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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

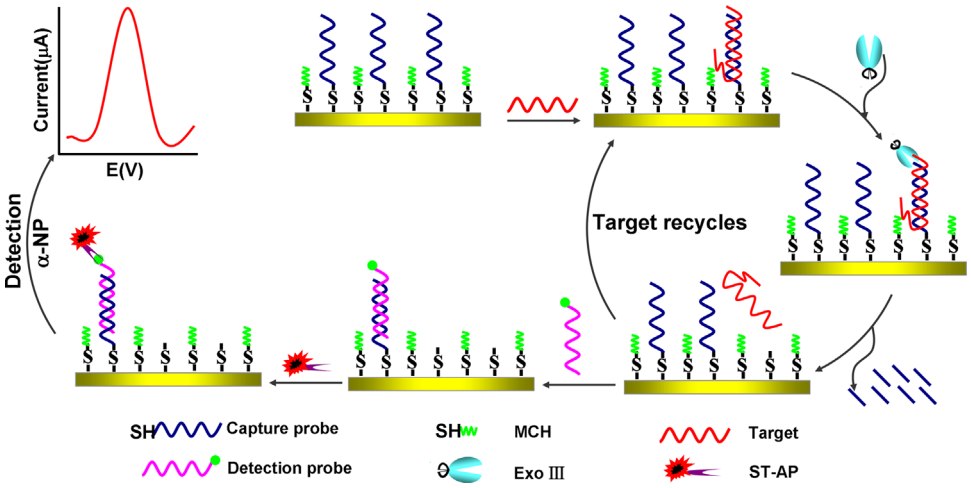
The bare gold electrode was polished with 0.05 μm alumina slurries and ultrasonically treated in ultrapure water for a few minutes, followed by soaking in piranha solution (H_2SO_4: H_2O_2=3:1) for 10 min to eliminate other substances. The pretreated gold electrode was rinsed with ultrapure water and 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



Scheme 1. Schematic representation of the electrochemical biosensor via Exo III-assisted signal amplification strategy for sequence-specific DNA detection.

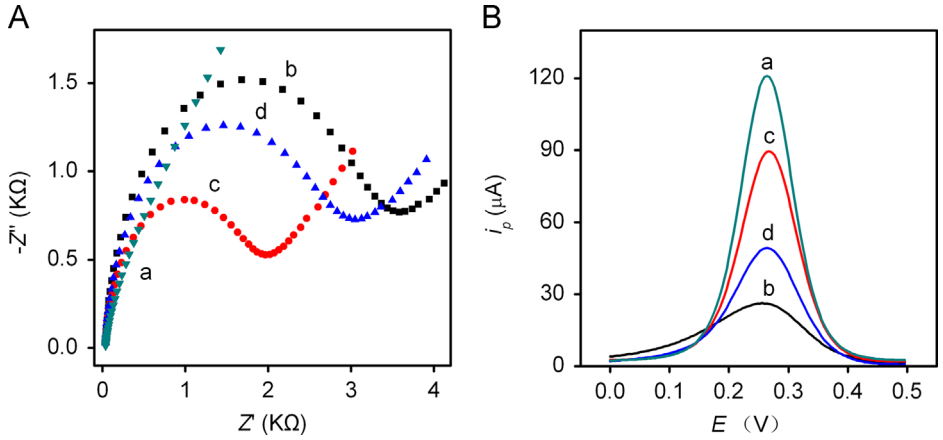


Fig. 1. EIS (A) and SWVs (B) in 0.4 M KCl containing 0.5 mM $Fe(CN)_6^{3-/4-}$ at (a) bare electrode, (b) capture DNA modified electrode, (c) capture DNA modified electrode after reaction with target DNA and Exo III, and (d) further reaction with detection probe.

to produce enzymatic electrochemical signal for quantitative detection of *Enterobacteriaceae* bacteria.

3.2. Characterization of biosensor fabrication

EIS and square wave voltammetric (SWV) measurements were used to characterize the electrochemical DNA biosensor (Zhang et al., 2011). In the terms of EIS, $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ was utilized as the redox probe and the semicircle diameter was equal to electron-transfer resistance, R_{et} (Fig. 1A). In 0.4 M KCl containing 0.5 mM $Fe(CN)_6^{3-/4-}$, bare electrode exhibited an almost straight line (curve a), which was characteristic of a mass diffusion limiting step of the electron-transfer process. When the thiolated capture DNA was self-assembled onto the bare electrode via Au-thiol binding, the R_{et} increased (curve b). This was because that the negatively charged phosphate backbone of the oligonucleotides produced an electrostatic repulsion force to $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$. The R_{et} decreased significantly (curve c) when the biosensor was hybridized with target DNA and reacted with Exo III, which was attributed to the fact that considerable capture DNA strands were digested, which proved the successful implement of Exo III-assisted target recycling amplification. Afterwards, upon the hybridization of the biotinylated detection probe with the remaining capture DNA, the R_{et} further increased (curve d). These results were in a good agreement with those obtained from SWV measurements (Fig. 1B). The SWV at the bare gold electrode showed the highest peak current (curve a), self-assembled monolayer of the capture probe on the gold electrode surface lead to a large decrease of the peak current due to the increased electron transfer resistance (curve b). When the biosensor was reacted with the mixture of Exo III and target DNA, the peak current greatly increased due to the successful implement of Exo III-assisted target recycling amplification (curve c). The peak current further decreased after the detection probe hybridized with the remaining capture probe (curve d). As the above figure shows, both results of EIS and SWV proved that the biosensor worked indeed as described in the principle scheme.

3.3. Signal amplification performance of designed biosensor

In this strategy, Exo III selectively digested the capture DNA strand, and the target DNA would be released to bind another capture DNA to undergo a new cleavage reaction, which produced a large amount of digested capture probes. As shown in Fig. S1, the DPV response in the presence of Exo III was obviously smaller than that in the absence of Exo III due to the Exo III-assisted target recycling. As a result, the biosensing platform was established for the specific and sensitive determination of target DNA based on Exo III-assisted signal amplification.

3.4. Effect of length of 3′-overhang ends on Exo III activity

To investigate the effect of length of 3′-overhang ends on Exo III activity, seven DNA targets including the perfect complementary target and 1-base, 2-base, 3-base, 4-base, 5-base, 6-base recessed DNAs (see Table S1 for details) were prepared to react with capture DNA modified gold electrode, respectively. The DPV peak currents increased monotonously from T1 to T7, and then trended the maximum value that was obtained in the absence of target DNA (Fig. S2). Thus the optimal 3′-overhang length in the duplex structure appeared to be of at least 5 bases which were protected from Exo III digestion. Those results were coincident with the information of commercial product Exo III.

3.5. Optimization of experimental conditions

To obtain excellent analytical performance, the different experimental conditions were optimized (Fig. 2). ST-AP concentration was an important parameter affecting the signal readout. With the increasing concentration of ST-AP, the DPV response rose gradually and then tended to a constant value at 0.9 μg mL^{-1}, which was chosen as the optimized ST-AP concentration. At the ST-AP concentration of 0.9 μg mL^{-1}, the signal increased gradually with the increasing concentration of α-NP and reached the stable value at 1.0 mg mL^{-1}. Therefore, 1.0 mg mL^{-1} was adopted as the optimal α-NP concentration.

The DPV response also increased with an increasing concentration of biotinylated detection probe and tended to a maximum value at 500 nM (Fig. 2C), which was selected as the appropriate concentration of the detection probe. The concentration of Exo III strongly influenced the response of the biosensor. The peak current decreased with the increase of Exo III concentration and reached the minimum at 0.5 U μL^{-1} for the incubation time of 2 h (Fig. 2D). Due to the limitation of incubation time, the response signal did not obviously decrease when the Exo III concentration beyond 0.5 U μL^{-1}. So, 0.5 U μL^{-1} Exo III was used in the following experiments.

3.6. Analytical performance of designed biosensor

Under the optimal experimental conditions, the DPV responses for synthetic target oligonucleotides at different concentrations were shown in Fig. 3. The DPV response linearly decreased with the logarithm of target DNA concentration (0.01 pM to 1 nM), and the corresponding regression equation was i_p (μA) = 10.07 − 2.14 × lg C (pM) with a correlation coefficient of 0.9988. The detection limit of this method, estimated as three times the standard deviation of the blank sample measurements, was about 8.7 fM. Compared to other methods reported previously for the detection of *Enterobacteriaceae* bacteria, the electrochemical DNA biosensor based on Exo III-assisted signal amplification strategy showed significant improvement of the detection limit (Li et al., 2012). To evaluate the repeatability of the developed biosensor, the synthetic target DNA at 500 pM was examined 5 times. The relative standard deviation was less than 5%, which indicated an acceptable reproducibility of this method.

In order to investigate the specificity of the biosensor, six different non-complementary synthetic DNAs and three different PCR products corresponding to 4×10^8 CFU mL^{-1} of bacterial samples (*Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, α-*hemolytic streptococcus*) were examined. The DPV response for T1 was the lowest among other mismatch target DNAs, including T8 (1-base mismatch), T9 (2-base mismatch), T10 (3-base mismatch) and T11 (4-base mismatch) (Fig. 4). The responses for T12 (5-base

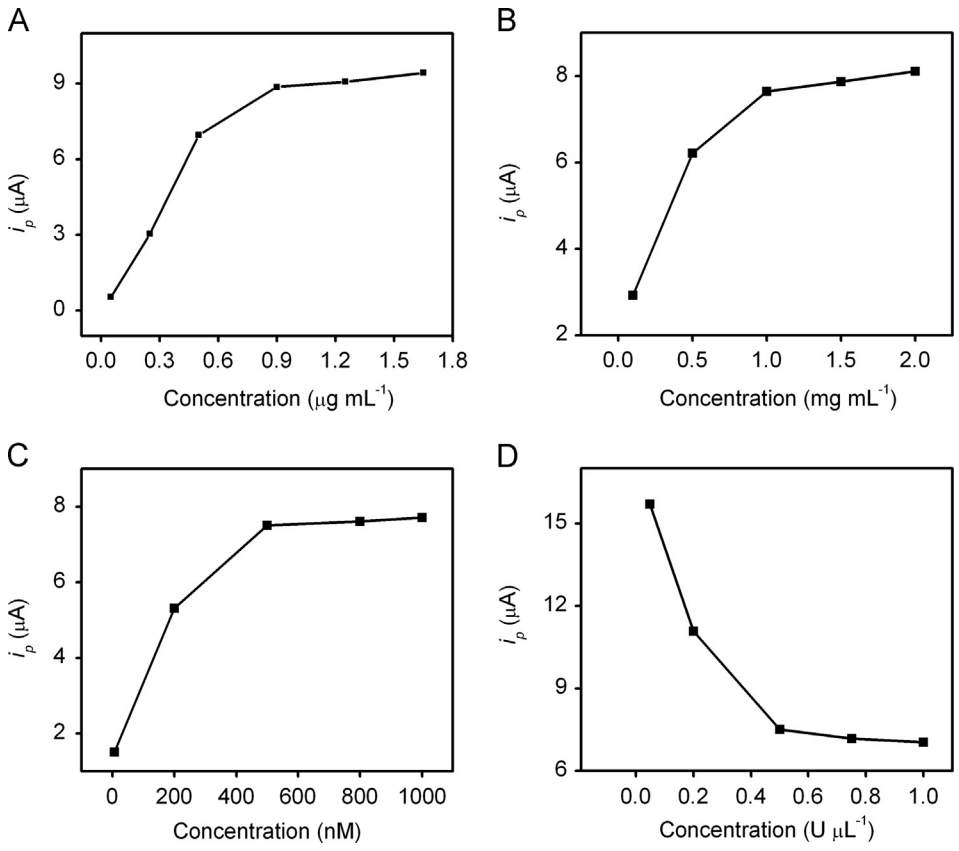


Fig. 2. Dependences of DPV peak currents on ST-AP concentration (A), α-NP concentration (B), detection probe concentration (C), and Exo III concentration (D). When one parameter changed and the others were under their optimal conditions.

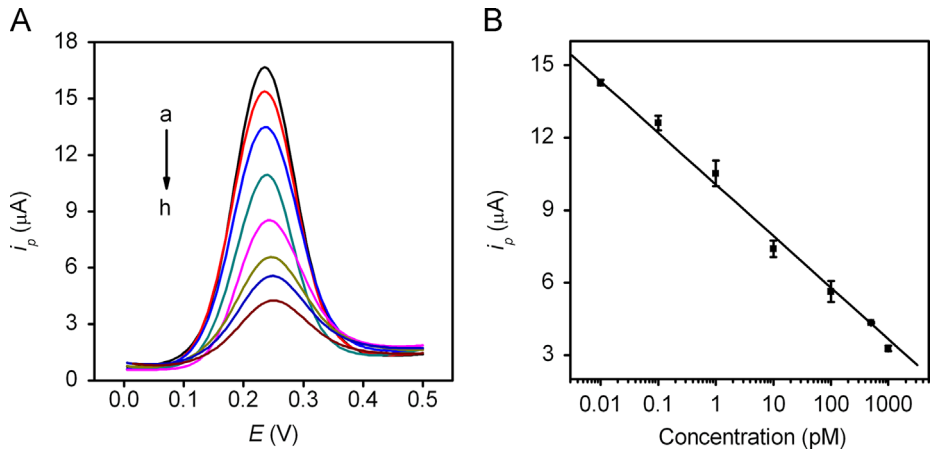


Fig. 3. (A) DPV response to 0, 0.01, 0.1, 1, 10, 100, 500 and 1000 pM target DNA (from a to h). (B) Plot of DPV peak current vs logarithm of target DNA concentration. The error bars represent the standard deviations in three different measurements for each concentration.

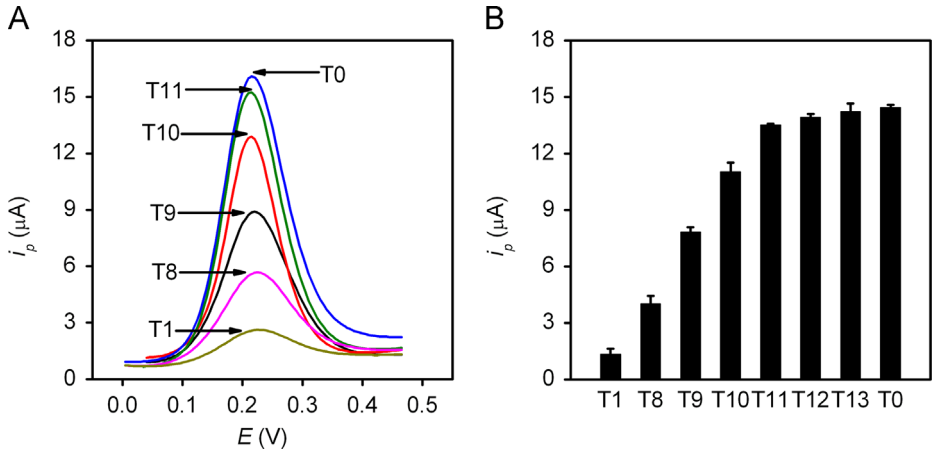


Fig. 4. (A) DPV curves for detection of T1, T8, T9, T10, T11 and blank and (B) DPV peak currents for T1, T8, T9, T10, T11, T12, T13 and blank.

mismatch) and T13 (6-base mismatch) were close to that for T11. These results demonstrated that the designed biosensor could discriminate different DNA sequences effectively and displayed excellent selectivity.

Six PCR products were assayed with the designed biosensor and compared with the agarose gel electrophoresis of the PCR products (Fig. 5A) to prove the specificity of the proposed DNA detection method. The PCR products were 4×10^8 CFU mL^{-1} of *E. coli*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *α-hemolytic streptococcus* and their mixture respectively. The DPV response of *E. coli* (a) was much smaller than those of (c), (d), (e), (f), while the signal of (b) was close to that of (a) (Fig. 5B), which were consistent with the electrophoresis results, demonstrating that the designed biosensor displayed excellent specificity for the detection of *Enterobacteriaceae* bacteria.

3.7. Detection of E. coli cells in milk samples

To prove whether the fabricated biosensor could detect actual sample sensitively and specifically, the cultured *E. coli* cells were inoculated into milk at the concentrations from 0 to 4×10^8 CFU mL^{-1}. After a pretreatment step for the milk samples, PCR was then carried out using the genomic DNA extracted from each concentration of *E. coli* cells. The agarose gel electrophoresis detection of PCR products with the right size (264 bp) was shown in Fig. 5C. The gel electrophoresis could not effectively identify the PCR products for *E. coli* cells less than 4×10^5 CFU mL^{-1} due to the low EtBr straining efficiency for ssDNA (Mao et al., 2006; Gong et al., 2008). The proposed electrochemical DNA biosensor was then applied to analyze the denatured PCR products for *E. coli* cells ($0–4 \times 10^8$ CFU mL^{-1}). The responses of the biosensor to different PCR samples were shown in Fig. 5D. The fabricated DNA biosensor could detect *E. coli* concentration as low as 40 CFU mL^{-1} in real milk samples, which was much lower than the PCR-ELISA system (Daly et al., 2002) and ELISA (≥10^5 CFU mL^{-1}) (Galikowska et al., 2011). PCR method has distinct advantages in sensitivity, but it often encounters false positivity, and the low resolution of post-PCR analysis by gel electrophoresis also limits its routine use in many laboratories. The proposed method overcomes the inherent disadvantages of traditional PCR method due to the specific capture probe. Furthermore, the detection process of the proposed method could be finished in 3.5 h. Compared with conventional culture method (at least 24–48 h) (Sun et al., 2009), this method is rapid and simple due to the integration of a simple DNA extraction and specific PCR with a high sensitive electrochemical DNA sensor for *Lac Z* gene. The proposed strategy exhibited high specificity, sensitivity and speediness, showing the potential as a pragmatic tool for *E. coli* detection in real samples. Moreover, the methodology could be used to detect other pathogenic bacteria by the use of appropriate oligonucleotides.

4. Conclusions

In summary, we developed a simple and ultrasensitive electrochemical DNA biosensing method for convenient detection of

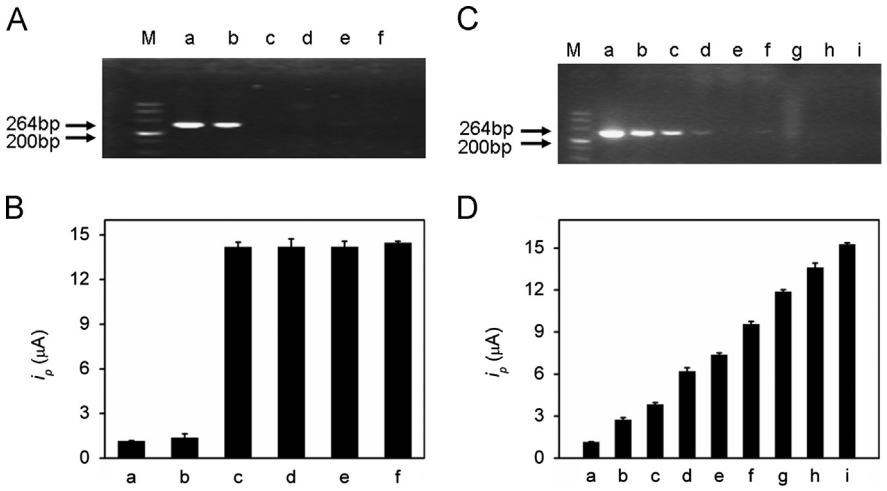


Fig. 5. (A) Gel electrophoresis photos of 500 bp size marker (M), and PCR products of 4×10^8 CFU mL^{-1} *E. coli* (a), mixture of the four strains (b), *Streptococcus pneumoniae* (c), *Pseudomonas aeruginosa* (d), α-*hemolytic streptococcus* (e) and blank (f). (B) DPV peak currents responding to PCR products obtained from a, b, c, d, e and f, respectively. (C) Gel electrophoresis photos of 500 bp size marker (M), and PCR products of 4×10^8 (a), 4×10^7 (b), 4×10^6 (c), 4×10^5 (d), 4×10^4 (e), 4×10^3 (f), 4×10^2 (g), 4×10^1 (h) CFU mL^{-1} *E. coli* and blank (i). (D) DPV peak currents responding to PCR products obtained from serial dilutions of *E. coli* in the range of 4×10^1–4×10^8 CFU mL^{-1}. The error bars represent the standard deviations calculated from three different spots.

bacteria through integrating Exo III-assisted signal amplification with enzymatic signal readout. The amplification strategy greatly improved the sensitivity for detection of a complementary target DNA down to 8.7 fM only within 3.5 h. The developed method had also been demonstrated to be suitable for specific and sensitive detection of PCR amplified products from *E. coli* bacterial cultures based on the highly specific DNA probes for *Lac Z* gene sequence. Meanwhile, this method could detect *E. coli* as low as 40 CFU mL^{-1} in real milk samples. Therefore, the proposed technique provided a simple, versatile and powerful tool for food safety, biothreat detection and environmental monitoring.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.03.084.

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