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Short communication

Electrochemical Detection of Kinase Activity by Measuring the Surface Charge Change of Peptide Substrate on Gold Electrode

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Kinase can be used in clinical analysis as the biomarker for disease diagnosis. Herein, we proposed a simple and label-free electrochemical method for kinase detection. The method was based on the charge change of peptide substrate immobilized on the gold electrode. The change was measured by electrochemical impedance spectroscopy and the value was proportional to the concentration of kinase. The method shows satisfactory results for the analysis of kinase inhibitors, thus exhibiting great potential for diagnosis of kinase-related diseases and discovery of novel inhibitor drugs.

Keywords: Phosphorylation; post-translational modification; kinase; electrochemical impedance spectroscopy; carbodiimide

1. INTRODUCTION

Post-translational modifications (PTMs) regulate the function of most proteins in cells and plays a crucial role in many physiological processes. Protein phosphorylation as one of PTMs is catalyzed by kinase. Abnormal phosphorylation and over-expression of kinase are often associated with many diseases, such as cancers, diabetes and Alzheimer's disease [1, 2]. Therefore, it is very important to determine the activity of kinase and screen its inhibitors for basic biomedical research and discovery of novel drugs. The traditional methods for determination of kinase kinase include radioisotope labeling, mass spectrometry and immunoassay. These methods promote the assay of kinase activity, but they have some inherent shortcomings [3]. For example, ATP labeled with radioactive elements endows the direct detection with high sensitivity, but it has the limitation of radioactive contamination. Mass spectrometry requires the utilization of complex and expensive

instruments, and immunoassay has the requirement for high-cost and instable antibody to recognize phosphorylation group. Therefore, it is necessary to develop a rapid, simple, economic, sensitive and label-free method for kinase activity detection and high-throughput screening of the potential inhibitors.

Electrochemical biosensors have been widely used in bioassays because of the simple instrument, high sensitivity and rapid response. In recent years, many electrochemical biosensors have been proposed for the determination of kinase activity and screening of its inhibitors [4]. For example, the γ-phosphate group in adenosine triphosphate (ATP) can be transferred to the peptide chain and then recognized by antibody or other recognition elements [5-8]. ATP can be labeled at the terminal phosphate group by biotin or thiol and used as the phosphate source; the labeled phosphate group was transferred to the peptide chain after phosphorylation and then detected by various recognition elements through the avidin-biotin or metal-thiol interaction [3, 9-13]. These methods are sensitive but require the use of expensive biological reagents, labeled substrate or recognition elements and nanomaterials for signal amplification, thus increasing the operation complexity and analysis cost.

Self assembled monolayer (SAM) provides a convenient, flexible and simple way for investigating the interface behavior of metal, metal oxide and semiconductor. The electrostatic interaction between the groups of SAM and the redox mediators in solution can be characterized by electrochemistry based on the difference in electron-transfer resistance [14-16]. Thus, the direct electrochemical analysis of phosphorylation by measuring the charge change of peptide on the SAM-covered electrode is simple and label-free for detection of kinase activity. After phosphorylation, the phosphorylated peptide on SAM may limit the electron transfer of $[Fe(CN)_6]^{3-/4-}$ due to electrostatic repulsion effect, thus leading to the increase in electrochemical impedance. However, the method is less sensitive since the slight change in the structure of SAM can not cause the significant difference in the electron-transfer resistance. Our group found that the N-(3-(dimethylamino)propyl)-N′-ethylcarbodiimide (EDC)-activated carboxyl groups on SAM can faciliate the electron transfer of $[Fe(CN)_6]^{3-/4-}$ via electrostatic interaction between the positively charged EDC intermediate and the negatively charged $[Fe(CN)_6]^{3-/4-}$ [17]. Kinase can transfer the phosphate group of ATP to tyrosine (Tyr), threonine (Thr) or serine (Ser) residue in the peptide chain. Recently, it has been demonstrated that EDC can activate the hydroxyl group in tyrosine [18]. For this view, we suggest that the positively charged tyrosine-EDC intermediate can facilitate the electron transfer of $[Fe(CN)_6]^{3-/4-}$, thus improving the sensitivity by decreasing the impedance of background. However, once the hydroxyl group in tyrosine residue was phosphorylated by kinase, it would not be activated by EDC to form the intermediate. The negatively charged phosphate group on the SAM will repel $[Fe(CN)_6]^{3-/4-}$, thus producing a high electrochemical impedance.

2. EXPERIMENTAL

2.1 Chemicals and materials

The thiolated peptide of Mpa-GPPYIYGSFK-NH_2 was provided by ChinaPeptides Co., Ltd (Shanghai). Tyrosine kinase Src was ordered from R&D Systems, Inc. (Minneapolis). Protein kinase A

(PKA), BACE-1, thrombin, 6-mercapto-1-hexanol (MCH), ATP, okadaic acid, 4-amino-5-(4-chlorophenyl)-7-(tert-butyl)pyrazolo[3,4-d]pyrimidine (PP2), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich (Shanghai). Other reagents were analytical-grade and used without additional treatment.

2.2 Preparation of sensor electrode

The peptide substrate was immobilized on gold electrode by the formation of Au-S bonds. In brief, gold electrode with a diameter of 2 mm was polished with 0.05 μm Al_2O_3 powder. After being washed with ethanol/water under ultrasound, the cleaned electrode was immersed in 50 μM peptide overnight. After that, the electrode was placed in 0.5 mM MCH solution to incubate for 1 h. The unmodified gold surface was blocked by MCH. Finally, the electrode was rinsed thoroughly with ethanol/water to remove any non-specifically adsorbed substances.

2.3 Procedure for Src detection

The sensor electrode was placed in the HEPES buffer (20 mM) containing 5 mM $MgCl_2$, a known concentration of Src and 1 mM ATP. After incubation at 37 ^oC for a given time. The electrode was washed with water and then exposed to 0.1 M EDC solution. Electrochemical measurements were performed on a CHI-660E electrochemical workstation in a homemade plastic cell. To probe the inhibition efficiency, Src was respectively pre-incubated with two kinase inhibitors (PP2 and okadaic acid) for 5 min. The other procedures were the same as those for the determination of Src without the addition of inhibitor.

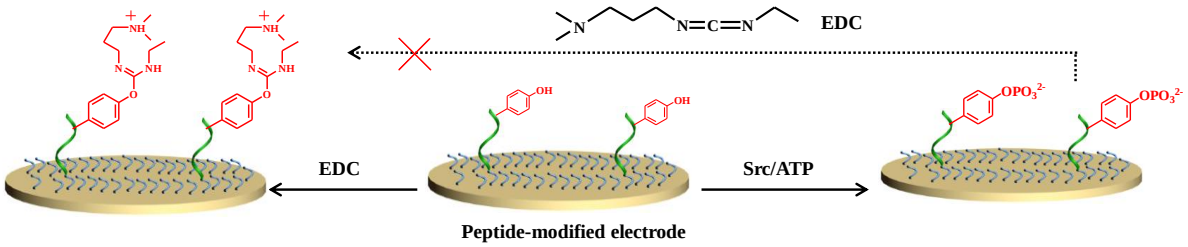

Figure 1. Scheme representation of this method for kinase detection based on the surface charge change of peptide substrate immobilized on gold electrode.

3. RESULTS AND DISCUSSION

3.1 Principle of the method

The detection principle of the method is shown in Fig. 1. It was based on the surface charge change of peptide substrate immobilized on the gold electrode. The negatively charged $[Fe(CN)_6]^{3-/4-}$ was used as the redox mediator. Src for tyrosine was used as the kinase mode. In the absence of Src, the hydroxyl group in tyrosine residue was activated by Src to form the positively charged O-

acylisourea intermediate, thus decreasing the electron-transfer resistance and facilitating the redox reaction of $[Fe(CN)_6]^{3-/4-}$. In the presence of Src, the hydroxyl group in tyrosine residue was phosphorylated, thus limiting the formation of O-acylisourea intermediate. More importantly, the negatively charged phosphorylated peptide on the electrode can form a barrier to prevent the electron transfer, thus producing a higher electron-transfer resistance. The change in electrochemical impedance is related to the level of phosphorylation that is strictly dependent on the concentration and activity of Src.

3.2 Feasibility of this method

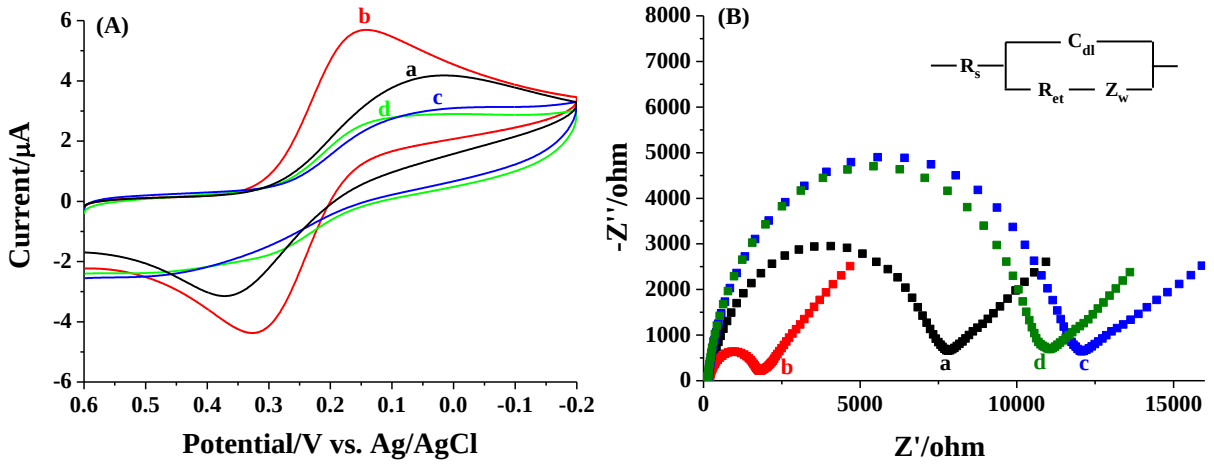

Figure 2. CVs (A) and EIS responses (B) of the peptide-modified electrode before (curve a) and after treatment by EDC (curve b), Src (curve c) and Src as well as EDC (curve d). The concentration of Src was 200 ng/mL.

To prove the feasibility of this biosensor, the voltammetric behaviors of the peptide-modified electrode in the absence and presence of EDC and Src were studied in $[Fe(CN)_6]^{3-}$ solution. As shown in Fig. 2A, the peptide-modified electrode exhibits a couple of quasi-reversible redox wave (curve a), which is attributed to the redox reaction of $[Fe(CN)_6]^{3-}$. When the electrode was treated by EDC, the redox waves were reversible and the peak currents were intensified (curve b). The result indicated that the formation of O-acylisourea intermediate facilitated the electron-transfer due to the electrostatic interaction [17]. However, when the electrode was treated by Src, the redox wave became inreversible, which is accompanied by the decrease in the peak current (curve c). This demonstrated that the phosphate group on the electrode surface resisted the access of $[Fe(CN)_6]^{3-}$ to the electrode surface. Note that small change in the CV response was observed when the Src-treated electrode was inbubated with EDC (curve d), indicating the phosphorylated peptide can not be activated by EDC to form the intermediate. The current change of the phosphorylated electrode treated by EDC is higher than that without treatment by EDC. Thus, the signal can be amplified by the EDC-mediated reaction. These results were also indicated by electrochemical impedance spectroscopy (EIS) in $[Fe(CN)_6]^{3-/4-}$ solution. As shown in Fig. 1B, the electron-transfer impedance decreased when the peptide-modified

electrode was treated by EDC (curves and b) but increased when it was treated by Src (curve c). There is a slight change when the electrode was treated by Src and then treated by EDC (curve d). These results demonstrated that the can facilitate the electron transfer but the phosphate group can prevent the electron transfer and can not be activated by EDC to form the positively charged intermediate. Thus, the activity of Src can be monitored by the impedance change induced by the charge change.

3.3 Sensitivity

To obtain a turn-on signal, the analytical performances of this method was investigated by EIS. As shown in Fig. 3A, the impedance increased accordingly with increasing concentration of Src. Thus, high concentration of Src can facilitate the phosphorylation of peptide, thus leading to the increase in the level of phosphate group on the electrode surface. The method shows a wide linear range from 1 to 100 ng/mL and shows a low detection limit down to 1 ng/mL. This value is lower than that achieved by the direct oxidization of tyrosine residue [11, 19, 20], and is comparable to that by the electroactive Fc-labeled phosphate substrate or the signal amplification of AgNPs [9, 21]. The sensitivity of the electrochemical method is much higher than that via measuring surface charge change of peptide-modified gold nanoparticles by Nano Zetasizer [22]. The lower detection limit can be attributed to the signal amplification of EDC-mediated reaction and the high sensitivity of EIS method. Moreover, the method obviated the use of expensive reagents, labeled substrated and enzymes or nanomaterials for signal amplification.

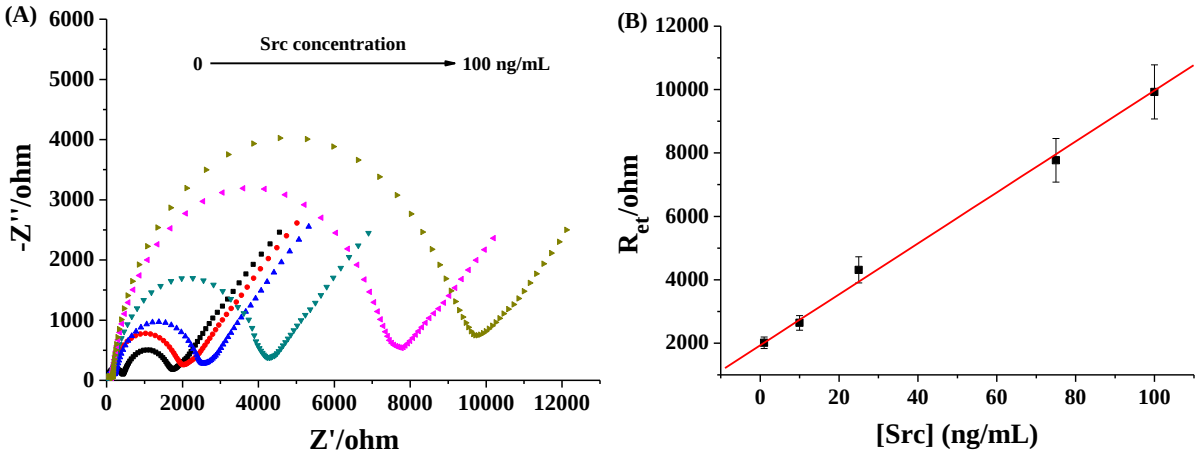

Figure 3. (A) EIS responses of the method for different concentrations of Src. (B) Dependence of Src concentration on the impedance value.

3.4 Selectivity

To explore the selectivity of this biosensor for Src detection, the electrode was challenged with kinase PKA and protease BACE-1 and thrombin. Consequently, the signals for the tested proteins are closed to the that of the blank control. The result suggested that the method shows excellent selectivity for Src detection. More importantly, the serum showed low background signal and had no influence on

the detection of Src. Thus, the biosensor exhibits a great potential for the analysis of complex biological samples.

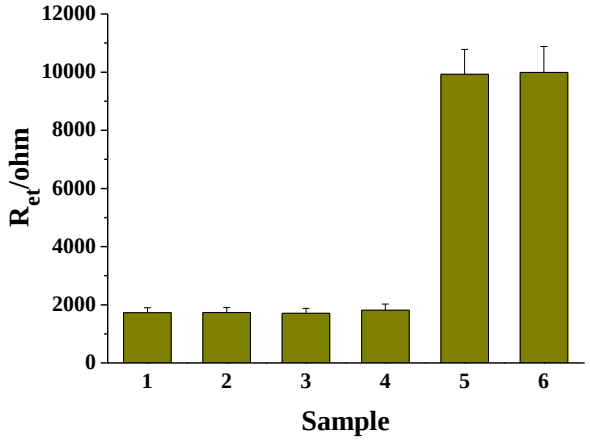

Figure 4. Selectivity of the method: bar 1, PKA, bar 2, BACE-1, bar 3, thrombin; bar 4, serum; bar 5, Src in buffer; bar, 6, Src in serum-containing buffer. The final concentrations of PKA, BACE-1, thrombin, Src and serum are 1 U/mL, 0.1 μM, 0.1 μM, 100 ng/mL and 10%.

3.5 Inhibition analysis

To explore the amenability of this sensor for inhibitor discovery, the effect of PP2 (a well-known Src inhibitor) was analyzed. It can be seen that the impedance value decreased with the increase of PP2 concentration. The result indicated that higher concentration of PP2 can inhibit the activity of Src more effective. The half-maximum inhibition value (IC_{50}) was found to be 30 nM based on the concentration change. The value is consistent with that obtained by other methods [9, 23]. Note that no significant change in the impedance was observed for okadaic acid. The result is understandable since okadaic acid is the Type 1 and Type 2A but not Src phosphatase inhibitor. Thus, the proposed method exhibits promising applications for detection of Src activity and discovery of the potential inhibition-drugs.

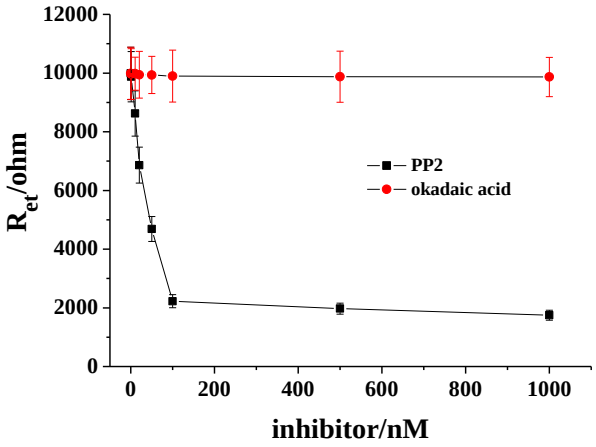

Figure 5. Dependence of impedance value on PP2 or okadaic acid concentration. Src concentration was 100 ng/mL.

4. CONCLUSION

In summary, we proposed a simple and label-free electrochemical method for kinase detection based on the surface charge change of peptide-modified gold electrode. In contrast to the reported methods, this work obviated the requirements of expensive reagents, labeled-substrate and enzymes or nanomaterials for signal amplification. The strategy by EDC-mediated reaction should be valuable for the development of novel electrochemical biosensors for determining different enzymes including protease and kinase.

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