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Short communication

Electrochemical Determination of Protease with Improving Sensitivity by Electrochemical-chemical-chemical Redox Cycling

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Redox-labeled peptides attached on the electrode surface have allowed for the determination of various proteases. Herein, we reported a simple method to improve the sensitivity of redox-labeled electrochemical protease biosensors by employing an electrochemical-chemical-chemical redox cycling. Specifically, ferrocene (Fc) conjugated to the peptide substrate immobilized onto the electrode surface was oxidized into ferrocenium (Fc^+); then, Fc was regenerated from Fc^+ by ascorbic acid. In the presence of tris(2-carboxyethyl)phosphine (TCEP), ascorbic acid was also regenerated immediately after its oxidation, therefore resulting in the further increase in the oxidation current. To demonstrate the feasibility of this amplified strategy, thrombin was tested as a model analyte.

Keywords: Proteases; Electrochemistry; Redox-label; Signal amplification; Redox cycling

1. INTRODUCTION

Protease can catalyze the degradation of protein to peptide. It shows pivotal functions in some biological processes, including cell differentiation and growth, blood coagulation, angiogenesis, wound healing, apoptosis, and lipid metabolism [1, 2]. The method for quantifying the protease content and probing of its catalytic activity is valuable for the discovery of new inhibitor-type drugs. Currently utilized techniques for protease detection are high-performance liquidchromatography (HPLC), fluorescence and gel electrophoresis [3, 4]. The techniques are time-consuming and lack sensitivity for high throughput screening of protease inhibitors. In the past decade, electrochemical sensors have attached particular attention due to their high sensitivity and selectivity, low cost, fast

response, and compatibility with miniaturization [5, 6]. Redox-labeled peptide site-specifically immobilized onto the gold electrode surface facilitates the simple and rapid detection of protease. Specifically, protease-catalyzed cleavage of peptide substrate made the redox moiety release from the electrode surface, thus leading to the deterioration in the Faradaic current. Lin and co-workers first demonstrated the electrochemical detection of matrix metalloproteinases (a zinc-dependent endopeptidases) using ferrocene (Fc)-labeled peptide attached to a gold electrode [7]. Recently, the similar detection principle was used to determine other protease, including caspases, plasmin, legumain and cathepsin B [8-12]. Although these redox-labeled methods are simple, fast and inexpensive for protease detection, they show low sensitivity since the electrochemical signal strictly depends upon the amount of immobilized probe.

It has been demonstrated that ferrocene can electrochemically catalyze the oxidation of ascorbic acid, that is to say, ferrocene can be regenerated by ascorbic acid after its electrochemical oxidation [13]. Based on this fact, Liu's group developed an electrochemical-chemical-chemical redox cycling system in which ferrocene derivative is used as the redox mediator and ascorbic acid as well as tris(2-carboxyethyl)phosphine (TCEP) are reducing reagents [14, 15]. The three additional critical factors for this design are that (1) both ascorbic acid and TCEP show no electrochemical signal on self-assembled monolayers (SAMs)-covered electrode, (2) ascorbic acid could be regenerated by TCEP after its oxidation by ferrocenium (the electro-oxidation production of ferrocene), and (3) TCEP shows no or slow reaction with ferrocenium. Inspired by these results, herein, we suggested that the electrochemical signal of Fc-labeled peptide probe could be improved by the electrochemical-chemical-chemical redox cycling, which would facilitate the amplified determination of protease. The amplified strategy is very simple since it only requires the addition of ascorbic acid and TCEP to the electrolyte solution without changing the detection procedure of the aforementioned redox-labeled methods. The feasibility of our design was demonstrated with thrombin as the model analyte.

2. EXPERIMENTAL

2.1 Chemicals and reagents

6-Mercapto-1-hexanol (MCH), thrombin, tris(2-carboxyethyl)phosphine (TCEP) hydrochloride, bovine serum albumin (BSA), immunoglobulin G (IgG), serum, and lysozyme were purchased from Sigma–Aldrich. Peptide (Fc-SGFPRGRYC) was prepared via solid phase Fmoc chemistry. The peptide can be cleaved by thrombin into two fragments of Fc-SGFPR and GRYC [16]. All other reagents were provided from Beijing Chemicals, Ltd. (Beijing, China). Thrombin and peptide stock solutions were prepared with water and then diluted with phosphate-buffered saline solution (PBS buffer, 10 mM, pH 7.4).

2.2 Preparation of peptide-covered electrode

The gold electrodes were polished with diamond pastes and then sonicated in ethanol and water. The peptide-covered electrodes were obtained by incubating the cleaned electrodes with a 20

μM peptide solution containing 100 μM TCEP overnight. Then, the electrodes was rinsed thoroughly with methanol/water and soaked in a 1 mM MCH solution for 30 min to block the unreacted gold surface. After that, the electrodes were washed with ethanol/water to remove any non-specifically adsorbed contaminants.

2.3 Assay of thrombin

For the assay of thrombin, the peptide/MCH-covered electrodes were incubated with solution with different content of thrombin for a given time. After having been washed with the PBS solution, the sensing electrodes were placed in the PBS buffer containing 50 mM Na_2SO_4 for electrochemical measurements. The electrochemical workstation used in the experiment is CHI 660E (CH Instruments, Shanghai, China). The auxiliary electrode is a platinum wire. The reference electrode is a Ag/AgCl. The cyclic voltammograms (CVs) were collected from -0.1 V to 0.4 V with a scanning rate of 20 mV/s. The amperometric currents were obtained at a potential of 0.3 V.

3. RESULTS AND DISCUSSION

3.1 Principle of the amplified strategy

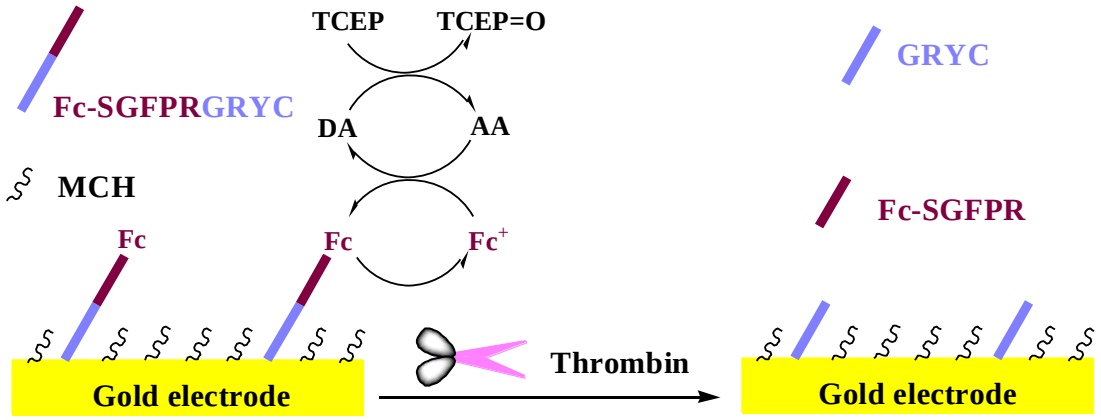

Figure 1. Schematic illustration of the electrochemical method for thrombin detection based on the signal amplification of electrochemical-chemical-chemical redox cycling.

The schematic representation of the electrochemical strategy for thrombin detection based on the signal amplification of electrochemical-chemical-chemical redox cycling is shown in Fig. 1. Peptide Fc-SGFPRGRYC was immobilized on the electrode surface via the Au/S interaction. Once Fc tag in the peptide was electrochemically oxidized into Fc^+, it would be regenerated by ascorbic acid (AA) in the solution; then, AA would also be regenerated from dehydroascorbic acid (DA, the oxidation state of AA) by TCEP. This redox cycling resulted in the enhancement in the oxidation current of Fc. However, once the peptide was cleaved by thrombin at the site between Arg and Gly, the Fc-containing peptide fragment (Fc-SGFPR) would release from the electrode surface, resulting in a

deteriorative current from the oxidation of Fc. The magnitude of the decrease in the oxidation current depends upon the activity and content of thrombin.

3.2 Feasibility

Fig. 2A presented the Amperometric responses of the peptide/MCH-covered electrodes in different solutions before and after incubation with thrombin. A couple of well-defined redox peak was observed at the peptide/MCH-covered electrode, which is attributed to the redox of Fc congugated to the peptide [5]. However, its oxidation current increased in the presence of AA, suggesting that Fc was regenerated from Fc^+. Furthermore, a greater enhancement in the oxidation current was observed after the addition of TCEP, demonstrating the electrochemical–chemical reaction between Fc^+ and AA was promoted by the chemical–chemical reaction between AA and TCEP. However, once the electrode was immersed in the solution of thrombin, a significant deterioration in the current was observed, implying that the Fc tag was released from the electrode surface because of the thrombin-promoted hydrolytic degradation of peptide. Amperometric biosensor is a simple and sensitive technique used in the electrochemical assays. The result obtained by amperometry is in good agreement with that achieved by cyclic voltammetry (Fig. 2B).

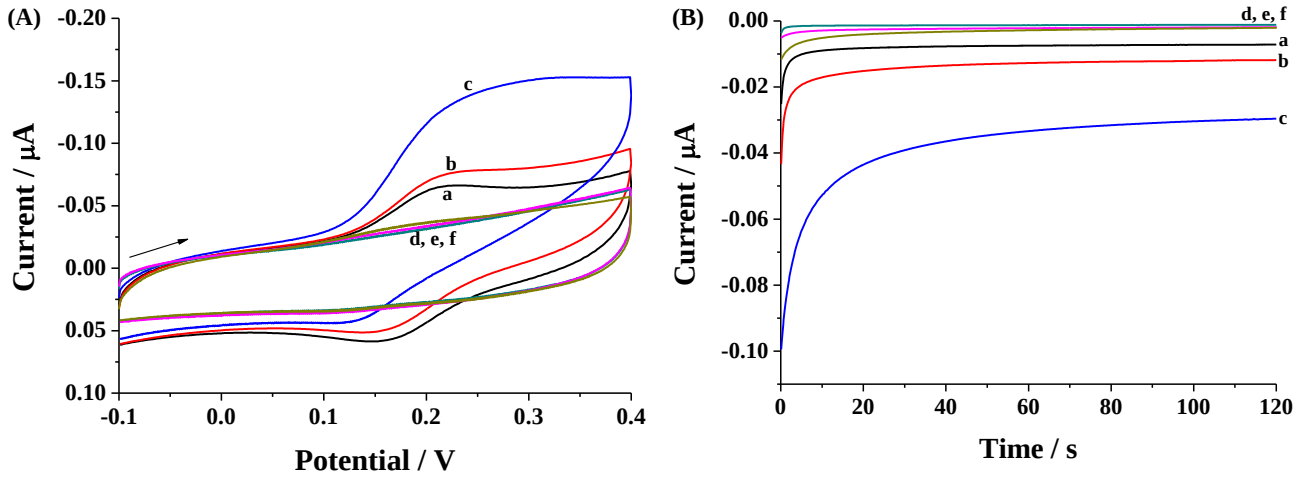

Figure 2. CVs (A) and amperometric responses (B) of the peptide/MCH-covered electrodes before (curves a ~ c) and after (curves d ~ f) incubation with thrombin at pH 7.4 for 90 min, which were collected in the solution of PBS in the absence (curves a and d) and presence of AA (curves b and e) or AA/TCEP (curves c and f). The concentrations of thrombin, AA and TCEP were 500 ng/mL, 20 μM and 500 μM, respectively.

3.3 Optimization of experimental conditions

It has been suggested that the thrombin activity is dependent upon solution pH. We found that ΔI (I^0-I, where I^0 and I represent the current of the sensing electrode before and after incubation with thrombin) reached to the maximum in the pH range of 7.4 ~ 8.1 (Fig. 3A), demonstrating thrombin

shows high-activity at this pH range. Herein, 7.4 was used for the enzymatic clipping reaction. We also studied the impact of cleaving time on the electrochemical signal and found that Δ*I* increased with the increased incubation time and reached the maximum beyond 60 min (Fig. 3B), indicating that almost all of the peptide molecules anchored on the electrode surface have been clipped by thrombin after 60 min. Thus, 60 min was chosen as the optimal reaction time for the enzymatic cleavage reaction.

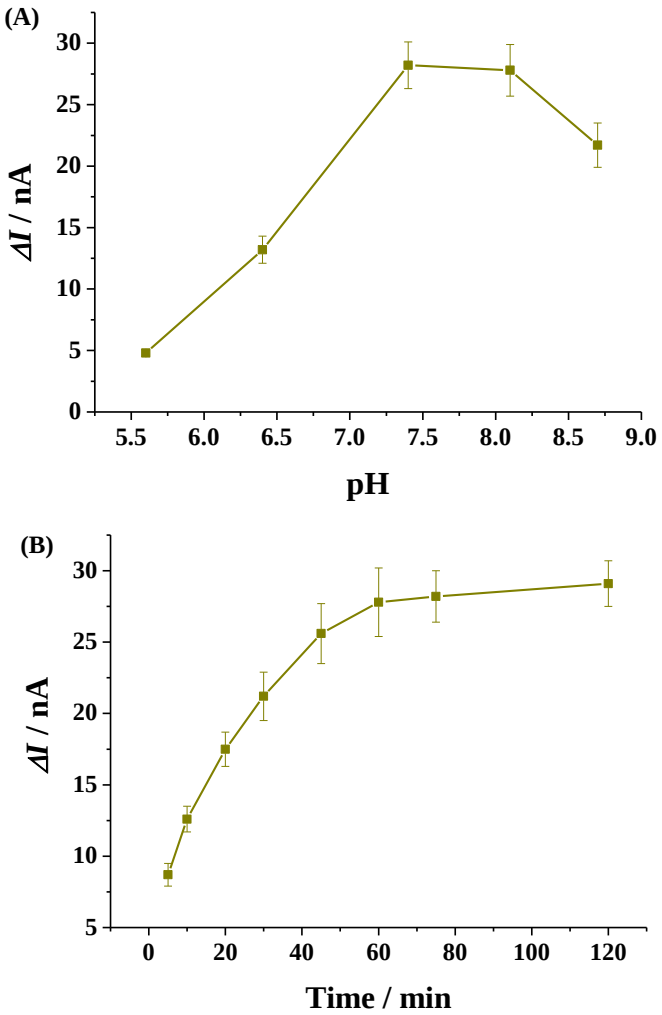

Figure 3. Dependence of Δ*I* on the pH (A) and the incubation time (B).

3.4 Sensitivity

Under the optimal assay condition, we investigated the other analytical performances of the sensor. Fig. 4A shows the amperometric responses after incubating the electrodes in solution with various content of thrombin. The currents decreased with increasing concentrations of thrombin. The linear regression equation is expressed as Δ*I* (nA) = 2.22 + 0.13 [thrombin] (ng/mL) (R = 0.993) in the concentration range of 2 ~ 200 ng/mL. The detection limit of the method was 1 ng/mL. The analytical performances were compared to those achieved by the signal amplification of manomaterials (Table 1).

However, our amplification strategy is simple and low-cost because this work only required the addition of AA and TCEP to the detection solution.

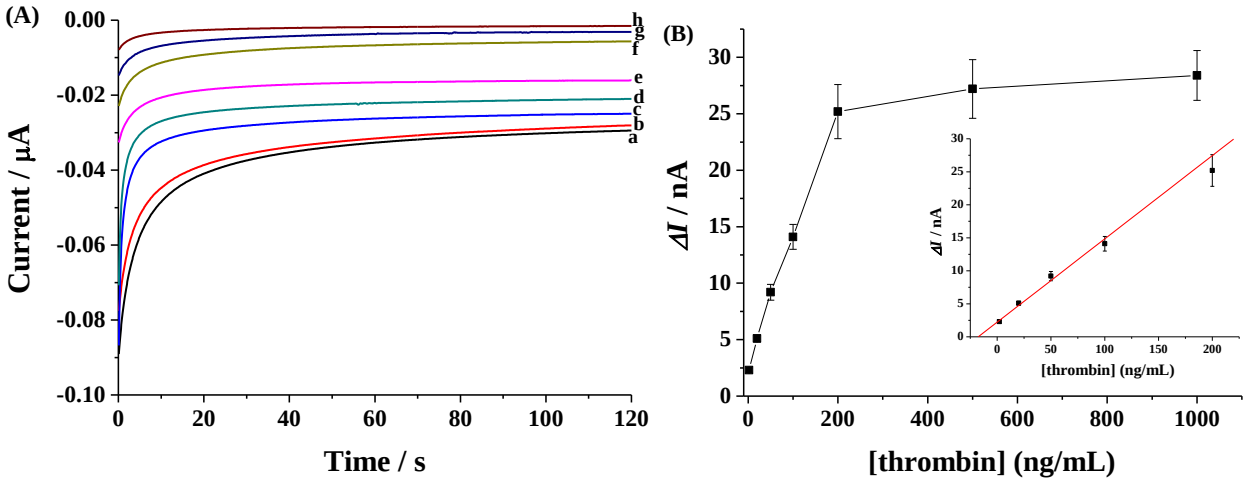

Figure 4. (A) Amperometric responses of the sensor electrodes after treatment with various concentrations of thrombin. The incubation time was 60 min and the solution pH was 7.4. (B) Dependence of Δ*I* on the thrombin concentration. The plots were obtained at 120 s.

Table 1. Comparison of analytical performances of this redox cycling-amplified method and other electrochemical thrombin biosensors by the signal amplification of nanomaterials

Materials	Methods	Detection limits	Linear ranges	References
SWNT-graphene /nano-Au/NiHCFNPs	DPV	2 pM	0.01 ~ 50 nM	[17]
FcSH/SiNCs	DPV	0.06 nM	0.1 ~ 5 nM	[18]
CoPt/GO	DPV	0.34 nM	0.001 ~ 50	[19]
Au-PEDOT@SiO2	DPV	0.02 pM	2 pM ~ 20 nM	[20]
Pd–AuNPs/HRP	DPV	0.003 nM	0.05–50 nM	[21]
Fc-peptide	Amperometry	1 ng/mL	2 ~ 200 ng/mL	This work

Abbreviations: nano-Au, gold nanoparticles; SWNT, single wall nanotube; NiHCFNPs, nickel hexacyanoferrates nanoparticles; ferrocenylhexanethiol loaded silica nanocapsules (FcSH/SiNCs); GO, graphene oxide; PEDOT, poly(3,4-ethylenedioxythiophene); HRP, horseradish peroxidase

3.5 Selectivity

To demonstrate the selectivity of this electrochemical to thrombin, three interfering proteins (BSA, IgG and lysozyme) were tested. As shown in Fig. 5, compared to the control, the three interferences showed no apparent change in the current, indicating that the presented electrochemical method showed extraordinary selectivity towards thrombin. The result is acceptable since the peptide substrate is specific to thrombin [22]. To demonstrate the application of this biosensor, determination of thrombin in a serum sample was carried out. As shown in Table 2, the found contents of thrombin

were closed to the spiked values, demonstrating that no thrombin in the serum sample was detected. The result is understandable since no coagulation factor is contained in healthy serum sample [23]. Our results also demonstrated that the spiked recoveries varied from 98.4 to 107 % with the relative standard deviation (RSD) below 7.5 %.

Table 2 Assay of thrombin in a serum sample

Sample	Spiked (ng/mL)	Found (ng/mL)	Recovery	RSD
Serum	20	21.4	107 %	7.5 %
	50	52.5	105 %	6.3 %
	200	196.8	98.4%	4.7 %

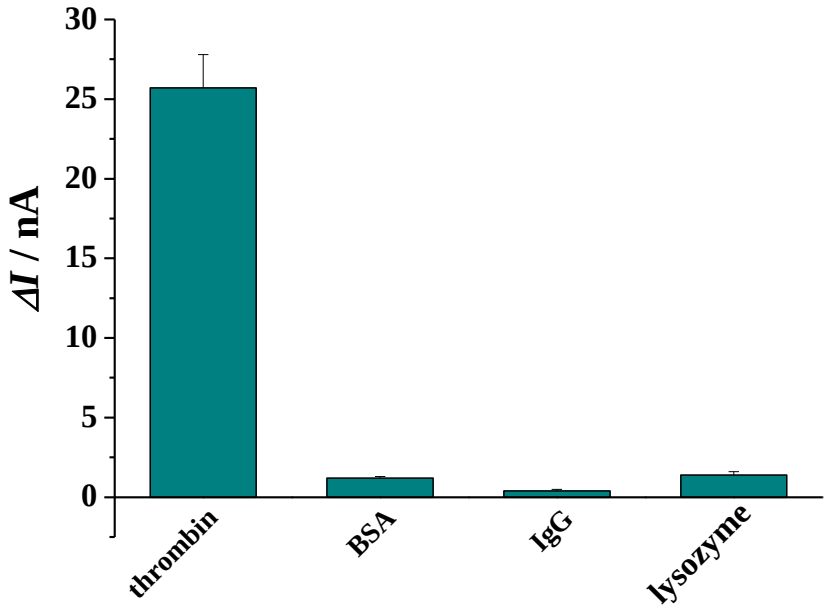

Figure 5. Selectivity of this sensor to thrombin. The concentration of thrombin was 200 ng/mL and that of BSA, IgG and lysozyme was 5 μg/mL. The other experimental conditions kept at those in Figure 4.

4. CONCLUSION

This paper presented a simple method for improving the detection sensitivity of the redox-labels-based electrochemical protease biosensors by employing the electrochemical-chemical-chemical redox cycling. A signal-amplified detection of thrombin as the model analyte is achieved by monitoring the current change. In contrast to the other amplified electrochemical strategies, our method requires simple sample handling procedure and obviates the utilization of nanomaterials and/or enzymes for signal amplification, thus reducing the detection cost and complexity of operation.

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