Biosensors and Bioelectronics 53 (2014) 295–300

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A gold nanoparticles colorimetric assay for label-free detection of protein kinase activity based on phosphorylation protection against exopeptidase cleavage

Jiang Zhou, Xiahong Xu, Xin Liu, Hao Li, Zhou Nie *, Meng Qing, Yan Huang, Shouzhuo Yao

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, PR China

ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form
17 September 2013
Accepted 26 September 2013
Available online 8 October 2013

Keywords:
Gold nanoparticles
Protein kinase
Protein phosphorylation
Carboxypeptidase Y
Cell lysate

ABSTRACT

Protein kinases are significant regulators in the cell signaling pathways, and it is still greatly desirable to achieve simple and quick kinase detection. Herein, we present a novel colorimetric gold nanoparticles (AuNPs)/peptide platform for probing the activity and inhibition of protein kinases based on phosphorylation-induced suppression of carboxypeptidase Y (CPY) cleavage. This AuNPs/peptide platform can easily monitor the kinase activity by a UV–vis spectrometer or even by the naked eye. The feasibility of the method has been demonstrated by sensitive measurement of the cAMP-dependent protein kinase (PKA) activity with a low detection limit of 0.232 mU/μL and assessment of kinase inhibition by H-89 with an IC_{50} value of 18.13 nM. The assay was also successfully put into practice for the detection of kinase activity in cell lysate. Because of its label-free, homogenous and colorimetric merits, the proposed assay presents great potential in high-throughput screening for kinase-targeted drug discovery.

1. Introduction

Protein phosphorylation catalyzed by kinase is a universal regulatory mechanism in the cell signaling pathways, which plays a crucial role in various vital cellular physiological processes including cell growth, metabolism, differentiation, and apoptosis (Manning et al., 2002; Cohen, 2002a, 2002b; Kalume et al., 2003). The over-expression and aberrant activity of protein kinases are closely related to a number of severe diseases such as cancer, diabetes, inflammation, cardiac diseases, and Alzheimer's disease (Mark and Joseph, 2010; Cohen, 2002a, 2002b; Hanger et al., 2007). Therefore, the development of kinase assays capable of monitoring kinases activity, identifying their substrates and screening potential inhibitors is important for the research of fundamental biochemical pathways, clinical diagnosis and drug discovery (Cohen, 2002a, 2002b; Noble et al., 2004).

Traditional method for assessing kinase activity relied on radioactive isotope-labeled ATP, which is general but hampered by the hazardous effect of radioactive materials (Turk et al., 2006; Hastie et al., 2006; Houseman et al., 2002). To overcome this shortcoming, various kinase assays, including electrochemical (Wieckowska et al., 2008; Ji et al., 2009; Xu et al., 2009; Kerman et al., 2008), surface-plasmon resonant(Yoshida et al., 2000; Viht et al., 2007), and mass spectroscopic techniques (Mann et al., 2002; Watts et al., 1994) have been developed as the alternatives of radioactive assay. Most of these techniques involve a surface-confined process of substrate peptide, heterogeneous enzymatic reaction at peptide-modified surface, and multistep-washing procedure. Compared with those heterogeneous kinase assays, the approaches for homogenous detection of kinase activity possess intrinsic merits such as the short detection time, facile detection without separation, and readiness for high-throughput screening (HTS). Great progress has been achieved on the design of homogenous fluorescence measurements of kinase-catalyzed phosphorylation (Sato et al., 1999; Sharma et al., 2007; Agnes et al., 2010; Xu et al., 2011; Bai et al., 2013; Zhou et al., 2013). Because the color change can be directly monitored by naked eye without sophisticated instruments, the colorimetric biosensing for protein phosphorylation has attracted much attention because of its low cost, simplicity, and practicality. Although it is a promising technique for developing homogenous kinase assay, the existing colorimetric assays for kinase activity are still scarce in comparison with well-developed fluorescent counterparts.

Gold nanoparticles (AuNPs) represent a potent colorimetric probe mainly due to their unique optical properties, particularly their size-dependent surface plasmon resonance absorption (Lim et al., 2007; Mirkin et al., 1996; Sato et al., 2003). There are only a few AuNPs-based methods currently available for homogenous activity detection of protein kinase. Brust et al. developed a colorimetric kinase assay in which the biotin-labeled ATP was

* Corresponding author. Tel.: +86 731 88821626; fax: +86 731 88821848.
E-mail address: niezhou.hnu@gmail.com (Z. Nie).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.09.070

used to probe phosphorylation process on peptide-modified AuNPs, and consequently the phosphorylated AuNPs with biotin label reacted with avidin-capped AuNPs to generate cross-linking and corresponding color-change (Wang et al., 2006; Wang et al., 2005). Similar cross-linking strategy has been designed by Stevens' group using AuNPs conjugated with substrate peptide and phospho-specific antibody, respectively, as dual probes to assess the activity of kinase (Gupta et al., 2010). Recently, Katayama group reported an intriguing kinase assay using phosphorylation-induced peptide charge change to mediate AuNPs aggregation (Oishi et al., 2008). Among these methods, most of them relied on phosphorylation-induced interparticles cross-linking of AuNPs, which are effective but suffer from the complicated modification of AuNPs, the high cost of labeled substrate, and relative long responsive time for observing the aggregation-induced color change (Lévy et al., 2004). Hence, it is still desirable to develop new label-free strategy to fulfill the phosphorylation-responsive non-crosslinking AuNPs aggregation.

In this paper, we present a novel colorimetric AuNPs/peptide platform for probing the activity and inhibition of protein kinase. This AuNPs/peptide platform is composed of the unmodified AuNPs and the rationally designed substrate peptide probe. The phosphorylation recognition is dependent on the phosphorylation protection against carboxypeptidase digestion. The carboxypeptidase refers to a kind of exopeptidase that hydrolyzes peptide bond from the carboxyl terminal (C-terminal) of a protein or peptide (Low and Yuan, 1996). It has been de monstrated that the activity of exopeptidase are susceptible to modification on the amino acid (Dass and Mahalakshmi, 1996). With the aid of carboxypeptidase, the phosphorylated peptide could be discriminated from unphosphorylated one by its resistance to enzymatic cleavage, leading to retain the peptide fragment to mediate the aggregation of AuNPs. The cAMP-dependent protein kinase (PKA) and carboxypeptidase Y (CPY) were used as typical models. Because of its label-free property and no modification required for AuNPs and peptides, this method represents a promising application of AuNPs in protein kinase analysis.

2. Experimental methods

2.1. Materials and measurements

Cyclic adenosine 3′,5′-monophosphate-dependent protein kinase (PKA, catalytic subunit) and Casein kinase II (CKII) were purchased from New England Biolabs (Beverly, MA, USA). Generally, PKA was diluted using the storing solutions (50 mM NaCl, 1 mM EDTA, 2 mM DTT, 50% glycerol in 20 mM Tris–HCl buffer (pH 7.5, 25 °C)) and stored in the refrigerator at −80 °C. Substrate peptide probe (S-pep, CGGRRGLRRASLG) for PKA was synthesized by GL Biochem (Shanghai, China). ATP was bought from Generay (Shanghai, China). $HAuCl_4 \cdot 4H_2O$ was supplied from Shanghai Reagent Company (Shanghai, China). H-89 was obtained from EMD Biosciences (Calbiochem-Novabiochem. La Jolla, CA, USA). Carboxypeptidase Y (CPY), forskolin and 3-isobutyl-1-methylxantine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The improved Bradford protein assay dye reagent kit was purchased from Sangon (Shanghai, China). The rest of the chemical reagents including bovine serum albumin (BSA), Tris, glycerol, DTT, and EDTA used in this study were obtained from Bio Basic (Ontario, Canada). Human breast cancer cells (MCF-7) were bought from the Cell Bank of Xiangya Central Laboratory of Central South University (Changsha, China). The ultrapure water (18.3 MΩ cm) from the Millipore Milli-Q system was used in all experiments.

The UV–vis absorption spectra were recorded on a Beckman DU800 spectrophotometer in a wavelength range from 400 to 800 nm. The absorbance measurements of each sample were conducted in three independent times ($n=3$). High-resolution transmission electron microscopy (HRTEM) measurements were made on a JEOL JEC-3010 electron microscope. The samples of TEM were prepared by placing a drop of solution (10 μL) on carbon-coated copper grid and drying at room temperature in drying cabinet. Cell-breaking was performed using a JY92-IIN ultrasonic cell disruption system (Scientz, Ningbo, China). The photographs were recorded with digital camera.

2.2. Synthesis of gold nanoparticles (AuNPs)

AuNPs (13 nm) were synthesized according to the previously reported method (Jin et al., 2003). Generally, 38.8 mM sodium citrate solution (5 mL) was rapidly added into 1 mM boiled $HAuCl_4$ solution (50 mL) under vigorous stirring in a 100 mL round flask. The mixture was maintained boiling for 10 min, and the corresponding color change from yellow to deep wine red. The obtained gold nanoparticles solution was stored at 4 °C. The final concentration of AuNPs was calculated to be 11 nM using the UV–vis absorption spectrum based on Lambert–Beer's law and extinction coefficients (ε) of $2.7 \times 10^8\ M^{-1}\ cm^{-1}$ at λ_{520} for 13 nm AuNPs. The so-prepared AuNPs dispersion was diluted 2.5 times by ultrapure water and used as stock solution in the subsequent absorbance experiments. The absorption ratio (Abs_{520}/Abs_{620}) of the AuNPs dispersion was chosen as the respond signal of the AuNPs dispersion stability.

2.3. Peptide-induced AuNPs aggregation

A series of 40 μL S-pep samples at different concentrations (0, 0.001, 0.01, 0.1, 1, 2.5, 5, 10, 20 and 40 μM) were prepared in 10 mM Tris–HCl buffer (pH 7.5, 25 °C), then an equal volume of the unmodified AuNPs dispersion (4.4 nM) was added and mixed for 5 s. After mixing, UV–vis spectroscopic measurements at the 400–800 nm wavelength region were made at room temperature.

2.4. Treatment of S-pep by carboxypeptidases Y (CPY)

The S-pep (1 μM) was treated with the CPY (2.632 U/mL) for 45 min. The resulting solution (40 μL) was mixed with an equal volume of AuNPs dispersion (4.4 nM) and then measured by spectroscopy. A range of concentrations (0–4 U/mL) of CPY were exploited to optimize the quantity of CPY. For optimization of the digestion time, the same experiments were conducted incubating with 2.632 U/mL CPY for different incubation times (0—90 min).

2.5. Detection of activity and inhibition of PKA

For PKA-catalyzed phosphorylation, 200 μL of the PKA reaction solutions composed of PKA (0–1 U/μL), S-pep (1 μM), $MgCl_2$ (1 mM), ATP (0.1 mM) in 20 mM Tris–HCl buffer (pH 7.5, 25 °C) was incubated for 60 min at 30 °C, and the resulting phosphorylated solution was incubated with 2.632 U/mL CPY for 45 min at 25 °C, then 40 μL of the mixture was mixed with the AuNPs dispersion (40 μL, 4.4 nM) and measured by spectroscopy.

For PKA inhibition assays, 250 mU/μL PKA and H-89 at different concentrations (0–0.5 μM) were added into the reaction solutions, and the experiment procedures were set under the above-mentioned conditions.