A Rapid and Sensitive Aptamer-Based Electrochemical Biosensor for Direct Detection of *Escherichia Coli* O111

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Abstract
A sensitive and specific electrochemical biosensor based on target-induced aptamer displacement was developed for direct detection of *Escherichia coli* O111. The aptamer for *Escherichia coli* O111 was immobilized on a gold electrode by hybridization with the capture probe anchored on the electrode surface through Au-thiol binding. In the presence of *Escherichia coli* O111, the aptamer was dissociated from the capture probe-aptamer duplex due to the stronger interaction between the aptamer and the *Escherichia coli* O111. The consequent single-strand capture probe could be hybridized with biotinylated detection probe and tagged with streptavidin-alkaline phosphatase, producing sensitive enzyme-catalyzed electrochemical response to *Escherichia coli* O111. The designed biosensor showed weak electrochemical signal to *Salmonella typhimurium*, *Staphylococcus aureus* and common non-pathogenic *Escherichia coli*, indicating high specificity for *Escherichia coli* O111. Under the optimal conditions, the proposed strategy could directly detect *Escherichia coli* O111 with the detection limit of 112 CFU mL^{-1} in phosphate buffer saline and 305 CFU mL^{-1} in milk within 3.5 h, demonstrated the sensitive and accurate quantification of target pathogenic bacteria. The designed biosensor could become a powerful tool for pathogenic microorganisms screening in clinical diagnostics, food safety, biothreat detection and environmental monitoring.

Keywords: Biosensor, Aptamer, Escherichia coli O111, Differential pulse voltammetry

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1 Introduction

One of the most common causes of persistent infantile diarrhea and mortality is enteropathogenic *Escherichia coli* (EPEC), which is the most important pathogen infecting children worldwide because of its high prevalence in both the community and hospital settings [1,2]. It is estimated that EPEC is the second leading cause of death in children younger than 5 years globally, accounting for 1.3 million deaths annually [3]. Therefore, it is still an urgent demand to develop fast, highly sensitive, and easy-to-use methods for the detection of EPEC.

Various methods have been used for the detection of pathogenic bacteria, including conventional culture methods, enzyme-linked immunosorbant assay (ELISA) and polymerase chain reaction (PCR). Conventional culture methods are reliable but labor-intensive and time-consuming (at least 1–2 day) [4]. ELISA usually requires a minimum time of 24–48 h, and its sensitivity ($\geq 10^5$ CFU mL^{-1}) is insufficient to detect low levels of pathogen [5]. PCR method has distinct advantages in sensitivity, but it often encounters false positivity [6] and complicated pretreatment process [7]. In comparison to above three methods, the biosensor technologies for the detection of pathogenic bacteria, including optical [8], magnetic [9], FET [10], quartz crystal microbalance [11] and electrochemical biosensor techniques [12], have attracted considerable interest for their intrinsic advantages such as high-throughput analysis, high sensitivity and specificity [13].

Among the available biosensor platforms electrochemical biosensor techniques possess a series of advantages, such as rapid response, ease of use, low-cost and small-sized commercial detectors [14,15]. Various electrochemical sensors for pathogenic bacteria detection have been reported. Electrochemical immunosensor using antibody as molecular recognition element can rapidly and directly detect pathogenic bacteria with high affinity and specificity [16]. However, the drawbacks associated with antibody such as production, stability and modification limit its application [17]. Electrochemical DNA sensor is sensitive for detection of the specific gene of a certain pathogenic bacteria [18]. But this strategy generally needs the complicated PCR process and cannot detect pathogenic bacteria cells directly.

Aptamer is short, single-stranded, functional DNA or RNA strand from random-sequence nucleic acid libraries by in vitro evolution process called SELEX (systematic evolution of ligands by exponential enrichment) [19–21]. Compared with traditional antibody, aptamer possesses outstanding features, including high specificity, strong binding affinity, simple synthesis, easy labeling, good stability, high resistance against denaturation, and wide applicability [22–24]. Moreover, aptamers can bind with a wide array of targets, thus they have been applied as recognition probes in many fields, such as small molecules detection [25,26], protein analysis [27,28], cancer diagnosis [29], and bacteria detection [30].

Enteropathogenic *Escherichia coli* O111 (*E. coli* O111) aptamer (L9F) developed against lipopolysaccharide (LPS) from *E. coli* O111 has strong binding affinity to *E. coli* O111 surface component LPS, which is a critical structural, specific antigenic, and pathogenic component of the outer membrane of gram-negative bacteria [31,32]. To our best knowledge, no aptamer-based electrochemical biosensor has been reported to detect *E. coli* O111. In this work, a rapid and sensitive electrochemical biosensor was developed for direct detection *E. coli* O111 based on a target-induced aptamer displacement strategy [33]. This biosensor showed excellent performance and could be used for preliminary analysis of real samples.

2 Experimental

2.1 Materials and Reagents

DNA oligonucleotides were synthesized by Sangon Inc. (Shanghai, China), and their sequences were expressed as follows:

capture probe:
SH-$(CH_2)_6$-TTTT TTTT TTTA TACG GGAG CCAA CACC ACAT AACT TCCT

aptamer:
ATCC GTCA CACC TGCT CTAC TGGC CGGC TCAG CATG ACTA AGAA GGAA GTTA TGTG GTGT TGGC TCCC GTAT

detection probe:
Biotin-TTTT TTTT TTTA GGAA GTTA TGTG GTGT TGGC TCCC GTAT

6-Mercapto-1-hexanol (MCH), streptavidin-alkaline phosphatase (ST-AP), α-naphthyl phosphate (α-NP), bovine serum albumin (BSA) and salmon sperm DNA were obtained from Sigma-Aldrich (USA). All other reagents used were of analytical reagent grade. All solutions were prepared using Millipore-Q water ($\geq$18 MΩ). A 20 mM Tris-HCl buffer containing 0.10 M NaCl, 5.0 mM $MgCl_2$ and 0.005 % Tween-20 (PH 7.40) was used as washing buffer. PBST buffer contained PBS and 0.05 % Tween-20.

2.2 Apparatus

All electrochemical measurements were performed 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. Differential pulse voltammetric (DPV) measurements were performed using 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.

2.3 Preparation of Pure Microbial Sample and Artificial Specimen

The bacterial cultures, including *E. coli* O111, *Salmonella typhimurium*, *Staphylococcus aureus* and common non-pathogenic *E. coli*, were obtained from the Chongqing Municipal Center for Disease Control and Prevention. The pure cultures were grown in Luria-Bertani medium at 37 °C for 16 h. Then, the samples were washed twice in 5 mL sterile ultrapure water by centrifugation at 12000 rpm for 10 min; after removal of the supernatant fluid, the pellets were resuspended in 10 mL sterile phosphate buffer saline (PBS) buffer. The cultures were serially diluted to 10^{-8} with sterile PBS buffer. Viable counts were performed by surface plating 100 μL dilutions of 10^{-6}, 10^{-7} and 10^{-8} on plate count agars. After incubating the plate at 37 °C for 24 h, the concentrations were estimated by calculating the average number of CFU mL^{-1}. Finally, different concentrations of bacteria were heat-killed in a boiling water bath for 15 min and stored at 4 °C until used.

For the detection of real samples, the cultured *E. coli* O111 cells were inoculated into fresh pasteurized milk and then mixed with 1 mL PBST buffer, vortexed, centrifuged at 12000 rpm for 5 min to eliminate lipids and proteins [34]. The bacteria pellets were resuspended in sterile PBS buffer. The following steps were done according to above method.

2.4 Preparation of the Electrochemical Biosensor

A 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 electrode was then rinsed with ultrapure water and allowed to dry at room temperature. 10 μL of 1.0 μM thiolated capture probe was carefully dropped onto the surface of pretreated gold electrode and incubated overnight at 4 °C. After rinsed with washing buffer, the electrode was immersed into 100 μL of 1 mM MCH for 1 h to obtain well-aligned DNA monolayer. The electrode was further treated in salmon sperm DNA and 1 % BSA for 30 min respectively to avoid non-specific adsorption of DNA and enzyme on the electrode

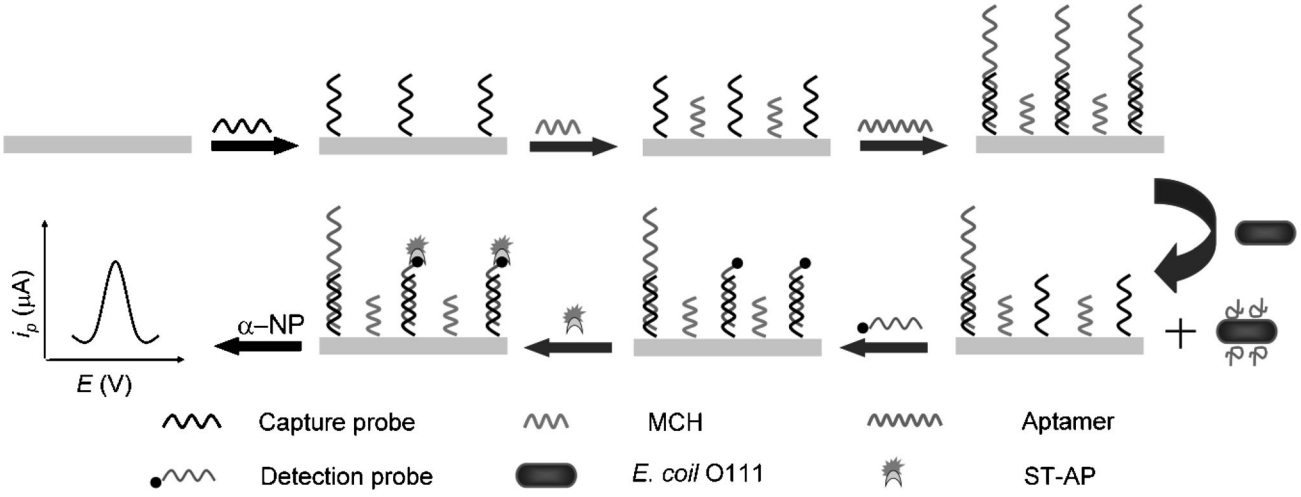

Fig. 1. Principle of the electrochemical biosensor for detection of *E. coli* O111.

surface. After the capture probe-modified electrode was washed with washing buffer, 10 μL of 2.0 μM aptamer solution was dropped on its surface and incubated for 1 h at 37 °C to obtain the electrochemical biosensor.

For the detection of *E. coli* O111, the biosensor was firstly incubated in PBS containing different concentrations of *E. coli* O111 for 2 h at 37 °C, and rinsed thoroughly with washing buffer. Biotinylated detection probe was then dropped onto biosensor and incubated for 1 h at 37 °C. After the biosensor was washed with washing buffer, 10 μL of 0.5 μg mL^{-1} ST-AP was dropped onto its surface and incubated at 37 °C for 30 min. Finally the biosensor was washed with diethanolamine buffer containing 0.05 % Tween-20 thoroughly to perform DPV detection in diethanolamine buffer containing 1 mg mL^{-1} of α-NP.

3 Results and Discussion

3.1 Electrochemical Biosensor

The principle of the electrochemical biosensor was shown in Figure 1. The biosensor contained three DNA probes: the capture probe, aptamer and the biotinylated detection probe. The capture probe with a thiol group at the 5′-terminal was immobilized on gold electrode surface via Au-thiol binding and then hybridized with the aptamer. In the presence of *E. coli* O111 as a target analyte, aptamer preferred to form *E. coli* O111-aptamer complex rather than aptamer-DNA duplex, resulting in the dissociation of aptamer from the biosensor surface. Thus the biotinylated detection probe could be hybridized with the capture probe. Upon the recognition of biotin to ST-AP, AP was introduced into electrode surface to catalyze the hydrolysis of α-NP substrate, which produced electroactive product and thus electrochemical signal. On the contrary, in the absence of *E. coli* O111, the detection probe could not displace aptamer at 37 °C because the temperature (37 °C) was much lower than the melting temperatures (85 °C) which were calculated using Oligo 6 software , so the biotinylated detection probe could not be assembled on the gold electrode surface and no electrochemical signal was observed.

The used aptamer has high affinity to bind *E. coli* O111 cell surface component LPS, which consists of gram-negative bacteria specific O antigen [35]. Thus, this electrochemical biosensor could provide a direct and simple biosensing platform for the detection of *E. coli* O111 cells.

3.2 Electrochemical Characterization of the Biosensor

Electrochemical impedance spectroscopy (EIS) was employed to monitor the interface properties of the gold electrode surface during stepwise modifications [36]. For EIS measurements, $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ was utilized as the redox probe and Nyquist plots were used to calculate the electron transfer resistance, R_{et}, at different electrodes (Figure 2A). The bare gold electrode exhibited an almost straight line (curve a), which was characteristic of a mass diffusion limiting electron-transfer process. When the capture probe was self-assembled onto the gold electrode, the R_{et} increased (curve b), because the negatively charged phosphate backbone of the oligonucleotides produced an electrostatic repulsion force to $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ [37]. The R_{et} further enhanced to a much larger value after aptamer hybridized with the capture probe (curve c) due to the increasing negative charge. When the biosensor was incubated with *E. coil* O111, the value of R_{et} decreased (curve d). This was attributed to the fact that the aptamer was induced to dissociate from the electrode surface due to the formation of the aptamer- *E. coli* O111 complex. Afterwards, upon the hybridization of the biotinylated detection probe with the remaining capture probe, the R_{et} was further increased (curve e). These results were in a good agreement with the square wave voltammetric (SWV) measurements (Figure 2B). These experiments proved the successful

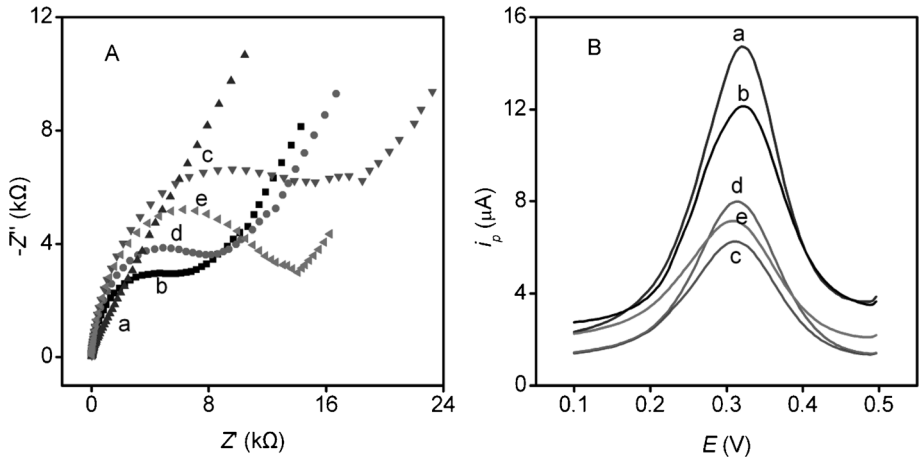

Fig. 2. EIS (A) and SWVs (B) at different electrodes in 0.4 M KCl containing 0.5 mM $Fe(CN)_6^{3-/4-}$. (a) Bare, (b) capture probe modified and (c) dsDNA modified gold electrodes. dsDNA-modified gold electrodes (d) after reaction with *E. coli* O111; and (e) further with detection probe.

modifications and reactions as described in the principle scheme.

3.3 Optimization of Experimental Conditions

In order to achieve the perfect assay performance, the different experimental conditions were optimized. Figure 3A shows the effect of incubation time of aptamer with *E. coli* O111 on DPV response. The DPV signal sharply increased with the increasing incubation time up to 120 min and then trended to a constant value, which suggested that 120 min was enough for the *E. coli* O111-induced dissociation of aptamer from the surface. Thus 120 min was chosen as the optimized incubation time, at which the signal increased gradually with the increasing concentration of biotinylated detection probe, and finally reached a plateau at 500 nM (Figure 3B). Therefore, 500 nM was selected as the optimum concentration of biotinylated detection probe in the following experiments.

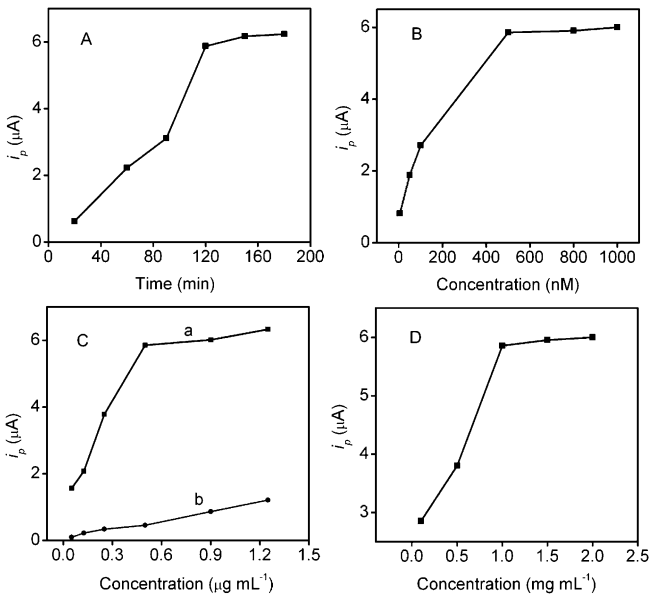

Fig. 3. Dependences of DPV peak currents on incubation time (A), detection probe concentration (B), ST-AP concentration (C), and α-NP concentration (D). When one parameter changes the others are under their optimal conditions.

The concentration of ST-AP obviously influenced the signal (Figure 3C, line a) and the blank (Figure 3C, line b) responses of the biosensor. With the increasing concentration of ST-AP, the DPV response increased rapidly up to 0.5 μg mL^{-1} and then trended to a stable value, while the blank response increased slightly. Thus, 0.5 μg mL^{-1} was the optimal concentration to obtain high ratio of signal to background.

Figure 3D shows the DPV responses at different α-NP concentrations. Due to the limited amount of enzyme, the response trended to a maximum value at 1.0 mg mL^{-1} α-NP, which was chosen as the appropriate concentration for the following experiments.

3.4 Analytical Performance of the Electrochemical Biosensor

The electrochemical detection of *E. coli* O111 was carried out under the optimal experimental conditions. Six *E. coli* O111 concentrations, from 2×10^2 to 2×10^7 CFU mL^{-1}, were prepared by serial dilution in PBS buffer. The DPV peak current increased with the increasing *E. coli* O111 concentration (Figure 4A). The plot of peak current vs. the logarithm of concentration in the rang from 2×10^2 to 2×10^6 CFU mL^{-1} showed a linear relationship with a correlation coefficient of 0.9989 (Figure 4B).The limit of detection (*LOD*), which was defined as three times the standard deviation of the blank sample measurements, was estimated to be 112 CFU mL^{-1} in PBS buffer. The detection process of the proposed method could be finished

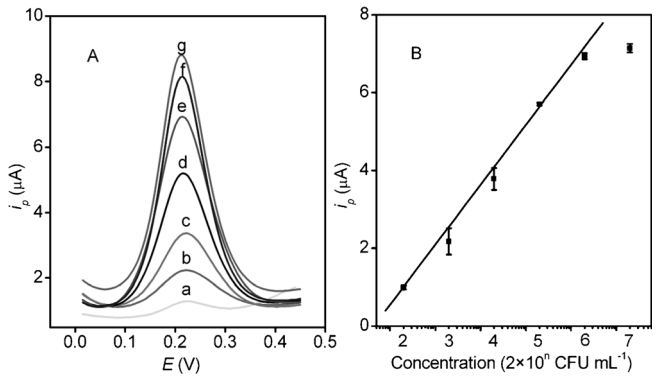

Fig. 4. (A) DPV responses to (a) 0, (b) 2×10^2, (c) 2×10^3, (d) 2×10^4, (e) 2×10^5, (f) 2×10^6, and (g) 2×10^7 CFU mL^{-1} *E. coli* O111. (B) Plot of DPV peak current vs. *E. coli* O111 concentration.

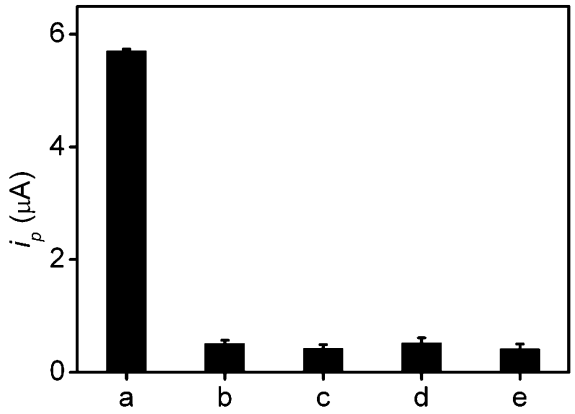

Fig. 5. Responses of the electrochemical biosensor to 2×10^5 CFU mL^{-1} (a) *E. coli* O111, (b) *Salmonella typhimurium*, (c) *Staphylococcus aureus*, (d) common non-pathogenic *E. coli* and (e) blank.

in 3.5 h. Compared with conventional culture method [4], this method was easy to use and time-saving.

To prove the specificity of the biosensor, three bacteria were tested under the same experimental conditions as those for *E. coli* O111. The concentrations of the test bacteria were 2×10^5 CFU mL^{-1} for *Salmonella typhimurium*, *Staphylococcus aureus* and common non-pathogenic *E. coli* respectively. The results were shown in Figure 5. The DPV responses of these three bacteria were only as small as the background, indicating that the electrochemical biosensor had a good specificity for the direct detection of *E. coli* O111.

3.5 Detection of *E. coli* O111 Cells in Real Samples

The cultured *E. coli* O111 cells were inoculated into milk at the concentrations of 0, 1×10^3, 10^4, 10^5, 10^6 and 10^7 CFU mL^{-1}. In the real sample the DPV showed linear response to *E. coli* O111 from 1×10^3 to 1×10^6 CFU mL^{-1} with a correlation coefficient of 0.9985 and a limit of detection of 305 CFU mL^{-1}, which was superior to the value $\geq10^5$ CFU mL^{-1} for ELISA [5]. So this biosensor held promise as a sensitive and viable technique for *E. coli* O111 detection in real samples. Meanwhile, due to the loss of target bacteria in sample pretreatment process, the detections in buffer and milk sample showed apparent difference of signals (Figures 4 and 6). Therefore, standardized sample pretreatment is necessary for the application of this proposed method.

4 Conclusions

An aptamer-based electrochemical biosensor for direct detection of *E. coli* O111 has been successfully developed. The biosensor shows wide linear range, low detec-

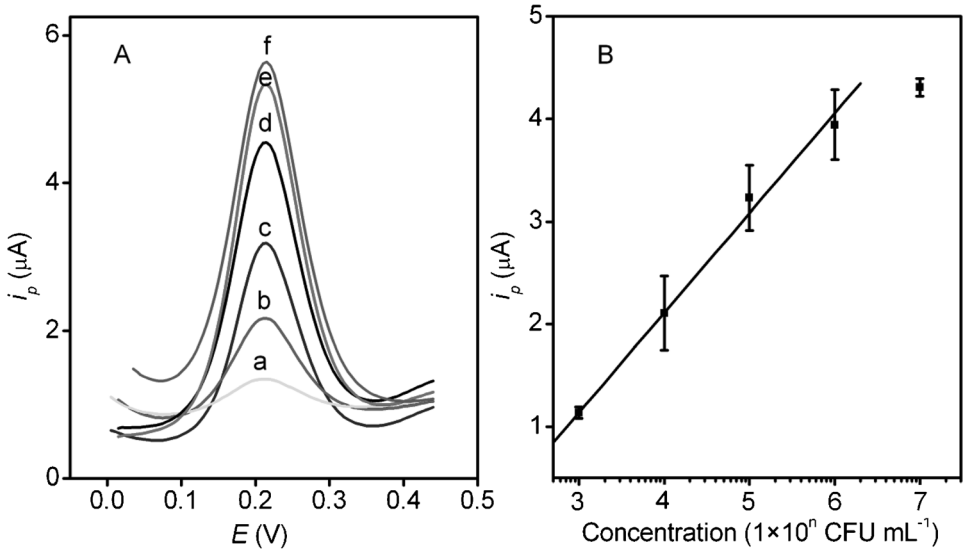

Fig. 6. (A) DPV from the electrochemical biosensor after reaction with different concentrations of *E. coli* O111 in milk. (a) 0; (b) 1×10^3; (c) 1×10^4; (d) 1×10^5; (e) 1×10^6; (f) 1×10^7 CFU mL^{-1}. (B) Plot of DPV peak current vs. *E. coli* O111 concentration.

tion limit and high specificity, and can be used for detection of *E. coli* O111 in real samples. Importantly, the target-induced aptamer displacement strategy can provide a direct sensing platform for detection of *E. coli* O111 and the whole analytical process can be finished in 3.5 h. The biosensing strategy could be used to develop other biosensors for pathogenic bacteria and would become a powerful tool for pathogenic microorganism screening in clinical diagnostics, food safety, biothreat detection and environmental monitoring.

Acknowledgement

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