flow are integrated onto the flow channel of the cloth-based device. Second, the detection zone can be well-modified by chitosan (CS) and glutaraldehyde (GA) to provide the desirable immobilization of capture DNA (CP-DNA). Third, the HCR integrated with hemin/G-quadruplex DNAzyme has been demonstrated to exhibit a very strong signal enhancement. Fourth,

the proposed biosensor exhibits an excellent analytical performance for the *hlyA* gene determination, such as high sensibility, good selectivity, wide linear detection range, and receivable reproducibility and stability. Finally, the biosensor can be applied to determine the DNA target from *Listeria monocytogenes* in real milk samples.

Experimental section

Preparation of target DNA samples

Firstly, genomic DNA samples were extracted from the cultured *Listeria monocytogenes* according to the kit's protocol. The extracted DNA could be used for PCR amplification, where a 25-μL reaction volume included 1× PCR buffer, 0.2 mM dNTPs, 0.025 units/μL Taq polymerase, 1 μM of each primer, and 2 μL genomic DNA samples. And, a PCR amplification procedure was performed, including a 3-min pre-denaturation process at 94 °C, and next 30 cycles of 94 °C for 30 s, 47 °C for 30 s, 72 °C for 60 s, and a 3-min final elongation process at 72 °C. After that, the agarose gel electrophoresis (2%) was applied to observe the double-stranded PCR products for 225-bp fragment of *hlyA* gene (see Electronic Supplementary Material (ESM) Fig. S1). Next, the amplified products were purified, and the resulting DNA sample concentration was measured, followed by denaturing the double-stranded products for 5 min at 95 °C and rapidly cooling the denatured products at − 20 °C over 1 h to obtain the desired single-stranded target DNA (T-DNA).

Design and preparation of signal probe

For the preparation of signal probe (S-DNA), three auxiliary DNA strands (A-DNA1, A-DNA2, and A-DNA3) (ESM Table S1) should be well-designed. To this end, the sequence of A-DNA1 at the 3′ end is complementary with T-DNA, while the sequence of its 5′ end is the same as that of A-DNA3 at the 3′ end, and complementary to the sequence of A-DNA2 at the 3′ end. In addition, the sequence of A-DNA2 is composed of three parts. The first part is a sequence of G-quadruplex at the 5′ end, which can bond with hemin to generate the hemin/G-quadruplex DNAzyme; the second part is a short middle sequence that is completely complementary with A-DNA3 at the 5′ end; and another part is completely complementary with A-DNA1 at the 3′ end.

Further, the preparation of hemin/G-quadruplex DNAzyme-contained S-DNA was performed and its process was schematically described in Fig. 1a. Firstly, three auxiliary DNA strands (0.5 μM of each) were treated at 90 °C for 5 min to eliminate the intermolecular interaction, and then slowly cooled down to room temperature before being used. Secondly, 2 μL of A-DNA1, A-DNA2, and A-DNA3 was respectively added into a reaction tube filled with 14 μL of hybridization buffer solution (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 7.4), and then hybridized at 37 °C for 50 min for a continuous HCR between alternating A-DNA2 and A-DNA3, followed by being cooled to 4 °C. As a result, the double-stranded HCR products were formed. Thirdly, 20 μL Tris-HCl buffer solution (20 mM Tris-HCl, 40 mM KCl, 0.06% (v/v) Triton X-100, pH 7.4) was added into the solution of HCR products, and the mixed solution was further reacted at 95 °C for 40 min to allow the folding of A-DNA2 in HCR products to form a great amount of G-quadruplex structure. Subsequently, 40 μL of 0.11 mM hemin solution, which was prepared in DMSO, was added, and the resulting solution further was reacted at 37 °C for 2 h to form the desired S-DNA.

DNA biosensor preparation and hybridization reaction

In this work, the wax screen-printing method is introduced for manufacturing the cloth-based devices (ESM Fig. S2). Using the as-fabricated cloth-based devices, the modification and hybridization process of DNA biosensor could be performed, and its detailed process is shown in Fig. 1b. Firstly, 6 μL of 0.25 g/L CS solution (in 0.1 M HAc) was used to modify the detection zone and dried at room temperature, followed by being activated with 6 μL of 2.5% (v/v) GA solution (in 10 mM PBS, pH 7.4) at room temperature for 60 min. Subsequently, the unreacted CS and GA were removed by washing four times with DI water. Next, for covalent immobilization of CP-DNA, 6 μL CP-DNA (0.6 μM) was added to the detection zone and further reacted at 37 °C for 30 min, followed by removing the excess CP-DNA by washing four times with Tris-HCl buffer solution (10 mM Tris-HCl, 50 mM KCl, 0.08% (v/v) Tween 20). Subsequently, the detection zone was treated with 6 μL BSA blocking buffer (BSA-BB) for 30 min at 37 °C to effectively reduce the potential nonspecific adsorption, followed by four times of washing with 20 μL Tris-HCl buffer solution. Afterwards, the CP-DNA-modified detection zone was reacted with 6 μL T-DNA for 40 min at 37 °C, and then washed using 20 μL Tris-HCl buffer solution to remove nonspecific DNA-DNA hybrids. Finally, 6 μL S-DNA was hybridized with T-DNA for 40 min at 37 °C. After hybridization, the similar washing and drying procedures were performed to remove unhybridized S-DNA. It should be noted that the cloth-based device was fixed on a plastic holder and then this holder was kept into a 37 °C incubator for the hybridization reaction. In addition, for the abovementioned washing procedures, DI water or Tris-HCl buffer solution was dropped onto the center of the detection zone, and then, a piece of blotting paper was in contact with the edge of the detection zone to absorb excess washing water or buffer solution.

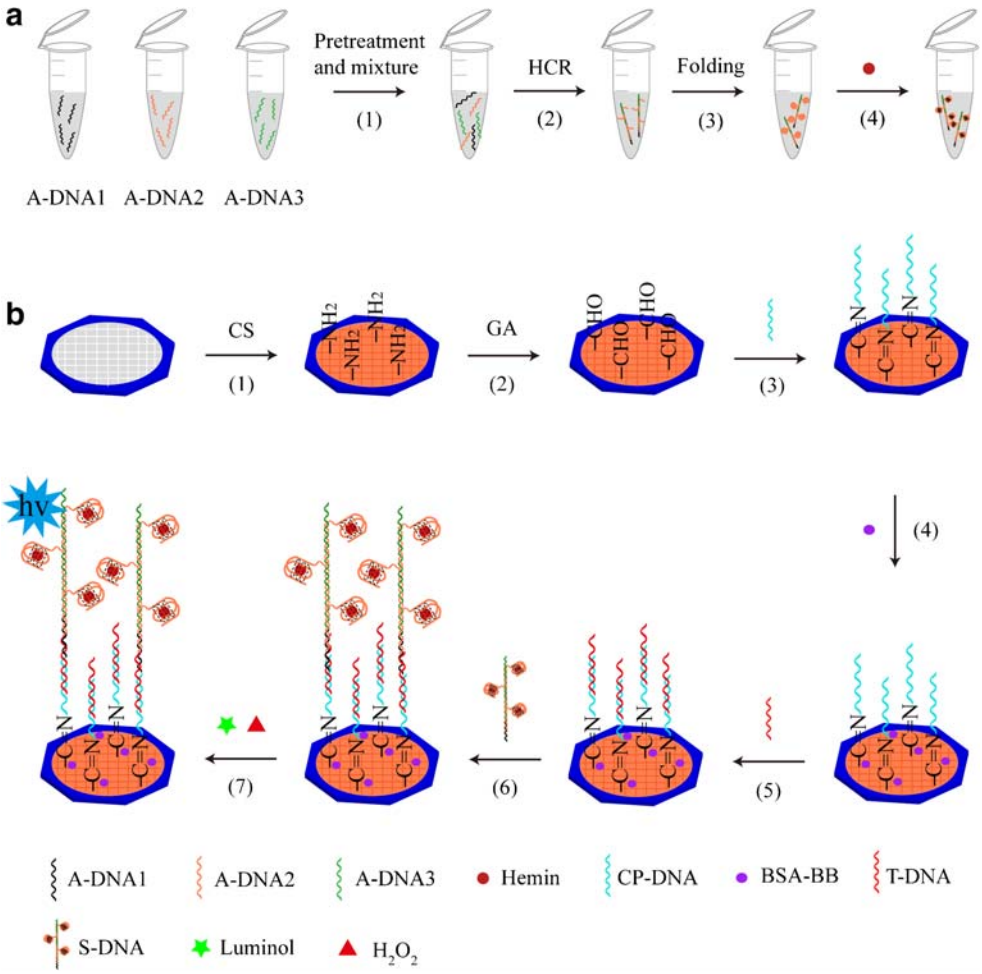

Fig. 1 Schematic representation of the preparation of S-DNA (**a**) and the preparation and hybridization reaction procedure of the presented biosensor (**b**). Here, A-DNA1, A-DNA2, and A-DNA3, three auxiliary DNA strands; HCR, hybridization chain reaction; CS, chitosan; GA, glutaraldehyde; CP-DNA, capture DNA; BSA-BB, BSA blocking buffer; T-DNA, target DNA; and S-DNA, hemin/G-quadruplex DNAzyme–contained signal DNA

CL measurement procedure

The assay procedures of the DNA biosensor are schematically shown in Fig. 2 and are described below in detail. Firstly, the cloth-base biosensor was fastened onto a folder-type holder (step 1 in Fig. 2), where the cloth-based device could be folded along the fold line, and then, an adequate angle of inclination between the horizontally laid detection zone and flow channel could be obtained. A typical assembled cloth-based biosensor is shown in ESM Fig. S3. Secondly, the holder was put into the black box that was equipped with the CL measuring and analyzing system, during which the CCD automatic imaging function was turned on and the camera could image the detection zone clearly (step 2 in Fig. 2). Thirdly, 40 μL of substrate solution (10 mM Tris-HCl, 1 mM EDTA, pH 9.0, containing 2 mM luminol and 0.035 M H_2O_2) was rapidly added to the loading zone of the cloth-based device (step 3 in Fig. 2). Since the gravity and capillary flow worked, the solution quickly passed the flow channel and then passed through the narrow wax barrier to arrive at the detection zone (step 4 in Fig. 2). Subsequently, the CL reaction took place, and the real-time CL signals were collected with a CCD camera connected with the computer (step 5 in Fig. 2).

Data acquisition and analysis

The CL images were gathered by a CCD camera and saved in the form of videos in the WMV format. To analyze these images, the VGIF software was applied to tailor the video images to the pictures with an interval of 100 ms. After that, these pictures were batch-cut using the software nEO iMAGING 4.4.1 (Shenzhen Thunder Network Culture Co., Ltd., Shenzhen, China). As a result, the size of each picture was 450 × 450 pixels, containing the whole luminous zone. Next, an automatic image-processing program exploited in MATLAB R2012a (MathWorks Company, USA) was applied to analyze the acquired batch pictures. Therefore, it was easy to acquire the picture with the maximum luminescence intensity by comparing the gray values at the whole luminous zone.

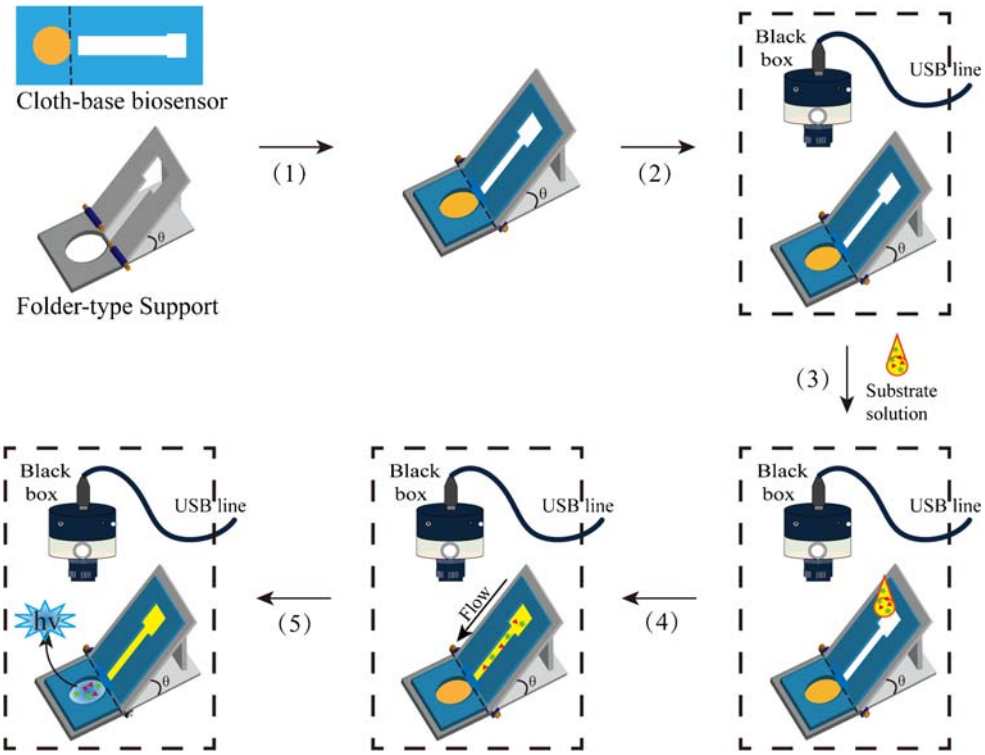

Fig. 2 Schematic illustration of the assay procedures of the proposed DNA biosensor

In this work, the mean gray value of each chosen CL picture that could be obtained by MATLAB was used to quantify each assay. Finally, the achieved values were analyzed with the Origin 7.0 (MicroCal Software Inc., MA, USA), and the desired curves or bar graphs could be easily obtained.

Results and discussion

Characterization of HCR

To verify whether the designed auxiliary DNA strands worked for the HCR, the polyacrylamide gel electrophoresis was performed. The results are displayed in ESM Fig. S4, where the lanes of 1–6 represented the images of A-DNA1, A-DNA2, A-DNA3, A-DNA1/A-DNA3, A-DNA2/A-DNA3, and A-DNA1/A-DNA2/A-DNA3, respectively. It can be seen that one single band could be observed in the case of A-DNA1/A-DNA3 (lane 4), which was similar to the case of A-DNA1 or A-DNA3 alone (lane 1 or 3), indicating that both A-DNA1 and A-DNA3 could not cause the self-hybridization. When A-DNA2 was incubated with A-DNA3, some discontinuous bands were observed (lane 5), implying that the hybridization between A-DNA2 and A-DNA3 could take place and generate the duplex DNA complexes. In the presence of A-DNA1/A-DNA2/A-DNA3, more discontinuous bands were achieved (lane 6), which should be attributed to the formation of long duplex HCR products. These results indicated that the HCR among three auxiliary DNA strands could be carried out successfully.

Characterization of cloth-based devices

A simple wax screen-printing process was successfully utilized to make the cloth-based devices (ESM and Fig. S2), and Fig. 3a shows a typical cloth-based device. It should be noted that the size of the loading zone was about 4 mm × 4 mm and the dimension of the flow channel was approximately 3 mm × 10 mm. Furthermore, the width of the wax barrier crossing the flow channel was about 0.5 mm, and the diameter of the detection zone was about 8 mm. To clearly observe the structure of the cloth-based devices, the SEM images were applied to characterize two different functional zones. It can be seen from Fig. 3b that the pure cellulose cloth exhibited a smooth porous structure, which indicated that the pure cloth could form a favorable hydrophilic channel. Owing to the porosity of the pure cloth, the wax of the screen-printing could effectively penetrate into the porous structure of the cloth to generate the good hydrophobic barrier (Fig. 3c). These results demonstrated that the desirable hydrophilic zones and hydrophobic barrier have been formed on the cloth.

Optimization of the experimental conditions

To obtain the optimal experiment conditions, several parameters including the concentration of CP-DNA ([CP-DNA]), incubation time between T-DNA and CP-DNA (or S-DNA) (t_T-

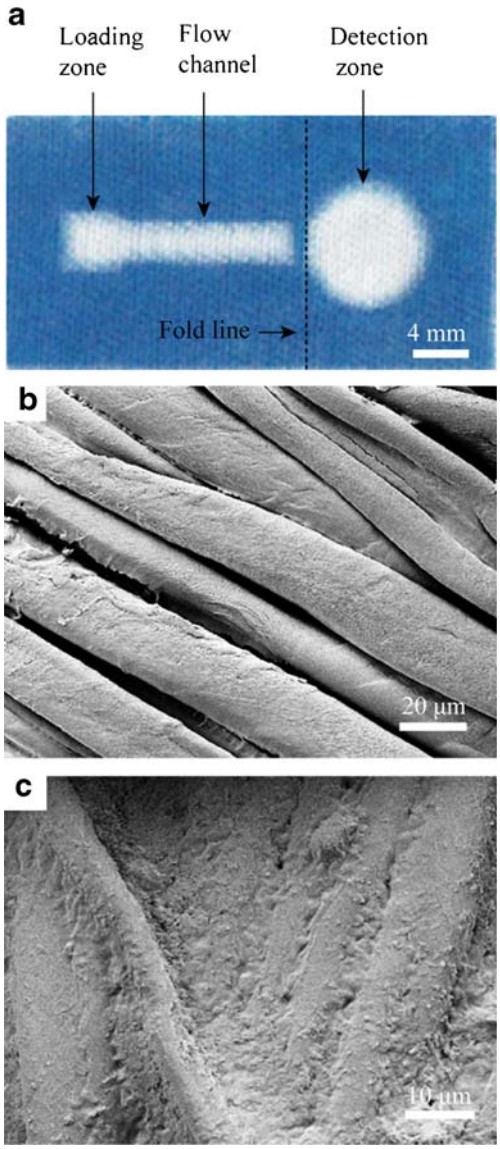

Fig. 3 Photo (**a**) and SEM characterizations (**b**, **c**) of a typical wax screen-printed cloth-based device. In these SEM images, panel **b** represents the porous structures of the pure cellulose cloth (for the hydrophilic zone), while panel **c** indicates the wax screen-printed cloth (for the hydrophobic zone)

$_C$ or $t_{T\text{-}S}$), and concentrations of hemin, H_2O_2, and luminol ([Hemin], [H_2O_2], and [Luminol]) have been investigated systematically. Here, unless indicated otherwise, the concentration of T-DNA was set as 200 pM for each optimization experiment.

The effect of [CP-DNA] on the CL was firstly studied. As seen in Fig. 4a, the CL intensity enhanced step by step as the increase of [CP-DNA] in the range of 0.2–0.6 μM. However, further increase of [CP-DNA] could give rise to the mild decline of CL intensity. This phenomenon can be interpreted by the steric hindrance of CP-DNA where the CP-DNA molecules are tightly close to each other in the detection zone, and those taking part in the target hybridization reaction were less exposed [40]. Thus, 0.6 μM was used for the following experiment.

As is well-known, the incubation time often greatly affects the DNA hybridization efficiency. To study this effect, seven different lengths of $t_{T\text{-}C}$ were investigated for the hybridization between T-DNA and CP-DNA. As shown from Fig. 4b, the CL signal increased along with the increasing $t_{T\text{-}C}$ and then attained saturation at more than 40 min, and it almost kept constant when the reaction time was further prolonged. The reason for this phenomenon may be that the applied DNA molecules have fully hybridized in the case of about 40 min. Similarly, the intensity of CL signal increased rapidly as the extended $t_{T\text{-}S}$ from 10 to 40 min, and then seemed to reach a plateau at more lengths of $t_{T\text{-}S}$ (Fig. 4c). Hence, 40 min was chosen as the optimal $t_{T\text{-}C}$ or $t_{T\text{-}S}$ in this work.

To better investigate the effect of [Hemin] on the CL intensity, CL measurements with seven different concentrations of hemin were carried out. As can be seen from Fig. 4d, there was a weak signal at a lower concentration. Upon gradually increasing the hemin concentrations from 0.06 to 0.11 mM, the CL signals were found to be increased correspondingly, indicating the generation of more hemin/G-quadruplex DNAzyme molecules caused a significant increase on CL intensity. However, when the hemin concentration exceeded 0.11 mM, the CL intensity almost maintained constant, which may be due to that the G-quadruplex concatemer formed during HCR bound hemin to reach saturation. Therefore, 0.11 mM hemin was used for later experiments.

For the luminol/H_2O_2-based CL systems, the concentrations of H_2O_2 and luminol should greatly affect the CL intensity. As depicted in Fig. 4e, the CL intensities were increased as the values of [H_2O_2] varied from 0.01 to 0.035 M, but showed no considerable changes in the case of H_2O_2 concentrations more than 0.035 M. This phenomenon has a similarity with that of other CL systems. So, 0.035 M H_2O_2 was used for the following experiments. Further, the luminol concentration was investigated in the range of 1–2.75 mM (Fig. 4f), which was also greatly affected the CL intensity. As increasing [Luminol] from 1 to 2 mM, the CL intensity increased correspondingly. However, the further increase of luminol concentration caused a reduction of the CL intensity. This phenomenon might be caused by the effect of self-inhibition at higher concentrations [41]. As a result, 2 mM luminol was opted for the final concentration.

CL signal enhancement in the proposed biosensor

In general, PCR is a technique that can amplify a specific piece of DNA. In theory, more than 10^9 copies of target piece can be obtained by PCR assay with 25–30 cycles [10]. For the proposed biosensor, the CL signals were first enhanced by the PCR process that could generate more desired T-DNA

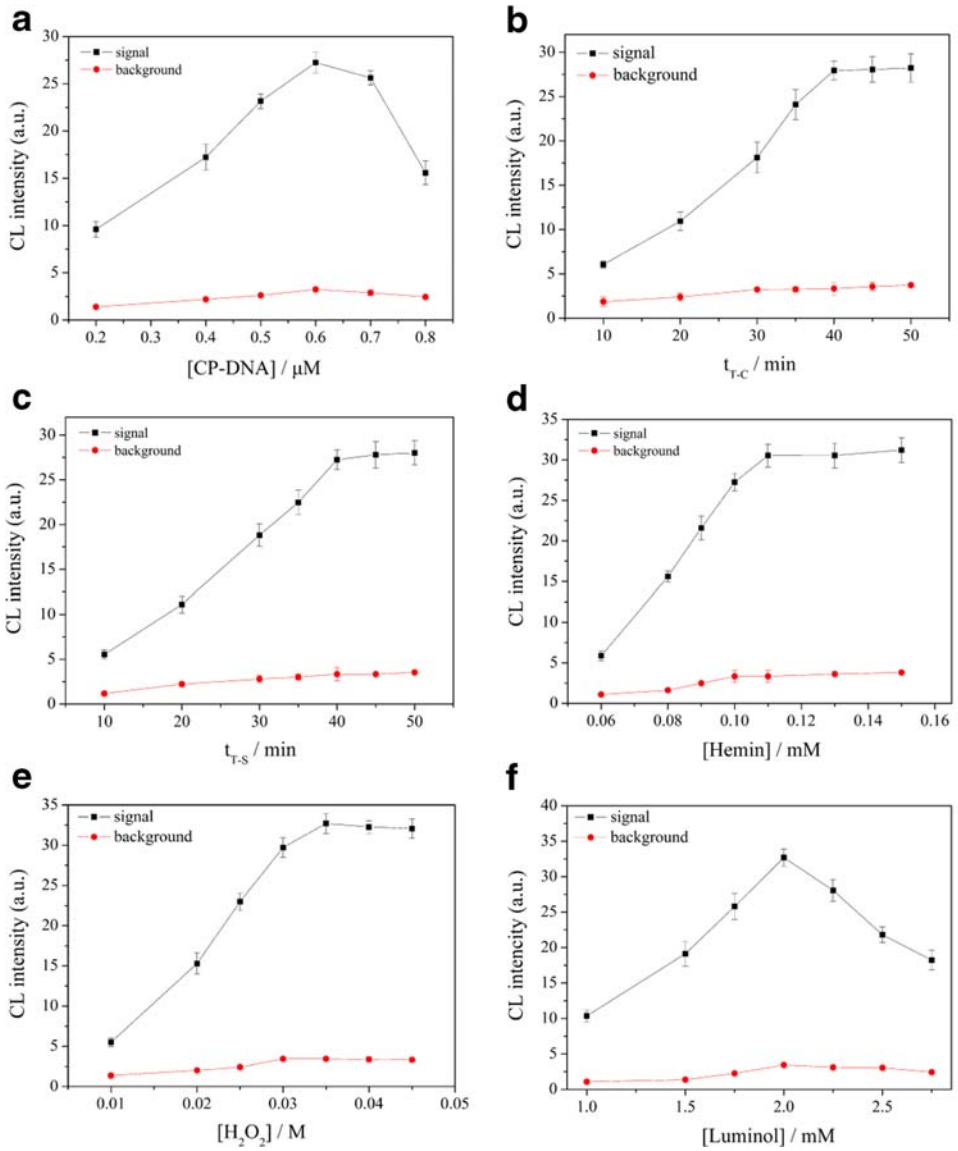

Fig. 4 Dependence of cloth-based CL intensities on the concentration of CP-DNA ([CP-DNA]) (**a**), incubation time between T-DNA and CP-DNA (S-DNA) ($t_{T\text{-}C}$ and $t_{T\text{-}S}$) (**b**, **c**), concentration of hemin ([Hemin]) (**d**), concentration of H_2O_2 ([H_2O_2]) (**e**), and concentration of luminol ([Luminol]) (**f**). In panel **a**, [CP-DNA] = 0.2–0.8 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.1 mM, [H_2O_2] = 0.03 M, and [Luminol] = 2 mM; in panel **b**, [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 10–50 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.1 mM, [H_2O_2] = 0.03 M, and [Luminol] = 2 mM; in panel **c**, [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 10–50 min, [Hemin] = 0.1 mM, [H_2O_2] = 0.03 M, and [Luminol] = 2 mM; in panel **d**, [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.06–0.15 mM, [H_2O_2] = 0.03 M, and [Luminol] = 2 mM; in panel **e**, [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.11 mM, [H_2O_2] = 0.01–0.045 M, and [Luminol] = 2 mM; and in panel **f**, [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.11 mM, [H_2O_2] = 0.035 M, and [Luminol] = 1–2.75 mM. For the panels **a**–**f**, 200 pM T-DNA was used. Error bars show the standard deviations of five independent experiments

sequences and reduced the negative impact of lengthy genome on the CL detection. In nature, the used PCR process amplified the CL signals by increasing the T-DNA concentration.

The proposed HCR-based sandwich protocol also could greatly enhance the CL signals under the optimized conditions. To evaluate this, a traditional sandwich protocol was designed for comparison, where a short auxiliary DNA sequence (A-DNA4) served as S-DNA. A-DNA4 consists of two parts (ESM Table S1): one part is completely complementary with T-DNA, and another part is a G-quadruplex sequence. The G-quadruplex sequence could be coupled with hemin to generate the hemin/G-quadruplex DNAzyme. As depicted in Fig. 5, relative to the traditional sandwich protocol, the HCR-based sandwich protocol could result in an obviously enhanced CL intensity. This phenomenon can be explained by the fact that in the traditional protocol, only one G-quadruplex sequence combined with hemin to produce one hemin/G-quadruplex DNAzyme molecule, and

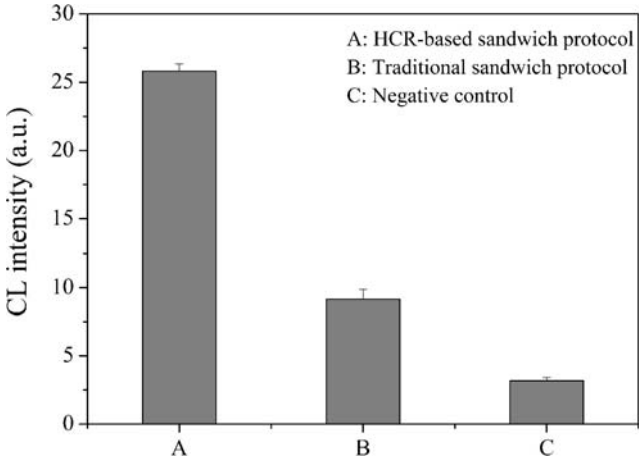

Fig. 5 Comparison of the CL intensities between the proposed HCR-based sandwich protocol and the traditional sandwich protocol. Experimental conditions: [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.11 mM, [H_2O_2] = 0.035 M, [Luminol] = 2 mM, and [T-DNA] = 20 pM. Error bars show the standard deviations of five independent experiments

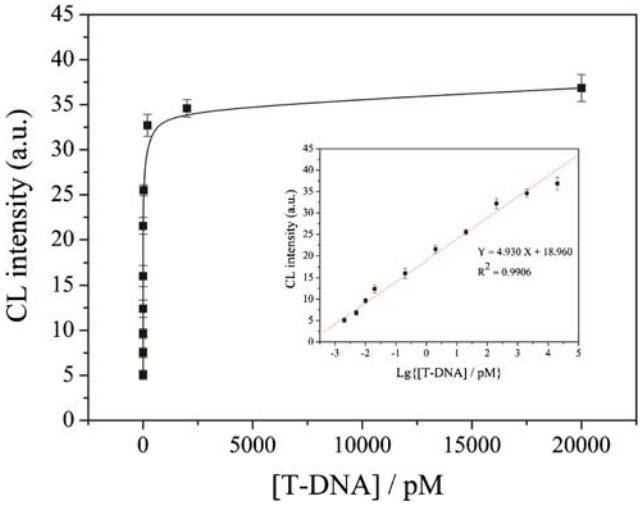

Fig. 6 Calibration curve for the detection of T-DNA using the proposed DNA biosensor under the optimal conditions. The inset revealed that the CL intensities were in linear correlation with the logarithms of T-DNA concentrations in the ranges of 0.002–20,000 pM. Experimental conditions: [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.11 mM, [H_2O_2] = 0.035 M, and [Luminol] = 2 mM. Error bars show the standard deviations of five independent experiments

thus a weak CL signal was generated, while a great deal of G-quadruplex sequences were generated in the HCR-based sandwich protocol, and thus a number of hemin/G-quadruplex DNAzyme molecules were produced, and reacted with luminol and H_2O_2 to cause a significantly enhanced CL intensity. Therefore, at a certain concentration of T-DNA, the HCR-based sandwich protocol enhanced the CL signals by increasing the number of luminescent substances on the biosensor. Here, it should be noted that in spite of HCR signal amplification, the proposed CL biosensor could not be used to perform the PCR-free detection of T-DNA sequences.

Analytical performance of the proposed DNA biosensor

Detectability and linear detection range of the biosensor

Under the above optimum conditions, the detectability and detection range of the proposed biosensor were researched. Figure 6 presents the corresponding changes of CL intensity at different T-DNA concentrations. Upon gradually increasing the T-DNA concentrations, the CL intensities were found to be increased correspondingly, demonstrating that the proposed biosensor could carry out quantitative T-DNA detection. As depicted in the inset of Fig. 6, in the range of 0.002–20,000 pM, the CL intensity had a favorable linear relationship with the logarithmic T-DNA concentration (R^2 = 0.9906, n = 5). The detection limit of the proposed biosensor, which could be defined as the T-DNA concentration yielding a signal three standard deviations larger than the mean blank, was evaluated to be 1.1 fM. This detectability bears comparison with or is higher than that acquired in some other CL DNA biosensors (ESM Table S2). Moreover, it can be shown from ESM Table S2 that the linear response range of the proposed biosensor is obviously wider than that obtained in these previous CL DNA biosensors.

Specificity, reproducibility, and stability of the biosensor

The specificity of the biosensor is a key factor for effectively distinguishing full-complementary T-DNA from non-complementary DNA sequence. To evaluate this, the DNA biosensor was investigated by comparing its CL intensities of single-stranded DNA sequences from four different pathogenic bacteria (including *Listeria monocytogenes*, *Salmonella enteric*, *E. coli* O157:H7, and *Staphylococcus aureus*). As depicted in Fig. 7, the CL intensity from the full-complementary T-DNA was much greater than those from the non-complementary DNA sequences. Especially, the CL intensity produced by the non-complementary DNA sequence was almost equal to that from the negative control. Therefore, the developed DNA biosensor had an excellent specificity, which might be put down to the process of specific recognition of CP-DNA, full-complementary T-DNA, and S-DNA [15].

The reproducibility of the proposed biosensor was also of great significance in practical application. In the presented case, it was investigated by inter-assay or intra-assay relative standard deviation (RSD) that was assessed from the CL intensity of 20 pM T-DNA in the cloth-based biosensor. The inter-assay was performed with five DNA biosensors from various fabrication batches, and its RSD was 4.00%. And the intra-assay was measured with the same batch of

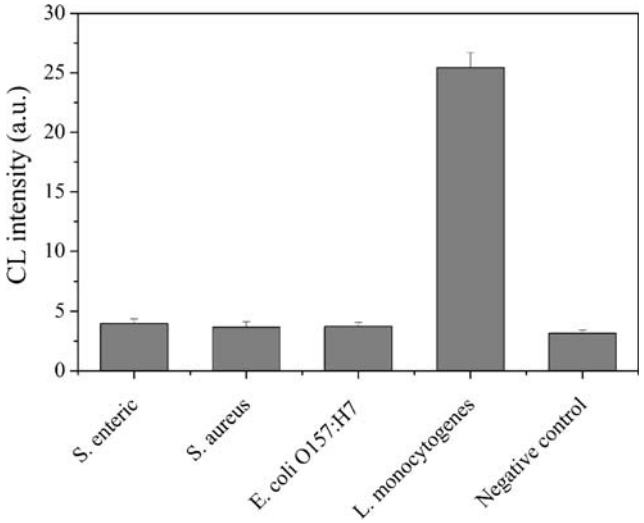

Fig. 7 Evaluation of specificity for detection of *Listeria monocytogenes hlyA* gene on the proposed biosensor. The used single-stranded DNA targets were prepared from *Listeria monocytogenes* (*L. monocytogenes*), *Escherichia coli* O157:H7 (*E. coli* O157:H7), *Salmonella enteric* (*S. enteric*), and *Staphylococcus aureus* (*S. aureus*). Experimental conditions: [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.11 mM, [H_2O_2] = 0.035 M, [Luminol] = 2 mM, and [T-DNA] = 20 pM. Error bars show the standard deviations of five independent experiments

biosensors, and the RSD of five parallel experiments was 4.68%. These results indicated that the reproducibility of the proposed DNA biosensor was acceptable.

Besides specificity and reproducibility, the stability of the proposed biosensor is also very important for its potential applications. To evaluate this, the storage capability of the DNA biosensor was investigated for about 3 weeks by measuring the CL intensity at intervals of 2 days. The as-prepared biosensors were stored within a sealed plastic bag in a 4 °C refrigerator when they were not in use. As can be shown from Fig. 8, after the biosensor was stored for 10 days, the CL intensity decreased slightly and remained 93.4% of its initial intensity. After 20 days of storage, 86.3% of the initial CL intensity could be maintained. These results proved that this biosensor had an acceptable stability and showed the great potential for analytical applications in remote regions.

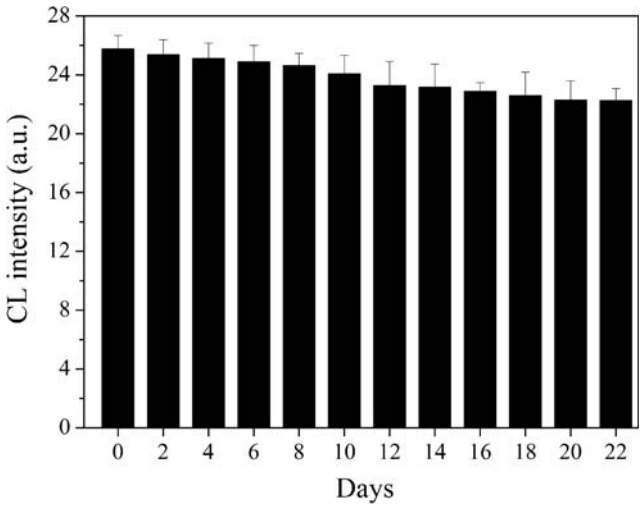

Fig. 8 Storage stability of the proposed DNA biosensor. Experimental conditions: [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.11 mM, [H_2O_2] = 0.035 M, [Luminol] = 2 mM, and [T-DNA] = 20 pM. Error bars show the standard deviations of five independent experiments

Detection of *Listeria monocytogenes* in real samples

To testify the practical application of the proposed biosensor, milk samples were investigated in this analytical case. Here, the pure milk samples were spiked with *Listeria monocytogenes*, with different concentrations of bacteria. The T-DNA sample was acquired from the spiked milk samples (see ESM) and was then subject to the CL measurement in the cloth-based biosensor. The CL intensities based on different spiked milk samples are shown in Fig. 9. The inset of Fig. 9 depicted that the CL intensities changed linearly as the logarithms of *Listeria monocytogenes* concentrations in the range from 1×10^2 to 1×10^7 CFU/mL ($R^2 = 0.9538$, $n = 5$), and the estimated detection limit was about 50 CFU/mL. Thus, the proposed biosensor might become a potential platform for bacterial DNA determination in real samples.

Conclusions

In summary, a cloth-based CL biosensor has been firstly demonstrated for highly sensitive T-DNA determination. The HCR-based sandwich protocol is introduced for enhancing the detectability, where the HCR products can serve as the

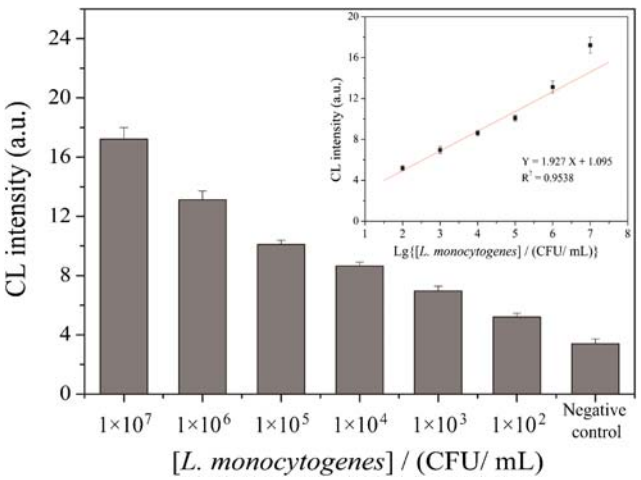

Fig. 9 CL intensities changing to PCR products acquired from serial dilutions of *L. monocytogenes*. The inset revealed that the CL intensities varied linearly with the logarithms of concentrations of *L. monocytogenes* spiked in milk samples. Experimental conditions: [CP-DNA] = 0.6 μM, $t_{T\text{-}C}$ = 40 min, $t_{T\text{-}S}$ = 40 min, [Hemin] = 0.11 mM, [H_2O_2] = 0.035 mM, and [Luminol] = 2 mM. Error bars show the standard deviations of five independent experiments

signal tag with a highly efficient catalytic ability benefiting from the associated generation of a great deal of hemin/G-quadruplex DNAzyme molecules. The cloth-based devices can be rapidly manufactured by wax screen-printing and are constructed into the effective biosensing interfaces by several facile modifications for simple, fast, and sensitive DNA determination. Under the optimal experimental conditions, this method has exhibited a good analytical performance toward a *Listeria monocytogenes hlyA* gene fragment, with the wide linear detection range of 0.002–20,000 pM and low detection limit of 1.1 fM, and as well acceptable specificity, reproducibility, and stability. Furthermore, this method can be successfully utilized to detect the *Listeria monocytogenes* in milk samples. Hence, the proposed biosensor may have broad application prospects in food security, environmental monitoring, and so on.

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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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