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Aptamer based fluorescence biosensor for protein kinase activity detection and inhibitor screening

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ABSTRACT

Herein, we firstly used double-strand-templated DNA Cu nanoclusters (Cu NCs) as a simple, lable-free and sensitive fluorescence (FL) probe to detect protein kinase (PKA) activity. This method was based on the strong interaction between adenosine triphosphate (ATP) aptamer and ATP. When ATP was added, fluorescent Cu NCs could not be formed due to the lack of effective substrate and the fluorescence of Cu NCs decreased. However, when PKA was added, the fluorescence of Cu NCs recovered because that ATP had been translated into ADP by PKA and ADP can not combine with ATP aptamer. We can effective monitored the activity of protein kinase according to the variation of fluorescence signal in the range of 0.1–1000 mU/μL. The detection limit (LOD) is 0.041 mU/μL. We also used this method to detect protein kinase inhibitor H-89. In addition, this method was also used to explore the activity of drug-induced PKA in Hela cells, which makes the sensor had a great application value in biochemistry and targeted kinase drug discovery research.

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1. Introduction

Protein kinase is a kind of enzyme which can transferring phosphate groups from nucleoside triphosphates (usually adenosine-5′-triphosphate, ATP) to specific amino acids, so that catalyzing the phosphorylation of proteins [1,2]. It plays a crucial role in many fundamental biological processes, such as cell growth, metabolism, differentiation, and apoptosis [3,4]. The overexpression and aberrant activity of protein kinases can cause abnormal protein phosphorylation processes and even cause a number of severe diseases such as cancer, diabetes, inflammation, cardiac diseases, and Alzheimer's disease [5,6]. Therefore, the development of simple and sensitive methods for PKA activity and inhibitors are of great significance for clinical diagnosis the research of fundamental biochemical pathways, clinical diagnosis and drug discovery [7,8].

Up to now, lots of methods had been used to identify and quantify kinases, such as electrochemistry [9–11], fluorimetry [12,13], colorimetry [14,15]. Among these methods, the fluorimetry had attracted more and more attentions of researchers due to its high sensitivity, simple, and high-throughput capability. However, most of these methods for protein kinases activity relied on the substrate peptides which were specific to the target kinase. For example, Wang and his coworkers used peptide modified graphene quantum dots to detect the activity of PKA [16]. After phosphorylated by PKA, peptide modified graphene quantum dots can be aggregated and cause the fluorescence quenching in the presence of Zr^{4+}. This method needs peptides as templates and it needs expensive raw materials, more complex design process. Therefore, it is of great significant to find a simple, low cost, and lable-free method for protein kinase activity detection.

Metal nanoclusters (NCs) which consist several to dozens of atoms, have now developed into a new class of fluorophores [17,18]. Its excellent physical and chemical properties have attracted more and more interests of researchers, such as high quantum yield, low toxicity, large Stokes shift, good photostability and excellent biocompatibility, etc [19]. Among fluorescent metal NCs, DNA-templated Cu NCs are of particular interest owing to its high specificity and easy operation without rigorously controlled temperature and arduous probe DNA design [20]. Now, the fluorescent Cu NCs has been wildly applied in the construction of biosensors [21], and biological imagings [22].

In this paper, we firstly used Cu NCs as a simple and sensitive fluorescence probe to detect protein kinase activity. This method was based on the strong interaction between ATP aptamer and ATP. When ATP existed, the fluorescence of Cu NCs would decrease due to the lack of effective substrate. However, when PKA was added, ATP could be translated into ADP by PKA and ADP can not combine

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with ATP aptamer. Thus the fluorescence of Cu NCs recovered, and the activity of protein kinase can be effective monitored according to the variation of fluorescence intensity.

2. Experiment

2.1. Reagents and instruments

The oligonucleotide (A Strand: 5′-ACCTGGGGGAGTATTGCGGAGGAAGGT-3′; B Strand: 5′-ACCTTCCTCCGCAATACT-3′) was synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) and purified by PAGE. Protein kinase A (PKA, catalytic subunit from bovine heart), Forskolin (from Coleus forskohlii, ≥98%), 3-isobutyl-1-methylxanthine (IBMX, BioUltra, ≥99%), Adenosine 5′-Triphosphate (ATP) disodium salt hydrate, Adenosine 5′-Diphosphate (ADP) sodium salt, Urease, Glucose oxidase (GOx), Exonuclease I (Exo I), Thrombin and H-89 were purchased from Sigma–Aldrich Co. $CuSO_4$ (99.99%) was obtained from Aladdin Co. Other inorganic reagents (analytically pure) were purchased from Beijing Chemical Co. and were used as received. Unless otherwise noted, all the stock and buffer solutions were prepared using ultrapure water (electric resistance ≥ 18 MΩ).

Fluorescence measurements were carried out by using a RF-5301 PC spectrofluorophotometer (Shimadzu, Japan) equipped with a xenon lamp using right-angle geometry. Ultraviolet spectra were performed on a Shimadzu UV-1700 UV–visible spectrophotometer (Shimadzu Co., Kyoto, Japan). Transmission electron microscopy (TEM) was conducted using a Hitachi H-800 electron microscope at an acceleration voltage of 200 kV with a CCD camera. All pH measurements were made with a PHS-3C pH meter (Tuopu Co., Hangzhou, China). All temperature measurements were accomplished using a water bath. In this experiment, a 0.7 mL quartz cuvette was used.

2.2. Synthesis of fluorescent Cu NCs

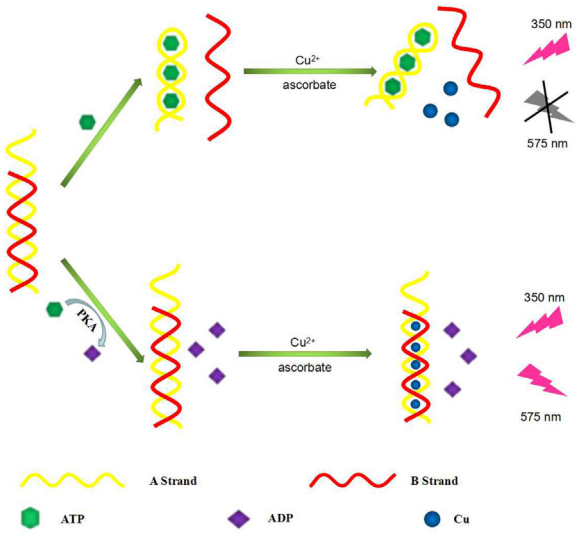
Briefly, 0.5 μM A strand, 0.5 μM B strand, and 1.8 mM ascorbate were dissolved in PBS-Buffer (10 mM, pH = 7.4) and incubated at ambient temperature for at least 15 min. Then, 100 μM $CuSO_4$ was added into the solution with PBS-Buffer (10 mM, pH = 7.4) to give final volumes of 400 μL. The system was allowed to react for 20 min under a gentle stirring. For optimization of copper ion concentration, different concentration of $CuSO_4$ was added. After 20 min, the fluorescence of the mixture was measured.

2.3. Fluorescence detection of ATP

0.5 μM A strand, 0.5 μM B strand and 1.8 mM ascorbate were added into a 2 mL test tube and incubated at ambient temperature for at least 15 min. Then, a certain amounts of ATP was added into the mixture. After 10 min, 100 μM $CuSO_4$ was added into the solution with PBS-Buffer (10 mM, pH = 7.4) to give final volumes of 400 μL. The final concentrations of ATP were 0, 5, 10, 20, 30, 40, 50, 60, 70, 80 μM, respectively. The fluorescence spectra were measured upon being excited at 350 nm. All of the measurements were performed three times.

2.4. Protein kinase activity detection and inhibitor screening

0.5 μM A strand, 0.5 μM B strand and 1.8 mM ascorbate were added into a 2 mL test tube and incubated at ambient temperature for at least 15 min. Then, 70 μM ATP and a certain amounts of PKA were added for another 60 min. After that, 100 μM $CuSO_4$ was added into the solution with PBS-Buffer (10 mM, pH = 7.4) to give final volumes of 400 μL. The final concentrations of PKA were 0, 0.1, 0.5, 1, 5, 10, 50, 100, 500 and 1000 mU/μL, respectively. The fluorescence of the mixture was then measured at room temperature. All of the measurements were performed three times.



Scheme 1. Schematic illustration of the strategy for the detection of PKA activity based on the fluorescent Cu NCs.

For a PKA inhibitor assay, the procedures were similar as above, except for different concentrations of inhibitor were added in the PKA reaction mixture.

2.5. PKA assay in HeLa cell lysates

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a humidified culture box containing 5% CO_2 for several days. Before stimulation, 4 mL serum-free medium has been used to replace the previous medium. Then, the mixture solution with various concentrations of forskolin and IBMX according to a certain proportion (1:2) and 10 μL PBS buffer were added into the medium to activate the intracellular PKA for 30 min. The final concentrations of forskolin were 0, 1, 2.5, 5, 10, 25, 50, 75 and 100 μM. For comparison, 10 μL of PBS buffer (10 mM, pH = 7.4) was added into the medium as a control. After that, the HeLa cells were clarified by centrifugation at 12,000 rpm for 20 min to remove the medium and then broken with an ultrasonic processor. Finally, the cell lysates were centrifuged again and ready for PKA detection.

For the detection of PKA activity in HeLa cell lysates, the procedures were similar as above PKA assay, except for 20 μL cell lysates were added in the PKA reaction mixture.

3. Results and discussion

3.1. Sensor design

In this paper, we established a simple and sensitive fluorescence probe to detect protein kinase activity based on Cu NCs. This method was based on the strong interaction between ATP aptamer and ATP. As shown in Scheme 1, fluorescent Cu NCs was formed by double-strand DNA (A and B) templates. When ATP was added, it can combine with aptamer (A strand) and fluorescent Cu NCs could not be formed due to the lack of effective substrate. Thus, the fluorescence of Cu NCs decreased. However, when PKA was added, the fluorescence of Cu NCs recovered because that ATP had been translated into ADP by PKA and ADP can not combine with ATP aptamer. We can effective monitored the activity of protein kinase according to the variation of fluorescence signal.

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