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Electrochemical biosensor for protein kinase A activity assay based on gold nanoparticles-carbon nanospheres, phos-tag-biotin and β-galactosidase

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ABSTRACT

A sensitive and selective electrochemical biosensor was fabricated for protein kinase A (PKA) activity assay. Multiple signal amplification techniques were employed including the nanocomposite of gold nanoparticles and carbon nanospheres (Au@C), the biocomposite of SiO_2 and streptavidin (SiO_2-SA), the composite of AuNPs and biotinylated β-galactosidase (AuNPs-B-Gal) and in situ enzymatic generation of electrochemical activity molecule of *p*-aminophenol. After peptides were assembled on Au@C modified electrode surface, they were phosphorylated by PKA in the presence of ATP. Then, biotinylated Phos-tag was modified on electrode surface through the specific interaction between Phos-tag and phosphate group. Finally, SiO_2-SA and AuNPs-B-Gal were captured through the specific interaction between biotin and streptavidin. Because the electrochemical response of *p*-aminophenol was directly related to PKA concentration, an innovative electrochemical assay could be realized for PKA detection. The detection limit was 0.014 unit/mL. The developed method showed high detection sensitivity and selectivity. In addition, the fabricated biosensor can be also applied to detect PKA in human normal gastricepithelial cell line and human gastric carcinoma cell line with satisfactory results.

1. Introduction

Protein phosphorylation is one of the most crucial post-translational modifications, which plays key regulatory role in many biological processes, including cell growth, cell signal communication, metabolism, cell differentiation, and apoptosis (Iakoucheva et al., 2004). The abnormal expression level of protein phosphorylation has been associated with a serious of diseases, such as diabetes, Alzheimer's disease, and cancer (Flajolet et al., 2007; Griner and Kazanietz, 2007). It is well-known that protein phosphorylation is catalyzed by protein kinase in the presence of ATP. The aberrant activity of protein kinase can lead to the change of protein phosphorylation. Thus protein kinases are a kind of vital drug target for therapy of serious diseases, which were related to the aberrant level of protein phosphorylation (Cho, 2013; Ohmachi et al., 2006). However, in order to understand the relationship between the disease and protein kinases, find out the molecular mechanism of clinical diagnosis and seek protein kinase-targeting drugs, a sensitive and specific method for protein kinase activity assay and inhibitor screening is needed.

Up to now, some methods have been developed for protein kinase activity assay, including radioanalysis (Houseman et al., 2002), mass spectrometry (Beausoleil et al., 2006), surface plasmon resonance (Mori et al., 2008), colorimetry (Zhou et al., 2014), fluorescence (Song et al., 2015a; Xu et al., 2014), electrochemiluminescence (Zhao et al., 2015), photoelectrochemistry (Yin et al., 2015), etc. Though these methods can detect protein kinase activity with acceptable results, there are still some deficiencies which need to be improved. For example, radioanalysis relies on the transfer of radioactive γ-^{32}P from ATP to the substrate peptide with the catalysis of protein kinase. However, the radioactive γ-^{32}P is harmful to the health of operator and environment. It also requires complicated multistep procedure. The detection sensitivity of colorimetry needs to be improved. Mass spectrometry not only requires large and expensive apparatus, but also need complicated operation process and skilled operator. Fluorescence-based techniques require expensive and large-volume instruments and multistep separation process, and some of these methods require fluorescence labeling. Due to the advantages of simple device, easy operation inexpensive cost, fast detection, micro-volume sample, high sensitivity, electrochemical methods have attracted many

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attentions on protein kinase activity assay and inhibitor screening (Chiku et al., 2010; Kerman and Kraatz, 2009; Shin et al., 2014). Itamar Willner' group performed an electrochemical analysis of casein kinase activity, which is accomplished by the voltammetric response of Ag^+ ions associated with the phosphorylated product (Wieckowska et al., 2008). Xu et al. (2009) employed a novel label-free electrochemical strategy for monitoring the activity and inhibition of protein kinase A (PKA), which was based on the linkage of two phosphate groups between the phosphorylated peptide and DNA functionalized AuNPs (DNA–AuNPs) by Zr^{4+} and the chronocoulometric response of $[Ru(NH_3)_6]^{3+}$ absorbed on the DNA–AuNPs. Kerman and Kraatz (2009) developed an electrochemical method to detect protein tyrosine kinase activity (PTK) using adenosine 5′-[γ-thio] triphosphate (ATP-S) as the co-substrate. Upon thiophosphorylation of the substrate peptides, the gold nanoparticles (AuNPs) can accumulate on the surface and the activity of PTK can be detected electrochemically by monitoring Cl^- oxidation signal at the AuNPs surface. Yang et al. (2011) detected the PTK activity using the electrochemical signal of tyrosine residue in the polypeptide, where $Os(bpy)_3^{2+}$ was used as electron mediator to increase detection sensitivity. Wang et al. (2010) reported a simple and sensitive electrochemical method for PKA activity assay based on the hindrance change of substrate peptides towards the redox probe of $[Ru(NH_3)_5Cl]^{2+}$, and this change was caused by phosphorylation reaction.

In order to detect protein kinase activity with high specificity, we must highlight the recognition strategy for phosphate group. Currently, the widely applied strategy is mainly focused on the following aspects, such as phospho-specific recognition antibody (Gupta et al., 2011; Kim et al., 2008), Zr^{4+} (Liu et al., 2015; Wang et al., 2014; Wang et al., 2015), TiO_2 (Bai et al., 2013), tyrosine (Li et al., 2013) and ATP derivates (such as γ-biotin-ATP (Wang et al., 2006), ATP-S (Liu et al., 2014b; Zhao et al., 2015)). Though these reagents or strategy can efficiently recognize the phosphorylation event and capture phosphate group, it is still need to overcome some negative factors. For instance, as a biological reagent, antibody is expensive and unstable. Zr^{4+} and TiO_2 can not only bind with phosphate group, but also with carboxyl group, which can interfere with the detection. The employment of the electrochemical signal of tyrosine residue can only detect the activity of PTK, which limits its extensive application. The synthesis and purification of ATP derivatives are difficult, less inexpensive and time-consuming.

According to the above shortages, it is necessary to employ new recognition reagent for protein kinase activity assay and inhibitor screening using electrochemical techniques. Recently, our group focuses on a kind of new recognition reagent for phosphate group with high binding force and specificity, phosphate-binding tag (Phos-tag). Phos-tag is alkoxide-bridged dinuclear metal (i.e., Zn^{2+} or Mn^{2+}) complexes, which were shown to preferentially capture phosphomonoester dianions bound to Ser, Thr, and Tyr residues at neutral pH (Kinoshita et al., 2006). So far, Phos-tag has been widely applied to separate and characterize phosphorylated proteins under conditions of neutral pH, near physiological temperature and ionic strength (Barbieri and Stock, 2008; Kinoshita et al., 2012). However, there are few reports on the application of Phos-tag in electrochemical techniques for protein kinase activity assay except several work have been done by our group. We once developed a selective electrochemical biosensor for PKA activity assay based on Phos-tag and horseradish peroxidase (HRP) catalytic signal amplification (Yin et al., 2014). In another work, we performed photoelectrochemical method to analyze PKA activity based on Phos-tag and alkaline phosphatase (ALP) in alkaline condition (pH 9.8) (Yin et al., 2015). Though the detection specificity is acceptable, the detection sensitivity of the above methods need to be further improved. In addition, the employment of H_2O_2 and alkaline condition might limit their application due to the harm for biomolecule.

In order to conquer the above disadvantages, herein we developed another Phos-tag-based electrochemical method for PKA activity assay and its inhibitor screening. Multiple signal amplification strategies based on functional nanomaterials were employed in this work due to their high efficiency on increasing detection sensitivity (Song et al., 2010; Xu et al., 2014), including AuNPs@carbon nanospheres hybrid material (Au@C), SiO_2-strepavidin nano-composite (SiO_2-SA), AuNPs-biotin-β-galactosidase (Au-B-Gal). To decrease the possible damage towards biomolecule, β-galactosidase catalytic system was selected, where β-galactosidase can catalyze the hydrolysis reaction of *p*-aminophenyl galactopyranoside (PAPG) to produce *p*-aminophenol (PAP) in pH 7.4 (Park et al., 2014; Zhao et al., 2014). Because the electrochemical signal of PAP was directly related to the phosphorylation level of peptide, PKA activity could be realized.

2. Experimental

2.1. Reagents and materials

The substrate peptide (NH_2-CGGALRRASLG-COOH), streptavidin and ATP were purchased from Sangon Biotech (Shanghai, China) Co., Ltd. cAMP-dependent PKA catalytic subunit, casein kinase I (CK1), casein kinase II (CK2), mitogen-activated protein kinases (MAPK) were purchased from New England Biolabs Ltd. (USA). Biotinylated Phos-tag (Phos-tag-biotin) was obtained from Wako Pure Chemical Industries, Ltd. (Japan). Carboxyl functionalized SiO_2 was purchased from BaseLine (Tianjin, China). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), tris(hydroxymethyl)aminomethane (Tris), ethanolamine and chloroauric acid ($HAuCl_4$) were purchased from Aladdin (Shanghai, China). Bovine Serum Albumin (BSA) was supplied by Solarbio (Beijing, China). Carboxypeptidase Y (CPY), forskolin, 3-isobutyl-1-methylxanthine (IBMX), 3-mercaptopropionic acid (MPA), 1-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), (3-aminopropyl)triethoxysilane (APTES), biotinylated β-galactosidase (B-Gal), PAPG and poly(diallyldimethylammonium chloride) (PDDA) solution (Average Mw=200,000–350,000, 20 wt% in H_2O) were purchased from Sigma (USA). HA-1077 and H-89 were offered by Selleck (USA). The preparation processes of AuNPs, AuNPs@carbon nanospheres hybrid material (Au@C), SiO_2-SA, and Au-B-Gal, cell culture and protein extraction process, and transmission electron microscope graphs were listed in Supplementary Materials.

The buffer solutions used in this work were as follows. Peptide immobilization buffer, 20 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM KCl, 1 mM TCEP, pH 7.4. PKA reaction buffer, 50 mM Tris–HCl, 70 μM ATP, 10 mM $MgCl_2$, pH 7.5. Phos-tag-biotin reaction buffer, 10 mM Tris–HCl (pH 7.0) containing 0.1 M NaCl, 0.1% Tween-20, 0.4 mM $Zn(NO_3)_2$. Streptavidin reaction buffer, 10 mM phosphate buffer saline (PBS, pH 7.4). CPY reaction buffer, 20 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM KCl, pH 7.5. B-Gal buffer, 0.05 M potassium phosphate, 1 mM $MgCl_2$, 0.1 M 2-mercaptoethanol, pH 7.8. Detection buffer, 10 mM PBS containing 50 mM PAPG. Washing buffer, 10 mM Tris–HCl containing 50 mM NaCl (pH 7.4). 10 mM phosphate buffer saline (PBS) was prepared by mixing the stock solution of 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4, and the pH was adjusted by 0.1 M HCl or 0.1 M NaOH.

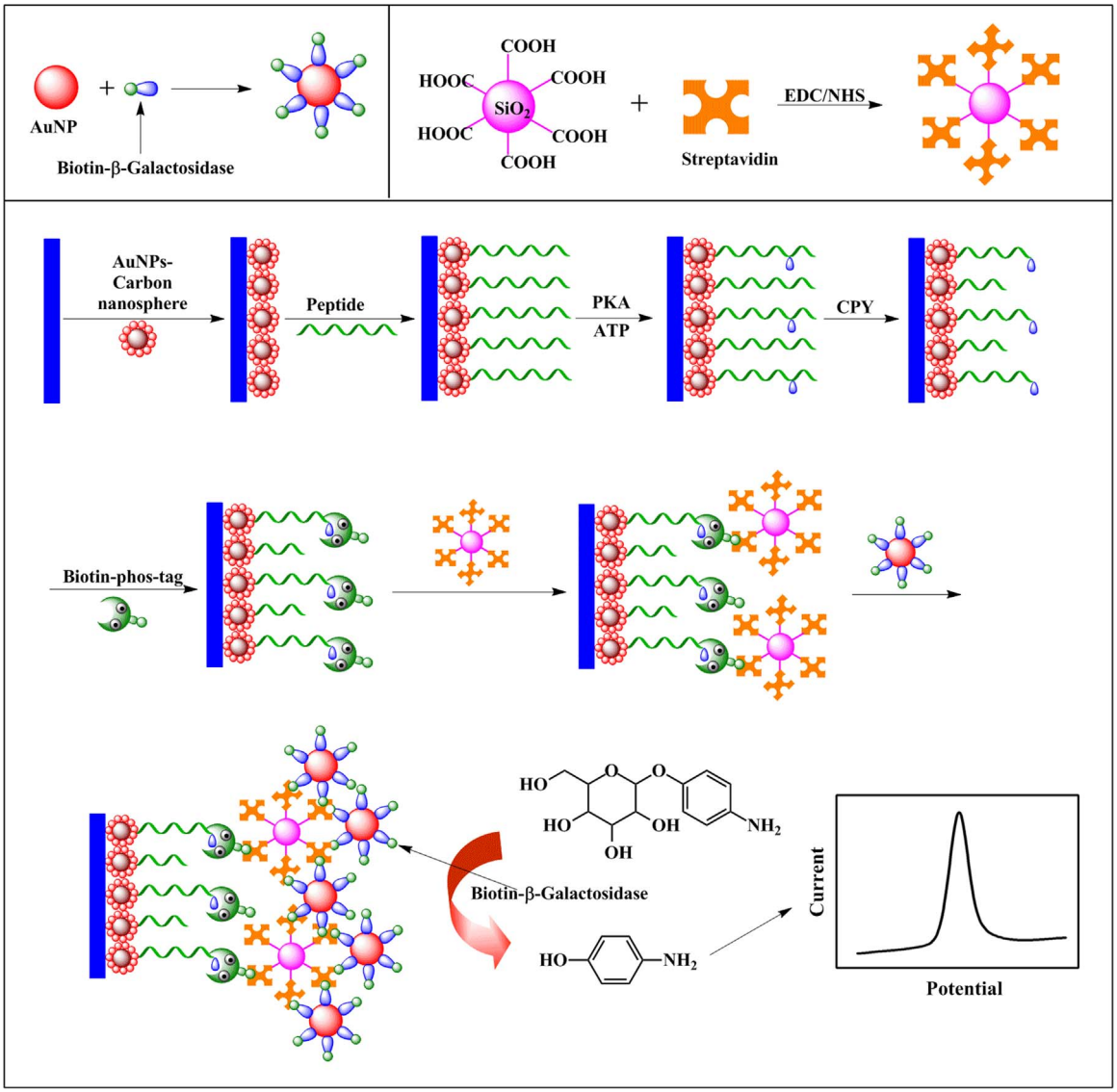
2.2. Biosensor fabrication

Before modification, the glassy carbon electrode (GCE, 3 mm in diameter) was firstly polished to a mirror-like surface with 0.3 μm

Al_2O_3 slurry, and then the electrode was washed under ultrasonication in double-distilled deionized water and anhydrous alcohol for 3 min, respectively. The cleaned GCE was rinsed thoroughly with double distilled deionized water and dried under nitrogen flow. After that, 10 μL of Au@C was dripped on GCE surface and dried under the irradiation of infrared lamp. The unimmobilized Au@C was removed from GCE surface by washing the electrode with double distilled deionized water. The obtained electrode was noted as Au@C/GCE. For peptide self-assembly, 10 μL of peptide immobilization buffer containing 1.0 μM peptide was dripped on the electrode surface and incubated for 12 h in a humid cell under ambient temperature. The electrode was then rinsed with washing buffer for three times and noted as Peptide/Au@C/GCE. Subsequently, 10 μL of peptide immobilization buffer containing 0.1 mM MPA was dripped on the electrode surface and incubated for 30 min to obtain a well-aligned peptide monolayer and remove the unspecific adsorbed peptide. After rinsed with washing buffer for three times, the electrode was further incubated with 10 μL of PKA reaction buffer containing different concentrations of PKA at 37 °C for 75 min under humidity condition. The obtained P-Peptide/Au@C/GCE was rinsed with washing buffer. Subsequently, 10 μL of CPY reaction buffer containing 150 unit/mL CPY was dripped on the electrode surface and incubated for 60 min at 37 °C in a humid cell. After rinsed with washing buffer, the electrode was further incubated with 10 μL of Phos-tag-biotin reaction buffer containing 15 μM Phos-tag-biotin for 40 min at ambient temperature under humidity condition. The obtained electrode was named as Phos-tag/P-Peptide/Au@C/GCE. Afterwards, 10 μL of SiO_2-SA was dripped on the electrode surface and maintained the reaction for 50 min at ambient temperature. The obtained electrode (SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE) was rinsed with washing buffer to remove the nonspecific adsorption of SiO_2-SA. Finally, the electrode was further incubated with 10 μL of Au-B-Gal for 40 min at ambient temperature in a humid cell. The electrode was then rinsed with washing buffer and marked as Au-B-Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE. For comparison, Gal/SA/Phos-tag/P-Peptide/Au@C/GCE, Au-B-Gal/SA/Phos-tag/P-Peptide/Au@C/GCE and Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE were also fabricated.

2.3. Electrochemical detection

See Supplementary Materials.



Scheme 1. The biosensor fabrication process and PKA detection strategy.

2.4. Inhibition investigation

In order to investigate the effect of inhibitors on the activity of PKA, different inhibitors with diverse concentrations were introduced into the PKA reaction solution containing 100 unit/mL PKA and incubated for 75 min at 37 °C. Then the electrode was rinsed thoroughly using the washing buffer. The following operation was same to Section 2.2.

The inhibition ratio was calculated according to the following equation of $(I_1-I_2)/I_1\times 100\%$, where I_1 is the current without inhibition, I_2 is the current after inhibition.

3. Results and discussions

3.1. Detection strategy

In this work, a sensitive electrochemical strategy was proposed for PKA activity assay and inhibitor screening based on the specific recognition effect of Phos-tag-biotin towards phosphate group, SiO_2-SA signal amplification unit and Au-B-Gal catalytic signal amplification. As shown in Scheme 1, Au@C was first immobilized on the GCE surface through physical adsorption, where Au@C was used as not only the signal amplification unit, but also as peptide immobilization substrate. Based on the formation of Au–S bond between AuNPs and –SH in peptide molecule, the peptide can be self-assembled on the electrode surface. Then, under the catalytic effect of PKA in the presence of ATP, a phosphate group can be transferred to the OH of serine residual and achieve the phosphorylation reaction. In order to decrease the hindrance effect of un-phosphorylated peptide and the fragment of amino acid at the side of phosphorylated serine towards the capture of Phos-tag-biotin, these sequences were cleaved by CPY, where CPY is a kind of proteinase with hydrolytic activity towards peptide linkage from the C-terminal of protein or peptide, and the activity of carboxypeptidase can be inhibited when the amino residue was phosphorylated (Zhou et al., 2013). The cleavage can not only decrease the hindrance, but also increase the detection sensitivity. Afterwards, Phos-tag-biotin can be captured on the electrode surface through the specific interaction between Phos-tag and phosphate group. Here, Phos-tag-biotin played the role of bridge to connect phosphorylated peptide and signal amplification unit. Subsequently, the two signal amplification units of SiO_2-SA and Au-B-Gal can be further assembled on the electrode surface through the specific interaction between biotin and strepavidin. β-Galactosidase can catalyze the hydrolysis reaction of PAPG to produce PAP with high reduction activity, and PAP can be further electrochemically oxidized to 4-quinone imine. Based on it, a well-defined electrochemical oxidation peak can be obtained and this oxidation peak current is related to PKA concentration. Therefore, a sensitive and specific electrochemical assay was established for PKA activity detection in neutral operation condition.

3.2. Detection feasibility assay

In order to prove the detection feasibility of the proposed method for PKA activity assay, several control experiments were performed and the results were illustrated in Fig. 1A. No electrochemical response was observed at SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE in the potential range of −0.4 to 0.7 V (curve a). These results indicated that (1) all the modifiers on the electrode surface are non-electrochemical activity in the selected potential range, which cannot interfere with the detection; (2) In detection solution, PAPG does not possess electrochemical activity. However, for Au-B-Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE, a well-defined oxidation peak was obtained with the oxidation potential of 0.03 V

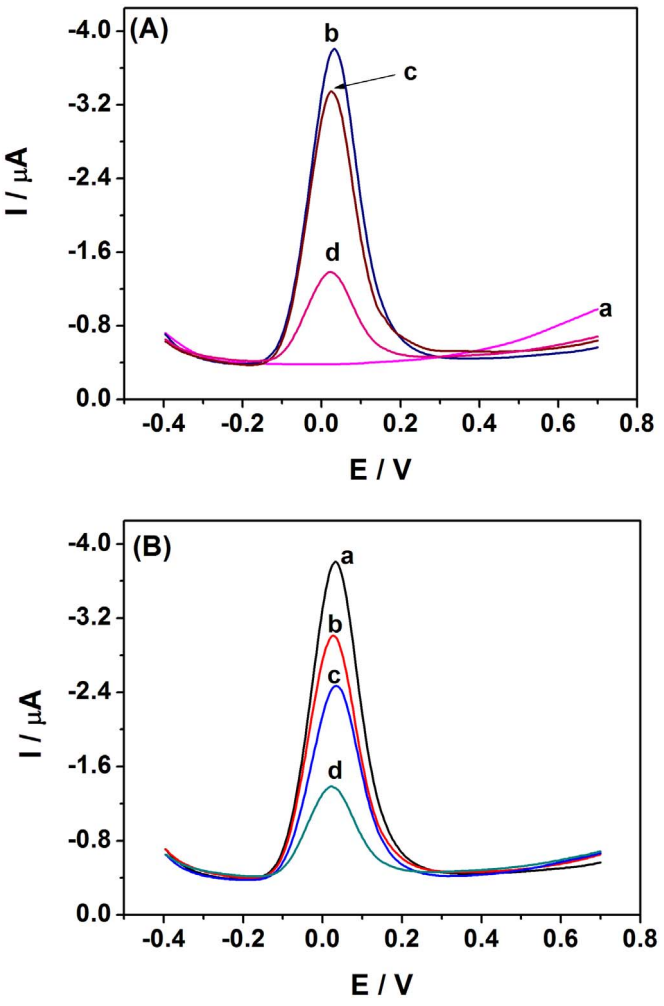


Fig. 1. The DPV response of different electrodes in detection buffer. (A) (a) SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE, (b) Au-B-Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE, (c) P-Peptide/Au@C/GCE was directly incubated with Phos-tag-biotin, SiO_2-SA and Au-B-Gal, successfully, (d) Gal/SA/Phos-tag/P-Peptide/Au@C/GCE. (B) (a) Au-B-Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE, (b) Au-B-Gal/SA/Phos-tag/P-Peptide/Au@C/GCE, (c) Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE, (d) Gal/SA/Phos-tag/P-Peptide/Au@C/GCE.

(curve b), which could be attributed to the oxidation of PAP. When the detection solution was incubated with Au-B-Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE, PAPG can be hydrolyzed under the catalysis effect of β-galactosidase to produce the electrochemical activity molecule of PAP. The produced electrochemical oxidation signal also demonstrated the successful fabrication of the biosensor. In this work, the digestion of peptide by CPY was performed to improve the detection sensitivity after PKA catalytic phosphorylation. To verify it, a contrast experiment was conducted. As also seen in Fig. 1A, Au-B-Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE showed a strong electrochemical oxidation peak with the oxidation peak current (I_{pa}) of 3.42 μA (curve b). However, when CPY digestion was deleted in the biosensor fabrication process, the oxidation signal of PAP decreased to 2.92 μA (curve c). Without CPY digestion, the steric hindrance of peptide could decrease the immobilization amount of Phos-tag-biotin, SiO_2-SA and Au-B-Gal, but also reduced the amount of PAP. Thus, a relative weak electrochemical signal was generated, which led to low detection sensitivity.

To ensure the signal amplification ability of SiO_2-SA and Au-B-Gal, several additional experiments were carried out and the results were compared. As shown in Fig. 1A, Gal/SA/Phos-tag/P-

Peptide/Au@C/GCE only presented a small electrochemical oxidation signal with the I_{pa} of 0.976 μA (curve d), which was much lower than that obtained at Au-B-Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE (3.42 μA, curve b). In the fabrication process of Gal/SA/Phos-tag/P-Peptide/Au@C/GCE, SiO_2-SA and Au-B-Gal was replaced by SA and B-Gal, respectively. That means, one phosphorylated peptide on the electrode surface can only capture one B-Gal to catalyze the hydrolysis reaction of PAPG. Compared with SiO_2-SA and Au-B-Gal, the capture efficiency for SA and B-Gal was low. As a result, the produced amount of PAP was low and only a weak electrochemical signal with low detection sensitivity was obtained.

In order to further testify the signal amplification effect of SiO_2-SA and Au-B-Gal, Au-B-Gal/SA/Phos-tag/P-Peptide/Au@C/GCE and Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE were fabricated. In the above electrodes, only one kind of signal amplification method was used for each electrode, SiO_2-SA or Au-B-Gal. In Fig. 1B, it is clear that the oxidation signals for PAP at Au-B-Gal/SA/Phos-tag/P-Peptide/Au@C/GCE (curve b) and Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE (curve c) were both weaker than that obtained at Au-B-Gal/SiO_2-SA/Phos-tag/P-Peptide/Au@C/GCE (curve a), and higher than that at Gal/SA/Phos-tag/P-Peptide/Au@C/GCE (curve d). These results indicated that the detection sensitivity was improved significantly in the presence of signal amplification unit. According to the above investigations, one can conclude that the developed method can be used to detect PKA activity with high sensitivity.

3.3. Detection performances

Under the optimal experiment conditions (see Supplementary Materials), the quantitative detection of PKA activity was performed. Fig. 2A showed the DPV response of biosensor with different concentrations of PKA. With increasing PKA concentration within the range of 0.05–100 unit/mL, the electrochemical oxidation peak current increased gradually with the linear regression equation of I_{pa} (μA) = −1.44 log$c$ (unit/mL) − 0.98 (R = 9961) (Fig. 2B). The detection limit was calculated to be 0.014 unit/mL (S/N = 3). The detection performances of our biosensor were compared with some of previous reports and results were listed in Table 1. The linear range of our biosensor was wider and the detection limit is lower than some of other biosensors, indicating that the detection performances were improved in our method.

The detection specificity is a key parameter for biosensor. In order to evaluate the specificity of the developed method, we selected CK1, CK2 and MAPK as possible interferents. For investigating it, the Peptide/Au@C/GCE was firstly incubated with the PKA reaction buffer containing 50 unit/mL PKA and 50 unit/mL other protein kinases, and then treated with the described process in Section 2.2. As illustrated in Fig. 2C, the electrochemical response of the biosensor for 50 unit/mL PKA was closed to that obtained at for PKA+CK1, PKA+CK2 and PKA+MAPK. This result demonstrated that CK1, CK2 and MAPK showed no effect on PKA detection and the proposed method possessed high detection selectivity.

The reproducibility is another important parameter for the applicability of the biosensor, thus it is also assessed by detecting 10 unit/mL PKA activity with seven group of biosensors (each group contained three biosensors), which are fabricated independently with the same process. As seen in Fig. 2D, the relative standard deviation (RSD) for the seven group of biosensors was 4.15%, demonstrating the acceptable detection reproducibility.

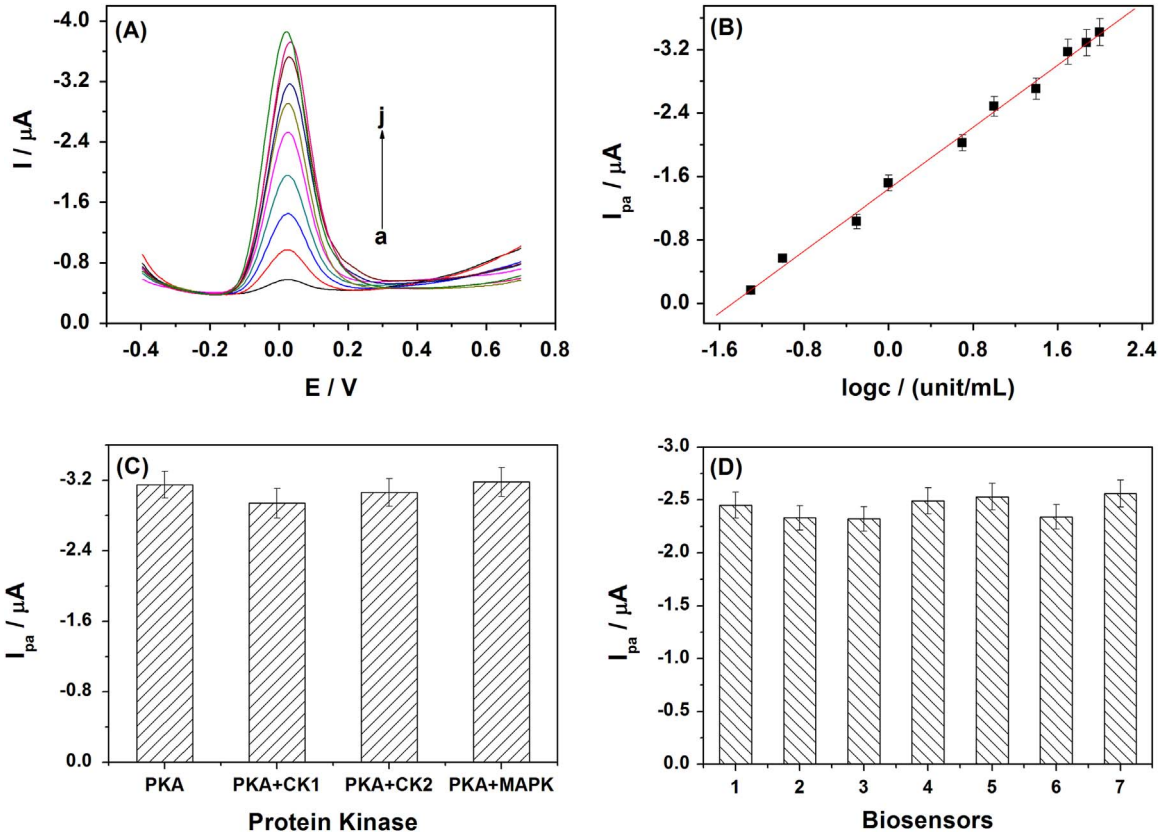


Fig. 2. (A) Differential pulse voltammograms of the biosensor with different concentration of PKA. a–j, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 75 and 100 unit/mL. (B) Calibration curve. (C) The histogram for the electrochemical response of the biosensors after incubated with different protein kinases. (D) The histogram for the electrochemical response of seven groups of biosensors.

Table 1
Comparison of detection performances of our biosensor with other biosensors.

Methods	Protein kinase	Linear range (unit/mL)	Detection limit (unit/mL)	Refs.
Electrochemistry	PKA	0.5–25	0.15	(Yin et al., 2014)
Electrochemistry	PKA	0.03–40	0.03	(Wang et al., 2014)
Electrochemistry	PKA	5–500	0.5	(Miao et al., 2012)
Fluorescence	CK2	0.08–2	0.027	(Song et al., 2015b)
Fluorescence	PKA	–	0.47	(Xu et al., 2011)
Fluorescence	PKA	0.1–10	0.05	(Liu et al., 2014a)
PEC	PKA	0.05–100	0.015	(Yin et al., 2015)
ECL	PKA	0.1–10	0.09	(Wang et al., 2015)
ECL	PKA	0.02–40	0.013	(Liang et al., 2014)
ECL	PKA	0.01–50	0.005	(Zhao et al., 2012)
Electrochemistry	PKA	0.05–100	0.014	This work

PEC: Photoelectrochemistry. ECL: Electrogenerated chemiluminescence.

3.4. Inhibition assay

In order to further evaluate the applicability of the developed method in PKA inhibition assay, two PKA inhibitors (HA-1077 and H-89) were selected as model inhibitors. The inhibition process was described in Section 2.4. As presented in Fig. 3, the DPV response of the biosensor decreased significantly after incubated with inhibitors. The half maximal inhibitory concentration (IC_{50}) was 1.35 μM and 41.5 nM for HA-1077 and H-89, respectively. These IC_{50} values were comparable with some of previous reports (Yin et al., 2014; Zhou et al., 2014; Zhou et al., 2015). These results indicated that the proposed detection strategy had great potential in the field of protein kinase inhibitor screening, which could provide some useful information for the discovery of the drugs targeting protein kinase.

3.5. Detection of PKA activity in cell lysates

PKA has crucial regulation roles in cellular process, such as transcription, apoptosis, and differentiation. In addition, it has also been found that the activity of PKA disordered in cancers, which might be indicated that PKA might represent a biomarker for tumor detection and identification, and might be a potential target for pharmacological treatment of tumors (Caretta and Mucignat-Caretta, 2011; Naviglio et al., 2009). Therefore, it is important to detect PKA activity in complex biological samples for better

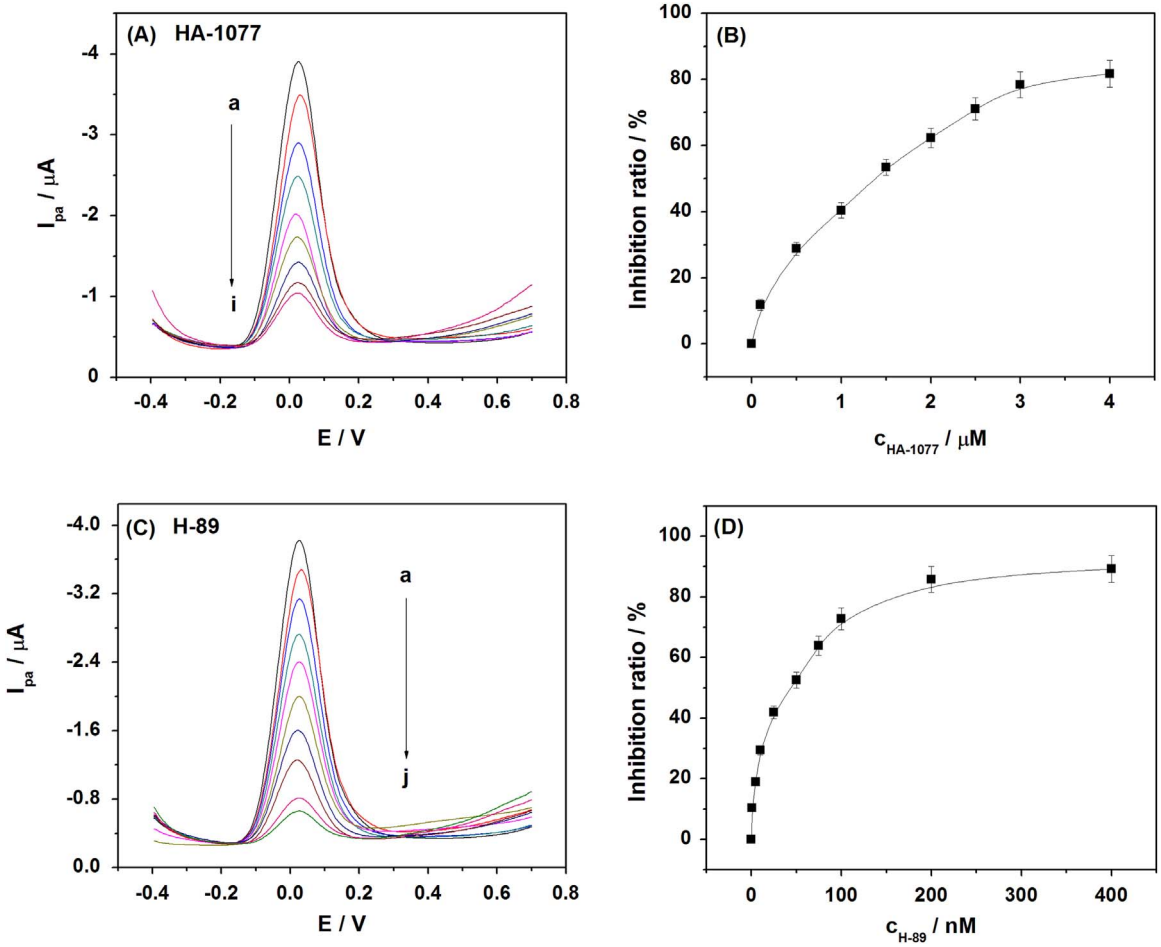


Fig. 3. (A) and (C) are the different pulse voltammograms of the biosensors after inhibited by different inhibitors. (B) and (D) are the relationship between inhibition ratio and inhibitor concentration. (A) and (B) HA1077, (C) and (D) H89. (A) a–i, 0, 0.1, 0.5, 1, 1.5, 2, 2.5, 3 and 4 μM. (B) a–j, 0, 1, 5, 10, 25, 50, 75, 100, 200 and 400 nM.

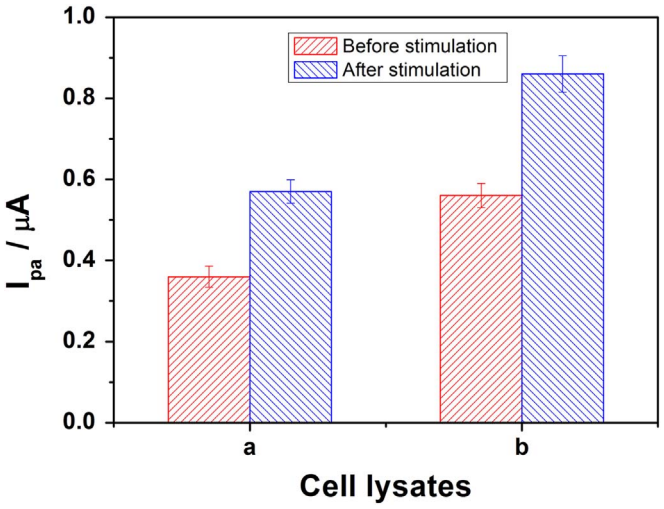


Fig. 4. Histogram for the electrochemical response of the biosensor incubated with different cell lysates. (a) Human normal gastricepithelial cell line GES-1, (b) human gastric carcinoma cell line BGC-823. The total protein concentrations of the cell lysates were 10 μg/mL.

understanding their functions and the role in cancer diagnosis, especially in cells or cell lysates. In order to testify the potential applicability of our method, PKA activity in the lysate of human gastric carcinoma cell line was investigated. As control, the activity of PKA in the lysate of human normal gastricepithelial cell line was also investigated. As shown in Fig. 4, the electrochemical response of the biosensor for PKA in the lysate of human gastric carcinoma cell line BGC-823 was higher than that for PKA in the lysate of human normal gastricepithelial cell line GES-1, indicating that the activity of PKA was increased in human gastric carcinoma cell line BGC-823, indicating that the activity disorder of PKA might be related to cancer development. These results also demonstrated that the developed method can be applied to detect PKA activity in complex biological samples.

It has also been reported that the combination of forskolin and IBMX can promote the concentration of cAMP, which can lead to the activity improvement of cAMP-dependent PKA in cells (Bai et al., 2013). For further verifying the applicability of our method, human normal gastricepithelial cell line GES-1 and human gastric carcinoma cell line BGC-823 were stimulated with 50 μM forskolin and 100 μM IBMX, and then PKA activity was detected. As illustrated in Fig. 4, the electrochemical response of PKA in both normal cell line and cancer cell line increased obviously after drug stimulation, indicating the increased activity of PKA. Therefore, the drug-induced improvement of PKA activity could be detected by the proposed detection strategy, which further proved that the fabricated biosensor can be applied to detect PKA activity in complex biological samples.

4. Conclusions

In summary, with the confluence of Au@C, SiO_2-SA and AuNPs-B-Gal, a sensitive electrochemical biosensor was fabricated and applied to detect PKA activity and its inhibitors. In the detection strategy, Au@C, SiO_2-SA and AuNPs-B-Gal can effectively improve the detection sensitivity, and B-Gal can catalyze the hydrolysis of PAPG to produce PAP under neutral conditions. Based on these favorable factors, the detection limit achieves to be 0.014 unit/mL. In addition, this detection strategy can also be used as a general platform for other protein kinase detection based on the simple change of substrate peptide. Moreover, the application of this method for PKA activity detection in cell lysates and PKA inhibition assay were also performed, indicating the potential application of the developed method in protein kinase-related drug screening and bio-functional researches.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.07.004.

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