Sensors and Actuators B 248 (2017) 178–186

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

Two sensitive electrochemical strategies for the detection of protein kinase activity based on the 4-mercaptophenylboronic acid-induced *in situ* assembly of silver nanoparticles

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ARTICLE INFO

Article history:
Received 23 December 2016
Received in revised form 7 March 2017
Accepted 27 March 2017
Available online 28 March 2017

Keywords:
Protein kinase
Electrochemical biosensors
Silver nanoparticles
Phenylboronic acid
Signal amplification

ABSTRACT

This work presents two sensitive electrochemical strategies for the detection of protein kinase activity *via in situ* formation of citrate-capped silver nanoparticles (AgNPs) aggregates as labels. In the first design, adenosine 5′-[γ-thio] triphosphate (ATP-S) was used as the co-substrate. After phosphorylation, the AgNPs were conjugated specifically to the thiophosphate peptides *via* the Ag-S interaction. Meanwhile, 4-mercaptophenylboronic acid (MPBA) in solution induced the *in situ* assembly of AgNPs on an electrode surface through the formation of Au-S bonds and the covalent interactions between α-hydroxycarboxylate of citrate and boronate of MPBA. In this process, MPBA acted as a cross-linker in the assembly of AgNPs. The electrochemical signal was thus amplified because of the formation of AgNPs-based network architecture. In the second strategy, tyrosine residues on electrode surface were first oxidized by tyrosinase to form o-diphenol moieties; then, MPBA molecules were immobilized on the electrode surface through the formation of boronate ester bonds. The MPBA molecules anchored onto the electrode surface and present in solution also induced the assembly of citrate-capped AgNPs through the Au-S and citrate-boronate interactions. However, once the tyrosine residues were phosphorylated by tyrosine kinase, the phosphorylated peptides could not be oxidized by tyrosinase. As a result, MPBA and AgNPs were not anchored onto the electrode surface. To demonstrate the applications and analytical merits of our methods, tyrosine kinase Src were measured as a model analyte. The detection limits of these two proposed strategies were 0.1 and 1.2 ng/mL. Furthermore, the inhibition ability of PP2 (a well-known tyrosine kinase inhibitor) was evaluated.

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1. Introduction

Protein kinases play crucial roles in many biological processes, including signal transduction, cell apoptosis, immune regulation, proliferation and differentiation and other important cellular pathways [1]. There are good evidences that aberrant activities of protein kinases may cause various human diseases, such as cancer, metabolic disorders and inflammation [1–3]. Furthermore, protein kinases have been considered as an important family of molecular targets for the discovery of new targeted drugs [2,3]. Thus, simple, sensitive and selective detection of the activities of proteins kinases is of great significance for clinical diagnosis, targeted therapy and drug discovery.

Protein kinases can catalyze the transfer of a phosphoryl group from adenosine triphosphate (ATP) to a protein/peptide substrate at the serine, tyrosine, or threonine residues. At present, the autoradiography technique with a radio-labeled ATP analog is the gold standard for determining kinase activity. However, the radiometric assay shows obvious drawbacks, such as radiological hazards and low-resolution sensitivity [4]. Thus, a few novel methods have been explored recently for the detection of kinase activity and the screening of potential inhibitors, including colorimetry [5–7,8], electrochemistry [9–14], fluorescence [15–18], resonance light scattering [19], quartz crystal microbalance [4], photoelectrochemistry [20], localized surface plasmon resonance (LSPR) [21], electrochemiluminescence [22,23] and mass spectrom-

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http://dx.doi.org/10.1016/j.snb.2017.03.122
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etry [24]. Moreover, electrochemical biosensors have shown great potential for determining protein kinase activities because of their simplicity, rapid response, high sensitivity as well as specificity, and compatibility with miniaturization. In a general detection format of electrochemical kinase biosensors, the kinase-specific peptide substrates are immobilized on an electrode surface. After phosphorylation, the resulting phosphorylated peptides could be recognized by special elements, such as anti-phosphorylated peptide antibodies [25], metal ions (Zr^{4+}, Fe^{3+}, Ag^{+} and Tb^{3+}) [26–29], bis(Zn^{2+}-dipicolylamine) [30,31], and TiO_2 nanoparticles [32]. Moreover, the use of ATP analogs as the co-substrates also facilitates the development of various novel electrochemical kinase biosensors. For example, Kraatz's group demonstrated that phosphorylation could be monitored directly by measuring the oxidation current of ferrocene by using adenosine-5′-[γ-ferrocene] triphosphate (Fc-ATP) as the co-substrate [33–35]. With adenosine-5′-[γ-biotin] triphosphate (biotin-ATP) as the co-substrate, the biotin moiety in the phosphorylated peptide allows for the attachment of streptavidin-conjugated nanoparticles [36]. Furthermore, when adenosine 5′-[γ-thio] triphosphate (ATP-S) was used as the co-substrate, the resulting thiophosphorylated peptide captured the unmodified gold nanoparticles (AuNPs) *via* the Au–S interaction [22,37–40]. All of these electrochemical methods have their own advantages and have made great advances for evaluating the activities of protein kinases. However, a majority of these methods are less sensitive and/or require the modification of nanoparticles. Therefore, it still remains a great challenge to develop a simple, rapid and sensitive electrochemical method for determining the activities of protein kinases and screening of the potential inhibitors.

Silver nanoparticles (AgNPs) have been applied recently as the electrochemical biosensing elements used for signal readout [41,42]. In particular, the AgNPs-based network architecture provides a well-defined and amplified electrochemical signal [43–48]. With AgNPs aggregates as the electrochemical reporters, several groups have demonstrated the design of electrochemical chem/biosensors. For example, Xu's group presented an ultrasensitive DNA assay with an attomolar detection limit by using AgNPs aggregates as labels which were prepared in advance through hybridization [46]. Also, they presented an ultrasensitive electrochemical aptamer sensor for protein detection with AgNPs aggregates tags formed *via in situ* hybridization of DNA [44]. Han's group reported the detection of sequence-specific DNA down to 10 fM on the basis of signal amplification of biotinylated AgNPs aggregate tags formed through the biotin–streptavidin interaction [43]. Recently, Dai's group proposed a novel concept for concerting colorimetric assay into electrochemical analysis based on the Hg^{2+}-induced formation of AgNPs aggregate tags on electrode surface [47]. All of their results indicated that the AgNPs aggregate tags could be used for the design of novel electrochemical chem/bio-sensors with high sensitivity, throughput and miniaturization although they need to be functionalized.

One of the most commonly used methods for the preparation of stable water-soluble AgNPs is based on the reduction of silver nitrate ($AgNO_3$) by sodium borohydride ($NaBH_4$) and is stabilized by citrate. Interestingly, phenylboronic acid derivatives can react with diol-containing biomolecules and α-hydroxycarboxylate acids (*e.g.*, citrate and tartrate) to form the boronate ester complexes [49,50]. This binding property have allowed for the selective sensing and specific purification of diol derivatives (*e.g.* RNA and glycoproteins) by using phenylboronic acid-functionalized materials [51,52]. In the present work, we demonstrated that 4-mercaptophenylboronic acid (MPBA) can induce the aggregation of citrate-capped AgNPs in solution through the formation of the Au-S bond and the covalent interaction between α-hydroxycarboxylate of citrate and boronate group of MPBA. With this fact, we demonstrated that the activities of protein kinases could be determined on the basis of MPBA-induced *in situ* formation of AgNPs aggregates as labels on electrode surface. In contrast to the previously reported nanoparticle-based signal-amplified strategies, the proposed method obviates the modification of nanoparticles, exhibits good electrochemical signal and high sensitivity, relies on a simple detection principle, and offers an easy operation procedure.

2. Experimental

2.1. Chemicals and reagents

Peptides with the sequences of Ac-IYGEFK and Ac-IYGEFKGGGGC were synthesized and purified by synpeptides Co., Ltd (Shanghai, China). Cyclic adenosine 3′,5′-monophosphate-dependent protein kinase A (PKA, catalytic subunit), β-secretase, thrombin, ATP-S, tyrosinase, okadaic acid potassium salt, 4-amino-5-(4-chlorophenyl)-7-(tert-butyl)pyrazolo[3,4-d]pyrimidine (PP2), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), ascorbic acid, $NaBH_4$, MPBA, trisodium citrate and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) were purchased from Sigma-Aldrich (Shanghai, China). Active tyrosine kinase Src was provided by R&D Systems, Inc. (Minneapolis, MN). Hexokinase was provided by Sangon Biotech. Co., Ltd. (Shanghai, China). Other reagents were analytical-grade and used without additional treatment. The peptide stock solution at the concentration of 1 mM was dissolved with deionized water and diluted with buffer solution before use. Unless otherwise noted, the reactions were conducted at room temperature.

2.2. Instruments

The UV–vis spectra were collected on a Cary 60 spectrophotometer using a 1 cm quartz spectrophotometer cell. Mass spectra were collected on a LCT Premier XE mass spectrometer (Waters, Milford, MA). The transmission electron microscope (TEM) images were taken by an FEI Tecnai G2 T20 TEM. The electrochemical experiments were carried out on a CHI 660E electrochemical workstation (CH Instruments, Shanghai, China). The auxiliary electrode is a platinum wire. The reference electrode is Ag/AgCl.

2.3. Preparation of citrate-capped AgNPs

Citrate-capped AgNPs were synthesized by the chemical reduction of Ag^{+} ions using $NaBH_4$ as the reducing reagent and citrate as the stabilizer. Briefly, 1 mL of a 10 mM $AgNO_3$ solution and 1 mL of a 10 mM trisodium citrate solution were added to 36.8 mL of deionized water under vigorous stirring. This step was followed by addition of 1.2 mL of freshly prepared $NaBH_4$ (10 mM). The solution color gradually changed to yellow, indicating the formation of AgNPs. After the reaction proceeded for approximately 10 min, the resulting colloid was aged for one week at 4 °C. The concentration of AgNPs was calculated according to the total Ag^{+} concentration and the average size of nanoparticles [48]. The prepared AgNPs were isolated by centrifugation for 15 min, washed and dissolved to 8 nM by a phosphate-buffered saline solution (PBS buffer, 5 mM, pH 7.2).

2.4. Mass spectroscopy

To confirm the formation of citrate-MPBA complex, the mixed solution comprising of 500 μM MPBA and 500 μM citrate was injected into the detector for mass measurement. To demonstrate the interaction between MPBA and o-diphenol in the tyrosinase-oxidized peptide Ac-IYGEFK (denoted as Ac-IoYGEFK), 1 mM Ac-IYGEFK in 200 μL of PBS (5 mM, pH 6.5) was first incubated with 1 kU/mL tyrosinase for 20 min, which was followed by

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