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A label-free electrochemical aptasensor for sensitive thrombin detection in whole blood

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ABSTRACT

In this paper, we reported a novel label-free electrochemical aptasensors for thrombin detection in whole blood using self-assembled multilayers with carboxymethyl-PEG-carboxymethyl (CM-PEG-CM) and thrombin-binding aptamer (TBA). In the sensing strategy, CM-PEG-CM and TBA were assembled on the electrode surface *via* covalent binding. In the presence of target, the TBA on the outermost layer of the self-assembled multilayer would catch the target on the electrode interface, which makes a barrier for electrons and inhibits the electro-transfer, resulting in the decreased DPV signals. Using this strategy, a wide detection range (1 pM–160 nM) for target thrombin was obtained, with a low detection limit of 1.56×10^{-14} M. The control experiments were also carried out by using bull serum albumin (BSA) and lysozyme in the absence of thrombin. The results showed that the aptasensors had good specificity, stability and reproducibility to thrombin. Moreover, the aptasensors could be used for detection of thrombin in whole blood which could provide a promising platform for fabrication of aptamer based biosensors in clinical application.

1. Introduction

Thrombin is a kind of serine protease, which plays an important role in the physiological and pathological process [1]. It is usually regarded as a tumor marker in the diagnosis of pulmonary metastasis, and the high or low concentration of thrombin in blood is associated with coagulation abnormalities [2,3]. Therefore, the specific recognition and quantitative detection of thrombin are extremely important in both clinical practice and diagnostic application. Recently, thrombin-binding aptamer (TBA) have successfully been demonstrated as molecular recognition receptors using various transducers [4–6]. Aptamers are artificial oligonucleotides (DNA or RNA) selected from systematic evolution of ligands by exponential enrichment process *in vitro* that bind to their target molecules (*e.g.* metal ions, small organic compounds, metabolites, proteins and even cells) with high affinity [7–9]. Aptamers have several advantages such as relative ease of isolation and modification, tailored binding affinity, and resistance against denaturizing [10]. Consequently, by incorporating these aptamers into biosensors, it is facile for aptamers to convert bio-recognition events into physically detectable electrochemical signals. Thus, aptamer-based biosensors are considered as prospective sensors for rapid and sensitive protein detections.

In recent years, a large number of strategies have been developed for the detection of thrombin such as fiber-optic sensor, fluorescence anisotropy and near-infrared fluorescence [4,11,12]. However, these methods are fluorescence-based, thus they require a laborious labeling process, qualified personnel and sophisticated instrumentation. Moreover, the fluorescent labels may cause steric hindrance, which will affect the binding ability. Accordingly, it needs to develop a quick and easy detection method. Electrochemical biosensors show promising performance for point-of-care due to low cost, label-free operation, ease of miniaturization, and ability for integration with multi-array for diagnostic tools. However, these detection methods of thrombin concentration are performed in serum which are isolated from whole blood [13,14]. As we know, when in direct contact with blood, the foreign materials are prone to initiate the formation of clots, as platelets and other components of the blood coagulation system are activated. At present, it is very

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difficult to design and prepare an electrochemical biosensor that can be used in whole blood just because the biofouling of electrode surface can be developed by platelet, fibrin and blood cell adhesion in the complex environment of whole blood media.

Our groups have designed and explored PU-F127 nanospheres as antibiofouling surface for preparing for electrochemical glucose biosensor that can be used directly in whole blood samples [15]. In this paper, the hydrophilic polymer coating, carboxymethyl-PEG-carboxymethyl (CM-PEG-CM), was chosen to modify the GCE for its antibiofouling effect that due to the coherence between biocompatibility [16,17] and antifouling property of PEG [18,19]. Results showed that this novel aptasensor by molecularly grafting TBA can be used for accurate quantification of the concentration of thrombin in whole blood directly. The aptasensor we prepared here provided a precise, convenient, sensitive and specific method for thrombin detection, which could be an accurate technique in the clinical laboratory.

2. Experiments

2.1. Reagents

Carboxymethyl-PEG-carboxymethyl (CM-PEG-CM, MW 2000) was obtained from Seebio Co. Ltd. (China). TBA (5′-NH_2-GGT TGG TGT GGT TGG-3′) was purchased from Sangon Biotechnology Co., Ltd. (China) with HPLC purification. Thrombin (human α-Thrombin), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimidobiotin (NHS) were purchased from Sigma–Aldrich (USA). (3-Aminopropyl)-triethoxysilane (APTES) (98%), bovine serum albumin (BSA) and lysozyme were purchased from Aladdin Chemistry Co. Ltd. (China). Crystal violet were purchased from Sinopharm Chemical reagent Co. Ltd. (China). All the above reagents were used without further purification. Phosphate buffer solution (PBS) was prepared by mixing stock standard solution of Na_2HPO_4 and NaH_2PO_4. All other chemicals were of analytical grade and were used as received. All solutions prepared with double-distilled water and high purity N_2 was applied for deaeration.

2.2. Apparatus and measurements

The existence of CM-PEG-CM and TBA/CM-PEG-CM was measured using on an UV–vis spectrophotometer (Cary 50 Conc, Australia). The spectra of CM-PEG-CM, TBA and TBA/CM-PEG-CM were proved by Fourier transform infrared (FTIR, VARIAN Cary 5000, USA).

2.3. Biocompatibility evaluation of the aptasensors

The biocompatibility of the aptasensors was characterized by water contact angle and circular dichroic (CD) measurements. The water contact angle for each modified electrode surface was measured five times by a sessile drop method using an optical contact angle goniometer (Rame-Hart-100, Rame-Hart Inc., USA) at room temperature ($25 \pm 2\,^{\circ}C$). Drops of purified water (3 μL) were deposited onto the electrode surface, and the readings were stabilized and taken in 60 s after dropping. Water sessile drops were deposited from a glass syringe onto the sample surfaces, and the water contact angle was measured after a defined period of time, in order to allow the establishment of equilibrium. The blank electrode surface was used as reference. The CD spectra were made on a JASCO Model J-810 dichrograph (Japan Spectroscopic Co. Ltd., Tokyo, Japan) at room temperature in a 1 cm quartz cuvette. CD spectra were obtained by taking the average of three scans made from 200 to 320 nm. All DNA samples at a final concentration of 1 μM were dissolved in 0.10 M PBS (pH = 7.4).

2.4. Construction of the thrombin/TBA/CM-PEG-CM/APTES aptasensors and thrombin measurements

The glassy carbon electrode (GCE, 3 mm in diameter) was polished to a mirror-like surface with 0.3- and 0.05-μm alumina slurry and successively sonicated in ethanol and doubly distilled water. 10 μL APTES was dispersed into 10 mL anhydrous alcohol to form 0.1% APTES ethanol solution. Then 8 μL of 0.1% APTES was dropped onto the surface of the GCE and dried under the infrared lamp. APTES was linked to the GCE surface through the silicon-oxygen bonds. 1 mg of CM-PEG-CM was dissolved in 1 mL double-distilled water to obtain 1 mg mL^{-1} CM-PEG-CM solution. 8.0 μL of the resulting CM-PEG-CM solution was dropped onto the pretreated electrode surface and dried in air. The carboxylic group of CM-PEG-CM was combined with the amino at the end of APTES molecule. After that, the surface of modified GCE was activated using EDC and NHS. Then, TBA was molecularly grafted onto the surface of modified GCE *via* covalent binding. In the presence of the target molecule of thrombin, a complex of quadruplex–thrombin was formed and such a complex increased the steric hindrance that greatly restrained access of electrons for a redox probe of $[Fe(CN)_6]^{3-/4-}$. When not in use, the electrodes were stored at 4 °C in a refrigerator.

The electrochemical experiments were conducted on a CHI 760D electrochemical analyzer (Shanghai Chenhua, China) at room temperature with a three electrode system, which consisted of GCE (Shanghai Chenhua, China) as a working electrode, a platinum as a counter electrode, and a saturated calomel electrode (SCE) as a reference electrode.

Cyclic voltammogram (CV) experiments were carried out in 5 mL of 0.10 M PBS at 100 mV s^{-1}, and the solution was purged with high purity nitrogen prior to and blanked with nitrogen during the electrochemical experiments. Differential pulse voltammetry (DPV) measurements were carried out with pulse amplitude of 0.05 V and pulse width of 0.2 s, and Nyquist plots of Faradic impedance were carried out in 0.10 M PBS (pH = 7.4) containing 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ and 0.1 M KCl.

The control fluorescence method was followed by the literature [20]. First, the fluorescence spectra of crystal violet were recorded on a fluorometer (Cary Eclipse, Varian, USA) with excitation at 590 nm and an emission range from 615 to 750 nm. Then, a certain volume of 1 μM TBA was added into the 5 mM crystal violet solution. The fluorescence of this mixture was recorded after incubating for 30 min. For the study of thrombin interaction with crystal violet, the solutions of thrombin varied from 0.001 to 0.1 μM were added into the above mixture for fluorescence detection.

3. Results and discussion

3.1. Characterization of the TBA/CM-PEG-CM

UV–vis and FTIR spectroscopy were used to confirm the binding function between TBA and CM-PEG-CM. In Fig. 1A, CM-PEG-CM showed no absorption peak from 200 to 600 nm (curve a) and TBA showed an absorption peak at 260 nm (curve b). After TBA was attached to CM-PEG-CM, an obvious absorption peak occurred at 260 nm as shown in curve c, which is a characteristic of the DNA strand, indicating the successful binding between TBA and CM-PEG-CM [21].

Furthermore, as shown in Fig. 1B, the peaks at 1101 cm^{-1} (curve a) correspond to the CH_2-etheric bonds of CM-PEG-CM [22]. Compared with CM-PEG-CM modified electrode, the