Fabrication and Performance Characterization of an Electrochemical Biosensor for Bacterial Endotoxin

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Abstract

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Full Text

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Construction and Characterization of an Electrochemical Biosensor for Bacterial Endotoxin Detection

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Abstract

Bacterial endotoxin is an exogenous pyrogen, and its detection is crucial in the production of biological products. This study constructed an electrochemical aptamer biosensor for detecting bacterial endotoxin. An amino-modified endotoxin aptamer EAQ2 was used as the ligand and immobilized on a gold electrode surface via a 3-mercaptopropionic acid (MPA) linker. The biosensor construction process was characterized using both cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The results showed that a 6-hour MPA assembly time formed a stable self-assembled monolayer on the gold electrode surface. The constructed biosensor achieved a detection limit of 0.001 EU/mL, which is lower than that of other reported endotoxin detection methods. It exhibited a good linear relationship in the endotoxin concentration range of 0.001-0.1 EU/mL with a correlation coefficient $R^2 = 0.9878$, demonstrating potential for application in actual biological sample detection.

Keywords: Aptamer, Endotoxin, Biosensor

Introduction

Bacterial endotoxin was first discovered by Richard Pfeiffer in 1892-1895 from heat-inactivated *Vibrio cholerae* lysates [1]. Detection and removal of bacterial endotoxin from non-enteral drugs, especially injectable preparations, has become a critical step in production processes [2]. Current methods for endotoxin detection mainly include the rabbit pyrogen test, Limulus amebocyte lysate (LAL) assay, enzyme-linked immunosorbent assay (ELISA), and biosensors [3]. Aptamers are often selected as biorecognition elements due to their advantages of easy modification, broad target range, and high specificity [4-6]. Electrochemical analysis technology, as an analytical method with high sensitivity, simple operation, and real-time detection capabilities, has been widely applied in biosensors. Combining it with aptamers to construct a new generation of electrochemical aptamer biosensors has become an important research area at the intersection of analytical and life sciences [7]. Voss et al. [8] reported a bacterial endotoxin biosensor using two polypeptide analogs of the CD14 endotoxin-binding protein structure. Priano et al. [9] developed an electrochemical method for endotoxin detection using endotoxin-neutralizing protein (ENP) as the recognition molecule. Zuo Mingyan [10] constructed a magnetic bead aptasensor for bacterial endotoxin detection through a sandwich structure formed by two aptamers and endotoxin. Although various biosensors have been developed domestically and internationally, electrochemical aptasensors targeting endotoxin have not been reported in China. The electrochemical aptamer biosensor constructed in this study uses an aptamer as the recognition element (sensitive element) and employs a known sequence aptamer EAQ2 [11] as a probe

to detect bacterial endotoxin based on electrochemical signal changes. By combining the advantages of aptamers and electrochemical analysis, this biosensor can convert subtle environmental changes into measurable electrical signals for qualitative and quantitative analysis of endotoxin, overcoming the drawbacks of current endotoxin detection methods such as complex operation, long duration, and low sensitivity.

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1 Materials and Methods

1.1 Materials

1.1.1 Reagents The amino-modified aptamer sequence: NH -(CH) -5 -ATGAGAGCGTCGGTGTGGTAT-GCTCACCCCCTGCGGCGCCGTTACGCGGTCCTTGTGTAGGAGGC 3 (Sangon Biotech). Potassium ferricyanide, potassium ferrocyanide trihydrate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Aladdin Reagents. 3-Mercaptopropionic acid (MPA) was obtained from Sinopharm Chemical Reagent Co., Ltd. Bovine serum albumin (BSA) was from Shanghai Shenhang Biological Technology Co., Ltd. Tris(hydroxymethyl)aminomethane (Tris) was from Shanghai Boao Biological Technology Co., Ltd. Alumina powder, polishing cloth, gold electrode (CHI101), platinum wire electrode (CHI115), and saturated calomel electrode (CHI111) were from Shanghai Chenhua Instrument Co., Ltd. All other reagents were of analytical grade, and water used in the laboratory was double deionized. Aptamer buffer (pH = 7.4): 50 mmol/L Tris-HCl, 120 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl , 1 mmol/L CaCl .

1.1.2 Instruments Electrochemical workstation (CHI760E model; Shanghai Chenhua Instrument Co., Ltd.), ultrasonic cleaner (Tianjin Autoscience Instrument Co., Ltd.), and benchtop high-speed refrigerated centrifuge (Thermo Fisher Scientific).

1.2 Experimental Methods

1.2.1 Gold Electrode Surface Treatment Electrochemical cleaning was used to treat the gold electrode surface. Prior to treatment, the gold electrode was polished sequentially with 1.0 m, 0.3 m, and 0.05 m alumina powder. The electrode surface was rinsed with double deionized water, then ultrasonically cleaned twice with absolute ethanol and twice with double deionized water for 5 min each. After drying, the electrode was placed in 0.5 mol/L H SO solution and subjected to cyclic voltammetry scanning from 0 to 1.6 V at a scan rate of 100 mV/s until the cyclic voltammogram stabilized and approached the standard curve for a bare gold electrode (Figure 1 [Figure 1: see original paper]).

Figure 1. Standard cyclic voltammogram of a bare gold electrode in 0.5 mol/L

H SO

1.2.2 Assembly and Characterization of MPA-SAMs The polished bare gold electrode was immersed in 200 L of 200 mmol/L 3-mercaptopropionic acid and assembled in the dark at room temperature for several hours. After removal, the electrode surface was rinsed with absolute ethanol to remove physically adsorbed MPA molecules, yielding an MPA-SAM modified gold electrode. This modified electrode was placed in a PBS buffer solution (10 mmol/L, pH 7.4) containing 2 mmol/L K Fe(CN) and K Fe(CN) · 3H O as the electrolyte for cyclic voltammetry and electrochemical impedance spectroscopy characterization. Cyclic voltammetry parameters: potential range of -0.3 to 0.6 V, scan rate of 100 mV/s. Electrochemical impedance spectroscopy parameters: frequency range of 0.1 Hz to 100 kHz, amplitude of 5 mV.

1.2.3 Aptamer Immobilization and Characterization Aptamer pretreatment: To enable the aptamer to form a stable secondary structure, the synthesized dry-film aptamer was dissolved in aptamer buffer solution and stored at -20°C for later use.

The MPA-modified gold electrode was placed in an MES acidic buffer system containing 20 mmol/L EDC and 20 mmol/L NHS to activate the carboxyl groups, followed by incubation in 200 nmol/L NH -ssDNA solution for 40 min. After washing with buffer solution and drying, the electrode was characterized by cyclic voltammetry and electrochemical impedance spectroscopy. The biosensor modification process is shown in Figure 2 [Figure 2: see original paper].

Figure 2. Principle of aptamer immobilization

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2 Results and Discussion

2.1 Characterization of Bare Gold Electrodes

The treated bare gold electrode was electrochemically polished in 0.5 mol/L H SO and subjected to cyclic voltammetry scanning from 0 to 1.6 V, yielding the stable cyclic voltammogram shown in Figure 3 [Figure 3: see original paper]. After 10 cycles, the voltammogram was essentially consistent with the standard curve for a clean gold electrode, indicating that the electrochemical cleaning method achieved satisfactory cleaning results and the electrode was ready for subsequent modification.

Figure 3. Cyclic voltammograms of bare gold electrode in 0.5 mol/L H SO

2.2 Formation and Characterization of MPA Self-Assembled Monolayers

This study investigated various MPA-SAM assembly times of 1 h, 6 h, 9 h, 12 h, and 24 h, characterizing the results for different assembly durations using both cyclic voltammetry and electrochemical impedance spectroscopy. Figure 4 [Figure 4: see original paper] shows the cyclic voltammetry characterization. Comparison of the cyclic voltammograms reveals that after MPA self-assembly on the gold electrode surface, the peak current of [Fe(CN)]³ / in the electrolyte decreased, the peak potential difference increased, and reaction reversibility deteriorated. This is because the carboxyl groups of MPA are negatively charged in the electrolyte, which hinders electron transfer at the gold electrode surface to some extent, resulting in decreased peak current. Figure 5 [Figure 5: see original paper] shows the electrochemical impedance spectroscopy characterization. Generally, the high-frequency region of impedance spectra is kinetically controlled, while the low-frequency region is diffusion-controlled. Compared with the bare gold electrode (a), the MPA-modified gold electrodes (b-e) exhibited a larger semicircle diameter in the high-frequency region, indicating greater resistance of the modified electrode relative to the bare electrode, consistent with the cyclic voltammetry results. Self-assembled monolayer formation on gold substrates occurs in two stages [12]: the first step is a rapid adsorption process that completes in a short time, and the second step is reorganization of the film on the gold electrode surface, which requires a longer duration. The electrochemical characterization results show that as assembly time increased, surface coverage increased between 0-6 h, remained essentially unchanged between 6-12 h, and actually decreased after 24 h of assembly. Therefore, 6 h was selected as the optimal assembly time for MPA modification on the gold electrode surface.

Figure 4. Cyclic voltammograms of MPA-modified gold electrode in electrolyte

Figure 5. Nyquist plots of the MPA SAM electrode in PBS (10 mmol/L, pH 7.4) solution containing 2 mmol/L Fe(CN) ³ / a: bare gold electrode; b: 1 h assembly; c: 24 h assembly; d: 12 h assembly; e: 6 h assembly

2.3 Characterization of Immobilized Aptamer

Figure 6 [Figure 6: see original paper] shows cyclic voltammetry characterization of gold electrodes at different modification steps. Since aptamers are composed of nucleotides and are inherently negatively charged, their immobilization on the gold electrode surface enhances the barrier to electron transfer and increases resistance, leading to decreased peak current, though the effect is not pronounced.

Figure 7 [Figure 7: see original paper] shows electrochemical impedance spectroscopy characterization of gold electrodes at different modification steps. The biorecognition element used in this study is single-stranded nucleic acid, which produces relatively weak electrical signal changes upon interaction with bacterial endotoxin. Electrochemical impedance spectroscopy provides excellent

interfacial characterization, using small-amplitude sinusoidal potential as the perturbation signal to generate relevant linearity with the system without interfering with biomacromolecules. As shown in Figure 7, when the aptamer was immobilized on the gold electrode surface, the radius of the semicircle in the high-frequency region expanded. This is due to electrostatic repulsion from the negative charges on the nucleic acid phosphate backbone [13], which limits electron exchange at the gold electrode surface and increases resistance.

Figure 6. Cyclic voltammograms of gold electrodes under different modification conditions

Figure 7. Nyquist plots of gold electrodes under different modification conditions

2.4 Performance Characterization of the Aptamer Biosensor

Specificity testing of the aptamer for endotoxin primarily verifies whether the selected aptamer binds only to endotoxin and not to other chemical or biological substances. Endotoxin structure contains lipopolysaccharides and proteins. Bovine serum albumin (BSA) is a ubiquitous protein present in many biological products and biotechnological drugs, and like endotoxin, contains protein and polysaccharide structures. Figure 8 [Figure 8: see original paper] shows Nyquist plots of the biosensor after incubation in different BSA concentrations. The results demonstrate that when BSA concentration is below 5×10 g/mL, the biosensor shows no significant signal output. Since BSA concentrations in typical biological samples are far below 5×10 g/mL, the biosensor exhibits adequate selectivity in practical environments. Four electrodes modified in the same batch showed similar electrochemical response signals during characterization, indicating acceptable reproducibility of the constructed sensor. When the fabricated biosensor was immersed in a series of different bacterial endotoxin concentrations for 30 min, a good linear relationship was observed in the range of 0.001-0.1 EU/mL, as shown in Figure 9 [Figure 9: see original paper].

Figure 8. Nyquist plots of the aptasensor incubated in different concentrations of BSA a: 5×10 g/mL; b: 5×10 g/mL; c: 5×10 g/mL; d: no BSA

Figure 9. Linear relationship between ΔR and logarithm of endotoxin concentration

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3 Conclusion

In summary, this study constructed an aptamer biosensor for endotoxin detection. This method employs an aptamer as the recognition element of the sensor and combines two electrochemical analysis methods—cyclic voltammetry and electrochemical impedance spectroscopy—to achieve stepwise construction and characterization of the biosensor. Furthermore, the sensor is not interfered with by bovine serum albumin during endotoxin detection, exhibits high sensitivity

with a detection limit of 0.001 EU/mL, and shows a good linear relationship in the endotoxin concentration range of 0.001-0.1 EU/mL. The electrochemical aptamer biosensor constructed in this study has not been previously reported in China and features label-free operation and low detection limits, showing promise for endotoxin detection in actual biological samples.

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Abstract: A biosensor was constructed to detect endotoxin. The amine-terminated aptamer was immobilized on the gold electrode surface covered in advance with a self-assembled monolayer (SAM) of 3-mercaptopropionic acid (MPA). The modification of the gold electrode was confirmed by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). It was found that MPA assembly time at 6h on the gold electrode surface formed a stable self-assembled monolayer. The biosensor has a good linear relationship with concentration of endotoxin in the range of 0.001-0.1 EU/mL, and it is possible to be applied for quality control in actual biological samples.

Keywords: Aptamer, Endotoxin, Biosensor

Note: Figure translations are in progress. See original paper for figures.