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Aptamer based electrochemical assay for protein kinase activity by coupling hybridization chain reaction

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ABSTRACT

The present work reported a simple, lable-free and sensitive electrochemical method for the detection of protein kinase A (PKA) activity. This method was based on the specific recognition of aptamer and the aptamer-induced hybridization chain reaction (HCR) amplification strategy. The aptasensor was constructed by immobilizing capture probe on a gold electrode via an Au–S bond. When adenosine triphosphate (ATP) aptamer was introduced, its one terminus hybridized with capture probe and the other hybridized with the complementary region of an auxiliary probe, which other region triggered HCR between two hairpin DNA (H1 and H2) to form a long DNA concatamer. At last a large number of electroactive methyle blue (MB) molecules were assembled on the dsDNA concatamer, which generated a significantly amplified electrochemical signal. In the presence of ATP, the HCR would not be performed because the aptamer specifically bond to ATP and the electrochemical response would decrease. However, when ATP and PKA coexisted, the electrochemical response would recovery because that ATP had been translated into ADP by PKA. So the activity of PKA could be effectively monitored according to the change of electrochemical signal. Based on the HCR amplification strategy, the aptasensor showed a wide linear range ($4 - 4 \times 10^5$ U L^{-1}) and a low detection limit (1.5 U L^{-1}) for the detection of PKA. Furthermore, the method was applied to study the inhibitory effect of H-89 on PKA activity. The developed aptasensor was also used to the analysis of drug-induced PKA activity in cell lysates, indicating the potential application of the developed method in the fields of clinical diagnostics and discovery of new targeted drugs.

1. Introduction

Cyclic AMP (cAMP)-dependent protein kinases regulate multiple processes in eukaryotes by phosphorylating diverse cellular substrates, including metabolic and signaling enzymes, ion channels and transcription factors (Natasha et al., 2016). Due to their primary role in cell physiology and pathology, protein kinases have become major drug targets to counteract human diseases, such as heart failure and cancer (Kim et al., 2016). The function of protein kinase A is to transfer the g-phosphate of ATP to Ser/Thr/Tyr residues of substrates, thereby activating or deactivating various signaling pathways (Endicott et al., 2012). So the over expression and aberrant regulation of protein kinases may resulted in severe diseases such as cancer, diabetes, immune diseases, and cardiac diseases (Wang et al., 2012, 2017). Therefore, developing a simple, sensitive and low-cost method for the detection of protein kinase activity is of great importance in clinical diagnosis, targeted therapy and drug discovery (Liu et al., 2017).

At present, the standard method for analyzing protein kinase activity is the autoradiography technique by use of ^{32}P-labeled adenosine triphosphate (Houseman et al., 2002; Hutti et al., 2004). However, the radioactive labels are harmful to the environment and human health. Thus, a few non-radioactive methods have been developed recently for the detection of protein kinase activity, including fluorescence (Yin et al., 2015; Bai et al., 2013), flow cytometry (Ren et al., 2013), quartz crystal-microbalance (Xu et al., 2012), electrochemistry (Zhou et al., 2016; Wang et al., 2012; Marti´c et al., 2012), mass spectrometry (Kim et al., 2007), colorimetric methods (Wang et al., 2006; Oishi et al., 2007), magnetic resonance imaging (Shapiro et al., 2009) as well as electrogenerated chemiluminescence (Xu et al., 2010; Liang et al., 2016). However, most of these methods require laboratory-based expensive and sophisticated instruments such as mass spectrometry and fluorescence. Therefore, it is urgent to develop a simple, low-cost and sensitive method for the assay of protein kinase.

Recently, the electrochemical methods have attracted many attentions on protein kinase assay due to the advantages of simple device, easy operation, inexpensive cost, fast detection, micro-volume sample,

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and high sensitivity. In a general design of electrochemical kinase biosensors, the kinase-specific peptide substrates are immobilized on an electrode surface. After phosphorylation, the resulting phosphorylated peptides can be recognized by special elements, such as anti-phosphorylated peptide antibodies (Wilner et al., 2008), metal ions (Zr^{4+}, Fe^{3+}, Ag^{+} and Tb^{3+}) (Miao et al., 2012; Wang et al., 2013, 2014; Yang et al., 2015), TiO_2 nanoparticles (Ji et al., 2009) and ATP derivates (Wang et al., 2006; Liu et al., 2014; Zhao et al., 2015). These electrochemical methods with peptides as substrates have made great advances for evaluating the activities of protein kinases. However, there are still some deficiencies which need to be overcame. For example, most of these methods need specific peptides as templates, the design strategies are more complex, some biochemical reagents such as anti-phosphorylated peptide antibodies are expensive, and the synthesis of ATP derivatives is difficult and time-consuming.

Aptamers are artificial oligonucleic acids to bind target molecules with high affinity. Compared to other molecular recognition system, aptamers show a lot of advantages such as the simplicity of synthesis, excellent stability, and ease of labeling. Recently the electrochemical aptamer biosensors have attracted significant attention due to the excellent specificity, simple instrument, and fast response (Lu et al., 2008; Zhu et al., 2012; Yang et al., 2014). However, a challenge in electrochemical aptasensor design is to improve the sensitivity for low level target detection. Nowadays, many signal amplification strategies have been developed to improve the detection sensitivity, including hybridization chain reaction (HCR) amplification (Hou et al., 2015; Ge et al., 2014), rolling circle amplification (RCA) (Liu et al., 2013), and isothermal exponential amplification (Liu et al., 2012; Yu et al., 2014). Among them, HCR shows great potential in signal amplification because of its unique features such as simple, cost-effective, isothermal, and sensitive. HCR is an enzyme-free and triggered self-assembly strategy to construct one-dimensional DNA nanostructure. Since HCR was found in 2004, it has been widely used to fabricate a variety of biosensors for the sensitive detection of oligonucleotides (Jia et al., 2016), proteins (Xu et al., 2013), cancer cells (Sharma et al., 2012) and so on (Jia et al., 2018).

Inspired by the aforementioned works, we reported a novel electrochemical aptasensor for evaluating the activity and inhibition of PKA based on aptamer (Apt) recognition and HCR amplification strategy. The presence of Apt can lead to the formation of dsDNA polymers on the sensing surface through in situ HCR. Numerous indicators, methyle blue (MB), thus can be efficiently intercalated into the grooves of the dsDNA polymers to generate the signals. In the presence of ATP, the Apt would bind to ATP with high affinity, which prevented the assembly of HCR on the electrode surface. As a result, a significant decrease in electrochemical signal was observed. However, when PKA was added, the electrochemical signal would recover because that ATP would be translated into ADP by PKA and ADP can not combine with Apt. Based on this "signal-off" and "signal-on" mode, the quantitative assay for ATP and PKA can be readily realized. The traditional assays of PKA activities were typically performed by the phosphorylation of specific peptide substrate then the interaction of the substrates with various signal probes. However, to the best of our knowledge, few studies have been reported for the detection of PKA based on the aptamer recognition (Wang et al., 2017). Importantly, the aptasensor introduced HCR amplification strategy, which enhanced the response sensitivity and decreased the detection limit. Furthermore, the aptasensor was free-labeled and free-enzyme, which enabled the fabrication simple. So this work provided a new strategy for the assay of PKA activity.

2. Experimental

2.1. Materials

All oligonucleotides used in the experiment were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China), and their sequences (from 5′ to 3′) are listed in Table S1.

Protein kinase A (PKA, catalytic subunit from bovine heart), adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), uridine triphosphate (UTP), horse radish peroxidase (HRP), glucose oxidase (GOx), exonuclease I (Exo I), exonuclease II (Exo II), thrombin, bovine serum albumin was purchased from Sigma-Aldrich. Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), tris-(hydroxymethyl)-aminomethane, 6-mercapto-1-hexanol (MCH), and methyl blue (MB) were purchased from Aladdin Inc. (Shanghai, China). Forskolin, 3-isobutyl-1-methylxanthine (IBMX) and H-89 were purchased from Beyotime Institute of Biotechnology (Shanghai, China). One step animal cell active protein extraction kit (BSP022) was purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Other reagents such as $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, NaCl, KCl, $MgCl_2$, H_2SO_4, Na_2HPO_4 , NaH_2PO_4 were purchased from Shanghai Reagent Corporation (Shanghai, China). All these reagents were of analytical grade and without further purification. All oligonucleotides solutions were prepared with Tris buffers (25 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4). Furthermore, the capture probes were reduced in 2 mM TCEP for 1 h to cleave disulfide bonds. Electrochemical measurements was performed in 0.1 M PBS buffer (pH 7.4). All solutions were prepared with Milli-Q water (18 mΩ·cm resistivity) from a Millipore system.

2.2. Electrochemical measurements

Differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) were performed with a CHI 760 C electrochemical workstation (CH Instruments, Shanghai, China). All electrochemical experiments were performed with a conventional three-electrode system comprising of a gold working electrode, a platinum wire auxiliary electrode, and a Ag/AgCl reference electrode. The DPV measurements were performed in 0.1 M PBS (pH 7.4) by using a potential window from 0 to −0.5 V with scan rate of 100 mV s^{-1}. The potential increments was 4 mV, the amplitude and pulse width were both 0.05 V, and the pulse period was 0.2 s. Before the DPV measurements, the electrolyte solution should be thoroughly purged with high purity nitrogen to avoid the interference of oxygen. EIS measurements were carried out in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl with the frequency range from 0.1 Hz to10 kHz. The amplitude of the applied sine wave potential was 5 mV and the formal potential of the system was set at + 0.22 V.

2.3. Pretreatment of gold electrode

Briefly, a gold electrode (2 mm in diameter) was polished on a microcloth with 0.05 μm alumina slurry for 5 min, followed by sonicating in ethanol and water for 5 min, respectively. Then the polished electrode was electrochemically cleaned in 0.5 M H_2SO_4 solution by scanning from −0.3 V to + 1.5 V until a steady-state cyclic voltammogram was obtained. After being thoroughly rinsed with Milli-Q water and dried with nitrogen, the cleaned electrodes should be immediately used for DNA immobilization.

2.4. Fabrication of the sensing platform

First, 6 μL of 1.0 μM thiolated CP solution was dropped on the cleaned gold electrode surface and incubated for 3 h at 37 °C to form self-assembly monolayers by Au-S bond. Next, the electrode was immersed into 1 mM MCH for 0.5 h to remove the nonspecific DNA adsorption. After rinsed thoroughly and dried with nitrogen, 6 μL of 1.0 μM Apt solution was dropped on the electrode surface for 1.5 h at 37 °C. Then the electrode was covered with 6 μL of 1.0 μM AP solution. After 1.5 h incubation time, the electrode was immersed into the mixture solution containing 1.0 μM H1 and 1.0 μM H2 for 1.5 h to perform HCR. Finally, 6 μL of 1 mM MB was dropped on the electrode surface for

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