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Peptide-templated multifunctional nanoprobe for feasible electrochemical assay of intracellular kinase

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ABSTRACT

Protein kinases play a critical role in regulation of intracellular signal transduction, whose aberrant expression is closely associated with various dangerous human diseases. In this paper, we propose a feasible electrochemical assay of intracellular kinase by incorporating peptide nanoprobe-assisted signal labeling and signal amplification. Protein kinase A (PKA)-specific peptide P1 is self-assembled on the surface of a gold electrode, serine of which could be phosphorylated with catalysis of PKA in the presence of adenosine-5′-triphosphate (ATP). Another artificial peptide P2 contains a short template for preparation of copper nanoparticles-based nanoprobe (P2-CuNPs) and provides arginine residues for specific recognition of phosphorylation site. After PKA-catalyzed phosphorylation, phosphorylated P1 specially binds with P2-CuNPs through ultra-stable phosphate-guanidine interaction, and thus results in amplified electrochemical response from surface-attached CuNPs. Our method demonstrates a satisfactory sensitivity toward PKA detection with a detection limit of 0.0019 U/mL, which is also successfully applied in intracellular PKA assay and inhibitory study with high specificity comparable to ELISA. Therefore, the facile method suggests a promising potential use in kinase-related biochemical fundamental research, disease diagnosis and drug discovery in the future.

1. Introduction

Protein kinases (PKs) are a family of post-translational modification enzymes that catalyze transferring phosphate group of a nucleoside triphosphate (e.g. adenosine triphosphate, ATP) to free hydroxyl group of amino acid residues (e.g. serine, tyrosine, or threonine) within a peptide or protein substrate (Liu et al., 2015; Yin et al., 2015a; Yin et al., 2015b). PK-catalyzed phosphorylation plays a critical role in intracellular signal transduction pathways, and thus regulates various fundamental cellular processes, including gene expression, cell metabolism, proliferation and apoptosis (Manning et al., 2002; Sims et al., 2013; Zhou et al., 2013a). At the meanwhile, over-expression of PKs and following abnormal protein phosphorylation are closely correlated with several dangerous human diseases, such as diabetes, HIV, restenosis, Alzheimer's disease, endocrinological disorder, cardiovascular disease and even aggressive tumors (Melville et al., 2017; Perez-Gallegos et al., 2015; Russo et al., 2013). Therefore, understanding potential relationship between kinase activity and intracellular signaling transduction is very important to reveal fundamental biological metabolism processes, thereby driving advance in PKs-related disease diagnosis and drug discovery in practice (Song et al., 2015; Yin et al., 2015c; Zhou et al., 2016). Radioactive assay is a conventional method for phosphorylation determination using $\gamma - {}^{32}$P-ATP as a probe (Lehel et al., 1997; Park et al., 2007). Nonetheless, radioactive assay is always limited by hazardous effects, such as dangerous radioactive waste, complicated treatment procedures and high requirement for experimental environment. For this reason, radioactive assay has been gradually replaced by some emerging techniques, including surface-plasmon resonance (SPR) (Yoshida et al., 2000), mass spectroscopy (MS) (Ji et al., 2012; Kondo et al., 2009), quartz crystal microbalance (QCM) (Xu et al., 2012), fluorescence (Song et al., 2015; Wang et al., 2017a; Zhou et al., 2013a), colorimetry (Gupta et al., 2011; Lowe et al., 2011; Wang et al., 2006; Zhou et al., 2014), electrogenerated chemiluminescent (Xu et al., 2010) and electrochemical techniques (Martić et al., 2012; Shen et al., 2016; Sun et al., 2017; Yin et al., 2015a).

Polypeptide is a versatile and biocompatible building block for biological application, especially at the forefront of bio-analysis. Compared to whole protein, polypeptide does not require specific spatial structure and is thus highly stable and flexible for design, synthesis and modification (Miao et al., 2013; Zhao et al., 2016). In the

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past decades, evolution of screening technique pushes forward extensive use of functional peptide for the diversity of amino acids. For example, artificial short peptide is proven to mediate nanomaterial synthesis by modeling bio-mineralization, offering enhanced chemical stability, controllable ultrafine size and tailorable surface properties (Wang et al., 2013, 2016; Zhang et al., 2016). Active group that functionalizes nanomaterial surface via one-step synthesis promotes molecular recognition, signal labeling and strategy design, while controllable nanomaterial is beneficial to improvement of analytical performances due to intriguing physical, electrical, and optical properties. Recently, peptide-templated metal nanoclusters have been used as interesting nanoprobes in label-free fluorescent sensing protein post-translational modifications as well as imaging cancer cells (Das et al., 2015; Song et al., 2015; Wen et al., 2013; Zhang et al., 2016). However, fluorescent detection has obvious defects, which relies heavily on strict control of nanomaterial size and is usually interfered by external environment.

Given that metal nanomaterial is an ultrasensitive electrochemical signal other than a fluorescence one, we herein propose an electrochemical method to assay intracellular PK using peptide-templated copper nanoparticles (CuNPs) as a multifunctional nanoprobe (Zhou et al., 2013b). Scheme 1 illustrates the principle of our method using a representative kinase protein kinase A (PKA) as a target. A covalent-like interaction of a basic guanidine group within arginine and an acidic phosphate group within phosphorylated serine is exploited to identify PKA-catalyzed phosphorylation (Wang et al., 2017b; Woods et al., 2005). Accordingly, two artificial peptides are recruited in our method: one is a substrate peptide P1 containing a serine residue for phosphorylation and a cysteine residue for surface immobilization; the other is a template peptide P2 containing a short sequence CCY for bio-mineralization and arginine (Arg) residues for signal labeling. P1 is firstly immobilized on electrode surface through formation of Au-S bond, while P2 mediates preparation of CuNPs-based nanoprobe (P2-CuNPs) using CCY as both reducing and stabilizing agent. After PKA catalyzes phosphorylation of serine within P1 with co-existence of ATP, additional phosphate group not only reduces electrostatic repulsion between two artificial peptides, but also provides a specific binding site for coordination with guanidine groups. Based on covalent-like interaction of phosphate-guanidine binding, P2-CuNPs are captured on the electrode surface. Besides providing an active surface group for stable immobilization, P2-CuNPs also contribute to an amplified electrochemical signal. Surface-attached CuNPs release a large number of copper ions in an acid oxidizing environment, which could be quantitatively determined by a trace-level and reproducible electrochemical technique, stripping voltammetry (Mao et al., 2016; Zhao et al., 2015; Zhu et al., 2016). Dissolved CuNPs arouse noticeable electrochemical responses after accumulation on electrode surface, and thus achieve a purpose of sensitive and specific PKA assay and inhibitory study both in vitro and in vivo. Benefiting from peptide-templated nanoprobe assisted signal labeling and amplification, our method provides a promising potential for practice use in PK-related biological research, disease diagnosis and drug discovery in the future.

2. Material and methods

2.1. Materials and reagents

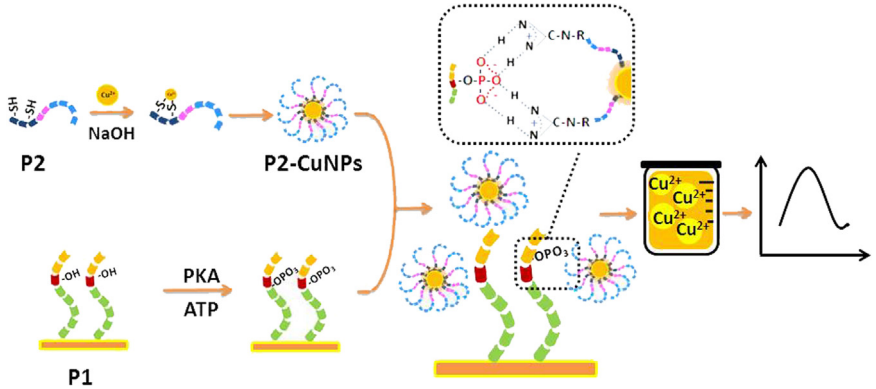
Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) was obtained from New England Biolabs. Artificial peptides including P1 (CLRRASLG), P2 (CCYGGVLRR) and P3 (CCYGGVL) were synthesized from GL Biochem Ltd. (Shanghai, China) and purified by HPLC. ATP, 6-mercapto-1-hexanol (MCH), tris(2-carboxyethyl)phosphine (TCEP), Tris base, sodium acetate were obtained from Sigma-Aldrich. Ellagic acid, forskolin (Fsk) and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sangon Biotech (Shanghai) Co., Ltd. Other reagents were of analytical reagent grade and used without further purification. Millipore Milli-Q Ultrapure water (18.2 MΩ) was used throughout all the experiments.

2.2. Preparation of P1-modified electrode

To achieve a mirror-like surface, gold electrode (Φ = 3 mm) was first carefully polished with 1.0, 0.3 and 0.05 μm alumina slurry in sequence. After rinsing thoroughly with double-distilled water, the electrode was dipped into piranha solution (H_2SO_4:H_2O_2 = 3:1) for 5 min. Then, the residual was completely removed by sonicating the electrode in ethanol and double-distilled water for 5 min, respectively. Finally, the electrode was electrochemically activated in 0.5 M H_2SO_4 by scanning from 0 V to 1.6 V until a stable cyclic voltammogram was obtained. After being rinsed thoroughly with double-distilled water and dried in nitrogen atmosphere, activated electrode was immersed into 10 mM PBS containing 50 μM P1, 50 mM TCEP (pH 7.4) for 16 h at 4 °C. After being thoroughly rinsed by double-distilled water, P1-modified electrode was finally prepared for further use. The modification process of the electrode was characterized by electrochemical impedance spectroscopy (EIS, Fig. S1).

2.3. Preparation of P2-CuNPs

P2-CuNPs was prepared based on previous report (Wang et al., 2013). Specially, 16 μL of $Cu(NO_3)_2$ aqueous solution (25 mM) was slowly added to 376 μL of P2 solution (1.0 mM) under vigorous stirring. Then, 8 μL of 0.5 M NaOH was used to adjust pH to about 9 in a quite short time. Finally, the mixture was sealed and stored for 10 h in dark environment under vigorous stirring. CCY domain facilitated nanoparticle formation using the phenolic groups of Y for reduction of Cu^{2+} to Cu^0, and the sulfhydryl groups of C for the capture of nanoparticles. To remove unbounded peptides, as-synthesized P2-CuNPs were



Scheme 1. Schematic illustration of electrochemical assay of intracellular kinase using peptide-templated multifunctional nanoprobe.