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Zr^{4+}-mediated hybrid chain reaction and its application for highly sensitive electrochemical detection of protein kinase A

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ABSTRACT

An electrochemical platform has been developed to detect protein kinase activity through the combined actions of Zr^{4+} mediated signal transition and hybridization chain reaction (HCR)-stimulated DNAzymes nanowires. First of all, protein kinase A (PKA) phosphorylates substrate peptides immobilized on gold electrode surface. Thereafter, the DNA1 containing 5′-phosphoryl ends is linked to the phosphorylated substrate peptide via the robust phosphate-Zr^{4+}-phosphate linkages. By the introduction of molecular beacons (MBs), the DNA1 can open the hairpin structures of MBs through toehold mediated strand displacement (TMSDR), leading to an autonomous stem-opening process and subsequent assembly of G-quadruplex-containing DNA chains by HCR. After the addition of hemin, the formed HRP-mimicking DNAzymes can catalyze the hydroquinone-H_2O_2 system to generate amplified electrochemical signals. As expected, this method can achieve ultrahigh analytical performance with a low detection limit of 0.02U/mL and exhibit high cost-savings potential without the need for antibody, protease and labeling. Therefore, this method can serve as a new tool for the assay of protein kinase A and its inhibitor screening in the future.

1. Introduction

It is of great importance that protein kinases catalyze phosphorylation in essential cellular processes including signal transduction and regulation of multiple cellular functions [1,2]. Protein kinase A (PKA) is one of the kinases that transfer the phosphate groups from adenosine triphosphate (ATP) to serine or threonine residues of specific target proteins for phosphorylation[3] and runaway PKA may be tightly linked to multiple human diseases such as cancers[4,5], diabetes[6], and neurodegenerative diseases[7,8]. Accordingly, the precise evaluation of protein kinase A activity and further research on its inhibition are important approaches to confirm the early diagnosis of diseases and drug discovery[9,10].

Generally, commonly used methods to prepare gold standard data of protein kinase activities is radioisotopic techniques that involve radioactive ATP to monitor phosphorylation reactions [11]. Although these methods enable the sensitive detection of protein kinase activity, they require expensive specialized instruments and increase the risk of radioactive contamination. To address this issue, various techniques and approaches in recent times have been proposed to detect protein kinase-catalyzed phosphorylation, including electrochemical[12–14], electrogenerated chemiluminescence[15,16], colorimetric[17,18] and fluorescent approaches[19–21]. However, in most protocols, the usage of antibody, protease and labeling is inevitable, which brings some drawbacks, such as high cost, high time consumption, and complicated operation. Therefore, an ideal method for the detection of PKA is more straightforward, inexpensive and sensitive.

For this goal, we propose a straightforward electrochemical approach for the PKA assay by fully utilizing Zr^{4+} mediated signal transition and high signal amplification of hybridization chain reaction (HCR). After phosphorylation by PKA, the DNA1 containing 5′-phosphoryl end is linked to the phosphorylated peptide probe for HCR via the robust phosphate-Zr^{4+}-phosphate linkages. The chelation of Zr^{4+} and phosphate groups first proposed by Lee et al. [22] has been widely used in biosensing recently[23,24], which is also elaborately applied to this biosensor. Afterwards, the Hairpin-MBs containing G-quadruplex regions can be opened and immobilized on the electrode surface by HCR. Upon hemin

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addition, the formed G-quadruplex-hemin DNAzyme can catalyze peroxidation reaction to amplify electrochemical signaling[25]. Our approach differs from prior signal amplification methods such as nanoparticle-mediated signal amplification and traditional polymerase chain reaction (PCR) with strengths in the following three areas: (i) This method can be employed at a constant temperature and so removes the need for thermostable DNA polymerase and sophisticated laboratory equipment; (ii) This method is cost saving without the need for antibody, protease and labeling; (iii) The elongated feature of DNA nanowires can satisfy the demand of in situ amplification.

2. Experimental section

2.1. Reagents and materials

Peptides in our experiments were synthesized by Shanghai Science Peptide Biological Technology Co., Ltd and the peptides sequences were as follows: substrate peptide, LRRASLGGGGC. The HPLC-purified oligonucleotide (DNA1, MB1, and MB2) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The base sequences can be seen in Table S1. Fetal calf serum was obtained from Gibico (NY, USA). cAMP, cAMP-dependent protein kinase (PKA), ethylenediaminetetraacetic acid (EDTA), ellagic acid, $ZrOCl_2$, mercaptohexanol (MCH) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were all obtained from Sigma-Aldrich Chemical Co. Ltd. PBS solution (0.1 M, pH 7.4) was prepared by dissolving 0.2 g NaH_2PO_4, 2.2 g Na_2HPO_4 and 8.5 g NaCl in 1 L of deionized water. HEPES (1 M, pH 6–8) was prepared by dissolving 119.15 g HEPES in 0.5 L of deionized water[26]. Other chemicals were of analytical grade without further purification. Deionized water supplied by the Milli-Q purification system (Barnstead) was used for the preparation of all the solutions.

2.2. Preparation of substrate peptide-binding gold electrode

First of all, a gold electrode (diameter D = 3 mm) was etched with piranha solution (3:1 98% H_2SO_4/30% H_2O_2) for 5 min and thoroughly washed with water. Next, the electrode was polished to a mirror-finishing with 3000-grit SiC papers and alumina slurry (1 μm, 0.3 μm, and 0.05 μm). The electrode was further bathed in a nitric acid solution (50%) for 0.5 h. Hereafter, it was sonicated in ethanol and deionized water for 5 min each, then electrochemically cleaned in 0.5 M H_2SO_4 to remove off impurities. The obtained clean electrode was taken to dryness by flushing nitrogen gas and incubated under room temperature with 0.2 mM peptide solution (pH 6.0) containing 20 mM HEPES and 10 mM TCEP for 12 h, followed by blocking with 0.1 mM MCH for 0.5 h to minimize nonspecific binding of proteins. Finally, the electrode was rinsed with PBS + 0.5% Tween (PBST), PBS and pure water one after another.

2.3. Phosphorylation catalyzed by PKA

PKA was diluted with 10 mM HEPES (pH 6.5) containing 200 μM ATP, 600 μM cAMP, 5 mM TCEP, and 10 mM $MgCl_2$ to achieve various concentrations. Next, the peptide-modified electrodes were submerged into above solutions and kept at 37 °C for 0.5 h. After that, they were washed thoroughly using PBS. Inhibition experiments were conducted by adding variable amounts of the ellagic acid to the PKA solution in this study.

2.4. Zr^{4+} mediated ligation of DNA1 to peptide and the initiation of HCR

The peptide-modified electrode treated with PKA in advance was soaked in 0.2 mM Zr^{4+} solution at ambient temperature for 60 min, resulting in the binding of Zr^{4+} to the phosphorylated site of the peptide via the chelation of Zr^{4+} and phosphate groups. Similarly, Zr^{4+} as a mediator can continue the combination with DNA1 containing 5′-phosphoryl ends by incubating the above electrode in 1 μM DNA1 solution in the same conditions. After being washed 5 times with HEPES buffer, the electrode was further incubated with the MB mixture (MB1 2 μM, MB2 2 μM) to initiate HCR and the resulted nanowires were subsequently assembled onto the DNA1[27]. After 1 h, the electrode was removed and rinsed thoroughly with deionized water prior to addition of 0.1 mM hemin in HEPES buffer (20 mM, pH 8.0) for 0.5 h to induce G-quadruplex-hemin formation[27].

2.5. Electrochemical Measurements.

A CHI600D electrochemical instrument (Chenhua Shanghai) was used to conduct the Differential Pulse Voltammetry (DPV) and Electrochemical impedance spectroscopy (EIS) with three-electrode system including working electrode (gold electrode), reference electrode (saturated calomel electrode) and counter electrode (platinum auxiliary electrode). The EIS study was realized employing a 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 1 M KNO_3 and the experiments were performed at a bias potential of 0.225 V, using 5 mV amplitude at frequencies between 0.1 Hz and 100 kHz. DPV measurement was carried out in 5 mL of 0.1 M PBS (pH 7.4) containing 1.0 mM H_2O_2 and 0.2 mM hydroquinone, with a scan range of − 0.1 to 0.2 V.

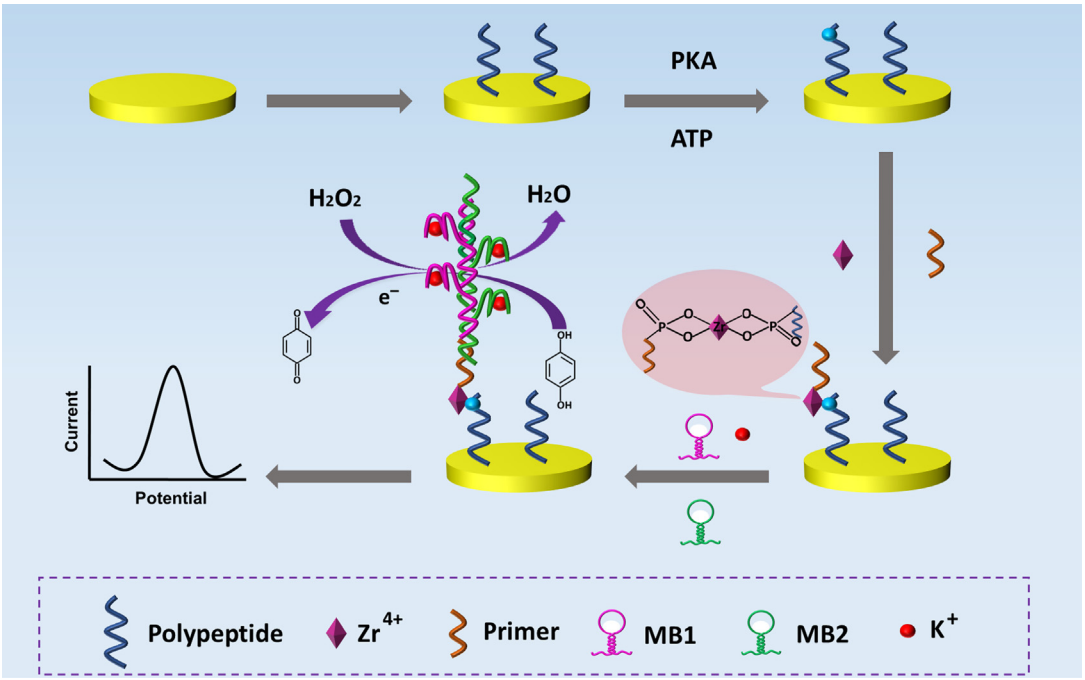
3. Results and discussion

3.1. Schematic overview of the biosensor

The rationale of fabricated biosensor is shown in Scheme 1. First, the substrate peptides are decorated on the gold electrode through their cysteine ends. Since the phosphate groups of ATP can be transferred to serine of the substrate peptides by PKA, Zr^{4+} are tightly attached to the phosphorylated sites via the well-established phosphate-Zr^{4+}-phosphate chemistry. Subsequently, Zr^{4+} perform as bridge to link the DNA1 with 5′-phosphoryl end in the same way. DNA1 can open the hairpin structure by hybridizing with the toehold of MB1. The arising structure subsequently opens MB2 via strand displacement reaction. The autonomous opening of MB1 and MB2 enables polymer nanowires consisting of the G-quadruplex units assemble on the electrode surface. As the MBs contain G-quadruplex forming region, G-quadruplex-hemin DNAzyme can be formed stably by addition of hemin and K^+, which could catalyze the oxidation of hydroquinone by hydrogen peroxide to generate a redox reaction on the surface of the electrode and produces an electrochemical signaling. Moreover, the signal response is proportional to PKA.

3.2. Biosensor feasibility assay

The biosensor for PKA activity based on HCR conducted in PBS (0.1 M, pH 7.4) containing 1.0 mM H_2O_2 and 0.2 mM HQ. As shown in Fig. 1A, when PKA is not present, only a weak peak current is shown (black line), close to the blank control (red line). But the current increases markedly after the PKA is introduced to electrode modified with peptides for 60 min (blue line). This increase in current is mainly attributed by the addition of PKA, which can catalyze



Scheme 1. Schematic representation of the electrochemical method for PKA assay.

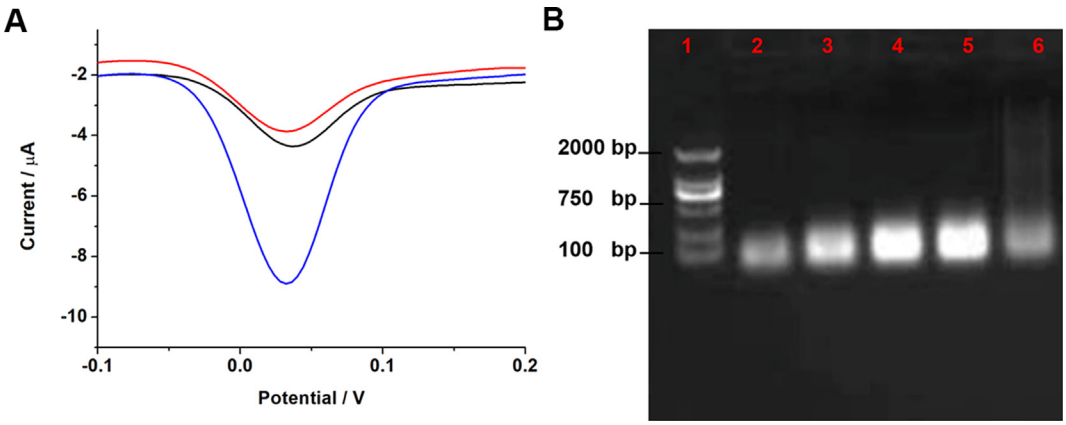


Fig. 1. (A) DPV curves of the biosensor for PKA activity. Red line: blank control; black line: in the absence of PKA; blue line: in the presence of PKA. (B) PAGE analysis of the products with lane 1: DNA marker, lane 2: 2 μM DNA1, lane 3: 2 μM MB1, lane 4: 2 μM MB2, lane 5: 1 μM MB1 + 1 μM MB2, and lane 6: 1 μM DNA1 + 1 μM MB1 + 1 μM MB2.

peptides phosphorylation. Zr^{4+} can double-link the phosphorylated peptides and DNA1 with 5′-phosphoryl end which will serve as an initiator to trigger the following HCR reaction. A large amount of G-quadruplex-Hemin HRP-mimicking DNAzymes generated can catalyze peroxidation reaction.

PAGE analysis is also used to investigate the viability of the sensing strategy. As can be seen from Fig. 1B, compared with the bands of DNA1 (lane 2), MB1 (lane 3), MB2 (lane 4), and MB1 + MB2 (lane 5), DNA1 + MB1 + MB2 (lane 6) appears a band with slower rate and dispersion, which indicates that DNA1 can successfully open hairpin probes of MB1 and MB2 for HCR, and there is no spontaneous reaction between hairpin probes MB1 and MB2. The above results indicate that the DNA1 and hairpin probes designed in this experiment could be successfully applied to the reaction.

3.3. Characterization of electrode modification process

Each step in the modification of the electrode has been characterized using Nyquist plot of impedance. As seen in Fig. 2, the bare electrode contains no semicircle portion (black line), indicating good electron transfer capability. A small semicircle can be observed when the substrate peptides are fully immobilized on the electrode surface (red line). A larger semicircle is present following phosphorylation of peptides by PKA (blue line) because of electrostatic repulsion between the negatively charged phosphorylation sites and the redox species ($[Fe(CN)_6]^{3-/4-}$). Besides, the trapped DNA1 and the subsequently generated DNA nanowires with phosphate backbones are negatively charged, resulting in the further suppression of interfacial charge transfer. Therefore, the semicircle diameter grows larger in size as the experiment

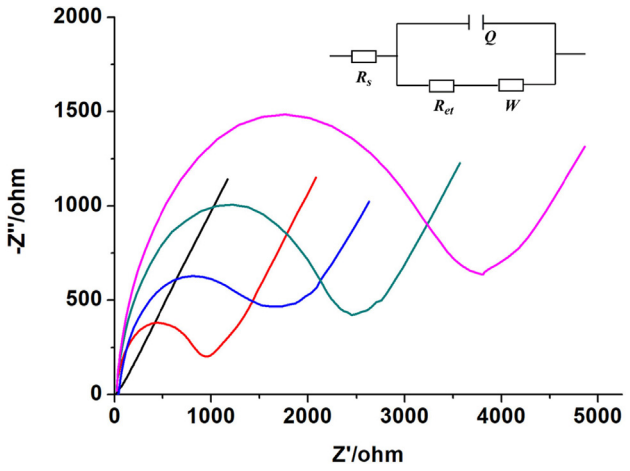


Fig. 2. EIS measurements for different modified electrodes. black line: bare electrode; red line: above electrode after substrate peptides modification; blue line: above electrode after incubation with PKA; green line: above electrode after incubation with Zr^{4+} and DNA1; pink line: above electrode after incubation with MB mixture.

proceeded (green line and pink line). The above results indicate the modified process is successful.

3.4. Optimization conditions

To harvest the best-performing biosensor, several significant experimental parameters including substrate peptide, Zr^{4+} and DNA1 concentrations as well as PKA phosphorylation time, Zr^{4+} incubation time and MB1 and MB2 hybridization time have been optimized. According to the results, the optimal conditions we employed are as follows, substrate peptide concentration, 0.2 mM (Fig. S1); PKA phosphorylation time, 30 min (Fig. S2); Zr^{4+} incubation time, 60 min (Fig. S3); Zr^{4+} concentration, 0.2 mM (Fig. S4); DNA 1 concentration, 1 mM (Fig. S5); hybridization time between MB1 and MB2, 60 min (Fig. S6).

3.5. Detection of PKA activity

Under optimum conditions, the current increases with PKA concentration growing from 0.05 to 100 U/mL (Fig. 3). Moreover, in the range between 0.05 and 100 U/mL, the current is proportional to the logarithm value of PKA concentration with the linear regression equation of y = 1.47x + 7.35 (R^2 = 0.991), which is shown as insert in Fig. 3. The detection limit of this biosensor is estimated to be 0.02 U/mL (S/N = 3), which is lower than most of other techniques, such as electrogenerated chemiluminescence (ECL) and fluorescence (Table 1). Therefore, this analytic approach for protein kinase activity has potential application value.

3.6. Specificity, reproducibility and stability of the biosensor

For detecting the specificity of the biosensor, the substrate peptides are incubated with several other protein kinases respectively, including HAT, DNMT1 and MAPK, which are incapable of recognizing and phosphorylating the serine residue in the substrate peptide of LRRASLGGGGC. The electrode is further treated the same as hereinbefore and the current is recorded. As can be seen in Fig. 4A, remarkable, other protein kinases fail to trigger a sequential series of reactions, so the current is much lower than that brought about by PKA, demonstrating acceptable detection specificity.

The reproducibility of the biosensor is confirmed by preparing five electrodes to detect 50 U/mL of PKA. The relative standard deviation (RSD) of triplicate measurements is 3.7% (Fig. 4B). This experimental result demonstrates acceptable reproducibility of the biosensor. The long-term storage stability of the biosensor is also evaluated. When the working electrode is stored at 4 °C for 14 days, the peak current remains 92.6% of its original value, implying good stability.

3.7. Assay of PKA activity in cell lysates

The PKA activities are determined in cell lysates of MCF-7 and MDA-MB-231 (10^6 cells) using our developed method and human hepatic cell (L-02) serves as control. In sequential order, the sub-

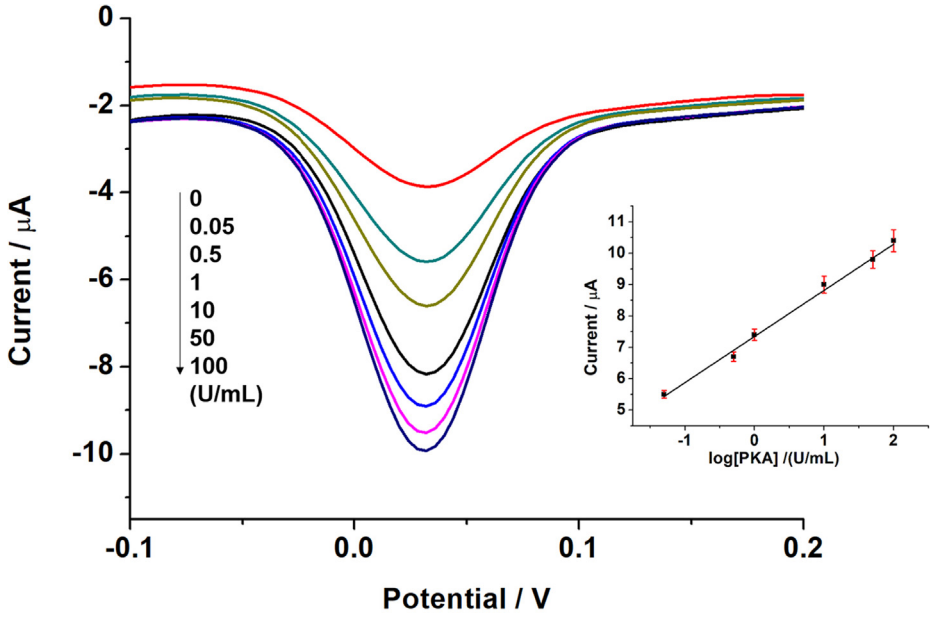


Fig. 3. DPV curves for different concentrations of PKA (U/mL). (a) 0, (b) 0.05, (c) 0.5, (d) 1, (e) 10, (f) 50, (g) 100 U/mL in 5 mL of 0.1 M PBS (pH 7.4) containing 1.0 mM H_2O_2 and 0.2 mM hydroquinone (HQ). The inset is the linear calibration curve of peak current value versus the logarithm of the concentration of PKA. Error bars indicate standard deviation of three independent experiments.

Table 1
A comparison of the method with previously reported ones.

Detection Method	Linear Range	Detection limit	References
Electrogenerated chemiluminescence	0.07U·mL^{-1} –32U·mL^{-1}	0.07U·mL^{-1}	[15]
Electrogenerated chemiluminescence	0.1U·mL^{-1} –10U·mL^{-1}	0.09U·mL^{-1}	[28]
Fluorescence	0.1U·mL^{-1}-5.0U·mL^{-1}	0.039U·mL^{-1}	[29]
Fluorescence	0.1U·mL^{-1}-2000U·mL^{-1}	0.039U·mL^{-1}	[30]
Electrochemical (Glassy carbon electrode)	0.1U·mL^{-1}-500U·mL^{-1}	0.053U·mL^{-1}	[13]
Electrochemical (Gold electrode)	0U·mL^{-1}-200U·mL^{-1}	0.083U·mL^{-1}	[31]
Electrochemical (Gold electrode)	0.01U·mL^{-1}-0.5U·mL^{-1}	0.01U·mL^{-1}	[32]
Electrochemical (Gold electrode)	0.05U·mL^{-1}-100U·mL^{-1}	0.02U·mL^{-1}	This study

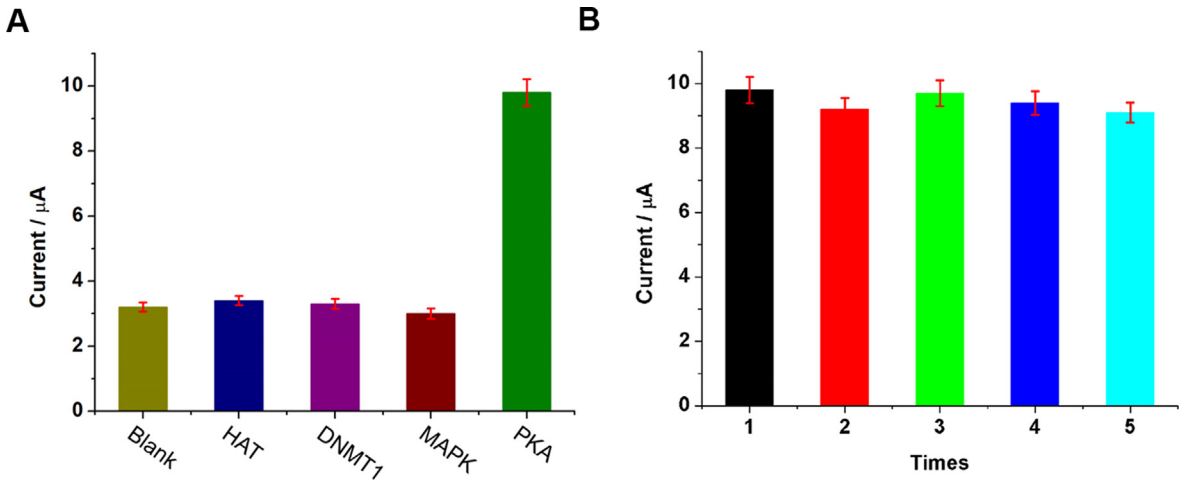


Fig. 4. (A) Specificity of the biosensor. Comparison of the DPV peak currents in the presence of HAT, DNMT1, MAPK and PKA, respectively, in which the blank is PBS buffer. The concentrations of PKA and interfering proteins are about 50 U/mL respectively. The error bars are the standard deviations from three independent experiments. (B) The histogram for the peak current of the biosensor for five parallel detections. The concentration of PKA is about 50 U/mL and the error bars are the standard deviations from three independent experiments.

strate peptides are first incubated with cell lysates prior to incubation with Zr^{4+}, DNA1, and MB, and the current response is recorded. As seen in Fig. 5, the current for MCF-7 and MDA-MB-231 cell lysates incubated electrode is significantly higher than that obtained at L-02 cell lysates, indicating that PKA in human cancer cells possess much higher activity than those in normal cells. Moreover, PKA activity in MDA-MB-231 is slightly higher than that in MCF-7, implying the higher malignancy of tumor, the higher activity of PKA. The above results coincide well with previous reports and indicate that our developed method to assay PKA activity is available for a complex biological system.

3.8. Inhibition experiments

Since aberrant protein kinase activity has been associated with many diseases including cancers, validation of PKA inhibitors appears valuable for protein kinase-targeted drug screen and tar-

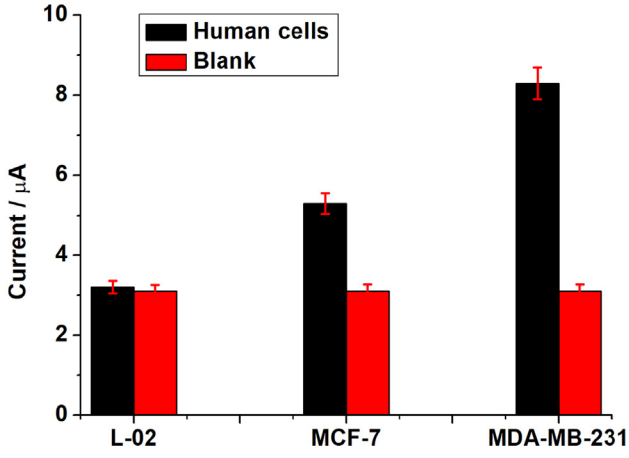


Fig. 5. Measurements of PKA levels in the lysates of breast cancer cells of MCF-7 and MDA-MB-231 and normal cells. The blank is PBS. The error bars are the standard deviations from three independent experiments.

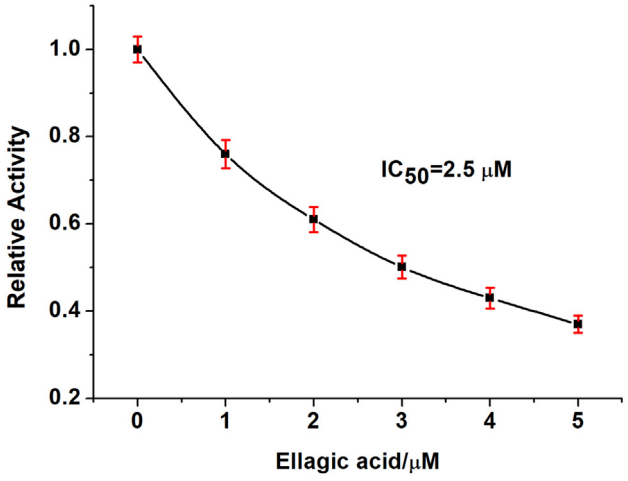


Fig. 6. The inhibition effect of ellagic acid on the PKA activity. The IC50 value is calculated to be 2.5 μM.

geted therapy for related diseases[33]. Therefore, the PKA activity is measured after treatment with various concentrations of PKA inhibitors. Herein, ellagic acid, a potent and cell-permeable inhibitor of PKA, is used[34]. As displayed in Fig. 6, the electrochemical reduction signal is attenuated in response to increasing concentrations of ellagic acid, which reveals that inhibiting PKA activity leads to less substrate peptide phosphorylation. Moreover, the IC50 value (half-maximal inhibitory concentration) is obtained to be 2.5 μM, basically consistent with previous reports[35].

4. Conclusion

To be concluded, in this work, we have designed a highly sensitive electrochemical method to detect PKA activity through the combined action of Zr^{4+} mediated signal transition and HCR-stimulated DNAzymes nanowires. Finally, a detection limit of as low as 0.02U/mL is estimated. Our proposed biosensor offers the prospects of both a new technology of monitoring phosphorylation and protein kinase activity and a new insight into hybrid chain reaction used for protein assays. Furthermore, this biosensor is successfully applied to assay protein kinase activity in cell lysates and confirmed by inhibition experiments, which shows great potential for kinase-related drug development, disease early detection and therapeutic efficacy assessment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2021.107796.

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