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Rapid and ultra-sensitive detection of viable *Enterobacter sakazakii* by a continual cascade nanozyme biosensor

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ABSTRACT: Recent outbreaks of life-threatening neonatal infections linked to *Enterobacter sakazakii* (*ES*) heightened the need to develop rapid and ultra-sensitive detection strategies, especially those capable of determining the viable cells. This study introduced a continual cascade nanozyme biosensor for the detection of viable *ES* based on propidium monoazide (PMA), loop-mediated isothermal amplification (LAMP) and Nanozyme-strip. The *omp*A gene of *ES* was determined using FITC-modified and BIO-modified primers in LAMP process. LAMP combined with PMA treatment was applied for distinguishing the viable from the dead state of *ES*. Then, using Fe_3O_4 magnetic as a nanozyme probe, a MNP-based immunochromatographic strip (nanozyme strip) was further employed for amplifying signal to allow visual detection and quantification by a strip reader. The LAMP products were sandwiched between the anti-FITC and the anti-BIO, and the accumulation of Fe_3O_4 magnetic enabled the visual detection of *ES*. The detection limit of the nanozyme biosensor was improved by 10 CFU/mL compared with previously reported techniques and the whole manipulation process was much faster (within 1 hour) and simpler (without specialist facilities). Hence, the developed continual cascade nanozyme biosensor has provided a rapid, ultra-sensitive, and simple tool for on-site detection of viable *ES*.

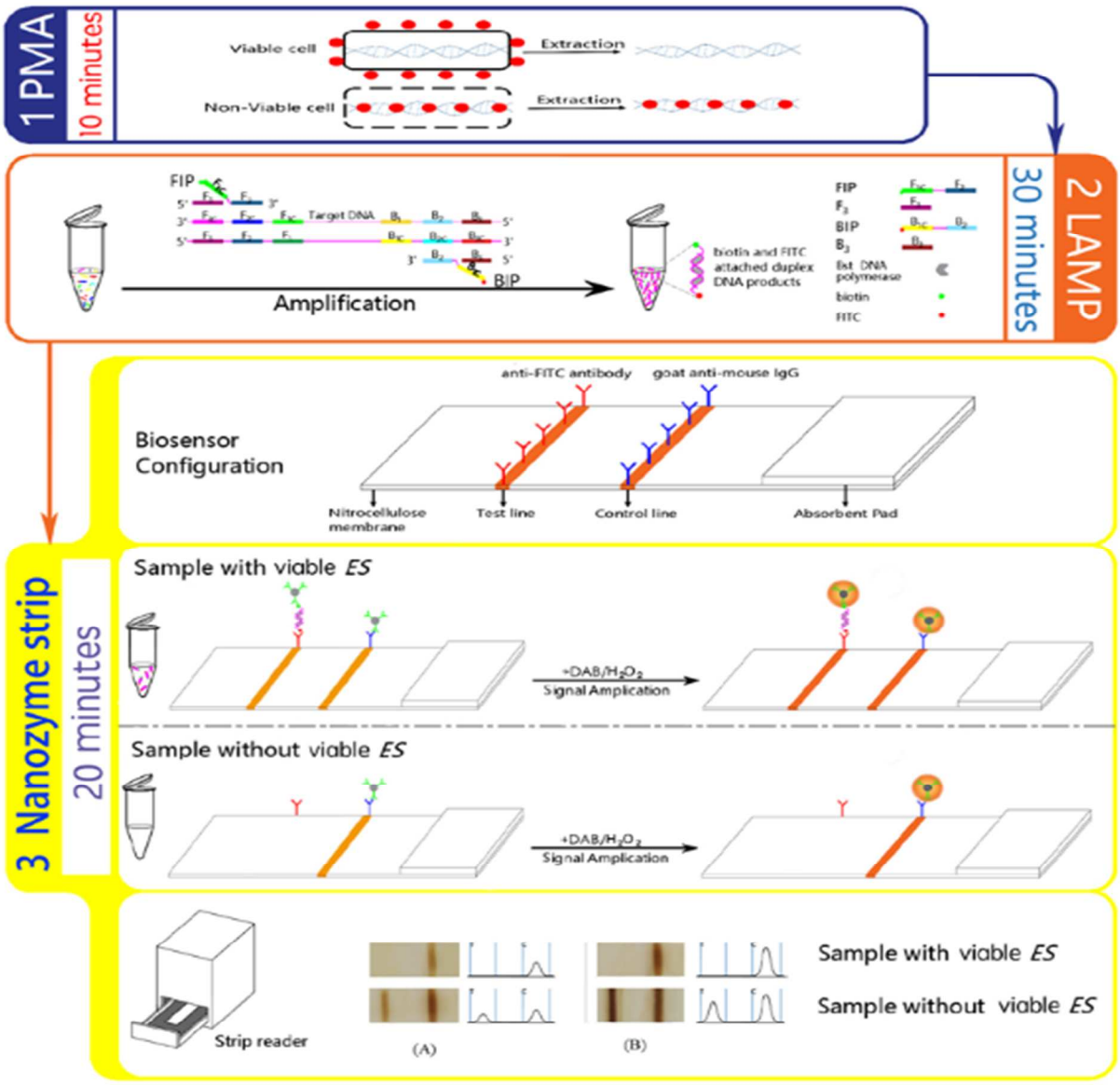
Enterobacter sakazakii (*ES*) is a Gram-negative, non-sporulating, motile, rod-shaped bacterium belonging to the *Enterobacteriaceae* family and an emerging food-borne pathogen that causes severe meningitis, meningoencephalitis, sepsis and necrotizing enterocolitis in neonates and infants.[1-4] The case-fatality rate of neonatal *ES* infections has been reported to be as high as 50%, with half of the reported patients dying within one week on diagnosis.[5-9] Although *ES* has been found in cheese, minced beef, sausage, and vegetables, dried infant formula has been implicated to be involved in both outbreaks and sporadic cases of *ES* meningitis and is considered the principal route of transmission to infants.[10-14] Therefore, rapid, ultra-sensitive and visual detection methods are in urgent need to detect potential *ES*. The pathogenic mechanism of *ES* has not yet been determined, but the secreted enterotoxin-like compounds and the *omp*A gene products of viable cells were considered to be the important virulence factors. Therefore, the detection methods for *ES* that can determine cell viability are especially required.

Conventional culture-based protocols are considered as a gold standard for detection of *ES*, but they are time-consuming, which usually take up to 7 days and show relatively low sensitivity.[15] Immunoassays have been developed for *ES* detection, such as enzyme-linked immunosorbent assay (ELISA). However, low sensitivity and specificity has limited their use. Recently, many biomolecular methods have been reported for detection of *ES*, such as PCR, and real time PCR.[16-19] However, they are not routinely used due to the requirement for an expensive thermal cycler. More recently, loop-mediated isothermal amplification (LAMP) has emerged as a promising alternative gene-amplification method that intergrates rapidity, simplicity, high specificity and efficiency. There are four or six primers in LAMP to produce repetitive stem-loop DNA products (10^9 copies) using the Bst DNA polymerase within one hour.[20, 21] However, it is not able to decide the viability of the detected bacteria. In fact, the genome derived from dead cells may also serve as a temple for amplification. Many molecular approaches have been used to distinguish the viable cells from dead, in which mRNA plays an important role as its instability in dead bacteria. However, the residual mRNA in dead cells can give false-positive results.[22] Ethidium monoazide (EMA) or propidium monoazide (PMA) are DNA-binding dyes which can penetrate damaged cytomembrane (dead or membrane-compromised bacteria) of the bacteria. When EMA/PMA is exposed to intense visible light, the photoreactive azide group on the dye can be converted to a highly reactive nitrene radical

embeded in dead bacterial DNA. [23] As a result, it is unavailable for the subsequent molecular amplification of DNA from dead cells. Combination of EMA/PMA and LAMP has been therefore widely used to detect the viable from dead bacteria, such as *Listeria monocytogenes, Salmonella* and *Vibrio parahaemolyticus*.[23-25] Moreover, it has been proved that PMA is advantageous over EMA in terms of excluding dead cells. In addition, LAMP combined with PMA typically does not immediately report the results in an on-site testing which is thus unable to achieve visual detection.

In order to meet different practical needs, Nanozyme-strip has attracted significant attention in recent years because of its high stability and can be reused. The Nanozyme-strip is based on the peroxidase-like activity of MNPs that produces detectable color reaction and has been successfully applied for biomedical screening, environmental analysis and Ebola detection.[26-29] The main objective of this study was to develop a continual cascade nanozyme biosensor for detection of viable *ES*. Using the BIO-and FITC-modified primers, the dual labelled products can be generated in LAMP process. After labeling with biotin antibody, this nanozyme probe is able to recognize, separate, and visualize *ES* on the strip. Owing to the catalytic properties of the probe, the detection sensitivity can be improved compared with the colloidal gold strip. Testing for *ES* on the biosensor can be completed within 1 hour and viable *ES* can be also successfully detected in dried infant formula.

Scheme 1. Schematic illustration of the design of the continual cascade biosensor. Typical photo images of 0 CFU/mL and 10^5 CFU/mL (sample solution) of target ES and recorded response signals of strip with a portable strip reader on (A) biosensor before catalytic reaction and (B) biosensor after catalytic reaction



Materials and methods

Reagents and instrumentation. PMA were purchased from Biotium, Inc (Hayward, USA). Bst DNA polymerase was purchased from New England Biolabs (Beijing, China).Bovine serum albumin (BSA), Anti-biotin, Goat anti-mouse IgG, anti-FITC, 1.5%LB agar, 20%LB media, EDC, NHS were purchased from Sigma Chemical Company (St. Louis, MO, USA). Betaine, DNA marker, and deoxynucleotide solution mixture (dNTPs) were purchased from TaKaRa Biotech (Dalian, China). $FeCl_3$ and NaAc were purchased from Beijing Chemical Reagents. All chemical reagents were of analytical grade or guaranteed reagents. Backing cards (HF0000MC100), glass fiber sample pads (CFSP001700), conjugation pads (GFCP000800), nitrocellulose membranes (135S) and absorbent pads (CFSP001700) were purchased from Millipore (Bedford, MA). A portable test reader (DT2050), which connected to a laptop was purchased from Shanghai Goldbio Tech.Co.,Ltd.(Shanghai, China)to collect assay signals of lines.

Strain and culture condition. A total of *ES* and 15 other bacterial strains were used to determine the specificity of biosensor (Table 1). All strains was stored in 20% (v/v) glycerol solution at -80 °C prior to use. It was subsequently streaked onto tryptone soy agar (TSA) and was incubated at 25 °C for 24 h. Single colonies from the TSA plates were transferred to 10 mL tryptone soy broth, and the cells were grown overnight at 25 °C. *ES* concentrations were determined spectrophotometrically. A 1 mL aliquot of the overnight cell suspension was centrifuged to harvest the cells and the pellet was re-suspended in 1.5% (m/v) NaCl. Adilution series (10^{-1}-10^{-6}) of the re-suspended cell suspension was prepared in 1.5% (m/v) NaCl, and the optical density of each dilution was determined with a spectrophotometer at 600 nm. Duplicate spread plates were prepared from the dilutions (10^{-1}-10^{-6}) on TSA, followed by incubation at 25 °C for 48 h. A standard curve was constructed of the cell counts (CFU/mL) against the corresponding optical density values.

Table1 Bacterial strains used in this study

Species	Sources
Enterobacter sakazakii	CICC21560
Enterobacter sakazakii	CICC21562
Enterobacter sakazakii	ATCC51329
Enterobacter sakazakii	ATCC29544
Enterobacter sakazakii	DSM18702
Enterobacter cloacae	CICC10011
Enterobacter aerogenes	ATCC51697
Enterobacter hormaechei	CICC10432
Salmonella spp.	CGMCC 1.0090
Yersinia	CO92
Pseudomonas aeruginosa	ATCC47085
Escherichia coli	ATCC43889
Vibrio parahaemolyticus	CMCC20001
Shigella	CMCC51572
Bacteroides fragilis	ATCC25285
Staphylococcus aureus	ATCC 25923
Bifidobacterium longum	CGMCC 2265
Klebsiella pneumoniae	CICC10013
Bacillus subtilis	CICC10002
Paenibacillus polymyxa	CICC10010

PMA treatment. Samples were treated as described previously with slight modifications.[30] Briefly, PMA was added at a final concentration of 10 ug mL^{-1}containing 1 uL viable or heat-killed cells. Samples were incubated in dark for 5 min with occasional mixing to allow penetration of PMA into dead cells. Then the sample tubes were laid horizontally on ice, 20 cm far from the 500W halogen light source and subsequently were exposed to light for 5 min. During this period, the tubes were shaken every 30 s to maintain homogeneous exposure to the light source.[31] Genomic DNA of *ES* was isolated using a bacterial DNA extraction Kit produced by Beijing Food Safety Tech.Co.,Ltd. (Beijing,China).The genomic DNA was amplified with LAMP assay immediately after preparation.

Primer design and PMA-LAMP reaction condition. The segments of *omp*A gene (GenBank DQ000206.1) from *ES* were selected as target sequence. A set of four primers (Table 2)was designed using Primer Explorer version 4 (https://primerexplorer.jp/lamp4.0.0/index.html). The forward inner primer (FIP) consisted of F1c (the complementary sequence of F1) and F2, and the reverse one (BIP) consisted of B1c and B2 (the complementary sequence of B2c). The 5' of FIP was modified with biotin and the 5' of BIP was modified with FITC. The outer primers consisted of the forward outer primer F3 and the reverse outer primer B3 (the complementary sequence of B3c). The primers were synthesized commercially by TaKaRa Biotech (Dalian, China).The optimized LAMP reaction was conducted with a 25 μL reaction mixture containing 1.6 μM (each) FIP and BIP, 0.2 μM (each) F3 and B3, 0.5 mM dNTPs, 3.6 mM MgSO4, 0.6 M betaine, 1×buffer, 8 U Bst DNA polymerase large fragment, 2 μL DNA template and ddH_2O to 25 μL. The mixture was incubated at 65 °C for 40 min. The LAMP products were analyzed by 2% agarose gel electrophoresis.

Table2 Primer sequences used in this study

Primers	Sequence(5'-3')
F3	GACCGCTAAACTGGGTTACC
B3	TGGTATTCCAGACGGGTAGC
FIP	AGAGGAATCAGCACGCCATACC-TAACCGACGATATGGACGT
BIP	GACCACGACACCGGCGTTTC-ATGTCGCGAGTCATTGCC

Preparation of MNPs and nanozyme probe. The synthesis of MNPs was carried out according to the hydrothermal methods.[29]The nanozyme probe was prepared as described previously.[32] Briefly, 5 mg MNPs were added to the mixed solution of EDC and NHS, and incubated at room temperature for 30 min. Functionalized MNPs were washed twice with ultrapure water, and then 100 μg mL^{-1} of the detection biotin antibody was added in NaAc buffer (50 mM, pH 6.0). The mixture was vortexed and incubated at 4°C overnight. The conjugate was washed twice with PBS (pH 7.0), and then incubated in Tris buffer (50 mM, pH 7.2) at room temperature for 30 min. Then, the nanozyme probe obtained was washed with PBS (pH 7.0), and then dispersed into 1 mL of 5% BSA-PBS solution.

Preparation of Nanozyme-strip. The nanozyme strip was prepared as previously described with some modifications.[29, 33] The test line(T line) and control line(C line) on

the nitrocellulose (NC) membrane were prepared by dispensing anti–FITC (1.0 mg mL^{-1}) and goat anti-mouse IgG (1.0 mg mL^{-1}), respectively. The distance between the two lines was 4.5 mm. Then the membrane was dried at 37 °C overnight and stored at room temperature. Finally, the absorbent pad was laminated onto the baking card with an overlap of approximately 2 mm to allow the sample to flow. Strips were cut with a 3.5 mm width using the cutting module and stored in a desiccated container at 4 °C.[34]

Detecting *ES* with the cascade biosensor. Following PMA treatment and LAMP process, the detection of *ES* was performed on the strip. Immunological hybridization and signal enhancement are the two important steps in a typical test strip biosensor. In the first step the 15 μL of MNP probe and different volume of samples diluted in the reaction buffer (50 mM Tris–HCl, pH 8.0,150 mM NaCl, 1% NP40 (v/v), 1% BSA (w/v)) were mixed in an Eppendorf (EP) tube. The strip was inserted vertically into the EP tube for 15 min. Next, the strip was taken out and put into substrates buffer (DAB and H_2O_2) in another tube for 5 min. Then, the strip was quickly washed with deionized water to terminate the reaction. As for the quantitative detection, the optical intensity of the T line was measured by a portable strip reader instrument equipped with preinstalled reader software. To further confirm that nanozyme biosensor can be applied in practical samples, performances were challenged in the infant formula. The samples was purchased from a local supermarket without any pretreatment and *ES* was quantified by standard culture and colony counting method. Then the *ES* was spiked into infant formula at the concentration of 10,10^2,10^3,10^4 and 10^5 CFU/mL.

RESULTS AND DISCUSSION

Design of the continual cascade biosensor. The design of this novel cascade is shown in Scheme 1. Firstly, the sample is treated with PMA (step 1). PMA selectively penetrates compromised membranes of dead bacteria to embed DNA inside the cells and make them unavailable for subsequent LAMP amplification, but has no effect on the intact membranes as in viable bacteria. Secondly, LAMP is used to generate massive BIO- and FITC-attached duplex DNA in a short period of time (step 2). In a typical assay the four primers recognize and hybridize with the target *omp*A regions. Finally, the nanozyme strip is applied as illustrated in Scheme 1 (step 3). The anti-FITC antibody and goat anti-mouse IgG are immobilized by physical adsorption to form the T line and C line on the NC membrane, respectively. In a typical assay, if a sample is positive, the sample solution rehydrates the nanozyme probe. The end of the positive product is labeled with nanozyme, and the other end is labeled with FITC. Then, the dual labelled products are captured by the anti-FITC antibody on T line. The free nanozyme probes continue to migrate and stopped in the C line through the reaction between the nanozyme probe and anti-mouse IgG. Upon applying DAB/H_2O_2 enzymatic substrate to the T line and C line, the enzymatic reactions between the nanozyme and DAB/ H_2O_2 enzymatic substrate will perform a color reaction to enhance visual effect. However, in the absence of viable *ES*, only one band can be observed in the C line.

Scheme1 presents typical photo images of the performance of Nanozyme-strip. As described in Scheme1 (step 3A), relatively weak bands were observed in the T line before catalytic reaction. However, the visual signal of T line was increased obviously after treatment with DAB/H_2O_2 enzymatic substrate (step 3B) Therefore, this nanozyme signal enhancement approach was very suitable for the ultra-sensitive detection of viable *ES*. The nanozyme probe is shown in Figure S1 as supporting information.

Performance of PMA. The efficiency of PMA treatment was analyzed firstly. When the *ES* bacteria were heat-killed, the membrane of *ES* facilitated the permeation of PMA. As a result, typical DNA band did not show up after PMA treatment (Fig. 1A) or during the LAMP reaction (Fig. 1B). This indicated that the nucleic acid dyes on agarose gel was not intercalated into DNA in the presence of PMA. PMA inhibited the LAMP amplification rather than indicating the viability status of bacteria. Most importantly, it indicated that PMA treatment was able to eliminate dead bacteria from the viable bacteria. The results also indicated that PMA treatment had high efficiency to eliminate the dead bacteria even at high concentrations (10^6 CFU/mL).

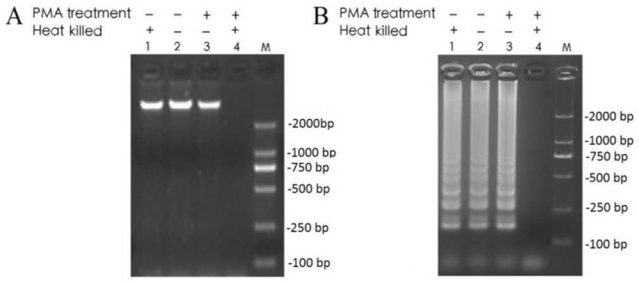


Figure 1 PMA treatment to distinguish viable and dead *ES*. (A) DNA extraction from viable or dead *ES* with (+) or without (-) PMA treatment. (B) The gel electrophoresis results of LAMP with (+) or without (-) PMA treatment. The concentration of *ES* each sample was 10^5 CFU/mL. In detail, PMA treatment (+) means the sample are treated with PMA.PMA treatment (-) means the sample are not treated with PMA. Heat killed (+) means the sample are treated under high temperature, that is to say the cells are dead. Heat killed (-) means the sample are not treated under high temperature, that is to say the cells are viable.

The specificity of LAMP. In order to verify the specificity of the proposed strategy, nineteen strains were used in this experiment, including five pure cultures of *ES* and fifteen different non-*ES* strains (Table 1). After DNA extraction and LAMP amplification, the products were analyzed by 2% agarose gel electrophoresis under the UV light. As shown in Fig. 2, only *ES* showed the positive ladder like pattern, indicating that the primers of *omp*A gene had good specificity. In contrast, non-*ES* strains did not generate any bands indicating no amplicons present.

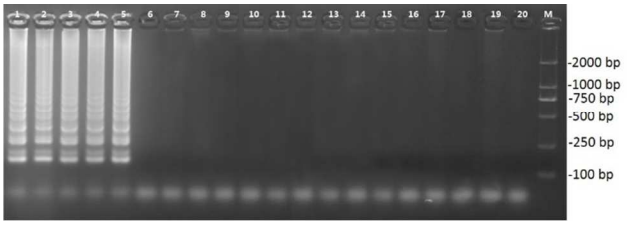


Figure 2 The electrophoretogram for determining specificity of the LAMP reaction.From left to right, bacteria strain are (1) *Enterobacter sakazakii* CICC21560; (2) *Enterobacter sakazkii* CICC21562; (3) *Enterobactersakazakii* ATCC513294; (4) *Enterobacter sakazakii ATCC29544*; (5) *Enterobacter sakazakii*

DSM18702; (6) *Enterobacter cloacae* CICC10011; (7) *Enterobacter aerogenes* ATCC51697; (8) *Enterobacterhormaechei* CICC10432; (9) *Salmonella spp.* CGMCC 1.0090; (10) *Yersinia* CO92; (11*) Pseudomonas aeruginosa* ATCC47085; (12) *Escherichia coli* ATCC43889; (13) *Vibrio parahaemolyticus* CMCC20001; (14) *Shigella* CMCC51572; (15) *Bacteroides fragilis* ATCC25285; (16) *Staphylococcusaureus* ATCC 25923; (17) *Bifidobacteriumlongum* CGMCC 2265; (18) *Klebsiella pneumoniae* CICC10013; (19) *Bacillus subtilis* CICC10002; (20) *Paenibacillus polymyxa* CICC10010

Optimization of cascade biosensor. To improve the sensitivity of the biosensor, various preparation and detection parameters were systematically investigated by comparing the analytical performances of biosensor including membrane materials, concentration of anti-FITC on the lines, the amount of nanozyme probe dispensed with the sample, reaction time. As shown in Fig. 3A, the best performance of biosensor was obtained with Millipore 135 S nitrocellulose membrane. The amount of nanozyme probe affected the hybridization efficiency between the nanozyme probe and sample and 10 μL of nanozyme probe was used as the optimal volume (Fig. 3B). Besides, the highest peak area was obtained with 1 mg mL^{-1} anti-FITC and 1 mg mL^{-1} anti-mouse IgG, and this combination was then used in the subsequent experiments (Fig. 3C). Moreover, the reaction time for strip detection was no less than 15 min (Fig. 3D).

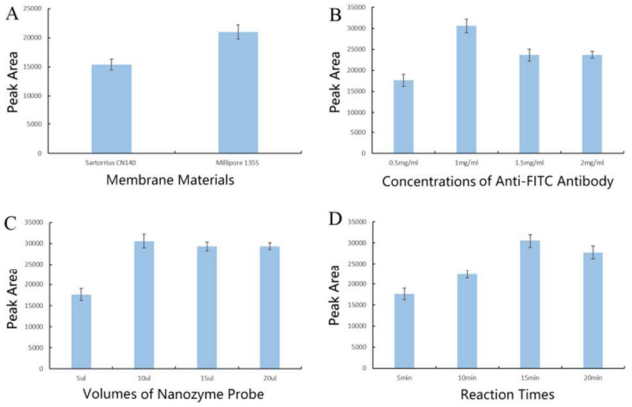


Figure 3 Optimizations of the biosensor. (A) Effect of membrane materials; (B) effect of concentration of anti-FITC on the line; (C) effect of volume of nanozyme probe; (D) effect of reaction times.

Analytical characteristics of the continual cascade biosensor. We evaluated the analytical performance of the continual cascade nanozyme biosensor in the following aspects. Reproducibility is one of the most import indicators in evaluating the biosensor. For the reproducibility test, 100 CFU/mL of viable *ES* was examined six times (Figure 4A). Each concentration was tested five times. The corresponding RSD values of optical responses were 1.5%, indicating excellent reproducibility. As samples in practice are always a mixture of different bacterial species, we tried to detect the target DNA of *ES* with high specificity. To determine the specificity of the cascade nanozyme biosensor, the performances were examined using ddH_2O, DNA of dead *ES* cells, DNA of viable *ES* cells and other *Enterobacter* bacterial strain DNA and other non-*Enterobacter sakazakii* bacterial strain DNA. As shown in Figure 4B, no false positive amplification was observed. The non-*ES* and dead *ES* had no signal as background. The high specificity of the biosensor was achieved by taking advantage of the specificity of the LAMP assay. For the stability study, the performances of the nanozyme biosensor were tested after one and a half month of storage at room temperature. As shown in Figure 4C, the responses of biosensor to 10^3 CFU/mL of *ES* remained almost the same after one month, indicating that the biosensor haddesirable stability.

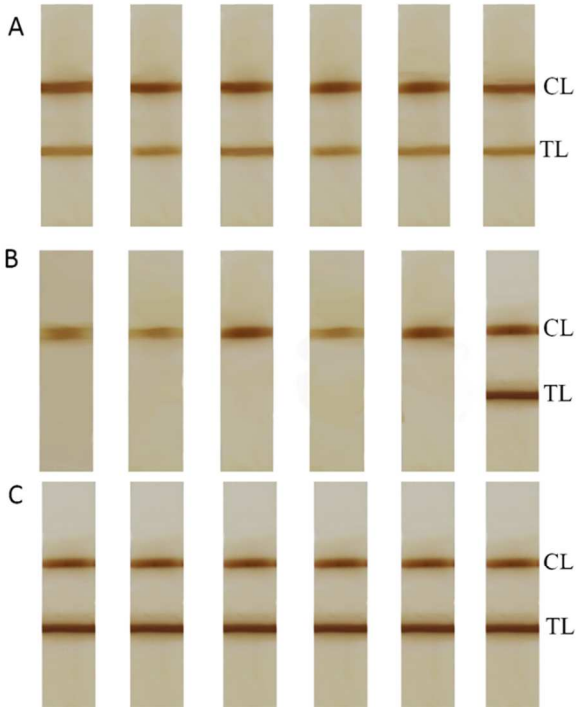


Figure 4. Representative images from analytical performances of biosensor. (A) Representative images for reproducibility. These tests were carried out every other week; (B) Representative images for specificity. From left to right, samples were produced by LAMP for ddH_2O, Other *Enterobacter*, other Non-*Enterobacter sakazakii* bacterial strain DNA, dead *Enterobacter sakazakii* bacterial strain DNA (CICC21560), viable *Enterobacter sakazakii* bacterial strain DNA (CICC21560); (C) Representative images for stability. These tests were carried out every other week.

Sensitivity is another important performance to evaluate the biosensor. Different concentrations of *ES* (ranging from 0 to 10^5 CFU/mL) were measured under the optimal nanozyme biosensor conditions. Figure 5 presents typical photo images of the biosensor in the presence of viable ES with different concentrations. For visual detection, the T line was observed with concentrations as low as 10 CFU/mL. In addition, the limit of detection (LOD) was tested with 2-fold serial dilutions of *ES* samples (ranging from 10 to 2 CFU /mL), and the LOD was 2 CFU /mL. The resulting plot of the response to ES concentration was linear over the min to max range, and the correlation equation was lg(peak area) = 1.2344 lg[(*ES*)CFU/mL]+0.0749 with a correlation coefficient of 0.9917, which was suitable for quantitative work.

The application in real samples was shown in Table S1 in supporting information. Recoveries of *ES* was in the range of 95.3±8.1% to 108.7±6.4%.It indicates that the biosensor is capable of detecting ES in infant powder.

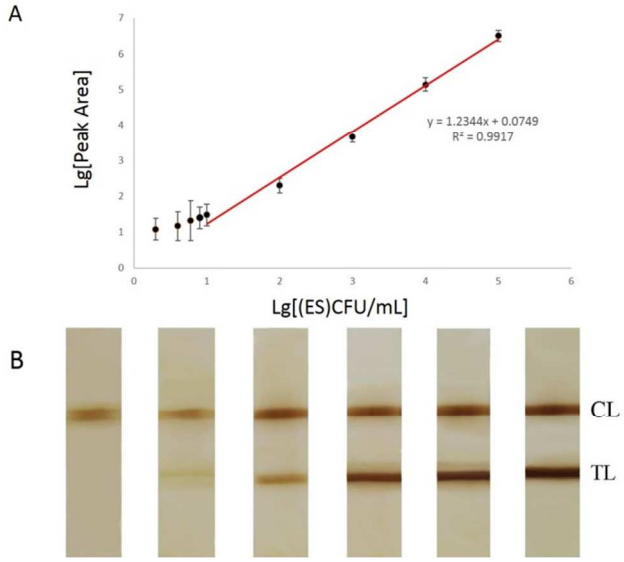


Figure 5. (A)Linear calibration curve for *ES* detection: changes in lg of peak area versus lg of *ES* concentrations. (B) Representative images from analytical sensitivity of biosensor. From left to right, concentrations of viable *Enterobacter sakazakii* (CICC21560) in samples were 0 CFU/mL, 10 CFU/mL, 100 CFU/mL, 10^3 CFU/mL, 10^4 CFU/mL, 10^5 CFU/mL.

CONCLUSION

In summary, a rapid and ultra-sensitive continual cascade nanozyme biosensor has been developed for viable *ES* detection. The highly optimized assay offers the following distinct advantages over other published methods: (1) PMA is applied to distinguish DNA in viable from dead cells. (2) The LAMP assay has high specificity and amplification efficiency.(3) The sensitivity is improved by employing Fe_3O_4 magnetic nanoparticles as nanozyme probe in place of colloidal gold introducing enzyme modification into the lateral flow biosensor, and the biosensor is able to detect 10 CFU/mL bacteria within 1 hour under optimal conditions. The proposed nanozyme biosensor not only has higher sensitivity than the traditional culture method, but also shows advances in many other aspects, such as cost, portability, rapidity and efficiency. In addition, the biosensor is capable of quantitative analyzing viable *ES* in infant powder by visual detection with simple sample pretreatment. Moreover, the nanozyme biosensor also has high potential for detecting other viable microorganisms by replacing the primers.

ASSOCIATED CONTENT

Supporting Information

Table S1 and Figures S1, as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript.

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