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Graphene based electrochemical biosensor for label-free measurement of the activity and inhibition of protein tyrosine kinase

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In this paper, we report a simple, ultrasensitive and label-free method to evaluate the activity of protein tyrosine (Tyr) kinase based on the electrochemical signal of Tyr residues at a graphene modified glassy carbon electrode. It was found that graphene could enhance the electrochemical response of Tyr through electrocatalytic oxidation reaction. After phosphorylation by kinase, the phosphorylated Tyr (pTyr) is electro-inactive and the electrochemical signal is reduced. Therefore, the electrochemical response of Tyr residues in peptides can be used as a signal reporter to assay kinase activity. In this study, using Src-catalyzed Tyr-phosphorylation as a model, the activity of kinase can be evaluated with a linear range from 0.26 to 33.79 nM and an extraordinarily low detection limit of 0.087 nM. Moreover, this electrochemical biosensor can also be utilized for monitoring the inhibition of kinase using 4-amino-5-(4-chlorophenyl)-7-(tert-butyl) pyrazolo [3,4-d] pyrimidine, a small molecule inhibitor. On the basis of the inhibitor concentration dependent Tyr oxidation signal, the IC_{50} value was estimated to be 99 nM.

Introduction

Protein phosphorylation by kinase is one of the most important molecular events of cell life and plays a crucial role in many cellular processes, including apoptosis, cell growth and differentiation.[1–3] During the process of phosphorylation, the γ-phosphoryl group of adenosine triphosphate (ATP) is transferred to the hydroxy group on the amino acid side chain of protein.[4,5] Protein phosphorylation mainly occurs on specific serine (Ser), threonine (Thr) and tyrosine (Tyr) residues. Among these events, Ser and Thr residues undergo phosphorylation much more than Tyr residues. Although the phosphorylation of Tyr residues only accounts for less than 5% of all the phosphorylation occurring in cells, it is the key physiological control mechanism.[6] Abnormal protein phosphorylation may cause many diseases, including cancer, diabetes and Alzheimer's disease,[7–9] especially Tyr phosphorylation. As a result, protein-kinase-catalysed phosphorylation and its inhibition play very important roles in understanding many signal transduction pathways and in the discovery of small molecule inhibitors targeting protein kinases for various human diseases.

Several strategies have been developed to investigate the kinase-mediated protein phosphorylation, such as radioisotopes,[10] mass spectrometry (MS),[11] surface plasmon resonance,[12,13] NMR,[14,15] HPLC[16] and so on. In general, these methods require costly labels, expensive instruments, or time-consuming procedures. On the other hand, there has been intense interest in developing fluorescence reporters that can measure protein Tyr kinase (PTK) activity in real time and screen inhibitors with high throughput. Martin *et al.*[17] reported the utility of fluorescent Pro-Q diamond phosphosensor dye technology for assaying protein phosphorylation status and protein kinase activity in microarrays. Freeman and colleagues[18] reported an investigation of the activity of casein kinase and alkaline phosphatase using CdSe/ZnS quantum dots as optical transducers. Phillips *et al.*[19] demonstrated the utility of single-cell capillary electrophoresis for the measurement of the kinetics of dephosphorylation and investigation of the effect of environmental toxins on protein Tyr phosphatase activity in A 431 cells using a fluorescent peptide substrate. Based on the chelation-enhanced fluorescence mechanism, a series of real-time fluorescent chemosensors were developed for measurement of PTK activity and inhibitor screening.[20–24]

Due to their simplicity, sensitivity and cost-effectiveness, electrochemical methods for kinase activity and inhibition assays have attracted much attention. Ekeroth and colleagues[25] reported an approach to differentiate between phosphorylated and nonphosphorylated amino acid analogues using electrochemical impedance spectroscopy. Kerman *et al.*[26] studied the effect of phosphorylation on the electro-oxidation of Tyr by differential pulse voltammetry using a screen-printed carbon electrode. Li *et al.*[27] demonstrated an electrochemical strategy to assay the activity of protein kinase based on HRP-linked

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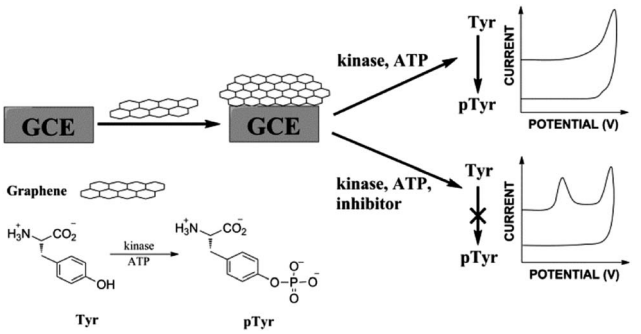
catalysis and gold nanoparticle amplification. An electrogenerated chemiluminescence biosensor was reported by Li's research group for profiling protein kinase activity and inhibition using gold nanoparticles for signal transduction.[28] Based on the observation of phosphorylation blocking tyrosinase-catalyzed oxidation of Tyr or tyrosyl residue in peptides, a reusable and label-free tyrosinase-based amperometric biosensor was developed for PTK activity measurement.[29] Using ferrocene labelled ATP as a co-substrate, Martić and coworkers[30] reported a method for monitoring Tau phosphorylation electrochemically by immobilzation of Tau protein films on gold surfaces. Taking advantage of the specific binding of phosphate groups with TiO_2 nanoparticles, a TiO_2/silver nanoparticle based biosensor was developed for kinase activity and inhibition assay using Ag nanoparticles as electrochemical signal reporters.[31] Using Zr^{4+} as a link between the phosphorylated site of a peptide and the 'signal reporter' of DNA functionalized gold nanoparticles, a DNA-based electrochemical method was employed for the facile and label-free monitoring of the activity of protein kinase.[32] Based on the electrocatalytic oxidation of Tyr by osmium bipyridine or phenoxazine complexes,[33,34] and gold nanoparticles,[35] various electrochemical approaches for detecting protein phosphorylation were developed. Despite the improvements of these electrochemical methods, sophisticated procedures for electroactive labelling and labour-intensive immobilization procedures for peptide substrates are needed and sometimes, the performances such as linear range, sensitivity, *etc.* are also limited. Therefore, it is still a challenge to develop simple, sensitive and label-free electrochemical methods for the profiling of kinase activity and inhibition.

Graphene with a unique one-atom thick structure is a novel and fascinating carbon material. Due to its large specific surface area, high mobility of charge carriers, excellent thermal and electric conductivity, graphene has attracted a great deal of interest in recent years.[36–38] Based on the affinity of phospholipids for the graphene oxide (GO)/carbon nanotube (CNT) surface, a novel method for the quantitative assay of phospholipase activity was developed using time-of-flight MS, in which the GO/CNT double layer was used as a substrate for laser desorption ionization.[39] Very recently, a sensitive and versatile fluorescent peptide/GO system for probing the activity and inhibition of protein kinases was reported based on phosphorylation protection against carboxypeptidase Y digestion and GO–peptide interaction-based signal transduction.[40] In this paper, we report a very simple and ultrasensitive electrochemical method for label-free detection of PTK activity and its inhibition. Based on the observation of electrocatalytic oxidation toward Tyr, a graphene based electrochemical biosensor was developed to measure the activity and inhibition of PTK (Scheme 1). In this work, a Src-catalyzed phosphorylation process was investigated as a model. While the hydroxy groups of Tyr residues in the peptide are replaced by phosphate groups, the phosphorylated Tyr (pTyr) cannot be oxidized electrochemically.[41] Therefore, the current signal is proportional to the unphosphorylated peptides, which is related to the activity of protein kinase. However, while the phosphorylation is inhibited by some inhibitors, the current signal recovers. In this way, the activity of kinase and inhibition can be evaluated by measuring the electrochemical signal of Tyr. To the best of our knowledge, there is no graphene-based electrochemical platform as yet reported for measuring the activity and inhibition of protein kinases.



Scheme 1 Illustration of the principle for the detection of Src-catalyzed phosphorylation based on electrochemical oxidation of Tyr.

Experimental section

Materials and reagents

Graphene was purchased from Nanjing XFNano Material Technology Company (Nanjing, China). Tyr, *o*-phospho-l-Tyr, ATP, 4-amino-5-(4-chlorophenyl)-7-(tert-butyl) pyrazolo [3,4-d] pyrimidine (PP2) were bought from Sigma-Aldrich. P60c-src substrate I (YIYGSFK) was synthesized by GL Biochemicals (Shanghai, China). Src kinase was synthesized by Life Technologies (Shanghai, China). All other reagents were reagent grade. All solutions were prepared and diluted using ultrapure water or as indicated in each experiment.

Preparation of graphene modified electrode

Firstly, graphene was dispersed in DMF with ultrasonic treatment for several hours to obtain an homogenous suspension (1 mg mL^{-1}). Then, 5.0 μL of the suspension was dropped on the surface of a freshly polished glassy carbon electrode (GCE) (denoted as G/GCE). Before each electrochemical measurement, the electrode was scanned over the range of 0.2–1.0 V in 0.01 M PBS buffer (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) until a stable cyclic voltammogram was obtained.

Characterizations

Fourier transform infrared (FTIR) spectra were recorded using a NEXLIS FTIR instrument (Nicolet, USA). X-ray diffraction (XRD) was performed using a Rigaku D/Ultima IV X-ray diffractometer (Rigaku, JAPAN) with monochromatized Cu Kα radiation (λ = 1.5418 Å). The morphology of graphene was investigated by scanning electron microscopy (SEM) using an HITACHI S-4800 (Hitachi, Japan). Transmission electron microscopy (TEM)

images were obtained using a JEM-2100F (JEOL, JAPAN) instrument at an accelerating voltage of 200 kV.

Peptide phosphorylation and inhibition

The activity of Src kinase was measured using specific kinase assay buffer with 50 mM Tris–HCl (pH 7.5), 2.8 mM $MnCl_2$, 30 mM $MgCl_2$ and 137 mM NaCl. Src stock solution (0.36 mg mL^{-1}) was kept in the storage buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.05 mM EDTA, 0.05% NP-40, 1 mM DTT, and 50% glycerol) at −80 °C until use. The reaction mixture containing 4.22 nM Src, 3 mM P60c-Src substrate I and 5 mM ATP was incubated at 30 °C for 2 h or as indicated in each experiment. The reaction was terminated by adding 1.5 μL acetic acid (1 M) into the reaction mixture.

For the inhibition experiment, different concentrations of PP2 were dissolved in the above kinase reaction mixture. PP2 stock solution (25 mg mL^{-1}) was kept in DMSO. After 2 h reaction at 30 °C, the procedure was terminated by adding 1.5 μL acetic acid (1 M).

Electrochemical measurements

All the electrochemical experiments were carried out using an Autolab TYPE III electrochemical workstation (Metrohm, The Netherlands) with a conventional three-electrode cell at room temperature. A saturated calomel electrode (SCE) was used as the reference electrode, a platinum wire electrode as the counter electrode, and a bare GCE (diameter, 3 mm) or the modified GCE as the working electrode. Cyclic voltammetry (CV) was performed between 0.2 and 1 V at a scan rate of 100 mV s^{-1}. For CV measurement, 20 μL kinase reaction mixtures were added into 10 mL PBS buffer. Electrochemical impedance spectroscopy (EIS) measurement was performed in 0.1 M KCl containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. EIS was carried out using the following parameters: scan rate 0.1 V s^{-1}, initial potential 0.18 V, frequency 0.1–10 kHz, amplitude 0.01 V.

Results and discussion

Characterizations of graphene

The morphology of graphene was investigated by SEM and TEM. As shown in Fig. 1a and b, graphene has a wrinkled paper-like structure. The wrinkles suggest that there are several layers in graphene. A broad diffraction peak at $2\theta = 26.5°$ (Fig. 1c) is assigned to (002) reflection of graphene, indicating some degree of re-aggregation.[42] From the FTIR spectrum (Fig. 1d), the absorption band at 3440 cm^{-1} is attributed to the hydroxyl groups of absorbed H_2O molecules.[43] The weak characteristic peak at 1380 cm^{-1} demonstrates that there some carboxyl groups remain in graphene.[44]

Fig. 2 shows the SEM images of bare GCE and G/GCE. As shown in Fig. 2a, the bare GCE exhibits a smooth and clean plane. After the modification with graphene (Fig. 2b), a paper-like crumpled and flaked structure can be observed.

EIS characterization

EIS provided detailed information on the changes of the surface property of the modified electrode. The Nyquist plot of the impedance spectrum includes a semicircular portion and a linear portion. The diameter of the semicircle at higher frequencies corresponds to the electron transfer resistance, and the linear part at low frequencies corresponds to the diffusion process. Fig. 3 shows the Nyquist plots for the bare GCE and G/GCE. The plot for the bare GCE has a semicircular domain with an electron transfer resistance (R_{ct}) value of 64.3 Ω. After modification with graphene, the R_{ct} decreases to 5.9 Ω, indicating that graphene could promote the electron transfer. Moreover, the change of R_{ct} also confirms the successful immobilization of graphene on the surface of the GCE.

Electrochemical behaviours of Tyr, pTyr and p60c-src substrate I

Fig. 4 depicts the CV of Tyr (10 μM) at the bare GCE and G/GCE. The oxidation peak potential is located at 0.637 and 0.586 V, respectively. Compared with the electrochemical behaviour for electrochemical reduction of the graphene oxide modified GCE, the oxidation potential of Tyr at G/GCE is decreased by about 30 mV.[45] In addition, the oxidation peak current for Tyr at G/GCE is about 9.4 μA, which is nearly 30 times higher than that at the bare GCE. The decrease of overpotential and increase of peak current reveal that the modification of graphene on GCE

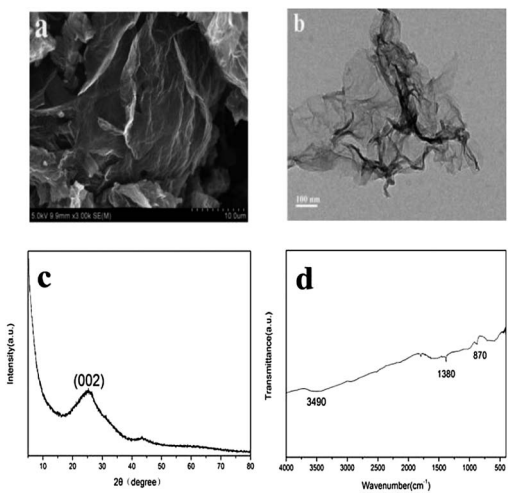


Fig. 1 (a) SEM, (b) TEM, (c) XRD and (d) FTIR of graphene.

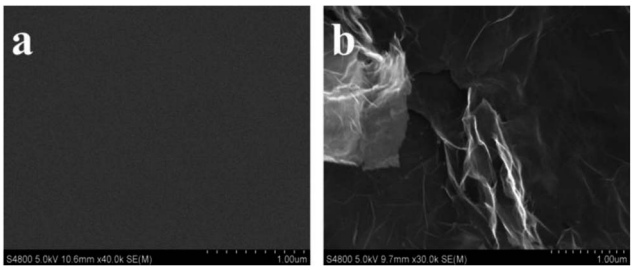


Fig. 2 SEM images of the (a) bare GCE and (b) G/GCE .

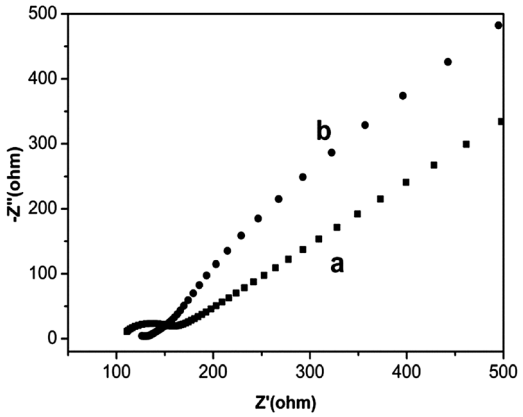


Fig. 3 EIS of (a) bare GCE and (b) G/GCE in 0.1 M KCl containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$.

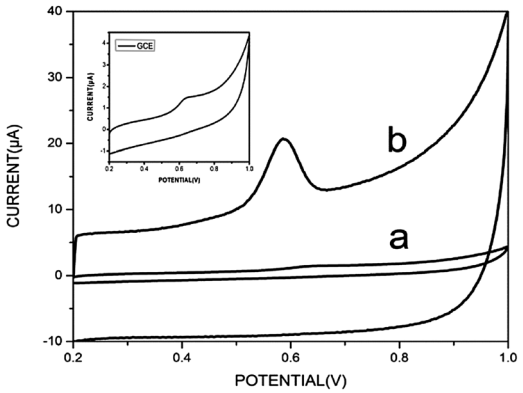


Fig. 4 CVs of 10 μM Tyr at (a) bare GCE and (b) G/GCE. Inset is the enlarged CV of 10 μM Tyr at the bare GCE.

can enhance the electrochemical response of Tyr due to electrocatalytic oxidation.

In order to test the possibility of using a G/GCE as an electrochemical biosensor to measure the activity of kinase, we verified whether the electrode could discriminate between Tyr and pTyr. It is well known that Tyr is electroactive and phosphorylation on Tyr residues could induce electroinactive pTyr. As is shown in Fig. 5, the oxidation peak current increases as the concentration of Tyr increases over the range of 0–10 μM ($R^2 = 0.997$) with a sensitivity of 0.9587 ± 0.0238 μA μM^{-1}. In the case of a mixture of Tyr and pTyr, a linear response is also observed with a sensitivity of 0.9306 ± 0.0218 μA μM^{-1}. Hence, we can draw a conclusion that pTyr cannot interfere with the response of Tyr at the G/GCE. Therefore, it is possible to measure kinase activity by detecting the electrochemical signal of Tyr in peptide.

P60c-src substrate I (YIYGSFK) is reported to be a specific and efficient substrate for the Src-family PTK, which contains two Tyr residues.[46] The electrochemical behaviour of p60c-src substrate I at G/GCE was also investigated. As is shown in Fig. 6, the peak current of 10 μM p60c-src substrate I is nearly 2 times higher than that of 10 μM Tyr, with a little negative shift of the oxidation potential. Though there are two Tyr residues in p60c-

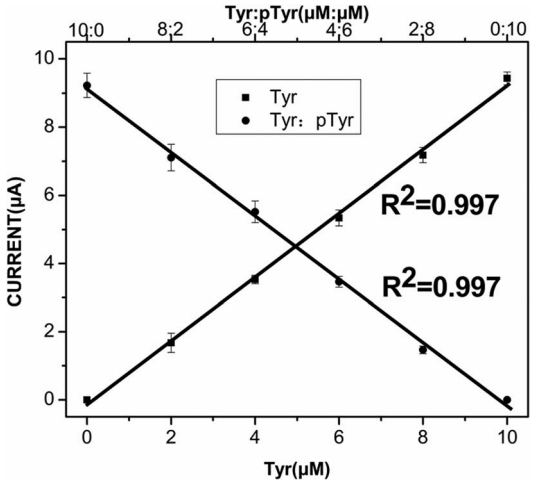


Fig. 5 A plot of current responses of 0, 2, 4, 6, 8, 10 μM Tyr and 10 : 0, 8 : 2, 6 : 4, 4 : 6, 2 : 8 and 0 : 10 Tyr: pTyr (μM μM^{-1}) at the G/GCE. Error bars indicate the standard deviation for three independent measurements.

src substrate I, the phosphorylation process mainly occurs on the N-terminus Tyr residue.[29]

Kinase activity measurement

Phosphorylation time optimization. To detect the phosphorylation, Src was used as the model kinase while p60c-src substrate I and ATP were employed as substrates. As the p60c-src substrate I is phosphorylated by Src, the electrochemical response decreases. The phosphorylation time was monitored by stopping the reaction at different time intervals and the electrochemical response of the solution was measured by CV. With an increase of the phosphorylation time, the electrochemical signal of tyrosine residues decreased and then reached a plateau after 60 min (Fig. 7). As the Tyr residue at the third position from the N-terminus is the major phosphorylation site,[29] the current cannot be blocked completely even after 120 min. Thus, a phosphorylation time of 120 min was selected.

Src activity measurement. As is shown in Scheme 1, an increase in the amount of Src in the reaction system results in an increase in the amount of peptides phosphorylated, and a

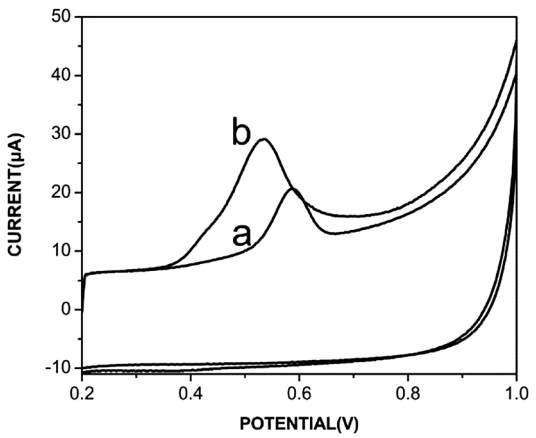


Fig. 6 CVs of 10 μM (a) Tyr and (b) p60c-src substrate I.

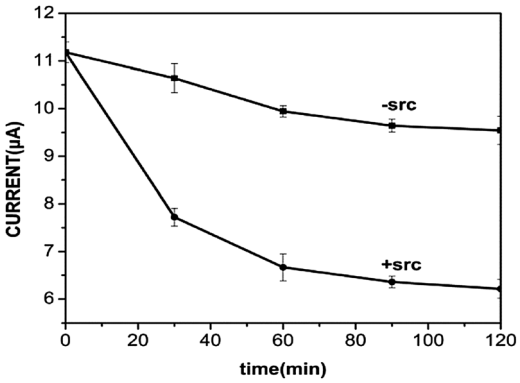


Fig. 7 Plots of the dependence of current signal on phosphorylation time in the presence of 4.22 nM Src, 3.0 mM p60c-src substrate I and 5.0 mM ATP at 30 °C. Error bars indicate the standard deviation for three independent measurements.

lower electrochemical response is obtained. The dependence of the electrochemical response on the Src concentration is shown in Fig. 8. The peak current decreases linearly with the logarithm of the Src concentration from 0.26 to 33.79 nM. The detection limit of Src is 0.087 nM ($S/N \geq 3$), which is lower than that of the previous reported electrochemical systems.[47] This suggests that the proposed method can be used to monitor the kinase activity with high sensitivity.

Inhibition assay. This method could also be used to quantitatively characterize the inhibition of kinase in the presence of small molecule inhibitors. PP2 is reported to be a potent and selective inhibitor of the Src family Tyr kinases.[48] As expected, with increasing concentrations of inhibitor, the electrochemical signal increased, indicating the reduction of kinase activity (Fig. 9). Based on the relationship between the electrochemical signal and inhibitor concentration, the half-maximal inhibition value IC_{50} was measured to be about 99 nM, which is in agreement with previous reports.[29]

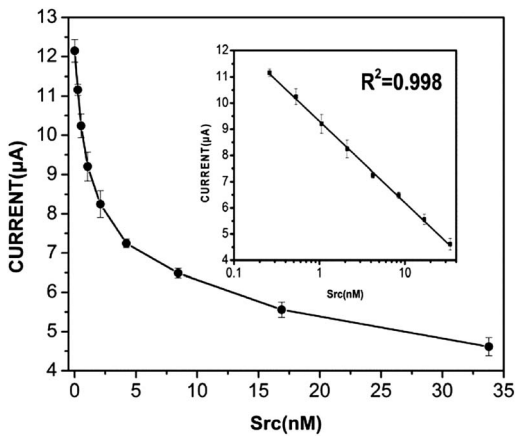


Fig. 8 Plot of the dependence of current signal on Src concentration. Inset is the linear relationship between the oxidation current of Tyr residues and the concentration of Src. Error bars indicate the standard deviation for three independent measurements.

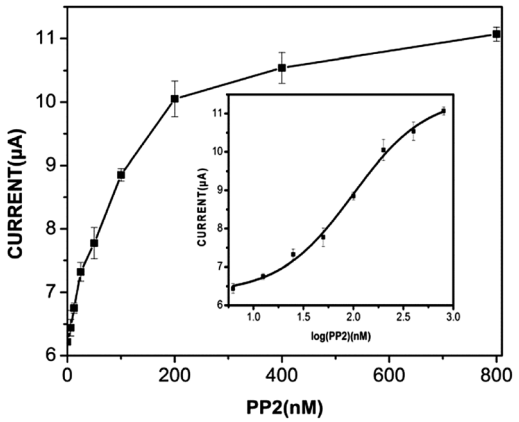


Fig. 9 Plot of the dependence of current signal on concentration of PP2. Inset is the linear relationship between the oxidation current of Tyr residues and the concentration of PP2. Error bars indicate the standard deviation for three independent measurements.

Conclusion

In this work, a graphene based electrochemical biosensor has been developed and the electrochemical behaviours of Tyr and pTyr at G/GCE were differentiated easily. Thus, the electrochemical sensor can be utilized to measure the activity of kinase and its relative inhibition with high sensitivity and a low detection limit. This method might have great potential applications in the evaluation of kinase activity and screening of inhibitors.

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