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# A single electrochemical biosensor for detecting the activity and inhibition of both protein kinase and alkaline phosphatase based on phosphate ions induced deposition of redox precipitates

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ABSTRACT

Protein kinase (PKA) and alkaline phosphatase (ALP) are clinically relevant enzymes for a number of diseases. In this work, we developed a new simple electrochemical biosensor for the detection of the activity and inhibition of both PKA and ALP. One common feature of the PKA and ALP catalyzing process is that PKA can hydrolysis adenosine-5′-triphosphate (ATP) and ALP can hydrolysis pyrophosphate, both reactions produce phosphate ions, and the amount of phosphate ion produced is proportional to enzyme activity. Our assay is based on the principle that phosphate ions react with molybdate to form redox molybdophosphate precipitates on the electrode surface, thus generating electrochemical current. The detection limit for PKA and ALP were much lower than existing assays. The biosensor has good specificity and was used to measure drug-stimulated PKA from lysates of HeLa cells. We also evaluated the use of the biosensor as a screening tool for enzyme inhibitors. To the best of our knowledge, this is the first report of a biosensor capable of detecting the activity of both PKA and ALP. This tool has the potential to simplify PKA and ALP clinical measurement, thereby improving diagnostics of relevant diseases. It also may serve as the basis for a simple screening method for new enzyme inhibitors for disease treatment.


## 1. Introduction

Enzymes are biological catalysts that participate in almost all metabolic processes in the cell (Luo et al., 2015; Sallmann and Limberg, 2015; Zhang and Houk, 2005; Kang et al., 2015). Enzymes serve a wide variety of functions inside living organisms. Monitoring activity of enzymes and discovering new enzyme inhibitors are of great importance in biomedical research, clinical diagnosis and treatment.

Among the various enzymes, protein kinase (PKA) and alkaline phosphatase (ALP) are two widely studied families of enzymes (Dong et al., 2015; Liu et al., 2014a; Qian et al., 2015a). PKA is responsible of transferring phosphate groups from nucleoside triphosphates (usually adenosine-5′-triphosphate, ATP) to specific amino acids, thus catalyzing the phosphorylation of proteins (Lee et al., 2015; Li et al., 2013b; Shin et al., 2014; Wang et al., 2013). ALP catalyzes the dephosphorylation of proteins (Barrozo et al., 2015; Yang et al., 2016; Yu et al., 2015). Phosphorylation and dephosphorylation processes lead to the functional change of the target proteins, which play vital roles in regulating intracellular processes involved in cell cycle, growth, apoptosis, and signal transduction (Chooi et al., 2014; Zhang et al., 2013).

Aberrant PKA and ALP levels are associated with several diseases (Sims et al., 2013). PKA is the best understood member of the serine-threonine protein kinase super family, and is involved in the regulation of a variety of cellular processes. It has been implicated in the initiation and progression of many tumors and, therefore, has been proposed as a novel molecular target for cancer therapy (Naviglio et al., 2009b; Sapio et al., 2014). A number of specific protein kinase inhibitors are already in use as cancer therapeutic agents (Davies et al., 2000). ALP is clinically important for liver, bone and kidney physiology (Weiss et al., 1988). ALP testing is used to help detect liver diseases (Stepien et al., 2016), cancer (Lopez et al., 1996) and bone disorders (Hessle et al., 2002). Because of the substantial clinical relevance of PKA and ALP testing, several analytical assays were developed to measure their activities.

The assays of PKA and ALP activities are typically performed by incubation of the enzymes with specific substrate and then the interaction of the substrates with various signal probes (Kang

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et al., 2014; Wang et al., 2015a). For example, Nie and coworkers reported a method for the detection of PKA activity by initially phosphorylation of peptide substrate using PKA, then the following interaction of the phosphopeptides with semisynthetic green fluorescent protein resulted in the fluorescence enhance of the proteins (Yin et al., 2015a). Upon the assay of enzyme activity, another important job is the screening of enzyme activity inhibitors as these inhibitors can be used as potential drugs for the treatment of relevant diseases (Li et al., 2013a; Wang et al., 2015c; Yin et al., 2015b). Different methods have been reported for PKA and ALP detection, such as chemiluminescence (Liang et al., 2014; Xu et al., 2010), electrochemical (Yin et al., 2015c, 2015d; Zhang et al., 2015), fluorescent (Deng et al., 2015; Qian et al., 2015b; Wang et al., 2015b) and surface-enhanced Raman spectroscopy (Liu et al., 2014b). Among these methods, electrochemistry has the advantages of relatively simple instrumentation, high sensitivity and mild operating conditions (Kerman et al., 2008; Luo et al., 2016; Miao et al., 2012). However, to the best of our knowledge, no study has reported the detection of both PKA and ALP using a single electrochemical biosensor.

Herein, we report an electrochemical biosensor for evaluating the activity and inhibition of both PKA and ALP. PKA can hydrolysis ATP to adenosine diphosphate (ADP), while ALP can hydrolysis pyrophosphate (PPi). These two processes resulted in the production of one common byproduct, phosphate ions (Pi). The following reaction of Pi with acidic molybdate could form a molybdophosphate precipitate on the electrode surface, generating electrochemical current that is proportional to the activity of PKA or ALP (Talarico et al., 2015; Xie et al., 2015). The inhibition effect of H-89 on the activity of PKA and NaF on the activity of ALP were also studied as well as the feasibility of the biosensor for the detection of PKA in HeLa cell lysates. The developed biosensor thus holds great promise for clinical application and biomedical research.

## 2. Experimental methods

### 2.1. Materials and apparatus

Cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase (PKA) was purchased from New England Biolabs Inc. (Beverly, MA). Adenosine-5′-triphosphate (ATP) was obtained from Generay Biotech Co., Ltd. (Shanghai, China). Adenosine-5′-diphosphate disodium salt ($ADPNa_2$), forskolin and H-89 were acquired from Beyotime Institute of Biotechnology (Shanghai, China). 3-isobutyl-1-methylxanthine (IBMX), sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$), alkaline phosphatase (ALP), sodium pyrophosphate decahydrate ($Na_4P_2O_7 10\ H_2O$, PPi), glucose oxidase (GOx), alcohol dehydrogenase (ADH) and β-Site Amyloid Precursor Protein Cleaving Enzyme 1 (BACE1) were obtained from Sigma-Aldrich. Glutathione(GSH) was received from Energy Chemical (Shanghai, China). Human serum albumin (HSA) was purchased from Zhenglong Biochem. Lab (Chengdu, China). Sodium fluoride (NaF) was obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). 10 mM Tris–HCl buffer (pH = 7.4, 100 mM NaCl) was used as the buffer solution. Other reagents were of analytical grade and used without further purification. All stock solutions were prepared with double-distilled water.

Electrochemical measurements were performed on a CHI-650D electrochemical workstation (Shanghai CH Instruments Co., China). A conventional three-electrode system was used with a gold electrode (Au, 2 mm in diameter) as the working electrode, an Ag/AgCl electrode as the reference electrode and a platinum wire as the auxiliary electrode. For testing PKA activity in cell lysates, the cells were broken with Fisher Model FB120 ultrasonic processor.

### 2.2. Electrochemical characterization of the molybdophosphate precipitate

For the modification of the electrode, 5 μL of graphene oxide (GO) solution (20 mg/mL) was dropped on the Au electrode surface. After dried, 15 μL of the solution containing 200 μM of $Na_2HPO_4$ and 6 mM of $Na_2MoO_4$ solution were dropped onto Au electrode surface for 20 min and then tested.

### 2.3. Electrochemical detecting of PKA activity and inhibition

To test the reaction of ATP with $Na_2MoO_4$, a specific concentration of ATP solution was reacted with 6 mM $Na_2MoO_4$ on GO modified Au electrode according to above mentioned procedure. For PKA test, different concentrations of PKA were mixed with 250 μM of ATP and incubated at 37 °C for 1 h. The resulting solution was then mixed with 6 mM $Na_2MoO_4$ on the electrode and then tested.

For assay of PKA inhibition, 50 U/mL of PKA was initially incubated with various concentrations of protein kinase inhibitor H-89 for 1 h, and then the activity of PKA was measured.

### 2.4. Electrochemical detecting of ALP activity and inhibition

For measurement of ALP activity, different concentrations of ALP were mixed with 100 μM PPi and incubated at 37 °C for 1 h. The resulted solution was then mixed with 6 mM of $Na_2MoO_4$ for 20 min and tested.

For assay of ALP inhibition, 200 U/L of ALP was incubated with the inhibitor NaF of various concentrations for 1 h. Then, the activity of ALP was measured.

### 2.5. Performance of the sensor for testing PKA activity in Hela cell lysates

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) supplemented with 10% fetal bovine serum. The cells were incubated under a humidified atmosphere containing 5% $CO_2$ at 37 °C in a cell culture box. The culture medium was then replaced by serum-free medium (pH = 7.4, Tris–HCl,10 mM NaCl) before stimulation. Various concentrations of forskolin and IBMX were added into the medium to activate the intracellular PKA for 30 min For comparison, 400 μL of Tris–HCl was added into the medium as a control, and 4 μM H-89 was added into one sample as another control. The cells ($3.75\times10^5$/mL ) were broken with an ultrasonic processor for 40 min, which was performed at 0 °C with ultrasonic interval time of 5 s The ultrasonic frequency was 20 KHz. The cell lysates were centrifuged at 2000 r/min, and the supernatant was ready for test.

## 3. Results and discussion

### 3.1. Electrochemical characterization of the molybdophosphate precipitate

The basic concept in this work is to develop an electrochemical sensor for monitoring the activity of both PKA and ALP based on phosphate ions induced deposition of redox precipitates. Fig. 1 addresses the schematic representation of the sensing principle. Initially, the reaction of phosphate ions with molybdate was studied. Graphene oxide (GO) was utilized to modify the gold electrode since the good conductivity of GO could enhance the electrochemical current of redox species (Brownson et al., 2015, 2014). GO was synthesized from natural graphite by Hummers' method according to literature reports (Dikin et al., 2007; Sun et al., 2013).

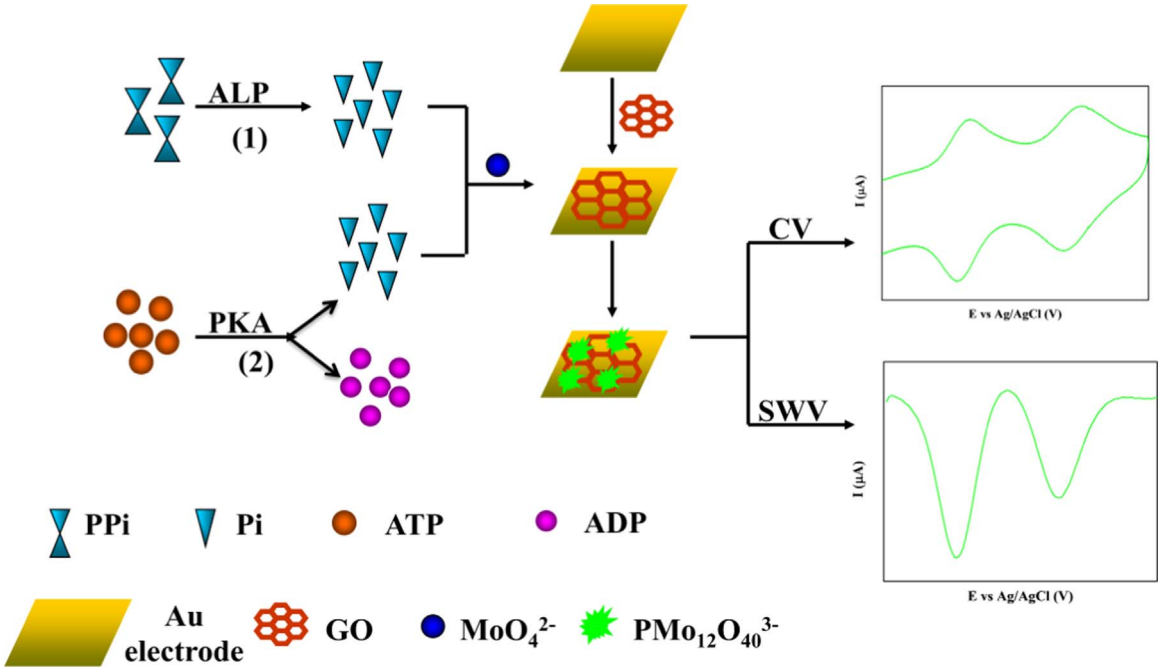



**Fig. 1.** Schematic representation of the sensing principle for detection of the activity of both PKA and ALP.

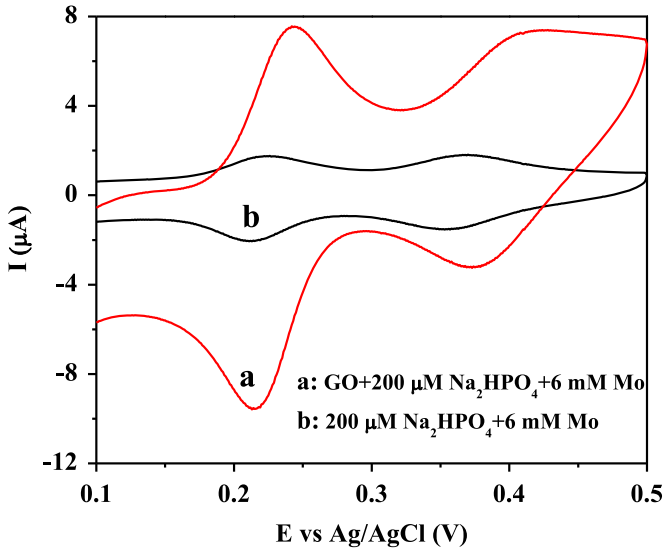



**Fig. 2.** CVs of the gold electrodes in 0.5 M $H_2SO_4$ after reaction of 200 μM of $HPO_4^{2-}$ with 6 mM of sodium molybdate at electrode surface.

The cyclic voltammograms (CV) of the electrodes in 0.5 M $H_2SO_4$ after deposition of molybdophosphate was shown in Fig. 2. The reaction of 200 μM of $HPO_4^{2-}$ with 6 mM of sodium molybdate solution resulted in two pairs of redox peaks on the electrode at around 0.22 V and 0.37 V, respectively, which are ascribed to electron-transfer between different states of Mo in the $PMo_{12}O_{40}$ precipitates (Xie et al., 2015). The corresponding equation regarding the reaction of Pi with molybdate (Eq. (1)) and redox reaction of molybdophosphate (Eqs. (2) and (3)) are displayed below (Xie et al., 2015):

$$12MoO_4^{2-} + 24H^+ + PO_4^{3-} = PMo_{12}O_{40}^{3-} + 12H_2O \quad (1)$$

$$PMo_{12}O_{40}^{3-} + 2e + 2H^+ = H_2PMo_2^{V}Mo_{10}^{VI}O_{40}^{3-} \quad (2)$$

$$H_2PMo_2^{V}Mo_{10}^{VI}O_{40}^{3-} + 2e + 2H^+ = H_4PMo_4^{V}Mo_8^{VI}O_{40}^{3-} \quad (3)$$

The redox current indicated the formation of molybdophosphate precipitate on the electrode surface. Compared to blank electrode (curve b), the GO modified electrode enhanced the redox current about 4 times (curve a). Control experiment with only molybdate resulted in no redox peaks.

### 3.2. Electrochemical detecting of PKA activity and inhibition

After testing the redox properties of molybdophosphate precipitate on GO modified electrode, we then applied the method for the detection of the activity of PKA. Formerly, we have reported a fluorescence assay for PKA based on ATP induced fluorescence enhancement of silver nanoclusters that does not involve the traditional phosphorylation process (Shen et al., 2015). Without the phosphorylation process, the assay was much simpler and cost effective. Here, the detection of the activity of PKA was also performed that does not need the phosphorylation process. As PKA can hydrolyze ATP into ADP and Pi, the redox current of the electrodes are expected to increase after the reaction of Pi with molybdate (Qian et al., 2015a). Since both ATP and ADP contain phosphate groups, the reaction of ATP and ADP with molybdate was studied. The reaction of ATP with molybdate resulted in similar CV curves to above discussed $HPO_4^{2-}$ with two pair of redox peaks (Supporting Information, Fig. S1). Fig. 3A displays the square wave voltammograms (SWV) of GO modified electrodes after incubation with ATP (or ADP) and molybdate. It can be seen the reaction of 250 μM ATP or ADP with molybdate resulted in no obvious current intensity difference (Fig. 3A, curve a and b). In addition, the peak current was increased accordingly with the increase of ATP concentrations from 0 to 250 μM (Fig. 3B). This is because increased concentration of ATP resulted in more molybdophosphate precipitate produced, leading to higher electrochemical current. These results demonstrated that both ATP and ADP can react with molybdate. There is no obvious current variation between ATP and ADP indicated that in both ATP and ADP molecules, only one exposed $PO_4^{3-}$ group can react with molybdate (The structure of ATP and ADP were seen in Supporting Information, Fig. S2). However, after ATP was incubated with 50 U/mL of PKA for 1 h, the following reaction of the resulted

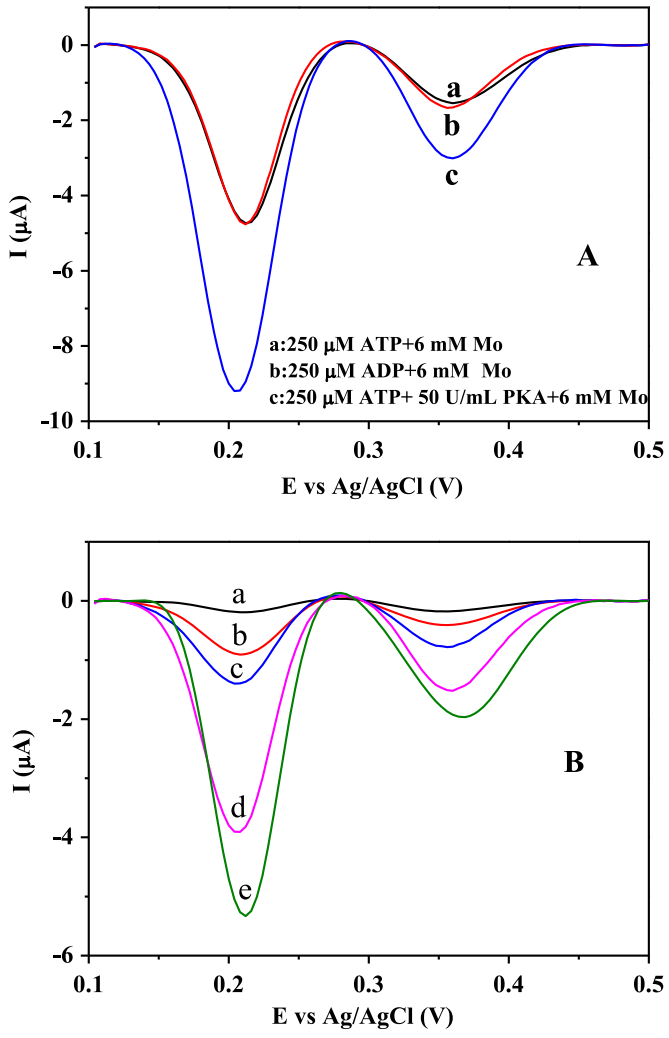



**Fig..3.** (A) SWV curves of different modified electrodes in 0.5 M $H_2SO_4$. (B) SWV curves of the electrodes towards the detection of different concentrations of ATP. From a to e, 0, 50, 100, 200, 250 μM.

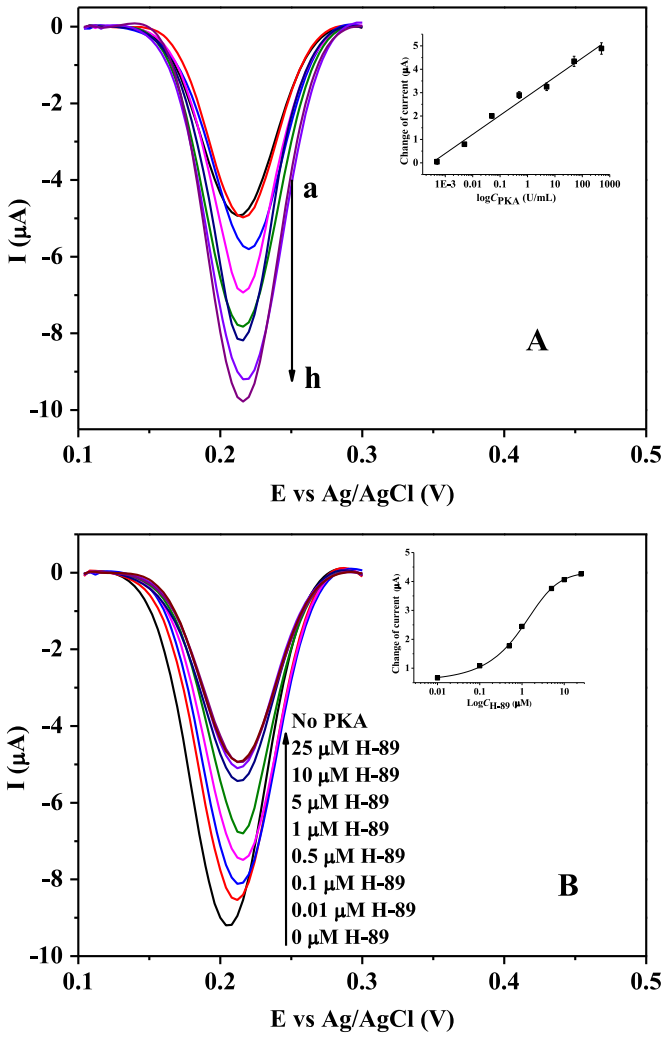



**Fig. 4.** (A) The SWV peak current of the sensor toward the detection of different concentration of PKA. From a to h, 0, 0.5, 5, 50, 500, $5 \times 10^3$, $5 \times 10^4$, $5 \times 10^5$ U/L. The inset is the calibration curve towards PKA. (B) The inhibition effect of different concentrations of H-89 on the response of the sensor. The inset is the dose response curve of inhibition of PKA activity by H-89.

solution with molybdate lead to a significant increase of the redox current (Fig. 3A, curve c). The enhanced electrochemical current was ascribed to the released Pi from ATP that induced the formation of additional molybdophosphate on the electrode surface.

The effect of the concentration of ATP on the sensitivity of PKA assay was then studied. The 50 U/mL of PKA were incubated with different concentrations of ATP and then tested. The current variation due to the introduction of PKA was considered as sensitivity to PKA. With the increase of ATP concentration from 50 μM to 250 μM, the sensitivity of the sensor towards 50 U/mL of PKA was increased (Supporting Information, Fig. S3). Further increase of ATP concentration, the background current will also be increased. So, the ATP concentration of 250 μM was selected for further experiments.

Under optimized ATP concentration, to test the linear range of the sensor towards PKA, different concentrations of PKA were reacted with 250 μM of ATP for 1 h and then tested. The SWV indicated the current peak of the electrodes at around 0.22 V was increased with the increase of PKA concentrations (Fig. 4A). This is because increased concentration of PKA results in more Pi produced. A linear relationship between the current change and the logarithm of PKA concentrations in the range from 5 to $5 \times 10^5$ U/L with 5 orders of magnitude was attained (inset of Fig. 4A). The detection limit was calculated to be 2 U/L based on signal to noise of 3. Such a detection limit was much lower than previously reported fluorescence assay for PKA based on semisynthetic fluorescent protein (500 U/L) (Yin et al., 2015a), graphene quantum dots (30 U/L) (Wang et al., 2013) and silver nanoclusters (500 U/L) (Shen et al., 2015), as well as reported electrochemical assay for PKA based on ferrocene (100 U/L) (Shin et al., 2014) and $[Ru(NH_3)_6]^{3+}$ (30 U/L) (Wang et al., 2014). Via the "mix and detect" procedure, the whole assay can be finished within approximately 1.5 h.

The developed sensor was also evaluated for testing PKA inhibitors as PKA inhibitors is of great importance for treating PKA related diseases, including cancer (Naviglio et al., 2009a; Nesterova et al., 2006). H-89 as the widely studied inhibitor, which can block PKA activity by inhibiting the ATP site of catalytic subunit, was selected as a model. The PKA solution of 50 U/mL was incubated with various concentrations of H-89 for 1 h, and then the activity of the treated PKA was measured. As displayed in Fig. 4B, the peak current of the electrodes was decreased with the increase of H-89 concentration, indicating the relative activity of PKA was inhibited gradually with the increase concentration of H-89. When the concentration of H-89 reaches 25 μM, the electrochemical current was close to that in the absence of PKA, demonstrating the activity of PKA was almost completely blocked. The relationship between current intensity and logarithm of H-89 concentrations

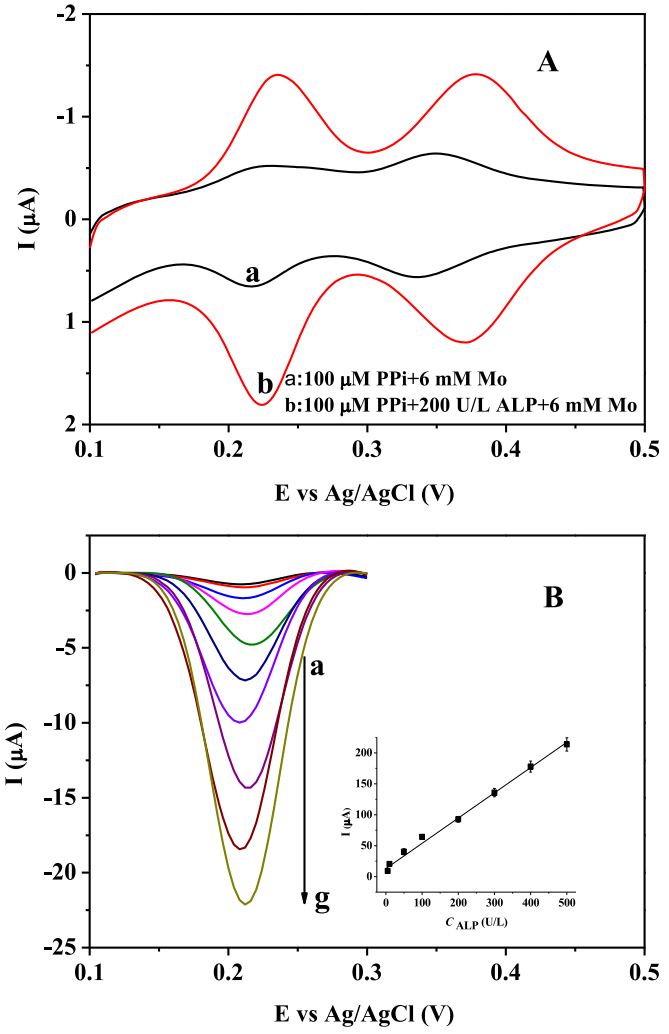
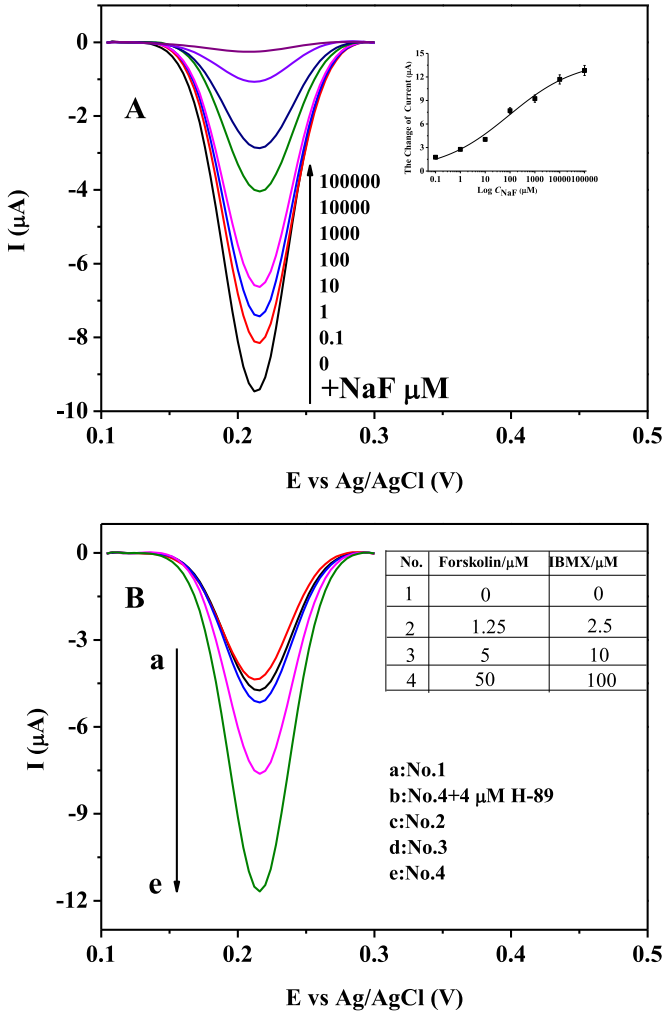



**Fig. 5.** (A) CV curves of different modified electrode in 0.5 M $H_2SO_4$. (B) SWV curves of the sensor for the detection of different concentrations of ALP. From a to g, 0, 1, 5, 10, 50, 100, 200, 300, 400, 500 U/L. The inset is the calibration curve to ALP.




| No. | Forskolin/μM | IBMX/μM |
|---|---|---|
| 1 | 0 | 0 |
| 2 | 1.25 | 2.5 |
| 3 | 5 | 10 |
| 4 | 50 | 100 |

**Fig. 6.** (A) The inhibition effect of different concentrations of NaF on the response of the sensor. The inset is the dose response curve of inhibition of ALP activity by NaF. (B) Electrochemical response of the sensor for PKA activity in Hela cell lysates stimulated by different concentrations of drugs (forskolin/IBMX).

resulted in a sigmoidal profile (insert of Fig. 4B). These results clearly demonstrated that the developed sensor is capable of screening kinase inhibitors.

### 3.3. Electrochemical detecting of ALP activity and inhibition

After successful detection of PKA activity, we then applied the sensor for measuring the activity of ALP. PPi was selected as substrate for ALP since ALP can hydrolysis PPi into 2 equiv of Pi (Kang et al., 2014; Zhang et al., 2013). The detection protocol for ALP was similar to the detection of PKA. When studying the reaction of the substrate PPi with molybdate, we found 100 μM of PPi generated much lower redox current on GO modified electrode than equivalent amount of ATP (Fig. 5A, curve a). However, after incubating 200 U/L of ALP with PPi, there is a significant increase of the peak current (Fig. 5A, curve b). The SWV peak current at around 0.22 V was also increased with the increase of ALP concentration (Fig. 5B). A linear relationship between the current intensity and ALP concentration in the range from 1 to 500 U/L was obtained and the detection limit was calculated to be 0.5 U/L based on signal to noise of 3. The detection limit was also much lower than previous work for ALP based on carbon quantum dot (1.4 U/L) (Qian et al., 2015a), fluorescence hydrogel (60 U/L), semiconductor quantum dot (10 U/L) (Jia et al., 2010) and DNA probe (100 U/L) (Miao et al., 2011).

To test ALP inhibition, NaF was selected (Sun et al., 2016). The activity of ALP was found to be decreased with the increase of NaF concentrations along with a gradual decrease of the response of the electrode. There is also a sigmoidal relationship between electrode current change and logarithm of NaF concentrations, again proving the sensor can be utilized for screening ALP inhibitors (Fig. 6A).

### 3.4. Selectivity and reproducibility of the electrochemical sensor

To study the selectivity of the sensor, the effect of different other proteins or enzymes, such as glucose oxidase (GOx), alcohol dehydrogenase (ADH), β-Site Amyloid Precursor Protein Cleaving Enzyme 1 (BACE1), glutathione (GSH) and human serum albumin (HSA) on the sensitivity of the sensor were tested (Supporting Information, Fig. S4). Compared to the response to PKA, only 50 U/mL of ADH caused a little current variation, indicating good selectivity of the sensor. In addition, for reproducibility of the sensor, 0.5 U/mL of PKA and 100 U/ mL of ALP were detected independently 4 times, and the relative standard deviation (RSD) of 1.02% and 2.713% were obtained.

### 3.5. Performance of the sensor for testing PKA activity in Hela cell lysates

To test the performance of the developed sensor in complex biological samples, we applied the sensor to detect PKA activity in HeLa cell lysates. The drugs of Forskolin and IBMX were very effective for the activation of PKA in cells (Bai et al., 2013; Yin et al., 2015a). HeLa cells were chosen and treated with different concentrations of Forskolin and IBMX for activation of PKA. The Hela cells were stimulated by different concentrations of Forskolin and IBMX for 30 min, and then the activity of PKA in the cell was tested. The electrode response was increased with the increase of Forskolin/IBMX used, indicating the PKA activity in the cell lysates was enhanced correspondingly (Fig. 6B). When the stimulated cell lysates was further treated with inhibitor H-89, the response of the sensor was almost decreased to that in the case of cell lysates without drug stimulation, again demonstrating the inhibition effect of H-89 on the activity of PKA. The results proved that the sensor can effectively monitor PKA activity from cells lysates induced by drugs.

## 4. Conclusion

In summary, PKA and ALP measurement have great clinical utility. For the first time, an electrochemical sensor was designed for highly sensitive detection of the activity and inhibition of both PKA as well as ALP. ATP and PPi were chosen as the substrate for PKA and ALP, respectively. Both of the two enzymatic hydrolysis processes resulted in the production of Pi, which can induce electrochemical current when reacted with molybdate to form redox precipitates on the electrode surface. Compared to existed methods, the developed assay has the advantages of simple operation, low cost, wide linear range and low detection limit. The good sensitivity enables the sensor for evaluating PKA activities in Hela cell lysates and for testing enzyme inhibitors. This study may find wide applications in clinical diagnosis, in biomedical research of phosphorylation as well as dephosphorylation relevant process and in pharmaceutical industry for screening enzymatic inhibitors as drugs. The new approach has the potential to simplify PKA and ALP clinical measurement and utilized as a potential screening method for new classes of enzyme inhibitors.

## Acknowledgments

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.05.025.

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