Development and Characterization of Electrochemical Biosensors for Bacterial Endotoxin

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Abstract

Abstract Bacterial endotoxin is an exogenous pyrogen, and its detection is crucial in the production process of biological products. In this study, an electrochemical aptamer biosensor for detecting bacterial endotoxin was constructed using amino-modified endotoxin aptamer EAQ2 as the ligand, which was immobilized on the gold electrode surface via a 3-mercaptopropionic acid (MPA) intermediate linker. The construction process of the biosensor was characterized by both cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The results demonstrated that a 6 h MPA assembly time could form 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 those reported for other endotoxin detection methods, and exhibited a good linear relationship within the endotoxin concentration range of 0.001-0.1 EU/mL with a correlation coefficient R^2=0.9878, indicating promising application prospects for the detection of actual biological samples.

Full Text

Construction and Characterization of an Electrochemical Aptamer-Based 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 through a 3-mercaptopropionic acid (MPA) linker. The biosensor assembly process was characterized using both cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). 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 previously reported endotoxin detection methods. The sensor 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 real biological sample detection.

Keywords: Aptamer, Endotoxin, Biosensor

Introduction

Bacterial endotoxin was first discovered by Richard Pfeiffer between 1892–1895 in heat-inactivated *Vibrio cholerae* lysates [1]. Detecting and removing bacterial endotoxin from non-enteral pharmaceuticals, particularly injectable drugs, has become a critical step in manufacturing processes [2]. Current endotoxin detection methods primarily include the rabbit pyrogen test, Limulus amebocyte lysate (LAL) assay, enzyme-linked immunosorbent assay (ELISA), and biosensors [3]. Aptamers are often selected as biological components due to their advantages of easy modification, broad target range, and high specificity [4–6]. Electrochemical analysis technology offers high sensitivity, simple operation, and real-time detection capabilities, and has been widely applied in biosensors. Combining electrochemical techniques with aptamers to construct next-generation electrochemical aptamer sensors 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. Prianò 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 using a sandwich structure formed by two aptamers and endotoxin. Although various biosensors have been developed domestically and internationally, electrochemical aptasensors for endotoxin have not yet been reported in China.

The electrochemical aptamer biosensor constructed in this study uses an aptamer as the recognition element (sensitive element). A known sequence aptamer, EAQ2 [11], was employed as the probe to detect bacterial endotoxin based on changes in electrochemical signals. By combining the advantages of aptamers and electrochemical analysis, this biosensor can convert subtle envi-

ronmental changes into measurable electrical signals for qualitative and quantitative endotoxin analysis, overcoming the limitations of current endotoxin detection methods such as complex operation, long detection times, and low sensitivity.

Materials and Methods

1.1 Materials

1.1.1 Reagents The amino-modified aptamer sequence was: NH -(CH) -5 -ATGAGAGCGTCGGTGTGGTAT-GCTCACCCCCTGCGGCGCCGTTACGCGGTCCTTGTGTAGGAGGC TGCGGAAGTA-3 (Sangon Biotech). Other reagents included potassium ferricyanide, potassium ferrocyanide trihydrate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2-(*N*-morpholino)ethanesulfonic acid (MES) (Aladdin); 3-mercaptopropionic acid (MPA) (Sinopharm Chemical Reagent); bovine serum albumin (BSA) (Shanghai Shenhang Biological Technology); Tris(hydroxymethyl)aminomethane (Tris) (Shanghai Bo' ao Biological Technology); alumina powder, polishing cloth, gold electrode (CHI101), platinum wire electrode (CHI115), saturated calomel electrode (CHI111) (Shanghai Chenhua Instruments). All other reagents were analytically pure, and water used in the laboratory was double-deionized. Aptamer buffer (pH = 7.4) consisted of 50 mmol/L Tris-HCl, 120 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl , and 1 mmol/L CaCl .

1.1.2 Instruments Instruments used included an electrochemical workstation (CHI760E; Shanghai Chenhua Instruments), ultrasonic cleaner (Tianjin Autoscience Instruments), and benchtop high-speed refrigerated centrifuge (Thermo Fisher).

1.2 Experimental Methods

1.2.1 Gold Electrode Surface Treatment Gold electrode surfaces were treated using electrochemical cleaning. Prior to treatment, gold electrodes were polished sequentially with 1.0 m, 0.3 m, and 0.05 m alumina powder. The electrode surfaces were rinsed with double-deionized water, then ultrasonically cleaned twice with absolute ethanol and double-deionized water for 5 minutes each. After drying, the electrodes were 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]).

1.2.2 MPA-SAMs Assembly and Characterization Polished bare gold electrodes were 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 surfaces were rinsed with absolute ethanol to remove physically adsorbed MPA molecules, yielding MPA-SAM-modified gold electrodes. The

modified electrodes were placed in PBS buffer (10 mmol/L, pH 7.4) containing 2 mmol/L K [Fe(CN)] and K [Fe(CN)] · 3H O as the electrolyte for CV and EIS characterization. CV parameters: voltage range of -0.3 to 0.6 V, scan rate of 100 mV/s. EIS parameters: frequency range of 0.1 Hz to 100 kHz, amplitude of 5 mV.

1.2.3 Aptamer Immobilization and Characterization Aptamer Pretreatment: To ensure the aptamer formed 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 acidic MES buffer system containing 20 mmol/L EDC and 20 mmol/L NHS to activate the carboxyl terminal, then incubated in a 200 nmol/L NH -ssDNA solution for 40 minutes. After washing with buffer and drying, the electrode was characterized by CV and EIS. The biosensor modification process is shown in Figure 2 [Figure 2: see original paper].

Results and Discussion

2.1 Characterization of Bare Gold Electrodes

The treated bare gold electrode was electropolished 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 gold electrode curve was essentially consistent with the standard curve for a clean gold electrode, indicating that the electrochemical cleaning method achieved good cleaning effects and was suitable for subsequent modification steps.

2.2 Formation and Characterization of MPA Self-Assembled Monolayers

This experiment investigated MPA-SAM assembly times of 1 h, 6 h, 9 h, 12 h, and 24 h, characterizing the results using both CV and EIS. Figure 4 [Figure 4: see original paper] shows the CV characterization results. 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 occurs because the carboxyl terminal of MPA is 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 EIS characterization results. 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), MPA-modified gold electrodes (b–e) exhibit a larger

arc diameter in the high-frequency region, indicating greater resistance in this region, consistent with CV results. Self-assembled monolayer formation on gold substrates occurs in two stages [12]: (1) a rapid adsorption process that completes in a short time, and (2) a reorganization process of the surface film on the gold electrode that requires longer time. Electrochemical characterization results show that as assembly time increased, surface coverage increased between 0–6 h, remained essentially unchanged between 6–12 h, and decreased after 24 h. Therefore, 6 h was selected as the optimal assembly time for MPA modification on gold electrode surfaces.

2.3 Characterization of Immobilized Aptamers

Figure 6 [Figure 6: see original paper] shows CV characterization of gold electrodes at different modification steps. Since aptamers consist of nucleotides that are inherently negatively charged, their immobilization on the gold electrode surface enhances the barrier to electron transfer, increasing resistance and causing a decrease in peak current, though this effect was not pronounced.

Figure 7 [Figure 7: see original paper] shows EIS characterization of gold electrodes at different modification steps. The biological element used in this study is a single-stranded nucleic acid, which produces relatively weak electrical signal changes upon interaction with bacterial endotoxin. Electrochemical impedance offers excellent interfacial characterization capabilities, using small-amplitude sinusoidal wave potential as the perturbation signal to produce relevant linearity with the system without interfering with biomacromolecules. Figure 7 shows that when the aptamer was immobilized on the gold electrode surface, the arc radius in the high-frequency region expanded due to electrostatic repulsion from the negative charges on the nucleic acid phosphate backbone [13], which limited electron exchange at the gold electrode surface and increased resistance.

2.4 Performance Characterization of the Aptamer Biosensor

Aptamer specificity for endotoxin was evaluated to verify that 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 for practical applications. Four electrodes modified in the same batch showed similar electrochemical response signals, indicating acceptable reproducibility of the constructed sensor.

When the fabricated biosensor was immersed in a series of bacterial endotoxin

solutions at different concentrations for 30 minutes, a good linear relationship was observed between 0.001 EU/mL and 0.1 EU/mL, as shown in Figure 9 [Figure 9: see original paper].

Conclusion

In summary, this study constructed an aptamer-based biosensor for endotoxin detection. This method employs aptamers as the recognition element and combines two electrochemical analysis methods—cyclic voltammetry and electrochemical impedance—to achieve stepwise characterization of biosensor construction. Furthermore, the detection process is not interfered with by bovine serum albumin, 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 detection and low detection limits, showing promise for endotoxin quantification in real biological samples.

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