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A sensitive electrochemical DNA biosensor for specific detection of *Enterobacteriaceae* bacteria by Exonuclease III-assisted signal amplification

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ABSTRACT

A specific and sensitive methodology was developed successfully for quantitative detection of *Enterobacteriaceae* bacteria by integrating Exonuclease III-assisted target recycling amplification with a simple electrochemical DNA biosensor. After target DNA hybridizes with capture DNA, Exonuclease III can selectively digest the capture DNA, which releases the target to undergo a new hybridization and cleavage cycle on sensor surface, leading to a successful target recycling. Finally, the left capture DNA is recognized by detection probe to produce the detectable signal, which decreases with the increasing target DNA concentration. Under the optimal conditions, the proposed strategy could detect target DNA down to 8.7 fM with a linear range from 0.01 pM to 1 nM, showing high sensitivity. Meanwhile, the sensing strategy was successfully used for detection of *Enterobacteriaceae* bacteria down to 40 CFU mL^{-1} in milk samples. This strategy presented a simple, rapid and sensitive platform for *Enterobacteriaceae* bacteria detection and would become a versatile and powerful tool for food safety, biothreat detection and environmental monitoring.

1. Introduction

Enteropathogenic bacteria infection is an important factor for the foodborne infectious diarrhea, especially in developing countries (Guerrant et al., 1990). It has been estimated that diarrheal disease causes approximately three million deaths worldwide per year (Guerrant et al., 2002). Therefore, a simple, specific, and sensitive method is urgently required for detection and identification of enteropathogenic bacteria efficiently in the clinical diagnostics, food safety, biothreat detection and environmental monitoring.

Conventional identification methods for enteropathogenic bacteria include culture and colony counting, polymerase chain reaction (PCR), and enzyme-linked immunosorbant assay (ELISA) (Li et al., 2012). Unfortunately, although these approaches are powerful and error-proof, most of them are labor-intensive, time-consuming, expensive and requiring highly trained personnel (Sanvicens et al., 2009). In recent years, researchers are looking for new biosensor-based methods for fast, inexpensive, simple and sensitive detection of pathogenic bacteria, including optical (Bahsi et al., 2009), magnetic (Gehring and Tu, 2005; Li et al., 2010), FET (Villamizar et al., 2008), quartz crystal microbalance (Mao et al., 2006; Pathirana et al., 2000) and electrochemical techniques (Liao et al., 2007; Salam and Tothill, 2009; Li et al., 2012; Luo et al., 2012). Among these techniques, electrochemical biosensor has attracted considerable attention for its intrinsic advantages, such as easy to use, rapid response, low-cost and inexpensive instrumentation (Li et al., 2011). Meanwhile, a series of signal amplification strategies have also been applied to improve the sensitivity of electrochemical methods by the use of nano-/micro-materials and isothermal polymerase amplification (Ma et al., 2011; Zhou et al., 2012), but these methods often suffer complex treatment procedures, easy contamination and high costs.

Recently, nicking endonucleases have been used to amplify the signal (Liu et al., 2011; Bi et al., 2010). These strategies have the advantages of simplicity and high sensitivity, but the enzymes require target DNA with a specific sequence for enzyme

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recognition, which limits the versatility. Exonuclease III (Exo III) is a sequence-independent enzyme and can assist signal amplification for DNA detection without a specific recognition site in the target DNA (Hsieh et al., 2010; Zuo et al., 2010; Luo et al., 2012). However, the previous methods require a complicated and expensive stem-loop hairpin probe. In this work, a specific electrochemical DNA sensing method was developed for sensitive "signal-off" detection of *Enterobacteriaceae* bacteria by integrating Exo III-assisted target recycling amplification on sensor surface with enzymatic signal readout. The sensing strategy could be successfully used for detection of *Enterobacteriaceae* bacteria down to 40 CFU mL^{-1} in milk samples.

2. Materials and methods

2.1. Reagents

DNA oligonucleotides were synthesized and purified by Sangon Inc. (Shanghai, China). Their sequences are listed in Table S1. 6-Mercapto-1-hexanol (MCH), streptavidin-alkaline phosphatase (ST-AP), α-naphthyl phosphate (α-NP), bovine serum albumin (BSA) and salmon sperm DNA were purchased from Sigma-Aldrich (USA). *Escherichia coli* Exonuclease III (Exo III) was obtained from New England Biolabs (China). Premix Taq Version 2.0, DL500 DNA Marker and agarose were purchased from Takara (Dalian, China). All other reagents were of analytical reagent grade. All aqueous solutions were prepared using Millipore-Q water (≥18 MΩ, Milli-Q, Millipore). 20 mM Tris-HCl buffer containing 0.10 M NaCl, 5.0 mM $MgCl_2$ and 0.05% Tween-20 (pH 7.40) was used as washing buffer. Hybridization buffer (pH 7.5) contained 0.3 M NaCl and 0.03 M sodium citrate. Diethanolamine (DEA) buffer (pH 9.6) contained 0.1 M diethanolamine, 1 M $MgCl_2$ and 100 mM KCl.

2.2. Apparatus

Electrochemical characterizations including differential pulse voltammetric (DPV) and electrochemical impedance spectroscopic (EIS) measurements were carried out on a CHI660D electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China) with a conventional three-electrode system composed of platinum wire as auxiliary, Ag/AgCl electrode as reference and a 3-mm-diameter gold electrode as working electrode. The PCR was carried out using a My Cycler thermal cycler (Bio-Rad Laboratories, USA). Gel images were recorded on an imaging system (Bio-Rad Laboratories, USA).

2.3. Preparation of DNA samples and PCR amplification

Escherichia coli O111 (*E. coli* O111) was obtained from Chongqing Municipal Center for Disease Control and Prevention. The pure culture of *E. coli* O111 was grown in Luria-Bertani medium at 37 °C for 16 h with shaking. The culture was then washed twice in sterile ultrapure water by centrifugation at 12,000 rpm for 10 min and resuspended in sterile ultrapure water. Viable counts were performed by plating 100 μL of appropriate 10-fold dilutions in sterile ultrapure water onto plate count agar. After incubating the plates at 37 °C for 24 h, the culture colonies on the plates were counted to estimate the number of viable cells in CFU mL^{-1}. The different concentrations of culture were incubated for 15 min at 100 °C in a water bath and immediately chilled on ice (Li et al., 2012). After centrifugation at 12,000 rpm for 7 min at 4 °C, the supernatant containing genomic DNA which was directly used as PCR template was transferred to a new tube.

PCR was performed in a final volume of 50 μL containing 5.0 μL of genomic DNA, 1.0 μL of 20 μM each primer, 25 μL of Premix Taq (1.25 U of DNA polymerase, 2 × Taq buffer, 0.4 mM of dNTPs) and 18 μL of water. *E. coli* genomic DNA was initially denatured at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing), 72 °C for 1 min (extension), and final 3-min extension. PCR products were identified by running 10 μL of PCR mixture in 2% agarose gel for 20 min and observed under ultraviolet light.

2.4. Preparation of electrochemical biosensor

The bare gold electrode was polished with 0.05 μm alumina slurries and ultrasonically treated in ultrapure water for a few minutes, followed by soaking in piranha solution (H_2SO_4: H_2O_2=3:1) for 10 min to eliminate other substances. The pretreated gold electrode was rinsed with ultrapure water and allowed to dry at room temperature. 10 μL of 1.0 μM thiolated capture probe was dropped on the pretreated gold electrode surface and incubated overnight at 4 °C. After washed with the washing buffer, the electrode was immersed into 100 μL of 1 mM MCH for 1 h to obtain well-aligned DNA monolayer and occupy the left bare sites (Cheng et al., 2012, 2007; Qian and He, 2009). The electrode was further rinsed with the washing buffer and treated in salmon sperm DNA and 1% BSA for 30 min to block the nonspecific binding sites on its surface to obtain the electrochemical DNA biosensor.

The PCR product was denatured by heating at 100 °C for 7 min in a water bath, and immediately chilled in ice for 5 min to obtain denatured ssDNA before the detection. The synthetic target DNAs were diluted to the desired concentration with tris-ethylenediaminetetraacetic acid (TE) buffer. The electrochemical DNA biosensor was firstly incubated in 10 μL of the mixture of 5 units Exo III and target DNA for 2 h at 37 °C (Cui et al., 2010). After washed with washing buffer, biotinylated detection probe was dropped on the biosensor surface and incubated for 1 h at 37 °C. Following washed by DEA buffer containing 0.05% Tween-20, the electrochemical DNA biosensor was reacted with 10 μL of 0.9 μg mL^{-1} ST-AP at 37 °C for 30 min, and washed thoroughly with DEA buffer containing 0.05% Tween-20. The DPV measurement was performed in DEA buffer containing 1 mg mL^{-1} of α-NP substrate with modulation time of 0.05 s, interval time of 0.017 s, step potential of 5 mV, modulation amplitude of 70 mV and potential scan from 0.0 to +0.6 V.

3. Results and discussion

3.1. Design of electrochemical biosensor

The identification of *Enterobacteriaceae* bacteria is usually performed using *Lac Z* gene that encodes the β-galactosidase enzyme as target DNA (Bej et al., 1990; Van Poucke and Nelis, 1995). As shown in Scheme 1, the target DNA firstly hybridized with specifically designed capture DNA to form double-stranded structure, which had unique characteristic 3′-blunt end at the capture DNA and 3′-overhang end at target DNA. Thus Exo III could recognize the formed structure to catalyze the stepwise removal of mononucleotides from 3′-hydroxyl termini of DNA duplexes with 3′-blunt or recessed (Zhang et al., 2011), which digested the capture DNA strand and led to the release of target DNA. The released target DNA hybridized with other capture DNA to lead to a new target recycling. After Exo III-assisted target recycling amplification, the capture DNA remained on sensor surface hybridized with biotinylated detection probe and then linked to ST-AP