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Electrochemical detection of the activities of thrombin and its inhibitor

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ABSTRACT

An electrochemical approach is described to assess the activities of thrombin and one of its inhibitors. Thrombin is a vital proteinase, which is one kind of serine protease, and plays an important role in coagulation cascade, thrombosis, and hemostasis. A gold electrode immobilized with *p*-aminodiphenylamine (pADA) modified peptide (H-D-Pro-Phe-Arg-*p*-aminodiphenylamine) was prepared for the analyses of thrombin and its inhibitor. The peptide was employed as the recognition and cleavage site for thrombin while pADA was used as an electroactive reporter. Experimental results showed that this method is sensitive as a low concentration of 5 fM thrombin can be detected. The inhibition of thrombin activity by argatroban was monitored and IC_{50} was found to be about 10 μM. The protocol was further applied for the measurement of a healthy human serum sample.

1. Introduction

Thrombin with a molecular weight of 34 kDa has significant importance in molecular biology, such as in the regulation of tumor growth, metastasis, and angiogenesis [1]. It is also the last enzyme protease involved in the coagulant cascade, which converts fibrinogen to insoluble fibrin that forms the fibrin gel [2]. Exact regulation of the blood coagulation is of great interest to pharmaceutical drug screening and much effort is being devoted to find effective thrombin inhibitors [3]. There are a diverse group of anticoagulant agents [4] that are either available or currently in development, including fondaparinux (an indirect Factor Xa inhibitor); dabigatran and argatroban (direct thrombin inhibitors); and rivaroxaban, apixaban, and edoxaban (direct Factor Xa inhibitors). To assess the activities of thrombin and its inhibitors is crucial in clinical treatment as well as for the discovery of efficient drugs with regard to thrombolytic risks or other blood coagulation diseases. The well established methods for the detection of thrombin and its inhibitors' activities are clot-based tests and chromogenic assays [5]. The clot-based tests include prothrombin time/INR (PT/INR) [6], dilute PT (dPT) [7], activated partial thromboplastin time (aPTT) [8], ecarin clotting time (ECT) [9], HepTest [10], and prothrombinase-induced clotting time (PiCT) tests [11]. Chromogenic assays [12] are based on the specificity of the chromogenic substrates and the resulting color development from the reactions. In principle, the chromogenic measurement requires a detection of the rate of color change with a colorimetric quantitation for the reaction. Efforts have also been carried out to develop sensitive sensors to monitor thrombin and its inhibitors. Gauglitz et al. [13] used a parallel and label-free method based upon reflectometric interference spectroscopy (ROIfs) technique for the detection of thrombin inhibitors. Song et al. [14] reported an LC–MS–MS method and an HPLC-UV method for the quantitative determination of a novel thrombin inhibitor in human fluids.

Herein, we developed a simple and sensitive electrochemical method for the detection of thrombin activity and inhibition by taking advantage of an electro-labeled tripeptide substrate (H-D-Pro-Phe-Arg-*p*-aminodiphenylamine), which is immobilized on gold electrode surface and can be specifically recognized and cleaved at the amide group connecting arginine and *p*-aminodiphenylamine (pADA) by thrombin [15]. Consequently, thrombin activity can be conveniently monitored by measuring the electrochemical signals ascribed to the remaining pADA as the electroactive reporter [16] on electrode surface. The strategy not only provides a facile readout of thrombin activity, but also offers a possible way to screen the throughput of its inhibitors.

2. Experiment

2.1. Reagents and chemicals

Thrombin (human-thrombin), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), mercaptoacetic acid (MAA), *N*-hydroxysuccinimide (NHS), and β-mercaptoethanol were purchased from Sigma-Aldrich (USA). H-D-proline-L-phenylalanine-L-arginine-*p*-aminodiphenylamine (H-D-Pro-Phe-Arg-pADA) was obtained from GL Biochem Ltd. (Shanghai, China). Argatroban was acquired from

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BrightGene (Suzhou, China). All chemicals were of analytical grade and used without further purification. Milli-Q water (18.2 MΩ cm) was used throughout the experiments.

2.2. Immobilization of the substrates on gold electrode

The procedures for the preparation of surface-modified electrode are summarized in Scheme 1. The gold electrode (3 mm in diameter) was polished carefully in sequence with alumina slurries (1.0, 0.3, and 0.05 μm), followed by sonication in deionized water. The electrode was then cycled between 0 and +1.5 V (vs. SCE) in 0.5 M H_2SO_4 till a clean cyclic voltammogram was obtained and dried afterwards with purified N_2. The Au electrode was immediately immersed in a solution of 10 mM MAA in absolute ethanol at 4 °C for 12 h and a self-assembled monolayer could be formed with carboxyl groups exposed on the surface. After the electrode was thoroughly rinsed with absolute ethanol and dried with purified N_2, the carboxyl groups were activated by immersing the electrode in an aqueous solution containing 0.4 M EDC and 0.1 M NHS for 60 min followed by rinsing with deionized water [17]. Subsequently, the electrode was immersed in 300 μM peptide solutions (100 mM phosphate buffer, pH = 7.4) for 6 h at room temperature. After rinsing with PBS buffer, 100 mM aqueous ethanolamine was added on the electrode surface and incubated for 30 min in a humidified chamber to deactivate the excess reactive succinimidyl groups, followed by β-mercaptoethanol (0.2% in ethanol) to block the remaining bare gold electrode surface. The peptide-immobilized gold electrode was rinsed with PBS buffer and stored at 4 °C prior to experiments.

2.3. Thrombin and inhibitor assay on peptide-modified gold electrode surface

Thrombin activity assay was performed with the surface-immobilized peptide substrates. Briefly, an aqueous mixture containing a desired amount of thrombin in 200 μL Tris–HCl buffer solution (50 mM Tris–HCl, 140 mM NaCl, 1 mM $MgCl_2$, pH = 7.4) was added on the H-D-Pro-Phe-Arg-pADA-modified gold electrode surface and incubated at 37 °C for 2 h in a humidified chamber. The reaction was terminated by rinsing the electrode thoroughly with Tris–HCl buffer solution. For the inhibition experiments, the inhibitor at a desired concentration was added into the mixture mentioned above to react with thrombin (500 fM) for 30 min. The same procedures were followed afterwards except the incubation time was 4 h.

2.4. Electrochemical measurements

Differential pulse voltammetry (DPV) was carried out at room temperature with the aid of an electrochemical analyzer (CHI 660c). The normal three-electrode system consisting of the saturated calomel reference electrode (SCE), the platinum wire counter electrode, and the working electrode (which is the surface-modified gold electrode in this case) was employed and immersed in 20 mM PBS buffer (pH = 7.4) for the measurements in a 5-mL electrochemical cell. The DPV scan was ranged from −0.2 V to +0.6 V to detect the pADA signals.

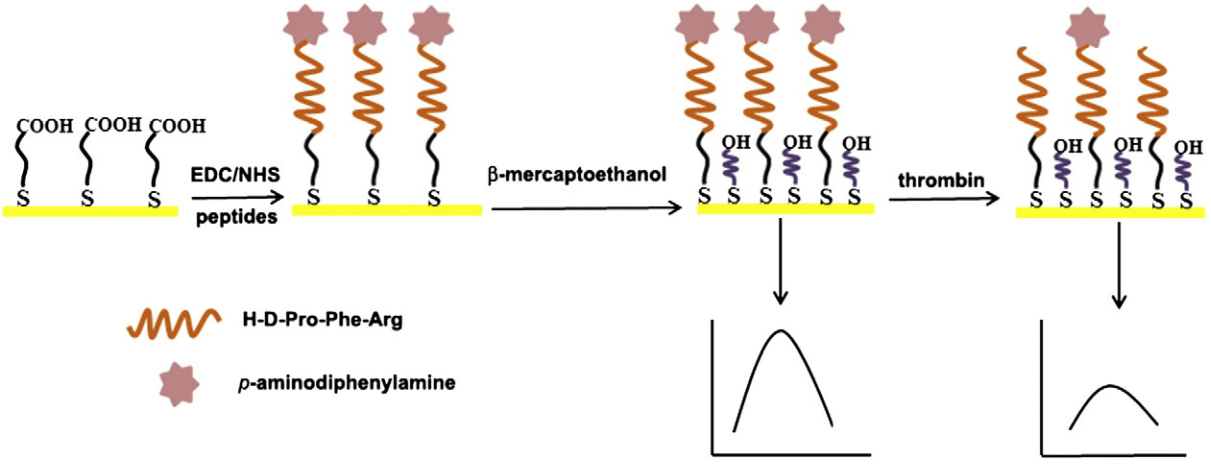
3. Results and discussion

3.1. Determination of thrombin activity by DPV

The immobilized H-D-Pro-Phe-Arg-pADA can be specifically recognized and cleaved at the amide group connecting Arginine and pADA by thrombin. The thrombin activity was detected by electrochemical measurements as shown in Fig. 1A, which gives the differential pulse voltammetry (DPV) results for the surface-modified Au electrode in 5 mL of 20 mM PBS solution (pH = 7.4) before and after the treatments with different concentrations (5 fM to 10 pM) of thrombin. It is observed that the DPV signal is inversely related to the amount of thrombin in the sample, which can cut loose pADA from the electrode surface. There was no obvious peak current decrease when a blank buffer was used for thrombin activity assay analysis. However, as the concentration of thrombin increased gradually, the intensity of the DPV peak corresponding to pADA decreased accordingly. A concentration as low as 5 fM of thrombin (RSD = 4.9% by using five sensors) was detected in the experiments and this sensitivity is a few orders better than the previous methods for thrombin detection [18] and also comparable to the most advanced aptamer biosensors [19]. The DPV peak current leveled off after the concentration of thrombin reached 500 fM, which could be considered as the saturation point and thus employed in the following analysis for thrombin inhibition assay.

3.2. Thrombin inhibitor assay measurements

The aforementioned biosensing protocol for thrombin activity assay could also be used to assess the inhibition of thrombin, which can be detected by measuring DPV signals for the surface-modified Au electrode after contacting thrombin samples incubated with the inhibitors. The half-maximum inhibition value IC_{50} would be determined by the corresponding electrochemical data. To examine the thrombin inhibition assay, argatroban was used as the model inhibitor, which is a small molecule anticoagulant that can directly inhibit thrombin [20]. With the capability of reversibly binding to the thrombin active site, argatroban does not require the co-factor antithrombin III for antithrombotic activity and can exert its anticoagulant



Scheme 1. Schematic illustration of the electrochemical biosensor for the detection of activities of thrombin and its inhibitors.