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Label-free electrochemical measurement of protein tyrosine kinase activity and inhibition based on electro-catalyzed tyrosine signaling

Yu Yang, Liang-Hong Guo*, Na Qu, Ming-Yuan Wei, Li-Xia Zhao, Bin Wan

State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Road, P.O. Box 2871, Beijing 100085, China

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ABSTRACT

A novel label-free electrochemical method for measuring the activity of protein tyrosine kinases (PTK) has been developed. Epidermal growth factor receptor (EGFR), a typical PTK associated with a large percentage of all solid tumors, was used as the model kinase. Poly(glu, tyr) (4:1) peptide, as a substrate of EGFR, was covalently immobilized on the surface of indium tin oxide (ITO) electrode by silane chemistry. The tyrosine (Tyr) residue in the polypeptide served as an electrochemical signal reporter. Its voltammetric current was catalyzed by a dissolved electron mediator $Os(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine) for increased sensitivity. Phosphorylation of the Tyr led to a loss of its electrochemical current, thus providing a sensing mechanism for PTK activity. Experimental conditions for the silanization of ITO surface and immobilization of polypeptide were investigated in details to facilitate the generation of Tyr electrochemical signal. The proposed biosensor exhibited high sensitivity and excellent stability. The limit of detection for EGFR was 1 U mL^{-1}. Furthermore, this biosensor can also be used for quantitative analysis of kinase inhibition. On the basis of the inhibitor concentration dependent electrochemical signal, the half-maximal inhibition value IC_{50} of three EGFR inhibitors, PD-153035, OSI-774 and ZD-1839, and their corresponding inhibition constants K_i were estimated, which were in agreement with those obtained from the conventional kinase assay. This electrochemical biosensor can be implemented in an array format for the high throughput assay of in vitro PTK activity and PTK inhibitors screening for practical diagnostic application and drug discovery.

1. Introduction

The reversible phosphorylation of protein is the most important post-translational modification that occurs in the cell (Ahn, 2001). It can regulate a wide range of biological processes including cellular proliferation, differentiation, development and apoptosis, signal transduction, nervous activity, muscle contraction, metabolism and oncogenesis. Especially for signal transduction pathways in cell response to outside stimuli, protein phosphorylation is the primary means presently known (Johnson and Lewis, 2001). The phosphorylation of protein occurs through the action of protein kinase. Protein kinases are ATP-dependent phosphotransferases that deliver a single phosphoryl group from the γ position of ATP to the hydroxyls of serine (Ser), threonine (Thr), or tyrosine (Tyr) in protein substrates. They can be categorized into two major types. One that phosphorylate Ser or Thr is termed protein Ser kinases (PSKs), and the other that phosphorylate Tyr is called protein Tyr kinases (PTKs) (Adams, 2001; Goldsmith et al., 2007). A significant number of PTKs are associated with cancer. Epidermal growth factor receptor (EGFR), as a member of PTKs family, is mostly involved in tumor cell proliferation (Haskell et al., 2001). A close examination of clinical tumors suggests that EGFR is associated with a large percentage of all solid tumors, such as breast, lung, colon, prostate, renal, bladder, head and neck, ovarian cancer and intracranial tumor, as over-expression of EGFR is found in all of them (Bridges, 2001; Pawar et al., 2010). Monitoring of kinase activity has become a critical issue, because the kinases have emerged as promising targets for cancer therapy. Meanwhile, the activities of protein kinases can be precisely regulated by small-molecule compounds. Thus, small-molecule inhibitors of kinases have attracted enormous research interest in the development of anticancer drugs for the clinics (Burkhard et al., 2009; Li et al., 2010).

Some analytical methods have been developed for the detection of protein kinase activity. These methods include the use of radioactive labeling assay (Houseman et al., 2002), Western blotting (Otto et al., 2001; Kaufmann et al., 2001; Maguire et al., 2002), mass spectrometry (Dunn et al., 2010; Beausoleil et al., 2006), fluorophotometry (Sato et al., 2002; Shults et al., 2005; Li et al., 2009; Allen et al., 2006), surface plasmon resonance (Mori et al., 2008; Takeda et al., 2010) that employed enzymatic

* Corresponding author. Tel.: +86 10 62849685; fax: +86 10 62849685.
E-mail address: LHGuo@rcees.ac.cn (L.-H. Guo).

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reactions or phosphor-specific antibodies to detect kinase activity. However, the above-mentioned methods are either labor- or time-consuming, or require highly specialized laboratory instruments and well-trained personnel. Recently, kinase-catalyzed biotinylation of substrate peptides and proteins has become a powerful alternative for phosphoprotein detection and identification (Green and Pflum, 2007; Wang et al., 2006). However, these methods require tedious post-labeling procedures. Electrochemical sensor, due to low cost, simple operation and miniaturization, exhibits its unique advantages. Recently, Willner et al. have developed a method to investigate the activity of a protein kinase (casein kinase, CK2) (Wilner et al., 2008). The phosphorylation of the sequence-specific peptide by CK2 was monitored by electrochemical impedance spectroscopy (EIS). Phosphorylation of the peptide monolayer assembled on a Au electrode yields a negatively charged surface that electrostatically repels the negatively charged redox label $[Fe(CN)_6]^{3-/4-}$, thus increasing the interfacial electron-transfer resistance. The phosphorylation process by CK2 is further amplified by the association of the anti-phosphorylated peptide antibody to the monolayer. Binding of the antibody insulates the electrode surface, thus increasing the interfacial electron-transfer resistance in the presence of the redox label. This method enabled the quantitative analysis of the concentration of CK2 with a detection limit of ten units. Whereas, it involved antibody synthesis and then led to high cost. Yao et al. have developed a label-free electrochemical strategy for a kinase activity assay (Xu et al., 2009). Protein kinase A (PKA), a typical serine/threonine protein kinase, was used as the model kinase. The PKA-specific peptide substrate (LRRASLGGGGC) was self-assembled onto the surface of the gold electrode through its cysteine end. After the phosphorylation reaction, Zr^{4+} was specifically attached to the phosphorylation sites (Step I) and then DNA-AuNPs were quantificationally bound with Zr^{4+} (Step II). The electrochemical detection recorded the chronocoulometric response of $[Ru(NH_3)_6]^{3+}$ absorbed on the DNA-AuNPs. Though this strategy does not require tedious label procedures or complicated protein recognition treatment, it needs multistep signal transition procedures. Li et al. have described a novel electrogenerated chemiluminescence (ECL) biosensor for the detection of kinase activity using gold nanoparticles as signal transduction probes (Xu et al., 2010). The gold nanoparticles were specifically conjugated to the thiophosphate group after the phosphorylation process in the presence of adenosine 59-[c-thio] triphosphate (ATP-s) cosubstrate. Due to its good conductivity, large surface area, and excellent electroactivity to luminal oxidization, the gold nanoparticles extremely amplified the ECL signal of luminol, offering a highly sensitive ECL biosensor for kinase activity detection. The detection limit for PKA was 0.07 U mL^{-1}. In brief, although a great deal of progress has already been made in the detection of protein kinase activity, much more work is needed to put acceptable detection platforms into microarray applications. To our knowledge, such platforms are too few due to the lack of appropriate label-free system. Besides, there have been few studies on the assay of protein kinase activity and its inhibition by small molecules using the electrochemical technique. Thus, working toward simple, cost-effective, label-free methods that can be readily adapted for multiplexed kinase detection will be desirable to enable kinase activity profiling for diagnostic applications and to accelerate the in vitro elucidation of cellular signal transduction pathways.

In this work, a novel label-free electrochemical sensing strategy for the measurement of PTK activity and their potential inhibitors has been developed. Tyr-containing polypeptide, as a substrate of a model kinase EGFR, was covalently immobilized on the surface of ITO electrode. Accompanied with the proceeding of EGFR-catalyzed polypeptide phosphorylation reaction, the proposed protocol for monitoring the PTK activity is feasible based on the electrochemical differentiation of phosphorylated and non-phosphorylated polypeptide by electro-catalyzed Tyr oxidation. On this basis, the effects of three small-molecule inhibitors on PTK activity were investigated and the corresponding inhibition constants K_i were estimated. This strategy provides a simple, sensitive, selective, and universal platform for PTK activity assay and inhibitor screening.

2. Materials and methods

2.1. Materials

Human epidermal growth factor receptor (EGFR), protein kinase A (PKA, catalytic subunit from bovine heart), poly(glu, tyr) (4:1) sodium salt, Kemptide, ellagic acid and (3-glycidoxypropyl)-trimethoxysilane (GPTMS) were obtained from Sigma Aldrich (St. Louis, MO, USA). PD153035, ZD-1839 and OSI-774 were purchased from Tocris Bioscience (St. Louis, MO, USA). The metal complex $Os(bpy)_3Cl_2$ was prepared according to the procedures described elsewhere (Hamann et al., 2005). Polypeptide was dissolved in 20 mM phosphate buffer (PB, pH 7.4). All reagents were of analytical reagent grade. Solutions were prepared in high-purity water from a Millipore milli-Q (Biocel) water purification system (Billerica, MA, USA).

2.2. Instruments

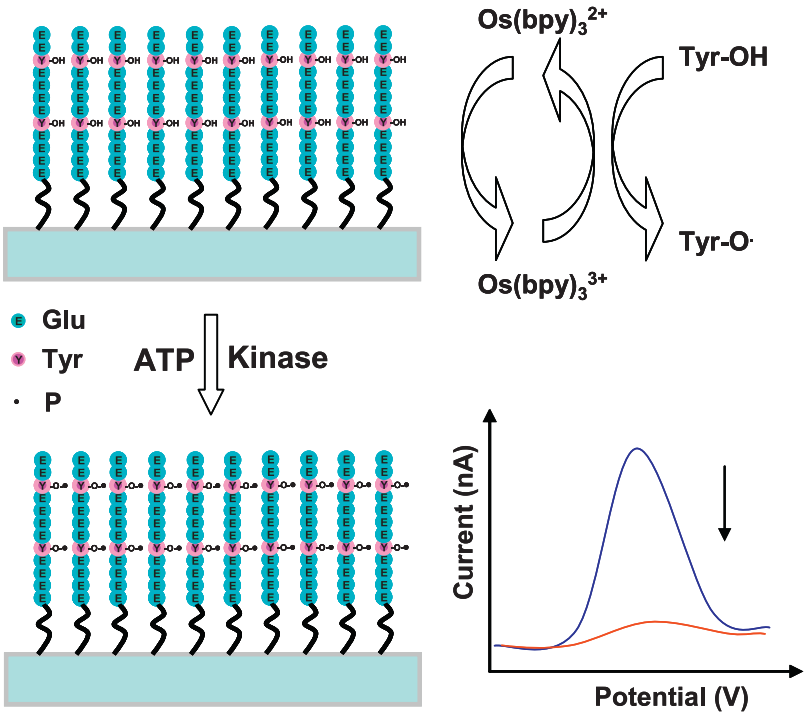
Cyclic voltammetry was performed on a CHI 660B electrochemistry analyzer from CH Instruments (Austin, TX) with a Pt counter electrode and an Ag/AgCl reference electrode. X-ray photoelectron spectrometer (XPS) measurements were made on a VG ESCALAB MkII spectrometer from Thermo VG Scientific (East Grinstead, West Sussex, UK) with a Mg Kα X-ray source (1253.6 eV photons). All binding energies (BEs) were referenced to the neutral C_{1s} peak at 284.6 eV.

2.3. Surface functionalization of ITO electrode

ITO conductive glass (film thickness: 900 ± 100 Å, conductivity: 19 ± 2.1 Ω/□) was obtained from Nanbo Corporation (Shenzhen, Guangdong, China), and was cut into 5 cm × 5 cm size slices. The slices were ultrasonically cleaned according to the published procedure (Guo and Qu, 2006), dried in an oven at 50 °C, followed by treating for 60 s with an Atmospheric Plasma Surface Treatment Systems CTD-1000z from Kulun Electronics Co., Ltd. (Nanjing, Jiangsu, China). The surface functionalization of ITO electrode was performed according to the following procedure (Wei et al., 2009). Firstly, the cleaned slices were immersed in a 1% (v/v) solution of GPTMS in dry toluene with shaking (40 rpm) at room temperature for the specified time. Secondly, after the reaction, the slices were washed ultrasonically in dry toluene (5 min, twice) and anhydrous ethanol (5 min, twice) to remove physically adsorbed GPTMS from the surface. Then, the modified slices were dried under a stream of nitrogen and cured at 115 °C for 20 h. They were stored in a sealed box till use. The modified ITO was denoted as ITO/GPTMS.

2.4. Polypeptide immobilization

ITO/GPTMS slices were cut into 2.5 cm × 0.5 cm electrodes, washed with purified water, and dried under a stream of nitrogen. A 10 μL polypeptide (0.2 mg mL^{-1}, 2.0 M PB pH 7.4) solution was spread evenly on the bottom of each electrode over a 0.5 cm × 0.5 cm area, and incubated in a moisturized container at 25 °C for 24 h. After the surface reaction, the electrodes were washed for 5 min each in 20 mM PB (with 0.1% Tween-20), 20 mM PB (with 150 mM NaCl) and 20 mM PB on a shaker, and then dried with nitrogen. Tris–HCl (pH 8.5, 1.0 M) was used to block the residual reacting sites on the electrodes. The reaction was kept for



Scheme 1. Schematic representation of electrochemical sensing strategy for PTK activity detection using electro-catalyzed Tyr oxidation as signal probe.

30 min at room temperature. The coated electrodes were denoted as ITO/GPTMS/Polypeptide.

As a control, polypeptide was immobilized on bare ITO or Tris-blocked ITO/GPTMS electrode according to the same procedure. The coated electrodes were denoted as ITO/Polypeptide or ITO/GPTMS/Tris/Polypeptide, respectively.

2.5. Assay of PTK activity and inhibition

PTK activity assay was performed according to the protocol provided by Sigma. Briefly, the reaction mixture containing 25 mM HEPES (pH 7.4), 2 mM $MnCl_2$, 5 mM $MgCl_2$, 50 μM Na_3VO_4, 50 μM ATP and kinase in a total volume of 10 μL was deposited onto the surface of the ITO/GPTMS/Polypeptide electrode and incubated in a moisturized container at 30 °C for 35 min. For the inhibition experiments, varying concentrations of inhibitors was included in the reaction mixture. After washing in PB, cyclic voltammograms (CVs) were measured in 3 μM $Os(bpy)_3^{2+}$/20 mM PB (pH 7.4).

3. Results and discussion

In our study, ITO electrode was chosen because its surface was relatively hydrophilic and chemically very inert. Therefore, there have a less tendency for large biomolecules to adsorb on the surface and complicate the situation (Armistead and Thorp, 2000), as is the case on the metal and carbon electrodes. Moreover, polypeptide is negatively charged and the ITO surface is also negatively charged due to a high number of hydroxyl groups on it. Thus, stronger electrostatic repulsions between the polypeptide and the ITO surface resulted in much less nonspecific adsorption. In addition, as illustrated later, the oxidation current of polypeptide on ITO is low even with such a large number of oxidizable Tyr residues. To increase the signal, an electrochemically reversible metal complex, $Os(bpy)_3Cl_2$ was employed as a redox mediator to shuttle electrons between the Tyr residues and the electrode. The developed electrochemical sensing strategy in profiling PTK activity is described in Scheme 1.

Considering polypeptide phosphorylation detected by electrochemical biosensor, polypeptide was needed to be immobilized on the surface of ITO electrode. First, the surface of ITO electrode was functionally modified. Generally, modified film on the surface of the electrode was mostly organic compounds with poor conductivity which could decrease or even fully inhibit the electrochemical response of electron mediator in solution. ITO electrodes were modified with GPTMS by the procedure described in Experimental Section. CVs were used to evaluate the assembly processes by monitoring the electron transfer ability tunneling through the barrier layer between the electroactive probes and ITO electrode. Film formation as a function of surface reaction time was characterized by CVs of two redox probes dissolved in solution, $Fe(CN)_6^{3-}$ and $Os(bpy)_3^{2+}$. A couple of well-defined redox peak in $Fe(CN)_6^{3-}$ solution was obtained on bare ITO, with peak separation of about 70 mV. As the surface reaction time was prolonged, peak current kept decreasing, with further increase in peak separation. When the reaction time exceeded 4 h, there was no electrochemical response for $Fe(CN)_6^{3-}$ solution in the voltammogram. Apparently, formation of GPTMS film on ITO impeded electrochemical reaction of $Fe(CN)_6^{3-}$. These results coincided with those of our previous work (Wei et al., 2009). Cyclic voltammetric measurements in $Os(bpy)_3^{2+}$ solution produced different results (Fig. 1). As can be seen, a couple of quasi-reversible redox peaks of the probe $Os(bpy)_3^{2+}$ were obtained on bare ITO electrode (curve a). With the increase of modification time, the GPTMS-modified layer on ITO electrode led to a slow decrease of peak current in $Os(bpy)_3^{2+}$ solution. However, the redox peak position of $Os(bpy)_3^{2+}$ remained unchanged. When the reaction time exceeded 24 h, there was no electrochemical response for $Os(bpy)_3^{2+}$ solution in the voltammogram. The results suggested that GPTMS surface coverage was dependent on reaction time and the electron inert feature of the GPTMS film formed could partially inhibit the electron transfer and mass transport process of $Os(bpy)_3^{2+/3+}$ ions on the surface of ITO electrode.

In the following experiments, the effect of silanization time on polypeptide immobilization amounts was investigated. After surface modification at desired time, polypeptide was immobilized on

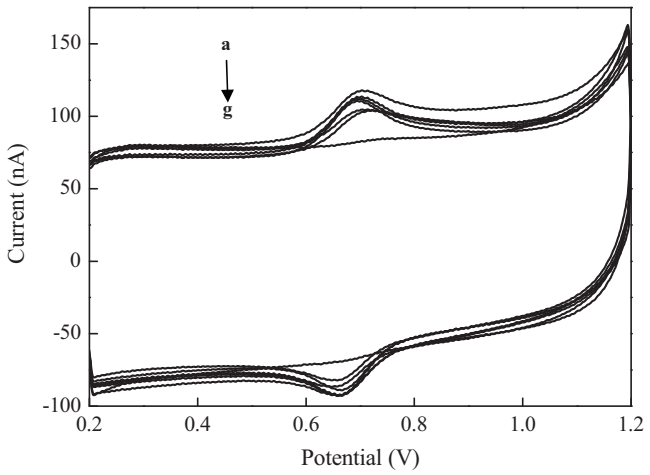


Fig. 1. Cyclic voltammograms of ITO/GPTMS electrode as a function of GPTMS modification time: (a) 0, (b) 30 min, (c) 1 h, (d) 2 h, (e) 3 h, (f) 4 h and (g) 24 h. Electrochemical measurement was carried out in 20 mM PB (pH 7.4) containing 3 μM $Os(bpy)_3^{2+}$. The electrode area in contact with the electrolyte was 25 mm^2. Scan rate: 30 mV s^{-1}.

ITO according to the procedure described in Experimental Section. X-ray photoelectron spectroscopy (XPS), as a unique surface-sensitive technique for chemical analysis, was used to characterize the assembling processes of polypeptide on ITO. Fig. S1 showed typical XPS spectra of polypeptide films and the change in intensity of XPS peak as a function of the GPTMS modification time (Fig. S1 in the Supplementary Material). A clear XPS peak of N_{1s} at 399 eV arising from surface-immobilized polypeptide appeared, as there was no such peak for either bare or GPTMS-modified ITO. Also, the intensity of the XPS peak was enhanced gradually with increasing GPTMS modification time, revealing more polypeptide successfully assembled onto the modified electrode.

Analyzing the above results, we found that a remarkable contradiction existed. That is, the increase of modified GPTMS film on ITO electrode enhanced the amounts of immobilized polypeptide. But in the meantime it also inhibited the electrochemical reaction of electron mediator $Os(bpy)_3^{2+}$. Therefore, the electrochemical response of ITO/GPTMS/Polypeptide electrode as a function of silanization time was investigated directly. As illustrated in Fig. S2, the electro-catalytic signal of ITO/GPTMS/Polypeptide electrode in $Os(bpy)_3^{2+}$ solution initially increased with GPTMS modification time, but decreased when the time exceeded 1 h. The increase of current at shorter time was due to the increase of immobilized polypeptide. The drop of current at longer time was ascribed to inhibition of $Os(bpy)_3^{2+}$ electrochemical reaction by the GPTMS film even though the amount of immobilized polypeptide was still increasing (as revealed by XPS in Fig. S1). Considering the effect of the modified GPTMS film on polypeptide immobilization and $Os(bpy)_3^{2+}$ electron transport rate, the optimal GPTMS surface reaction time was set at 1 h.

Salt-induced immobilization of polypeptide onto the epoxy-activated ITO electrode was found to be an effective route to the preparation of peptide-modified electrode (Wheatley and Schmidt, 1999; Bauer-Arnaz et al., 1998). Under the optimized GPTMS surface reaction time (1 h), the immobilization of polypeptide onto ITO/GPTMS electrode was investigated as a function of salt concentration. Initially the electrochemical current was enhanced with the increase of salt concentration, and then leveled off at 2.0 M phosphate salt and above (Fig. S3). Since the silane reaction time was kept constant, presumably the electrochemical reaction rate of $Os(bpy)_3^{2+}$ was the same, and the electrochemical current would correlate with the amount of polypeptide immobilized on ITO. The results seemed to be related with the facts that at low salt concentrations, the polypeptide exhibited lower reactivity with epoxy groups modified on the surface of the electrode. While, the polypeptide showed higher reactivity with epoxy groups at higher salt concentrations. As for high salt concentrations, the enhancement in the immobilization efficiency of polypeptide was attributed to a salt-induced hydrophobic interaction between the polypeptide and the epoxy groups. In order to obtain the maximal amount of immobilized polypeptide, the salt concentration was chosen to be 2.0 M.

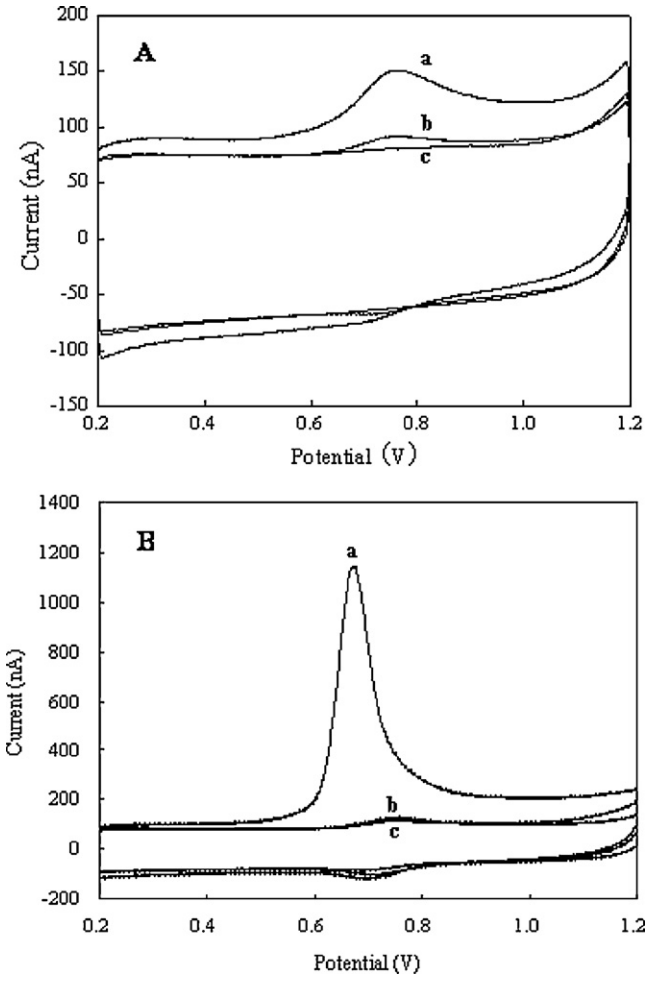


Fig. 2. Electrochemical responses of different polypeptide-immobilized electrodes in (A) 20 mM PB solution and (B) 3 μM $Os(bpy)_3^{2+}$ solution. (a) ITO/GPTMS/Polypeptide; (b) ITO/GPTMS/Tris/Polypeptide; (c) ITO/Polypeptide. Supporting electrolyte: 20 mM PB solution, pH 7.4. Scan rate 30 mV s^{-1}.

Under the optimized experimental conditions of GPTMS surface reaction time and salt concentration, the polypeptide was successfully immobilized on the ITO electrode. And then, a series of control experiments were also conducted by measurement of the Tyr electrochemical signal. As shown in Fig. 2(A), in PB solution, there was an oxidation peak at about 0.75 V on ITO/GPTMS/Tris/Polypeptide and ITO/GPTMS/Polypeptide, resulting from the oxidation reaction of Tyr residue in the polypeptide. Also, the oxidation current of ITO/GPTMS/Polypeptide was larger than those of the other two modified electrodes, which suggested that polypeptide was not non-specifically adsorbed but covalently immobilized on GPTMS-modified electrode. Interestingly, as can be seen from Fig. 2(B), in $Os(bpy)_3^{2+}$ solution, a couple of well-defined redox peaks appeared at ITO/Polypeptide, which was the same as that at bare ITO. It indicated that almost no polypeptide was immobilized on bare ITO. The redox current of ITO/GPTMS/Tris/Polypeptide was also weak

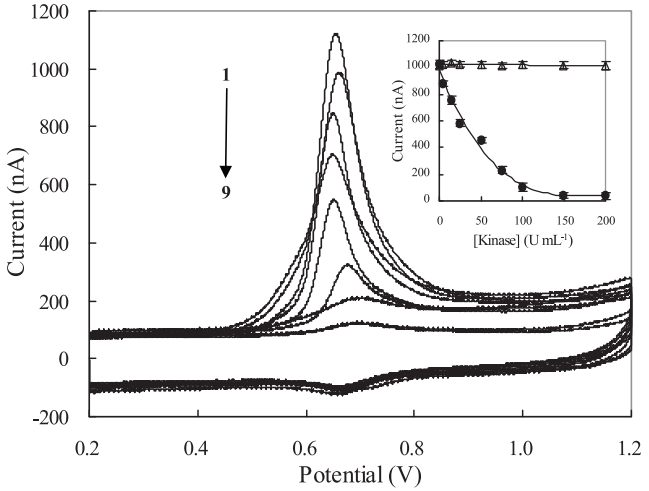


Fig. 3. Electrochemical sensor for the detection of polypeptide phosphorylation with increasing kinase concentrations at (1) 0, (2) 5, (3) 15, (4) 25, (5) 50, (6) 75, (7) 100, (8) 150 and (9) 200 U mL^{-1} in 20 mM PB (pH 7.4) containing 3 μM $Os(bpy)_3^{2+}$ solution. Inset: Dependence of electro-catalyzed Tyr oxidation signal on the concentration of kinase, EGFR (●) and PKA (Δ). Error bars indicated the standard deviation of measurements performed in triplicate (n = 3).

but showed a slight increase as compared to ITO/Polypeptide. The response might arise from the nonspecific adsorption of polypeptide by hydrogen bond interaction. However, the oxidation current of ITO/GPTMS/Polypeptide in the presence of $Os(bpy)_3^{2+}$ was drastically enhanced, which was nearly ten times larger than that in PB solution. Moreover, its oxidation potential in $Os(bpy)_3^{2+}$ solution (~0.67 V) shifted more negatively than that in PB solution (~0.75 V), which demonstrated that the addition of $Os(bpy)_3^{2+}$ could efficiently catalyze the oxidation of Tyr residue in polypeptide. The peak-shaped catalytic voltammogram was attributed to depletion of the limited amount of polypeptides immobilized on the ITO electrode.

The possible mechanism of electro-catalyzed oxidation of Tyr by $Os(bpy)_3^{2+}$ was proposed in Eqs. (1) and (2) (Qu et al., 2008):

$$Os(bpy)_3^{2+} \rightarrow Os(bpy)_3^{3+} + e \quad (1)$$

$$Os(bpy)_3^{3+} + TyrO^- \rightarrow Os(bpy)_3^{2+} + TyrO^{\bullet} \quad (2)$$

Tyr oxidation proceeded by the conversion of the aromatic phenol group into a phenoxonium group and then underwent further reactions to form several products including quinol and quinone products. In our study, the second-order rate constant between Tyr and $Os(bpy)_3^{2+}$ in solution was calculated as $2.2(\pm0.2) \times 10^5\ M^{-1}\ s^{-1}$, and for polypeptide $5.2(\pm0.2) \times 10^5\ M^{-1}\ s^{-1}$. The electro-catalytic effectiveness of the latter was a little higher because it was easier for the negative charged polypeptide to approach the positive charged $Os(bpy)_3^{2+}$ metal complex in solution, resulting in a faster electro-catalytic oxidization.

On the basis of the optimal condition, the activity of protein kinase was evaluated with different concentration of EGFR. According to the electro-catalytic mechanism mentioned above, when the hydroxyl of Tyr was replaced with a phosphate group during phosphorylation, the phosphorylated Tyr could not go through this

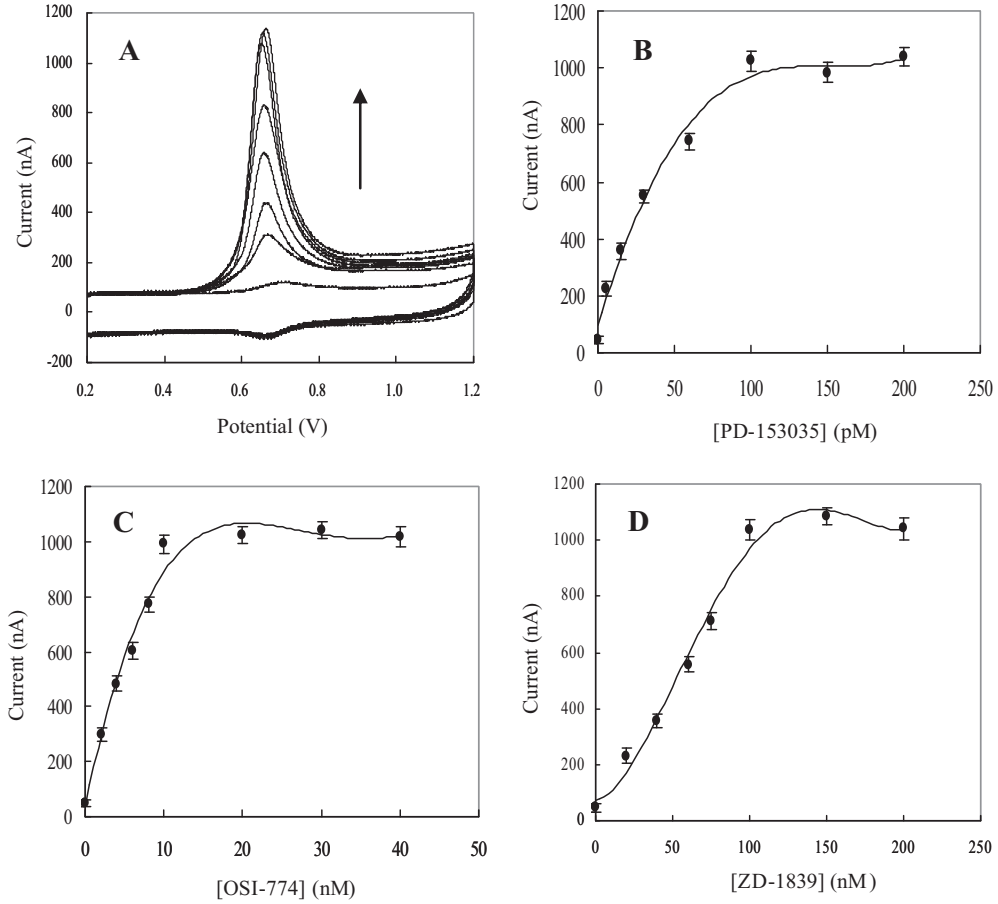


Fig. 4. Dose–effect curves of small-molecule inhibitors. The concentration of kinase was fixed at 150 U mL^{-1}. A. Cyclic voltammograms of ITO/GPTMS/Polypeptide electrode in the presence of PD-153035. B, C, and D showed dependence of electro-catalyzed Tyr oxidation current on the concentration of the small-molecule inhibitor in the phosphorylation reaction respectively. B. PD-153035; C. OSI-774; D. ZD-1839.

oxidation process, and the current response was suppressed. Fig. 3 showed a series of CVs recorded in the presence of various amounts of EGFR with ITO/GPTMS/Polypeptide electrode in 20 mM PB (pH 7.4) containing 3 μM $Os(bpy)_3^{2+}$ solution. When the EGFR units increased, the currents obtained from the electro-catalyzed Tyr oxidation decreased accordingly. It reached a saturation value once the concentration of EGFR was above 150 U mL^{-1} EGFR, indicating that all the accessible Tyr residues in polypeptide were phosphorylated. The dependence of the current response on the EGFR concentration was presented in the inset of Fig. 3. A linear relationship between current signals and the concentrations of EGFR was obtained in the range from 2 to 100 U mL^{-1}. The limit of detection for EGFR was 1 U mL^{-1} (signal-to-noise ratio of 3). The linear relationship can be represented as $I = -8.5197c + 905.71$ with the correlation coefficient of $R^2 = 0.9854$, where I is the current intensity and c is the kinase activity. Phosphorylation time was optimized to determine whether the polypeptide was allowed enough time to react with 150 U mL^{-1} EGFR. As the phosphorylation time was increased, the electro-catalyzed oxidation signal of Tyr decreased and then reached a plateau after 35 min (Fig. S4). Hence, 35 min was selected as optimal phosphorylation time. In addition, to demonstrate the specificity of the phosphorylation reactions, PKA was introduced instead of EGFR. With the increase of PKA concentrations, almost no changes in Tyr oxidation signals were observed (Fig. 3 Inset), indicating that the polypeptides were not phosphorylated. If a substrate peptide for PKA, Kemptide, was immobilized on the surface of ITO electrode, no electrochemical responses were still be observed. It was ascribed to the fact that Ser and Thr residues are not electroactive, and thus we cannot detect the phosphorylation of Ser and Thr using their electrochemical oxidation signals. These results demonstrated that the proposed biosensor can only be employed for Tyr-containing peptides as substrates of PTKs.

The reproducibility and repeatability of the electrochemical biosensor were also studied with intra- and interassay precision. The intraassay precision of the biosensor was evaluated by measuring the one level of EGFR activity using a batch of six modified electrodes. The interassay precision was assessed by assaying the same level of EGFR activity with different batches of six modified electrodes. The relative standard deviation values obtained from 50 U mL^{-1} EGFR were found to be 5.5% and 6.2%, respectively. Both the intra- and interassay of the biosensor enunciated acceptable reproducibility and repeatability.

The biosensor was also used to quantitatively evaluate the inhibition of PTK in the presence of small molecule inhibitors. In the inhibition assay, the EGFR activities were evaluated with EGFR inhibitors at different concentrations. Here, PD-153035, OSI-774 and ZD-1839, well-known as potent and selective inhibitors of EGFR, were employed as inhibitors. Fig. 4 showed the current responses obtained from the phosphorylation reactions performed in the presence of three kinds of small-molecule inhibitors including PD-153035, OSI-774 and ZD-1839. As the concentrations of inhibitors were increased, the electro-catalyzed oxidation currents of Tyr gradually enhanced. This suggested that the presence of inhibitors hampered the kinase-catalyzed phosphorylation reaction and then more Tyr residues in polypeptide became available for electro-catalyzed oxidation by $Os(bpy)_3^{2+}$. Also, the inhibition time was optimized. With increasing the inhibition time, the electro-catalyzed oxidation signal of Tyr increased, and then reached a plateau after 3 h (Fig. S5). Hence, 3 h was employed as optimal inhibition time. Furthermore, IC_{50} values for enzyme inhibition, and inhibition constants K_i were estimated. An IC_{50}, the concentration required to inhibit a biochemical process (enzyme activity or ligand binding) by 50%, was the most easily obtained from dose–effect relationship, which was a measure of potency for a potential drug in biochemical systems. IC_{50} could be further corrected to give an inhibition constant (Copeland, 2000), K_i, which was a measure of the thermodynamic binding of the ligand/inhibitor to the target protein. The results presented in Table 1 were in accordance with those reported in the literature obtained with conventional kinase assays (Fry et al., 1994; Wood et al., 2004; Wakeling et al., 2002), which verified effectiveness of our method. In addition, ellagic acid, a PKA inhibitor but not PTK inhibitor, was also added to evaluate the specificity of the biosensor. As expected, the electrochemical signals nearly had no change in the presence of PKA inhibitor with a concentration as high as 20 μM (Fig. S6), showing its excellent specificity to the PTK inhibitor profiling. These results indicate that the proposed electrochemical strategy has a potential in quantitatively screening the activity of the PTKs inhibitors.

Table 1
IC_{50} and inhibition constants of three inhibitors for EGFR.

Inhibitors	Obtained from our method		Cited from the literature	
	IC_{50}	K_i	IC_{50}	K_i
PD-153035	26.0 ± 4.7 pM	2.3 ± 0.4 pM	29.0 pM	5.2 pM
OSI-774	4.7 ± 0.5 nM	0.4 ± 0.1 nM	2.0 nM	0.7 nM
ZD-1839	49.6 ± 5.8 nM	4.5 ± 0.5 nM	23–78 nM	2.1 nM

4. Conclusions

A simple and label-free electrochemical strategy for the assay of PTKs activity and their inhibitors has been developed, based on the electro-catalyzed oxidation of Tyr in polypeptide. It is noteworthy that this strategy does not require complicated label procedures and can simplify the detection process in a single-step. Meanwhile, our label-free electrochemical technique can only be applied to Tyr-containing peptides or proteins as substrates for PTKs. In the future, this promising strategy will be used to an electrochemical microarray assay, which is suitable for practical diagnostic application and drug discovery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.07.033.

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