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An exonuclease-assisted amplification electrochemical aptasensor of thrombin coupling "signal on/off" strategy

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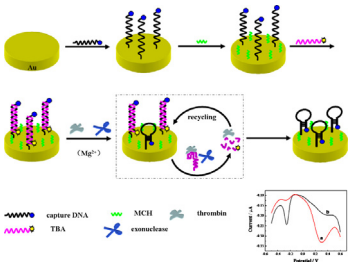
HIGHLIGHTS

- MB and Fc were labeled on two oligonucleotides separately to produce dual signals.
- Coupling "signal-on" and "signal-off" strategies.
- Exonuclease-catalyzed target recycling amplified two signals significantly.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, a dual-signaling electrochemical aptasensor based on exonuclease-catalyzed target recycling was developed for thrombin detection. The proposed aptasensor coupled "signal-on" and "signal-off" strategies. As to the construction of the aptasensor, ferrocene (Fc) labeled thrombin binding aptamer (TBA) could perfectly hybridize with the methylene blue (MB) modified thiolated capture DNA to form double-stranded structure, hence emerged two different electrochemical signals. In the presence of thrombin, TBA could form a G-quadruplex structure with thrombin, leading to the dissociation of TBA from the duplex DNA and capture DNA formed hairpin structure. Exonuclease could selectively digest single-stranded TBA in G-quadruplex structure and released thrombin to realize target recycling. As a consequence, the electrochemical signal of MB enhanced significantly, which realized "signal on" strategy, meanwhile, the deoxidization peak current of Fc decreased distinctly, which realized "signal off" strategy. The employment of exonuclease and superposition of two signals significantly improved the sensitivity of the aptasensor. In this way, an aptasensor with high sensitivity, good stability and selectivity for quantitative detection of thrombin was constructed, which exhibited a good linear range from 5 pM to 50 nM with a detection limit of 0.9 pM (defined as S/N = 3). In addition, this design strategy could be applied to the detection of other proteins and small molecules.

1. Introduction

Thrombin is a serine protease which converts soluble fibrinogen into insoluble fibrin during the coagulation progress. It plays an important role in many crucial physiological and pathological processes such as blood coagulation, thrombosis, inflammation and angiogenesis [1–4]. Thrombin concentration in blood varies from nanomolar to low micromolar levels [5], the concentration of thrombin is in connection with various coagulation abnormalities [6]. Therefore, quantitative detection of thrombin is significant in disease diagnosis and clinical practice [7]. So far, mass spectrometry [8], surface plasmon resonance [9] and fluorescence spectroscopy [10] have been used for the detection of thrombin. However, these methods are rather expensive, sophisticated and unsuitable

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for fast detection, while electrochemical methods have received lots of attention for the advantage of simpleness, fast response and in vivo detection.

Aptamers are single-stranded DNA or RNA oligonucleotides, which are synthesized by systematic evolution of ligands by exponential enrichment (SELEX) technology [11,12]. They can specifically recognize and bind to lots of targets, including small molecules, proteins and cells [13–16]. Compared with antibody, aptamers have sorts of advantages such as good stability, low cost, easy modification, long-term storage and high resistance against denaturation [17]. Aptamer based biosensor has many applications in food safety, pharmaceutical analysis, environmental monitoring and biochemical analysis [18–21]. For the great importance of thrombin, thrombin binding aptamer (TBA) is also been widely investigated [22]. TBA can bind to different epitopes of human α-thrombin and form a stable G-quadruplex structure with the excellent affinity and high selectivity towards thrombin, which has been widely used to construct biosensor for the detection of thrombin [23,24]. So far, two TBA has been founded: a 15 bases aptamer can bind to exosite I of thrombin, known as fibrinogen binding sites, and a 29 bases aptamer can bind to exosite II of thrombin, called heparin-binding aptamer. 29 bases aptamer has higher affinity towards thrombin than 15 bases aptamer [25,26]. In this work, 29 based TBA was chosen to construct proposed aptasensor. Generally, ferrocene (Fc) [27], methylene blue (MB) [28] and thionine [29] are applied to label aptamers to produce an electrochemical signal. The research of dual-signaling aptasensor is still a challenge [30].

Many signal amplification strategies have been used to improve the sensitivity of aptasensors, such as gold nanoparticles assisted amplification [31], DNAzyme assisted amplification [32], aptamer/graphene (graphene oxide) nanocomplex assisted amplification [33–35], rolling circle amplification [36], hybridization chain reaction amplification [37], enzyme labeling amplification [38], exonuclease-catalyzed target recycling [39], and so on. Among them, exonuclease can selectively digest aptamer which binds to target and release the analyte for target recycling, providing an excellent method for sensitive detection in aptasensors [40]. $RecJ_f$ exonuclease is processive single-stranded DNA specific enzyme, which functions unidirectionally at 5′-termini. It can degrade single-strand DNA in the direction of $5' \rightarrow 3'$ [41,42]. Exonuclease-catalyzed target recycling strategy amplifies the signal and improves the sensitivity of aptasensor significantly [43], which has been applied in the construction of aptasensor. However, most of aptasensor has only one signal, they are only "signal-on" or "signal-off" type, exonuclease can only enhance one signal. While the proposed aptasensor has two different signals, which can reach both "signal-on" and "signal-off" strategy, and exonuclease-catalyzed target recycling amplifies two different signals simultaneously.

Herein, we proposed a "signal on/off" electrochemical aptasensor for thrombin detection based on exonuclease-catalyzed target recycling. To construct the aptasensor, two different signaling molecules (MB and Fc) were labeled on capture DNA and TBA separately to produce two different signals. TBA formed aptamer–thrombin complex with thrombin and released from electrode, which led to the decrease of Fc signal, while the capture DNA formed hairpin structure, the signal of MB was increased. The aptasensor coupled signal-on and signal-off strategies, exonuclease amplified two different signals simultaneously, improving the sensitivity of the aptasensor significantly.

2. Experimental

2.1. Materials and reagents

Thrombin (≥95%, SDS-PAGE), 6-mercapto-1-hexanol (MCH), immunoglobulin G (IgG) and tris(2-carboxyethy)phosphine hydrochloride (TCEP) were purchased from Sigma–Aldrich Chemical Co. (USA). $RecJ_f$ exonuclease was obtained from New England Biolabs Ltd. (Beijing, China). BSA and Myoglobin were ordered from Aladdin Chemistry Co., Ltd. (China). Oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All other reagents were of analytical reagent grade. All solutions were prepared using ultrapure water ($18.25\ M\Omega\ cm^{-1}$) produced by Aquapro Ultra-pure water system (China). 20 mM tris-HCl buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$ was used to dissolve thrombin and DNA oligonucleotides.

The sequence of oligonucleotides were as follows:

capture DNA: 5′-HS-$(CH_2)_6$-AGTCACCCCAACCTGCCCTACCACG-GACT-MB-3′. The complementary four bases (underline showed) at both its 5′ and 3′ ends to make the capture DNA form a stem-loop structure.

TBA: 5′-AAAAGTCCGTGGTAGGGCAGGTTGGGGTGACT-Fc-3′. Three bases were added (underline showed) at the 5′ end of the thrombin binding aptamer, in order to make $RecJ_f$ exonuclease recognize the single strand in aptamer–thrombin complex more easily.

2.2. Apparatus

All electrochemical characterizations including cyclic voltammetry (CV), differential pulse voltammetric (DPV) and electrochemical impedance spectroscopic (EIS) measurements were carried out on a CHI660C electrochemical workstation (Shanghai Chenhua Instrument, China). A three-electrode system composed of platinum wire as the auxiliary electrode, saturated calomel electrode (SCE) as reference electrode and a 2-mm-diameter gold electrode as working electrode was used in the experiment. All the electrodes were purchased from CH Instruments, Inc., CV and DPV were performed in 10 mM PBS (pH 7.4) with a voltage range from 0.6 V to −0.5 V. Ultrasonic cleaners (Branson2000, USA) and 320-S acidity meters (Mettler-Toledo, Switzerland) were used in this experiment.

2.3. Preparation of the electrochemical aptasensor

The bare gold electrode (GE) was polished with 0.05 μm of Gamma-alumina powder and ultrasonic cleaning in water and ethanol separately. Then, the GE was electrochemical cleaned through scanned in 0.5 M H_2SO_4 by cyclic voltammetry for 10 scans from −0.3 V to 1.5 V at a scan rate of $100\ mV\ s^{-1}$. After drying with nitrogen, the electrode was immediately dipped into 50 μL capture DNA (0.5 μM) containing 0.2 mM TCEP for 12 h at room temperature (prior to use, the capture DNA was heated to 90 °C for 10 min and then immediately cooled down in ice-bath. In this way, the capture DNA kept single strand structure). Next, the modified electrode was soaked into 1 mM MCH for 1 h to block the free sites on GE. Then, the electrode was immersed into 50 μL TBA (1 μM) for 2 h at 37 °C. After that, the electrode was incubated in 50 μL of the mixture of different concentration of thrombin containing $0.03\ U\ \mu L^{-1}$ $RecJ_f$ exonuclease in 1× NEBuffer 2 (50 mM NaCl, 10 mM tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) for 90 min at 37 °C. Finally, the electrode was used for measurement in 10 mM PBS (pH 7.4). After each step, the electrode was rinsed with PBS (pH 7.4) to remove the adsorbate.

3. Results and discussion

3.1. Design strategy of the aptasensor

As shown in Fig. 1, capture DNA firstly self-assembled onto the electrode surface, and the Fc labeled TBA perfectly hybridized with capture DNA. The formation of double-stranded structure made