

Article

Development of an Electrochemical Biosensor for Rapid and Effective Detection of Pathogenic *Escherichia coli* in Licorice Extract

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Received: 30 November 2018; Accepted: 11 January 2019; Published: 15 January 2019



Abstract: An aptamer-based electrochemical biosensor was successfully developed and applied in the rapid detection of pathogenic *Escherichia coli* (*E. coli*) in licorice extract. The thiolated capture probes were firstly immobilized on a gold electrode, and then the biotinylated aptamer probes for *E. coli* were introduced by hybridization with the capture probes. Due to the stronger interaction between the aptamer and the *E. coli*, a part of the biotinylated aptamers will dissociate from the capture probes in the presence of *E. coli*. The residual biotinylated aptamer probes can quantitatively bind with streptavidin-alkaline phosphatase. Subsequently, α -naphthyl phosphate substrate was catalytically hydrolyzed to generate electrochemical response, which could be recorded by a differential pulse voltammetry. The dependence of the peak current on the logarithm of *E. coli* concentration in the range from 5.0×10^2 colony forming units (CFU)/mL to 5.0×10^7 CFU/mL exhibited a linear trend with a detection limit of 80 CFU/mL. The relative standard deviation of 5 successive scans was 5.3%, 4.5% and 1.1% for 5.0×10^2 , 5.0×10^5 and 5.0×10^7 CFU/mL *E. coli*, respectively. In the detection of the licorice extract samples, the results obtained from the proposed strategy and traditional culture counting method were close to each other, but the time consumption was only $\sim 1/30$ compared with the traditional method. These results demonstrate that the designed biosensor can be potentially utilized for rapid microbial examination in traditional Chinese medicine and relevant fields.

Keywords: aptamer; electrochemical; *Escherichia coli*; biosensor; licorice

1. Introduction

Pathogenic *Escherichia coli* (*E. coli*) is one of the most harmful bacteria related to food-borne diseases and can cause inflammation, abdominal pain, diarrhea and even severe cases like hemorrhagic enteritis and hemolysis, especially in infants and youngsters [1]. A systematic analysis indicated that *E. coli* infection has already become the second leading cause of death in children younger than 5 years old [2]. Therefore, a fast, sensitive, and easy-to-use method for the detection of *E. coli* is an urgent demand in clinical diagnosis, environment monitoring, pharmacy and food safety fields.

At present, *E. coli* detection methods mainly include the traditional culture counting method [3], molecular biological detection technology [4], immunological detection method [5], surface plasmon resonance (SPR) [6], Chemiluminescence immunoassay [7], etc. The traditional counting method for *E. coli* detection is based on culturing and isolation of bacteria in a specific medium, followed by observing

bacterial colony morphology, color change, and biochemical reactions for quantitative detection. This detection method is economical and relatively simple to operate, but the detection time is generally 48–72 h or even longer, which cannot meet the needs for rapid detection. As an effective molecular biological detection technology, polymerase chain reaction (PCR) and the derived technology of PCR has been widely used in detecting *E. coli* [4,7]. PCR is based on the principle that DNA templates can denature at high temperatures, reactivate and extend (one cycle) at low temperatures by using appropriate DNA polymerase and primers. As the template DNA amplification product increases exponentially, increasing the number of cycles can magnify the specified DNA sequence by several million-fold in a short time, which greatly improves detection sensitivity [8]. However, PCR technology is costly and prone to produce false negatives [9], which limits the extensive application of PCR. In addition, enzyme-linked immunosorbent assay (ELISA), as a common immunological detection technology, has also been used in bacterial test [5,10]. ELISA technology undergoes a color change by the catalysis of the enzyme in the specific recognition process. Owing to the immune activity between antigen and antibody, ELISA method has superior specificity, high sensitivity and short detection time, and has been used in detection of different subtypes of *E. coli* [11–13]. However, due to the costly and cumbersome preparation process of antibodies, the detection cost of ELISA has increased greatly [14,15]. Another disadvantage is their limited shelf lives, which limits the viability of antibody-based biosensors in ELISA technology [16]. Moreover, the SPR method is labor intensive and requires expensive instrumentations, which limits its usage in *E. coli* detection. Chemiluminescence immunoassay has the advantages of simple operation, short detection time, and has been used in rapid detection of microbial [17]. However, few stable and sensitive chemiluminescence systems hinder the development of chemiluminescence immunology in microbial detection. Compared with the above-mentioned methods, electrochemical methods have received considerable interest with their high sensitivity, miniaturized and low-cost devices, and simple operation, especially in medical, pharmaceutical, food and environmental rapid detection of *E. coli* [18–21]. Recently, great efforts have been devoted to the development of novel modified electrodes for *E. coli* rapid detection, because the direct oxidation current on the bare electrode was not attractive owing to the high overpotential and sluggish electron transfer processes [22,23]. To date, three kinds of materials, including enzymes [14,24], aptamers [25] and nano-composites [26,27], have been used to modify the electrode surface for better analytical properties. Among them, aptamers are expected to be a promising candidate for the design of electrochemical biosensors for *E. coli* for their easy preparation, low cost, high stability and strong affinity.

Aptamers are single-stranded oligonucleotides screened by systematic evolution of ligands by exponential enrichment (SELEX) [28]. The high affinity between aptamers and target molecules is based on the hydrogen bonding, electrostatic interactions, and Van der Waals interactions (spatial conformations) [29]. At present, many electrochemical sensors based on aptamers have been developed for *E. coli* detection. The aptamer-electrochemical sensor can be used for *E. coli* detection at gene, protein or cell levels. In the gene and protein detections, electrochemical-aptamer sensors exhibit good analytical performance because of the hybridization and strong binding ability with *E. coli* proteins. However, complex extraction and separation steps along with the high reagent cost are still challenging problems for practical detection of genes and protein. Therefore, an aptamer-electrochemical sensor for direct detection of *E. coli* cells with low detection limit but little pre-treatment is more and more attractive.

Bratov and his group used three-dimensional interdigitated electrode array (3D-IDEA) as the working electrode and constructed impedimetric transducer by composing of aptamer for *E. coli* cell detection. The electrochemical biosensor showed an extremely good analytical performance for *E. coli* O157:H7 cells with a low detection limit, good selectivity and short detection time [30], but the preparation of such a 3D-IDEA aptamer biosensor significantly increases the cost because of the preparation complexity. Without using these expensive electrodes, Luo et al. selected common gold rod electrode for achieving the rapid detection of *E. coli* cell by using aptamer displacement

strategy with three probes. Their method showed a linear relationship between the peak of current and the concentration of *E. coli* O111 cell in the range from 1.0×10^3 to 1.0×10^6 colony forming units (CFU)/mL with a detection limit of 305 CFU/mL in milk [31]. It is thus promising for the development of low-cost and practical electrochemical biosensor based on aptamer, but the sensitivity needs to be improved and the preparation process can be further simplified.

In this study, an aptamer (Seq.1) [32] with high affinity to pathogenic *E. coli* derived from the whole bacteria-SELEX strategy [33] was used as the identification probe, and a simple and sensitive method was developed for the rapid detection of *E. coli* in licorice extract. The proposed method showed high stability and sensitivity for the detection of *E. coli* with a low detection limit. In addition, in the detection of the *E. coli* existing in the licorice extractum samples, the results obtained from the proposed strategy are close to the plate counting results, while the detection time of our strategy has been greatly shortened. The method can be utilized as a promising tool for microbial examination in traditional Chinese medicine and other relevant fields.

2. Materials and Methods

2.1. Reagents

Thiol-modified capture probe and biotin-modified aptamer probe were synthesized by Sangon Inc. (Shanghai, China), and their base sequences were listed as follows:

Capture probe: SH-(CH₂)₆-CAG ATG CAC GAC TTC GAG TCT TAG GGC ACA CGA TGG CAG T

Aptamer probe: Biotin-CAG CTC AGA AGC TTG ATC CTA CCA GTA GAC TTT CAA CTT TAC TGC CAT CGT GTG CCC TAA GAC TCG AAG TCG TGC ATC TG

6-Mercapto-1-hexanol (MCH) and diethanolamine (DEA) were purchased from Tianjin HEOWNS Biochemical Technology Co. Ltd. Tris (2-carboxyethyl) phosphine (TCEP), salmon sperm DNA, brilliant green lactose bile (BGLB) medium and violet red bile agar (VRBA) were obtained from Beijing Solarbio Science & Technology Co. Ltd. (Beijing, China). Bovine serum albumin (BSA) and Luria-Bertani (LB) medium were purchased from Sangon Inc. (Shanghai, China). Streptavidin-alkaline phosphatase (ST-AP) was obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). α -Naphthyl phosphate (α -NP) was obtained from TCI Development Co., Ltd. (Shanghai, China). The washing buffer (pH = 7.4) was mainly composed of 20 mM Tris-HCl, 0.1 M NaCl, 5.0 mM MgCl₂ and 0.005% Tween-20. Licorice pieces (Lot NO. 1403008) were purchased from Hebei Qiyitang Pharmaceutical Co., Ltd. (Anguo, China). All the other reagents were of analytical reagent grade and all the solutions were prepared using sterilized ultrapure water.

2.2. Apparatus

All electrochemical experiments were performed using a CHI660E electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., Shanghai, China). A three-electrode system was used, which was composed of a saturated calomel reference electrode, a platinum wire counter electrode, and a gold electrode ($\varphi = 2$ mm) as the working electrode.

2.3. Preparation of Licorice Extract

Licorice is one of the most commonly used Chinese herbal medicines. Owing to its high sugar content, licorice extract is easily infected by bacteria, and especially with the prolongation of storage time, the risk of infection is higher. Therefore, it is necessary to select the appropriate method to detect the content of bacterial in the extract.

Licorice extract was prepared as follows: raw licorice pieces (20 g) and ultrapure water (160 mL) were placed into a flask for the 3 h reflux extraction. After filtering with a 200-mesh filter, the supernatant was collected and placed to settle for one day, and then the obtained mixture was centrifuged (6000 r/min, 10 min) to remove the sugar, protein and macromolecular substances. The

obtained licorice extract was unsterilized and the solid content was about 4% (g/mL). In order to obtain the sterilized licorice extract, the filtration should be heated to 121 °C for 20 min, and then cooled for use.

2.4. Preparation of Pure Microbial Sample and Artificial Specimen

The *E. coli* strain (CICC 10372) was obtained from the China Center of Industrial Culture Collection (CICC). Firstly, the bacteria were cultivated in sterilized LB liquid medium at 37 °C for 16 h. The resulted pure cell suspension concentration was then determined by the plate counting method [3]. Subsequently, the bacterial suspension was centrifuged for 15 min at 4 °C and $1079 \times g$. The supernatant was then removed and washed twice with sterile water and sterile phosphate buffer solution (PBS), the remaining bacteria were diluted to 5.0×10^2 CFU/mL, 5.0×10^3 CFU/mL, 5.0×10^4 CFU/mL, 5.0×10^5 CFU/mL, 5.0×10^6 CFU/mL, and 5.0×10^7 CFU/mL with sterile licorice extract.

2.5. Study on The Antibacterial Effect of Licorice Extract

The antibacterial effect of the sterilized licorice extract at different solid contents was investigated by filter-paper disk method [34,35]. Firstly, a 2% solid content of the licorice extract was diluted with the licorice extract prepared in Section 2.3, while the 8% and 20% solid contents of the licorice extract were concentrated from the licorice extract prepared in Section 2.3. Secondly, pieces of filter paper with a diameter of 6 mm were made by a punch, numbered and then put into the bottle for sterilization. Thirdly, those filter paper were added individually into the EP tubes containing 2 mL licorice extract at four kinds of solid contents, 2 mL mixture of penicillin and streptomycin (positive control) [36] and 2 mL sterile saline (negative control). After soaking for 30 min, the obtained pieces of medicinal paper were exposed to a stream of warm air for another 30 min. Finally, a 100 μ L, 5.0×10^4 CFU/mL *E. coli* suspension was spread onto the nutrient agar flat medium evenly and incubated for 15 min, and six pieces of medicinal paper were immersed into the medium equidistantly and incubated at 37 °C for 24 h. The average size of the inhibition zone was measured by repeating three 3 plates.

2.6. Preparation of the Electrochemical Biosensor

A bare gold electrode was polished twice to a mirror-like surface with 0.3 mm and 0.05 mm alumina slurry and rinsed thoroughly with sterilized ultrapure water between each polishing step. Then, it was washed successively by piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2 = 3:1$) and sterilized ultrapure water in an ultrasonic bath, and dried in the air. The polished electrode was electrochemically cleaned and characterized by potential cycling between -1.0 V and 1.0 V in 1.0 M H_2SO_4 until a stable cyclic voltammogram for the cleaned gold electrode was obtained. Then, 10 μ L of 2 μ mol/L thiol-modified capture probe solution was dropped onto the surface of the gold electrode and incubated overnight at 4 °C. After rinsing with washing buffer, the electrode was immersed into 100 mL MCH (2.0 mM) for 1 h to obtain capture probe-modified gold electrode. Subsequently, the modified electrode was immersed and slightly shook in 0.1 mg/mL salmon sperm DNA and 1% BSA solution (each for 15 min) to avoid nonspecific adsorption [37]. After that, the treated gold electrode was rinsed with washing buffer, and 10 μ L of 3.0 μ M aptamer probe solution was dropped on its surface and incubated for 1 h at 37 °C to obtain the electrochemical biosensor.

2.7. Detection of *E. coli* in Licorice Extract by Using the Electrochemical Biosensor

For the detection of *E. coli* in licorice extract, the prepared biosensor was firstly incubated in the licorice extract of different concentrations of *E. coli* for 2 h at 37 °C. After washing with washing buffer, 20 μ L of 0.5 mg/mL ST-AP aqueous solution was dropped onto the surface of the biosensor and incubated at 37 °C for 30 min. Finally, the obtained biosensor was rinsed with DEA solution containing 0.05% Tween-20 thoroughly and immersed in a 2.5% DEA solution containing 1.0 mg/mL of α -NP for differential pulse voltammetry (DPV) determination.

3. Results and Discussion

3.1. Principle of *E. coli* Detection by Electrochemical Biosensor

Figure 1 depicts the biosensing process of *E. coli* detection. Firstly, the thiol-modified capture probes (SH-Capture) are immobilized on the surface of the polished gold electrode by Au-SH covalent bond, and then the unreacted active sites on the electrode surface are blocked with MCH [38]. Secondly, excess biotin-modified aptamer probes (Bio-Aptamer) that are complementary to the capture probes are introduced onto gold electrode by DNA hybridization. Due to the stronger interaction between the biotinylated aptamer probes and *E. coli*, a part of the biotinylated aptamer probes can dissociate from the capture probes in the presence of *E. coli*. After that, quantitative ST-APs can be immobilized on the surface of gold electrode by linking to biotin on the residual aptamer probes. Under strong alkaline conditions, ST-APs can dissociate from the biotinylated aptamer probes and catalyze the hydrolysis of α -NP substrate generating the electrochemical signals [39]. As a result, the electrochemical response to *E. coli* can be measured by using DPV in the presence of α -NP.

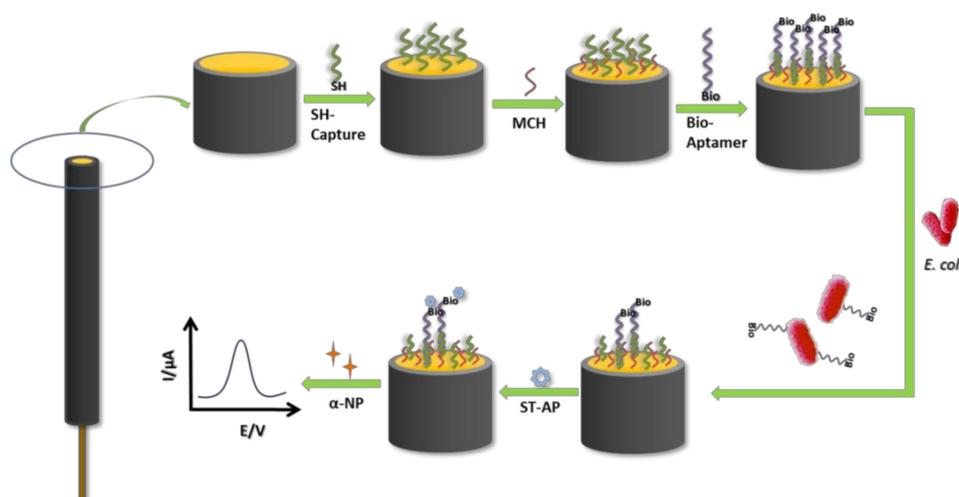


Figure 1. Schematic of the Electrochemical Biosensor for *E. coli* detection.

3.2. Electrochemical Characterization of the Biosensor

Electrochemical impedance spectroscopy (EIS) is used to determine the electron transfer properties of bare gold electrode and modified gold electrode surface. Figure 2 shows the Nyquist plots of the bare gold electrode (curve a), the capture probe modified gold electrode (curve b), the aptamer probe modified gold electrode (curve c) and the aptamer probe modified gold electrode after reaction with *E. coli* (curve d) in a solution containing 1.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl. The Randles circuit model (inset of Figure 2) is chosen to fit the impedance data obtained, where the element R_u is the electrolyte resistance, the resistance to charge transfer (R_{ct}) and the diffusion impedance (Z_w) were both in parallel with the interfacial capacitance (C_d). The R_{ct} at the electrode surface is equal to the semicircle diameter of EIS, which is used to describe the interface properties of the electrode [40]. For curve a, it presents an almost straight line, which demonstrates that there is a very high electron-transfer rate between the electrochemical probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and the bare gold electrode. When the capture probes are immobilized onto the gold electrode, the R_{ct} value increases at about 4100 Ω (curve b), which is attributed to an electrostatic repulsion force to $[\text{Fe}(\text{CN})_6]^{3-/4-}$ [41] produced by the combination of the negatively charged single-stranded oligonucleotides. For aptamer probes modified gold electrode (curve c), the R_{ct} further increases to a much larger value (nearly 38,000 Ω) after the aptamer probes hybridized with the capture probes because of the increased negative charge. Afterwards, the R_{ct} value (curve d, nearly 11,000 Ω) further decreased when the electrochemical biosensor is incubated with *E. coli*. This is due to the dissociation of the aptamer probes from the electrochemical biosensor,

which causes the reduction of negative charge. These Nyquist plot proves the successful modifications and reactions as described in the principle scheme.

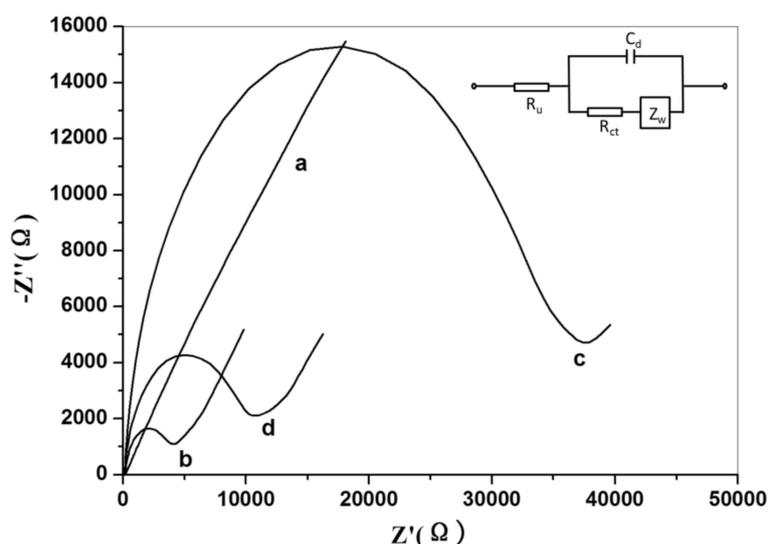


Figure 2. Nyquist diagrams of EIS at the bare gold electrode (curve a), the capture probe modified gold electrode (curve b), the aptamer probe modified gold electrode (curve c) and the aptamer probe modified gold electrode after reaction with 5.0×10^2 CFU/mL *E. coli* (curve d). EIS condition: frequency range: 100 kHz–0.01 Hz; potential: 0.175 V; amplitude: 5 mV; solution: 1 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ containing 0.1 M KCl. The inset of Figure 2 is the Randles circuit model.

3.3. Antibacterial Activity of Licorice Extractum

The antibacterial activity results of licorice extractum with 2% (D), 4% (C), 8% (E) and 20% (B) solid content, penicillin and streptomycin mixture (positive control, F) and sterile saline (negative control, A) are displayed in Figure 3. As shown in Figure 3, no antibacterial activity of licorice extractum of different solid content is observed except for the positive control group, indicating that the licorice extractum does not interfere with the *E. coli* testing.

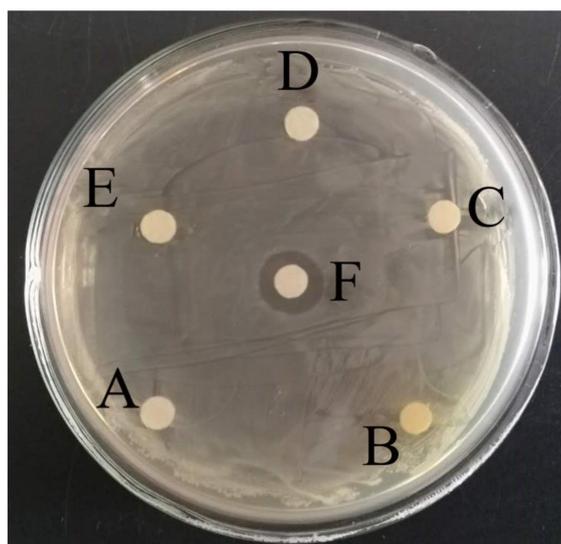


Figure 3. The antibacterial activity of filter paper immersed in licorice extractum in 2% (D), 4% (C), 8% (E) and 20% (B) solid content, penicillin and streptomycin mixture (positive control, F) and sterile saline (negative control, A).

3.4. Optimization of Experimental Conditions

3.4.1. The Effect of the Concentration of Aptamer Probe on the Peak Current

The influence of the concentration of aptamer probe on DPV response was investigated under the reaction with 5.0×10^2 CFU/mL *E. coli*. As shown in Figure 4, it can be seen that the peak current increases dramatically when the concentration of aptamer probe increases from 0 to 3 μ M. However, the peak current does not change significantly when the concentration of the aptamer probe exceeds 3 μ M. The results can be attributed to the fact that the low amount of the aptamer probes did not completely hybridize with the single-strand capture probes. Therefore, after incubating with *E. coli*, there were very few aptamer probes left on the gold electrode, leading to a low peak current. With increasing the amount of the aptamer probe, the peak current value gradually increases until reaching a maximum, after which the peak current stops increasing. Consequently, 3 μ M is selected as the optimum concentration of aptamer probe in the following experiments.

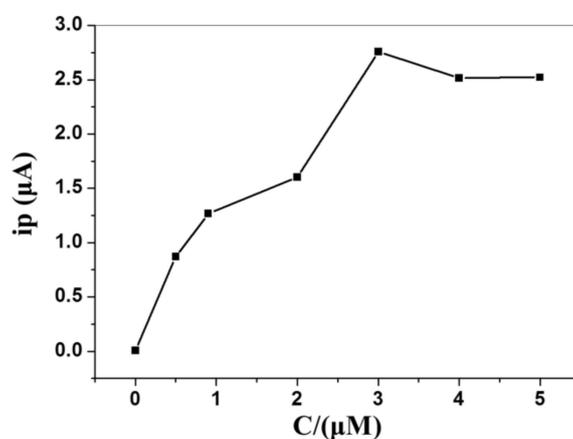


Figure 4. The relationship between the concentration of the aptamer probe and the peak current under the reaction with 5.0×10^2 CFU/mL *E. coli*.

3.4.2. The Effect of the Incubation Time on the Peak Current

The effect of the incubation time of electrochemical biosensor with *E. coli* on the peak currents is investigated. As shown in Figure 5, the peak current decreases sharply with increasing the incubation time up to 2 h and then become nearly constant. The dissociation time of the aptamer probes from the biosensor is dependent on the incubation time of *E. coli*. It is difficult for the aptamer probes to dissociate from the capture probes in a short period of time. Thus, the incubation time of the electrochemical biosensor with *E. coli* is chosen as 2 h to obtain the stable current value of α -NP.

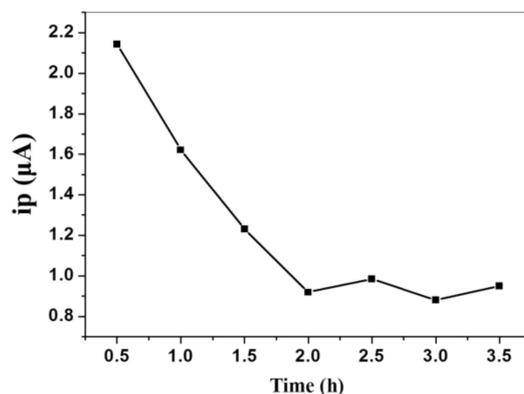


Figure 5. The relationship between the incubation time and the peak current under reaction with 5.0×10^3 CFU/mL *E. coli* and 3 μ M aptamer probe.

3.4.3. The Effect of ST-AP Concentration on the Peak Current

Figure 6 shows the relationship between the ST-AP concentration and the peak current of α -NP. It can be seen that the current value increases significantly when the ST-AP concentration increases from 0 to 0.7 $\mu\text{g}/\text{mL}$ and then reaches a stable value. Therefore, 0.7 $\mu\text{g}/\text{mL}$ is used as the optimal concentration of ST-AP in the following experiments.

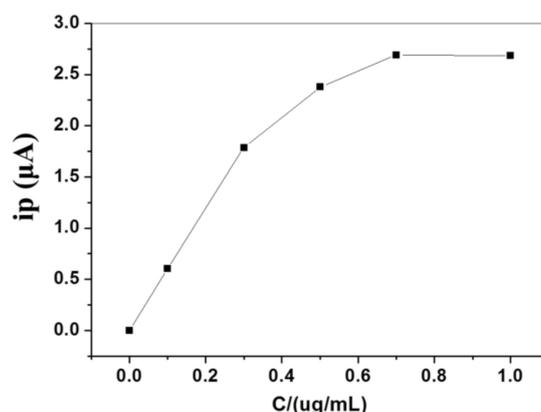


Figure 6. The relationship between the ST-AP concentration and the peak current under the reaction with 5.0×10^2 CFU/mL *E. coli*.

3.4.4. The Effect of α -NP Concentration on the Peak Current

The effect of the α -NP concentration on the peak current is investigated. As shown in Figure 7, the current value increases sharply when the α -NP concentration increases from 0 to 1.0 mg/mL, and then the increasing become much slower. It is because that the amount of α -NP is closely related to the amount of ST-AP. Therefore, 1.0 mg/mL of α -NP is chosen in the following DPV detection.

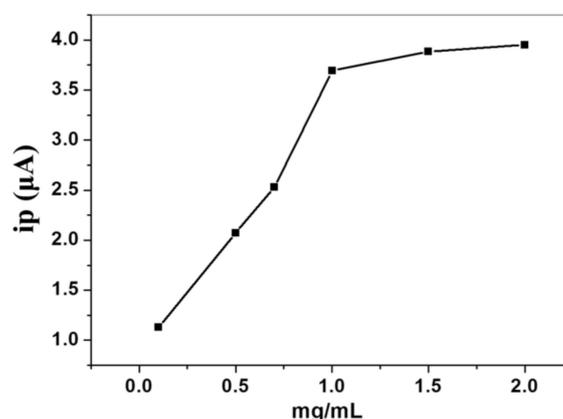


Figure 7. The relationship between the α -NP concentration and the peak current under reaction with 5.0×10^2 CFU/mL *E. coli*.

3.5. Sensitivity and Reproducibility of the Electrochemical Biosensor

Under the optimal experimental conditions, the DPV responses of the prepared electrochemical biosensor at different *E. coli* concentrations are shown in Figure 8. It is found that the DPV peak current decreases with the increasing concentration of *E. coli* from 5.0×10^2 CFU/mL to 5.0×10^7 CFU/mL. The prepared electrochemical biosensor has a good linear relationship between the peak current (i_p) value and the logarithm of *E. coli* concentration (n) in the range from 5.0×10^2 CFU/mL to 5.0×10^7 CFU/mL (Figure 9). The resulting linear equation is i_p (μA) = $-0.368n + 4.29$, with a correlation coefficient of 0.996. In addition, the limit of detection (LOD) is 80 CFU/mL at a signal-to-noise ratio of 3, which is lower than other methods reported previously for the detection of *E. coli* (Table 1) [6,42–51].

Moreover, the measurement time of this method is 150 min, which is shorter than that of PCR, ELISA, immunomagnetic bead sandwich assay and electrochemical immunoassay, not to mention the plate counting method (72 h). It is worth mentioning that the linear range is also wide, spanning 5 orders of magnitude.

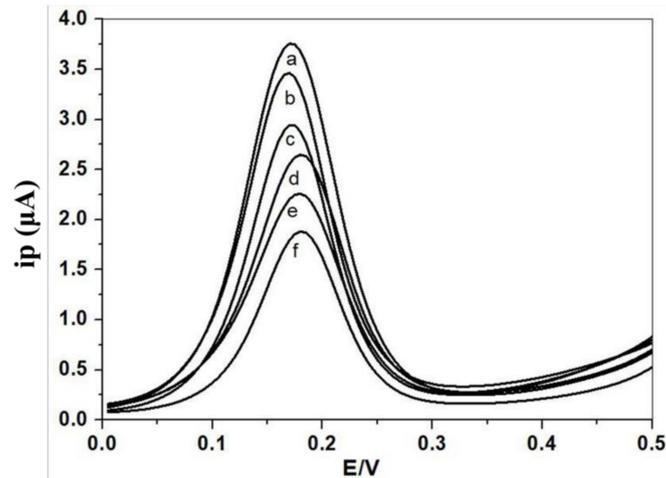


Figure 8. DPV responses in licorice extract of 4% solid content: (a) 5.0×10^2 CFU/mL, (b) 5.0×10^3 CFU/mL, (c) 5.0×10^4 CFU/mL, (d) 5.0×10^5 CFU/mL, (e) 5.0×10^6 CFU/mL and (f) 5.0×10^7 CFU/mL *E. coli*.

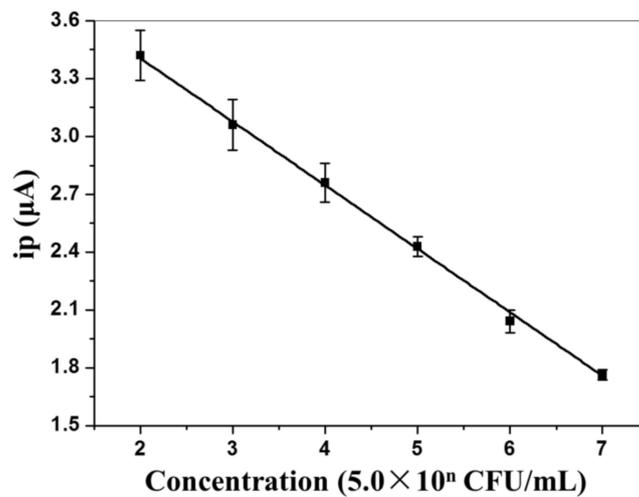


Figure 9. Calibration plot of peak currents versus concentration of *E. coli*.

Table 1. Comparison between the proposed method and other reported biosensors for the detection of *E. coli*.

No.	Biosensor Platform	Bio-Receptor	Linear Range	Measurement Time	LOD (CFU/mL)	Ref.
1	CL aptasensor	Aptamer	10^4 – 10^7 CFU/mL	63 min *	4.5×10^3	[42]
2	PCR	Primer	10^2 – 10^6 CFU/mL	180 min	10^2 (without pre-enrichment step)	[43]
3	ELISA	Bacteriophages	/	240 min *	10^6	[44]
4	Diffuse reflectance spectroscopy	Dye-labeled antibodies	5×10^5 – 5×10^8 CFU/mL	45 min	2×10^5	[45]
5	Immunomagnetic bead sandwich assay	Protein G-liposomal nanovesicles	10^2 – 10^8 CFU/mL	275 min *	100 (in pure cultures) 3.1×10^3 (in mixed cultures)	[46]
6	QCM (quartz crystal microbalance)sensor	Aptamer	10^2 – 10^5 CFU/mL and 10^5 – 10^7 CFU/mL	50 min	1.46×10^3	[47]
7	SPR biosensor	Fragment of tail protein J from phage lambda	2×10^4 – 2×10^9 CFU/mL	20 min	2×10^4	[6]
8	Electrochemical sensor	Antibody	6×10^3 – 6×10^5 CFU/mL	120 min	6×10^2	[48]
9	Light-addressable potentiometric sensor (LAPS)	Fluoresceinated antibodies	/	45 min	7.1×10^2 (heat-killed) 2.5×10^4 (live)	[49]
10	Electrochemical biosensor	ssDNA	10^3 – 10^8 CFU/mL	Less than 180 min	10^2	[50]
11	Electrochemical immunoassay	Monoclonal antibody against <i>E. coli</i> O157:H7 functionalized magnetic nanoparticles	3.5×10^3 – 3.5×10^8 CFU/mL	165 min *	1.83×10^2	[51]
12	Electrochemical biosensor	Aptamer	5.0×10^2 – 5.0×10^7 CFU/mL	150 min	80	This study

/ means not available in the text. * means not available in the text directly, the detection time is speculated based on the experimental steps in the paper.

To evaluate the repeatability of the developed electrochemical biosensor, the DPV responses were examined 5 times for each of 5.0×10^2 , 5.0×10^5 and 5.0×10^7 CFU/mL *E. coli*. The relative standard deviation (RSD) is 5.3%, 4.5% and 1.1%, respectively. The RSD value is only slightly greater than 5% at 5.0×10^2 CFU/mL concentration, and the rest are less than 5%, indicating that this method had an acceptable reproducibility.

3.6. Detection of *E. coli* in Licorice Extractum

The applicability of the proposed strategy is tested by the detection the *E. coli* in real licorice extractum samples at different concentrations, and validated by the plate counting method. In the electrochemical detections, the cultured *E. coli* at three unknown gradient concentrations are incubated with the electrochemical biosensor in the diluted licorice extracts with the solid content of 4%. At the same time, the parallel *E. coli* samples at the same unknown gradient concentrations are measured by the plate counting method. The results of DPV test and plate counting method are compared in Table 2. The amounts of strains obtained by these two methods are consistent in magnitude, but the specific values are slightly different. The electrochemical measurement results are higher than those measured by the plate counting method, which may be due to the sensitivity of the electrochemical test method. The *p*-values in the 10^5 and 10^6 concentration gradient groups are much larger than 0.1, indicating that our method and the plate counting method test results are not significantly different in these two groups, but in the 10^4 concentration gradient group, the difference between our method and the plate counting method is slightly significant. This may be ascribed to the large systematic error caused by the fewer repeated measurements. In order to clearly illustrate the consistency of these two methods, a large number of repetitive experiments are required. The detection time for plate counting method is 72 h, while the proposed method is only 2.6 h. The significantly shortened detection time will greatly improve the efficiency and expand its application areas in future.

Table 2. Comparison between the proposed method and the plate counting method for detection of *E. coli* at 95% confidence interval.

NO.	The Plate Counting Method (n = 3, $\bar{x} \pm SD$, CFU/mL)	This Study (n = 3, $\bar{x} \pm SD$, CFU/mL)	P (n = 3)
1	$(5.00 \pm 0.20) \times 10^4$	$(9.02 \pm 0.10) \times 10^4$	0.02
2	$(6.43 \pm 0.05) \times 10^5$	$(7.57 \pm 0.60) \times 10^5$	0.76
3	$(4.67 \pm 0.16) \times 10^6$	$(5.76 \pm 0.360) \times 10^6$	0.54

4. Conclusions

An aptamer-based electrochemical biosensor was fabricated successfully and applied in the direct detection of pathogenic *E. coli* in licorice extract based on the DPV method. Under the optimal conditions, the prepared electrochemical biosensor displayed a low detection limit, a good reproducibility and a wide linear response range for *E. coli*. Additionally, the proposed method was successfully used for the detection of *E. coli* in licorice extractum, and the test results were close to the plate counting method at three different gradient concentrations of *E. coli*. It is worth mentioning that the detection time of the proposed method has been greatly reduced compared with the traditional plate counting methods. We believe that the introduction of the electrochemical biosensing strategy can be used for developing other biosensors for pathogenic microorganism and become a powerful tool for pathogenic bacteria detection in the fields of food and drug safety, quality control, clinical diagnostics, environmental monitoring, etc.

Author Contributions: In this work, H.W. mainly carried out the article design, literature analysis and drafted the manuscript. Y.Z. conducted the electrochemical experimental analyses and drafted the manuscript. S.B. conducted the microbial cultivation. T.S. participated in the revision of the article. G.J. participated in the microbial cultivation and detection. B.L. participated in the revision of the article. R.Y. participated in the revision of the article. Z.L. participated in the article design and revision of the article.

Funding: This research was funded by the Science and Technology Program of Tianjin (15PTCYSY00030), Tianjin Education Program (2017KJ133), Natural Science Foundation of Tianjin, China (No. 16JCYBJC43900), the National S&T Major Project (No. 2018ZX09201011).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

<i>E. coli</i>	<i>Escherichia coli</i>
CFU	Colony forming units
SPR	Surface plasmon resonance
LAPS	Light-addressable potentiometric sensor
PCR	Polymerase chain reaction
ELISA	Enzyme linked immunosorbent assay
SELEX	Exponentially enriched ligand system evolution
3D-IDEA	Three-dimensional interdigital array electrode
DEA	Diethanolamine
TCEP	Tris(2-carboxyethyl)phosphine
BGLB	Brilliant green lactose bile
VRBA	Violet red bile agar
BSA	Bovine serum albumin
LB	Luria-Bertani
ST-AP	Streptavidin-alkaline phosphatase
α -NP	α -Naphthyl phosphate
CICC	China left of Industrial Culture Collection
PBS	Phosphate buffer solution
MCH	6-Mercapto-1-hexanol
DPV	Differential pulse voltammetry
SH-Capture	Thiol-modified capture probe
Bio-Aptamer	Biotin-modified aptamer probe
EIS	Electrochemical impedance spectroscopy
LOD	Limit of detection
QCM	Quartz crystal microbalance
RSD	Relative standard deviation
Rct	The resistance to charge transfer
Cd	The interfacial capacitance
Zw	The diffusion impedance

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