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Label-free fluorescence assay for protein kinase based on peptide biomineralized gold nanoclusters as signal sensing probe

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A label-free, sensitive and simple method to detect protein kinase based on the selective aggregation of phosphorylated peptide-gold nanoclusters (peptide-AuNCs) triggered by Zr^{4+} ion coordination is developed. The AuNCs were synthesized by peptide without any strong reducing agents, which prevent peptides from being disrupted. Under optimal conditions, a linear relationship between the decreased PL intensity of peptide-AuNCs and the concentration of casein kinase II (CK2) in the range of 0.08–2.0 unit mL^{-1} with a detection limit of 0.027 unit mL^{-1} (3σ) was obtained. The feasibility of this AuNCs-based sensor was further demonstrated by the assessment of kinase inhibition by ellagic acid, 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside, emodin, and quercetin in human serum. As expected, the PL intensity increased with increasing inhibitor efficiency in the presence of inhibitors. The IC_{50} value (inhibitor concentration producing 50% inhibition) for ellagic acid was estimated to be 0.045 μM. With more sophisticated design of the peptide substrate sequences, the detection of other enzymes will be realized. With characteristics of homogeneous, facile, universal, label-free, and applicable for kinase assay, the proposed sensor provides potential application in kinase-related biochemical fundamental research and inhibitor screening.

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1. Introduction

Gold nanoclusters (AuNCs) have attracted great attention in recent years for their unique and intriguing physical and chemical properties, such as discrete electronic transitions (Aikens, 2010; Wu and Jin, 2010), well-defined molecular structure (Zhu et al., 2008), magnetism (Zhu et al., 2009), quantized charging (Chen et al., 1998), strong photoluminescence (Yuan et al., 2013; Zheng et al., 2012), optical chirality (Jadzinsky et al., 2007; Qian et al., 2010), low toxicity and high biocompatibility (Lin et al., 2010). Due to these unique properties, the synthesis strategies of AuNCs in water solution by some soft landing such as arylthiolates (Arnold and Reilly, 1998; Pradeep et al., 2004), dendrimers (Bao et al., 2007; Gröhn et al., 2000) have been reported in large volumes, and the AuNCs have been widely used in the fields of chemistry, biology, and materials (Wu and Jin, 2010). Recently, AuNCs have been successfully prepared and widely applied to fluorescence assay of enzyme. For example, Gu et al. reported a peptide-templated method to synthesis fluorescent AuNCs beacons that were responsive to elastase (Gu et al., 2014). Dou et al. demonstrated that fluorescent gold/silver nanoclusters can be served as a novel probe for sensitive detection of deoxyribonuclease I (DNase I) (Dou and Yang, 2013). Moreover, Jiang's group developed a simple peptide-templated method for rapid synthesis of AuNCs beacons that were responsive to histone deacetylase 1 (HDAC 1) and protein kinase A (PKA) (Wen et al., 2013). Most of the previous AuNCs synthesis methods required strong reducing agents such as $NaBH_4$ to prepare fluorescent and stable AuNCs, and the strong reducing agents would potentially involve side effects in later biologic applications (Wang et al., 2012). Furthermore, the above mentioned methods of enzyme detection were all based on the fluorescence quenching of AuNCs caused by the destruction of AuNCs templates, which would disturb the biological functions of template biomolecules, unfavorable for further applications. Thus, green and simple synthesis methods of AuNCs without strong reducing agents and the detection methods without destruction of AuNCs templates are still in high demand to assist the development of this area of nanotechnology and nanobiology.

Protein kinase CK2 (formerly casein kinase II) catalyzes protein phosphorylation and plays a vital role in signal transduction pathways. CK2 is a multifunctional protein kinase that has a crucial role in cell survival, differentiation and proliferation (Ahmed et al., 2002). Abnormal expression of CK2 has been implicated in a number of diseases such as cancer (Wang et al., 2001), Alzheimer's disease (Flajolet et al., 2007) and HIV (Critchfield et al., 1997). Therefore, the establishment of good

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accuracy, high sensitivity, simple operation detection method of CK2 not only provide the basis for the early diagnosis of cancers, but also helps to treat cancer-related phosphorylation of discovery and screening of new drugs. There are various existing methods for the measurement of protein kinase activities including radio-active (Hutti et al., 2004; Lehel et al., 1997), fluorescent (Bai et al., 2013; Freeman et al., 2010; Kim et al., 2007; Wang et al., 2013b), electro-active (Kerman et al., 2008; Song et al., 2008), biotin (Wang et al., 2006, 2005) or thiol (Kerman and Kraatz, 2007; Xu et al., 2010a) labeling techniques and phosphorylation-specific recognition protein based methods (Ghadiali et al., 2010; Gupta et al., 2010, 2011; Herbst et al., 2011). Nevertheless, such assays are effective but require labor-intensive and time-consuming labeling procedures or complicated preparation of encoded reporters. In our recent research, a novel PL sensing strategy was proposed for profiling CK2 based on zirconium cation (Zr^{4+} ions) mediated signal transition by using graphene quantum dots (GQDs) as effective optical probes (Wang et al., 2013b). The sensing approach was sensitive but complex synthetic procedure of GQDs and conjugating peptide substrates to the GQDs surface via covalent coupling were needed. Although a lot of efforts have been devoted to overcome such shortcomings (Choi et al., 2007; Xu et al., 2010b; Zhou et al., 2014), there still remains a great challenge to realize disease diagnosis through a sensitive, reliable and cost-effective protein kinase assays and make high-throughput screening of kinase inhibitors possible.

Herein, we developed a label-free and antibody-free fluorescence assay for monitoring kinase activity based on zirconium cation (Zr^{4+}) mediated signal transition by using peptide-AuNCs as effective optical probes. As shown in Scheme 1, a one-step peptide biomineralization method is devoted to construct a peptide-AuNCs sensing platform without strong reducing agents. The substrate peptide reacted with CK2 via targeted phosphorylation, and followed by multicoordinative linking interactions between Zr^{4+} ions and phosphate groups, resulting in extensive AuNCs aggregation and effective PL quenching. Based on the PL quenching, the activity of protein kinase can be facilely monitored with high sensitivity.

2. Experimental section

2.1. Reagents and chemicals

CK2 was obtained from New England Biolabs (UK). The arginine-terminated peptide Cys–Cys–Tyr–Arg–Arg–Arg–Ala–Asp–Asp–Ser–Asp5 ($CCYRRRADDSD_5$) was purchased from GL Biochem (Shanghai, China). Adenosine 5′-triphosphate (ATP) disodium salt, 4,4′,5,5′,6,6′-hexahydroxydiphenic acid 2,6,2′,6′-dilactone (ellagic acid), 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB), and $ZrOCl_2$ were purchased from Sigma-Aldrich (USA). $HAuCl_4 \cdot 4H_2O$, NaOH, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-Flavone (quercetin), 1,3,8-trihydroxy-6-methyl-antraquinone (emodin) were purchased from Sinopharm Chemical Reagent Co., Ltd. Other regents of analytical grade were obtained from Beijing Chemical Company (China) and were used as received without further purification. All solutions were prepared and diluted using ultrapure water (18.2 MΩ cm) from the Millipore Milli-Q system. Human serum samples were provided by Jiangxi provincial people's hospital.

2.2. Preparation of peptide-Au nanoclusters

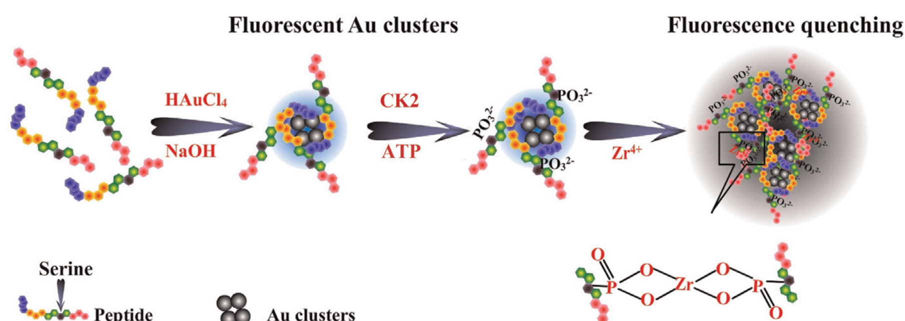
In a typical experiment, an aqueous solution of $HAuCl_4$ (25 mM, 16 μL) was slowly added to a solution of $CCYRRRADDSD_5$ (1.06 mM, 376 μL) in a 5 mL vial under vigorous stirring, and then NaOH (0.5 M, 8 μL) was added within 30 s to give a final pH of ~9. The sample was sealed and stored in the dark for 13 h under stirring without any disturbance to produce the peptide-AuNCs. The as-synthesized peptide-AuNCs were concentrated by a 3 kDa ultrafiltration device to remove free peptide (Cui et al., 2011).

2.3. Detection procedure

80 μL of 20 mM Tris buffer (pH 7.5, containing 50 mM KCl and 10 mM $MgCl_2$) was added into the 120 μL of 0.96 mM peptide-AuNCs solution, then mixed with 40 μL of a CK2 solution with the specified concentration, and 20 μL of 1.0 mM ATP. The mixture was diluted with ultrapure water to a volume of 360 μL. The phosphorylation reaction was performed by incubating the mixture at 37 °C for 60 min. Finally, 40 μL of 2.5 mM Zr^{4+} ions was added and mixed homogeneity to induce aggregation of the phosphorylated peptide-AuNCs. Subsequently, the PL spectra and light scattering (LS) spectra of the resulting solutions were recorded on an F-7000 spectrophotometer.

2.4. CK2 inhibition and IC_{50} assay

First, an aqueous solution of 80 μL of 20 mM Tris buffer (pH 7.5, containing 50 mM KCl and 10 mM $MgCl_2$) was added to the aqueous solution of 80 μL of 1% human serum sample, 40 μL of 20 unit mL^{-1} CK2, and 20 μL of 200 μM solution of the inhibitor (DRB, emodin, quercetin and ellagic acid). Next, 120 μL of peptide-AuNCs and 20 μL of 1.0 mM ATP were mixed with the inhibitor-pretreated CK2 solution, and the mixture was incubated at 37 °C for 60 min. Finally, 40 μL of 2.5 mM Zr^{4+} ions was added and mixed homogeneity to induce aggregation of the phosphorylated peptide-Au nanoclusters. Subsequently, the PL spectra were recorded. To measure the IC_{50} value of the potent inhibitor ellagic acid, 80 μL of 20 mM Tris buffer (pH 7.5, containing 50 mM KCl and 10 mM $MgCl_2$) was mixed with 80 μL of 1% human serum sample, 40 μL of 20 unit mL^{-1} CK2 and 20 μL of different concentrations of ellagic acid. Next, 120 μL of peptide-AuNCs and 20 μL of 1.0 mM ATP was added to the ellagic acid-pretreated CK2 solution and the mixture was incubated at 37 °C for 60 min. Finally, 40 μL



Scheme 1. Schematic representation of the CK2 kinase assay based on aggregation and PL quenching of phosphorylated peptide-AuNCs via Zr^{4+} ion linkage.

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