Short communication

Electrochemical Biosensor for the Detection of SARS-CoV-2 Main Protease and Its Inhibitor Ebselen

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This work reported an electrochemical method for the detection of SARS-CoV-2 major protease (Mpro). Specifically, ferrocene (Fc)-labeled peptide substrates were immobilized on the gold nanoparticles (AuNPs)-modified electrode. Cleavage of the peptides by Mpro led to the release of Fc tags and the decrease of the electrochemical signals. The analytical performance of the biosensor for analysis of Mpro was investigated. Inhibiting the activity of Mpro prevented the cleavage of the peptide substrates. The method was successfully used to evaluate the inhibition efficiency of a well-known inhibitor.

Keywords: new coronavirus; SARS-CoV-2 major protease; electrochemical biosensor; ferrocene

1. INTRODUCTION

The outbreak of pneumonia caused by new coronavirus 2019-nCoV began in a global pandemic in early 2020. The coronavirus is defined as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Classification Committee of viruses (WHO). Drugs currently approved for the treatment of infected patients show toxic and side effects [1]. Therefore, there is still an urgent need for more effective antiviral drugs. SARS-CoV-2 major protease (Mpro), also known as 3C-like protease (3CLpro), is involved in cleaving viral multiple proteins to produce essential viral proteins for viral replication and pathogenesis [2]. The protease plays a major physiological role in the life cycle of virus and is considered to be one of the ideal targets for the designing of antiviral drugs [3, 4]. The commercial kit for detecting Mpro mainly adopts fluorescence resonance energy transfer (FRET) method, in which Mpro catalyzes the hydrolysis of dual labeled peptide substrate through a pair of receptor and donor [5, 6]. Although this method is sensitive and rapid, it requires the use of expensive and complex peptide
substrate and advanced instruments. Therefore, it is very important to develop simple, sensitive, cost-effective and high-throughput methods to detect Mpro and screen its potential inhibitors.

Electrochemical biosensors have attracted more and more attention for bioassays due to rapid response, high sensitivity, less sample consumption and low cost [7-12]. The development of electrochemical peptide biosensors is one of the most active research topics. A classical electrochemical peptide biosensor usually contains a specific peptide sequence labeled with a redox reporter, in which the peptide is modified on the electrode surface [8]. The electrochemical reaction between redox reporter and electrode is promoted with the peptide as the connector. Ferrocene (Fc) and methylene blue (MB) are two electrochemically redox molecules, which are covalently connected to the end of the peptide as the redox reporters. Usually, cleavage of the peptide by protease can cause the loss of current response. The “signal off” biosensors have been proposed for determining various proteases, including matrix metalloproteinase, BACE1, caspase-3, prostate specific antigen and so on [13-18]. In this work, we proposed an electrochemical biosensor for the determination of SARS-CoV-2 Mpro based on the cleavage of a specific peptide labeled with Fc tag. The purpose of this work is to prove the sensitivity and practicability of the biosensor for the determination of Mpro. The sequence of peptide substrate (Fc-TSAVLQSGFRK) was designed according to the commercial substrate (Dabcyl-KTSAVLQSGFRKME-Edans) used for probing of Mpro activity by FRET. Immobilization of peptide on the plate electrode may limit the enzymatic cleavage of substrate due to the steric hindrance. Nanomaterials-modified interface and the well-designed surface chemistry may facilitate the interaction between peptide and protease [19, 20]. For this view, gold nanoparticles (AuNPs)-modified electrode was used for the immobilization of Fc-labeled peptide substrate. Cleavage of peptide by Mpro promoted the release of Fc tag from the electrode surface, thus causing the decrease in the peak current. The performances of the biosensor for analysis of Mpro in buffer and serum were demonstrated. When the activity of Mpro was suppressed by an inhibitor, the cleavage of peptide would be prevented and a stronger electrochemical signal was observed. Thus, the method can be used to evaluate the inhibition efficiency of potential inhibitors.

Scheme 1. Principle of the electrochemical biosensor for Mpro detection by the specific cleavage.

2. EXPERIMENTAL

2.1 Chemicals and materials

6-Mercaptohexanoic acid (MHA), bovine serum albumin (BSA), IgG, thrombin, protein kinase
(PKA) and serum were purchased from Sigma-Aldrich (Shanghai). Peptide was synthesized and purified by ChinaPeptide Co., Ltd. (Shanghai). Mpro kit was obtained from Beyotime Biotechnology (Shanghai). HAuCl₄ and other reagents were obtained from Aladdin Biochem. Tech. Co., Ltd. (Shanghai).

2.2 Fabrication of sensing electrode

The glass carbon electrode (GCE) was polished with 0.05 μm of alumina powder and then cleaned ultrasonically in 50% ethanol for a few seconds. The AuNPs/GCE was prepared by electrodeposition of AuNPs on the electrode surface with the previously reported procedure. Briefly, the cleaned GCE was placed in 1 mL of 1% HAuCl₄ to scan for 60 s with a constant potential of −0.25 V. After that, the AuNPs/GCE was washed with 50% ethanol and then incubated with 1 mM MHA for 4 h to form the self-assembled monolayers (SAMs) through the Au-S interactions. After being incubated with the mixture of EDC/NHS to activate the carboxyl groups, the SAMs-covered electrode was incubated with 0.1 mL of 0.1 mM Fc-TSAVLQSGFRK solution for 2 h. The peptide was immobilized on the activated SAMs through the standard amino covalent coupling reaction. The resulting peptide-modified electrode (Fc-AuNPs/GCE) was kept in a clean environment at 4 °C for use.

2.3 Electrochemical assays

Before the measurement, the Fc-AuNPs/GCE was incubated with 25 μL of Mpro at a given concentration for 30 min. For inhibition analysis, 10 μL of ebselen at a known concentration was added to 10 μL of 20 pM Mpro solution. After incubation for 5 min, the Fc-AuNPs/GCE was incubated with the mixture for 30 min. For the differential-pulse voltammetry (DPV) measurement, the electrode was placed in 50 mM Na₂SO₄ solution and the DPV curve was collected on a CHI 660E (CH Instruments, Inc.). A Pt wire and an Ag/AgCl electrode were used as the counter and reference electrodes, respectively.

3. RESULTS AND DISCUSSION

3.1 Feasibility of the method

To promote the enzymatic cleavage of peptide substrate by Mpro, AuNPs-modified electrode was used for the immobilization of peptide. The electrodes of GCE and AuNPs/GCE were characterized by scanning electron microscope (SEM). As shown in Fig.1A, the GCE is smooth, and many nanoparticles were found at the AuNPs/GCE, which confirmed the formation of AuNPs on the electrode. The size and morphology of the electrodeposited AuNPs was consistent with that in the previous report [21]. DPV technique was used to measure the electrochemical response of the sensing electrode because of its high sensitivity and low background signal. Fig. 1B depicts the DPV curves of the SAMs-covered AuNPs/GCE before (curve a) and after (curve b) modification with Fc-TSAVLQSGFRK. The apparent oxidation peak exhibited a potential of 0.44 V (vs. Ag/AgCl) is originated from the oxidation of Fc, demonstrating that peptide was successfully immobilized on the electrode surface. No significant change in the oxidation current (Iₚa) was observed when the modified electrode was incubated with buffer blank
(curve c), indicating the sensing electrode is stable. However, when the peptide-modified electrode was incubated with the Mpro solution, the current decreased greatly (curve d), which should be attributed to the cleavage of the peptide substrate. Thus, the sensing electrode can be used to determine Mpro.

![Figure 1. (A) SEM images of GCE before (top) and after (bottom) electrodeposition of AuNPs. (B) DPV responses of the SAMs-covered AuNPs/GCE (curve a) and the Fc-AuNPs/GCE before (curve b) and after incubation with buffer blank (curve c) or 20 pM Mpro (curve d).](image)

**3.2 Effect of incubation time**

The influence of incubation time on the DPV signal was investigated. As shown in Fig. 2, no significant change in the $I_{pa}$ was observed when the sensing electrode was incubated with the buffer blank, demonstrating the electrode exhibit excellent stability.

![Figure 2. Effect of incubation time on the $I_{pa}$ acquired at the Fc-AuNPs/GCE after incubation with buffer (black dotted curve) and Mpro (red dotted curve).](image)
However, in the presence of Mpro, the current decreased with the increase of incubation time and began to level off beyond 25 min. The platform is indicative of the cleavage completion. In this work, 30 min was chosen as the incubation time for the quantitative assay of Mpro.

3.3 Sensitivity

The sensitivity of the biosensor was investigated by determination of different concentrations of Mpro. Fig. 3 depicts the DPV responses of the biosensor for measuring different concentrations of Mpro. As can be seen, the DPV signal decreased when the Mpro concentration increased in the range of 0.1 – 25 pM. The change in the current ($\Delta I_{pa} = I_{pa}^0 - I_{pa}$) was used to evaluate the sensor performance, where $I_{pa}^0$ and $I_{pa}$ represent the current before and after incubation with Mpro. The linear correlation equation from 0.1 to 15 pM can be expressed as $I_{pa} = 0.023[Mpro] \text{ (pM)} + 0.016$. The lowest detectable concentration of 0.1 pM was lower than that achieved by the photoacoustic and fluorescent detection (50 nM) and the “covalent biosensing” strategy (1 pM) [6, 22]. This result can be attributed to the high sensitivity of the heterogeneous electrochemical biosensor and less sample consumption. We believed that the sensitivity may be improved by the use of nanomaterials for signal amplification.

![Figure 3](image)

**Figure 3.** (A) DPV responses for the analysis of different concentrations of Mpro. (B) Dependence of $I_{pa}$ on Mpro concentration. The inset depicts the linear portion of the curve.

3.4 Sensitivity

Selectivity of the biosensor was challenged by determining different enzymes and proteins, including BSA, IgG, thrombin, PKA and Mpro. As shown in Fig. 4, only Mpro caused the significant decrease in the $I_{pa}$, which is indicative of good selectivity of this method. Furthermore, the anti-interference ability of the method was challenged by determining Mpro in 5% serum. As a result, a significant decrease in the $I_{pa}$ was observed, indicating that other co-exist biomolecules showed no significant interference with the detection of Mpro. Thus, the biosensor exhibits great potential in biological sample analysis and clinical research.
3.5 Inhibition analysis

Ebselen is an organoselenium molecule which has been identified as a potential inhibitor of Mpro [3, 23-25]. To demonstrate the application of the biosensor, the sensing electrode was used to determine the activity of Mpro in the presence of ebselen. As shown in Fig. 5, the \( I_{pa} \) was intensified with the increase of Mpro concentration. Thus, high concentration of ebselen inhibited the Mpro activity more effectively. The half-maximal inhibitory concentration (IC\(_{50}\)) of ebselen for 10 pM Mpro (final concentration) was estimated to be 15.4 pM, indicating that the inhibitor exhibit excellent inhibition efficiency. Therefore, the biosensor can be used to develop novel inhibitor-type antiviral drugs. In contrast to the fluorescence kit, the proposed biosensor shows high sensitivity and less sample consumption.

![Figure 5](image)

**Figure 5.** Dependence of \( I_{pa} \) on the concentration of ebselen.
4. CONCLUSION

In summary, we developed an electrochemical biosensor for the assay of SARS-CoV-2 Mpro based on the cleavage of Fc-labeled peptide attached on the nanoscale electrode interface. The biosensor exhibits high sensitivity and selectivity, which can be designed into a point-of-care instrument for inhibitor screening and clinical research.

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References


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