Biosensors and Bioelectronics 64 (2015) 462–468

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

A signal "on" photoelectrochemical biosensor for assay of protein kinase activity and its inhibitor based on graphite-like carbon nitride, Phos-tag and alkaline phosphatase

Huanshun Yin [a], Bing Sun [b], Linfeng Dong [a], Bingchen Li [a], Yunlei Zhou [a,*], Shiyun Ai [a,*]

[a] College of Chemistry and Material Science, Shandong Agricultural University, 271018 Taian, Shandong, PR China
[b] Key Laboratory of Molecular Nanostructure and Nanotechnology, Institute of Chemistry, Chinese Academy of Sciences (CAS), Beijing 100190, PR China

ARTICLE INFO

Article history:
Received 4 July 2014
Received in revised form
22 September 2014
Accepted 24 September 2014

Keywords:
Photoelectrochemical biosensor
Protein kinase activity
Graphite-like carbon nitride
Phos-tag
Inhibitor
Cell lysate

ABSTRACT

A highly sensitive and selective photoelectrochemical (PEC) biosensor is fabricated for the detection of protein kinase activity based on visible-light active graphite-like carbon nitride (g-C_3N_4) and the specific recognition utility of Phos-tag for protein kinase A (PKA)-induced phosphopeptides. For assembling the substrate peptides, g-C_3N_4 and gold nanoparticles (g-C_3N_4-AuNPs) complex is synthesized and characterized. When the immobilized peptides on g-C_3N_4–AuNPs modified ITO electrode are phosphorylated under PKA catalysis, they can be specifically identified and binded with biotin functionalized Phos-tag (Phos-tag–biotin) in the presence of Zn^{2+}. Then, through the specific interaction between biotin and avidin, avidin functionalized alkaline phosphatase (avidin-ALP) is further assembled to catalyze its substrate of L-ascorbic acid-2-phosphate trisodium salt (AAP) to produce electron donor of ascorbic acid (AA), resulting an increased photocurrent compared with the absence of phosphorylation event. Based on the specific identification effect of Phos-tag, the fabricated biosensor presents excellent selectivity for capturing the phosphorylated serine residues in the substrate peptides. With the good photoactivity of g-C_3N_4 and ALP-catalyzed signal amplification, the fabricated biosensor presents high sensitivity and low detection limit (0.015 unit/mL, $S/N=3$) for PKA. The applicability of this PEC biosensor is further testified by the evaluation of PKA inhibition by HA-1077 with the IC_{50} value of 1.18 μM. This new strategy is also successfully applied to detect the change of PKA activity in cancer cell lysate with and without drug stimulation. Therefore, the developed PEC method has great potential in screening of kinase inhibitors and highly sensitive detection of kinase activity.

1. Introduction

Photoelectrochemical (PEC) biosensor, as a kind of new detection technique, has attracted great interests because it contains the merits of electrochemical and optical assay (Hu et al., 2013; Wu et al., 2013; Zhang et al., 2011a). For the application of PEC biosensor, one of the crucial influence factors is photoactive material. Up to now, many kinds of photoactive materials have been investigated, such as organic photoelectronic materials, conducting polymer and inorganic semiconductor nanomaterials. Among them, the inorganic semiconductor nanomaterials have been widely investigated and applied in the fabrication of PEC biosensors, such as TiO_2, ZnO, WO_3, Bi_2S_3, BiOI, CdS, CdSe, ZnS, ZnZe, etc (Zhang et al., 2013b; Zhang and Zhao, 2013). Though these materials show great potential in PEC biosensor, there are several disadvantages. Initially, some materials have the wide band gap (such as TiO_2 and ZnO), which limits their applications in PEC biosensing since it can only absorbs UV light ($\lambda < 400$ nm) that can damage some biomolecules or destroy the detected biosystems (Kang et al., 2010). In order to overcome these shortcomings and broaden the adsorbed visible range, modification with narrow-band gap semiconductors or organic photosensitizer is necessary (Tu et al., 2010; Wang et al., 2009). But this modification will increase the experimental complexity. Secondly, most of these materials are metal-contained, which might be harmful to environment. Additionally, several materials only present weak photocurrent response and it might decrease the detection sensitivity when the signal "off" mode is selected. Therefore, the development of new photoactive material is necessary with the merits of fast photoelectronic response, easy preparation, nontoxicity and visible light activity.

Graphite-like carbon nitride (g-C_3N_4) is the most stable allotrope of carbon nitride and has recently attracted great interest

* Corresponding authors. Fax: +86 538 8242251.
E-mail addresses: zhyl@sdau.edu.cn (Y. Zhou), ashy@sdau.edu.cn (S. Ai).

http://dx.doi.org/10.1016/j.bios.2014.09.070
0956-5663/

because of its semiconductor properties and the advantages of easy preparation, simple instrument, cheap reagents, metal-free, visible-light response. So far, g-C_3N_4 has been widely used as photocatalyst and applied in the fields of catalysis (Wang et al., 2010; Zhang et al., 2013a), energy storage (Park et al., 2011), photoelectronic devices (Zhang et al., 2011b), optical sensors (Lee et al., 2011, 2012; Tian et al., 2013), electrochemiluminescent sensor (Chen et al., 2014; Li et al., 2014) and electrochemical sensor (Zhang et al., 2014). Recent research has indicated the potential application of g-C_3N_4 in PEC biosensor. For instances, Xu et al. (2013) developed a novel PEC strategy for the detection of Cu^{2+} with visible-light-driven AgX/g-C_3N_4 (X=Br, I) hybrid materials. Dai et al. (2014) fabricated a sensitive and highly efficient PEC biosensor for the quantitative detection of arecoline based on g-C_3N_4 nanosheets with the assistance of carbon nanohorns. Among these PEC biosensors, g-C_3N_4 and its compounds illustrated good PEC activity.

Protein phosphorylation, which is catalyzed by protein kinases, plays important roles in many biological processes involving cellular proliferation, gene transcription, cell growth and differentiation (Montminy, 1997). Aberrant expression of protein kinase has been associated with a series of diseases (Critchfield et al., 1997; Flajolet et al., 2007; Wang et al., 2001). Therefore, the identification of kinase and their potential inhibitors is not only necessary for basic biology to clarify molecular mechanisms of signal transduction but also valuable for protein kinase-targeted drug discovery and therapy. Therefore, simple, rapid and sensitive assays for determining specific protein kinase activity are needed to facilitate disease therapy and drug discovery.

For protein kinase activity assay, one of the key steps is the valid identification of phosphate group in peptide or protein. Until now, many recognition reagents have been developed, including ATP labeled with radioactive γ-^{32}P or ferrocene or –SH (Houseman et al., 2002; Kerman and Kraatz, 2007; Song et al., 2008), phosphorylated amino acid specific antibody (Shults et al., 2005), carboxypeptidase Y (Zhou et al., 2013), TiO_2 and Zr^{4+} (Bai et al., 2013; Chen et al., 2013), etc. Unfortunately, radioactive ATP suffers from complicated multi-steps operation, harmful to human health and radioactive pollution. Ferrocene or –SH labeled ATP presents complex synthetic process and expensive cost. The application of phosphorylated amino acid specific antibody is beset by the high cost, complicated preparation process, low binding force and the dependence of the type of amino acids (Kinoshita-Kikuta et al., 2009). TiO_2 and Zr^{4+} have the deficiency of low identification specificity.

For overcoming these defects, a kind of dinuclear metal complex that acts as a specific phosphate-binding agent, commercially known as Phos-tag, has been synthesized and applied to assay protein phosphorylation (Inamori et al., 2005; Kinoshita et al., 2006). In the presence of Zn^{2+} or Mn^{2+}, Phos-tag can form a specific noncovalent complex with the phosphomonoester dianion at neutral pH with excellent stability. More importantly, Phos-tag can specifically interact with phosphorylated peptides or proteins containing phospho-Serine, phospho-threonine, phospho-tyrosine, and phospho-histidine residues (Kinoshita et al., 2006; Yamada et al., 2007). Since the synthesis of Phos-tag, it has been widely applied in various techniques for assay and purification of phosphorylated proteins such as fluorescence, mass spectrometry, chromatography, surface plasmon resonance, western blot analysis and gel electrophoresis (Barbieri and Stock, 2008; Takiyama et al., 2009). To date, no reports have described methods for applying Phos-tag-based technologies to analyze the activity of protein kinase. We strived for the assay of kinase activity using Phos-tag and its derivatives. To this end, we developed a novel signal "on" PEC biosensor based on in situ enzymatic product of electron donor under visible-light irradiation, where g-C_3N_4 is used as photoactive material, biotin functionalized phos-tag (Phos-tag–biotin) as phosphate capture molecule and avidin functionalized alkaline phosphatase (avidin-ALP) as signal amplification unit.

2. Experimental section

2.1. Reagents and instruments

The substrate peptide (s-peptide, CGGALRRASLG), the control peptide (c-peptide, CGGALRRAALG), avidin-ALP and adenosine 5′-triphosphate (ATP) disodium salt hydrate are purchased from Sangon Biotech (Shanghai) Co., Ltd. cAMP-dependent protein kinase A (PKA) catalytic subunit, casein Kinase I (CK1), casein kinase II (CK2) and mitogen-activated protein kinase (MAPK) are purchased from New England Biolabs Ltd. (Beverly, MA). Phos-tag–biotin is obtained from Wako Pure Chemical Industries, Ltd. (Japan). 6-Mercapto-1-hexanol (MPH) is supplied by Sigma (USA). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), Tris (hydroxymethyl)aminomethane (Tris), chloroauric acid ($HAuCl_4$) and ascorbic acid (AA) are purchased from Aladdin (shanghai, China). L-Ascorbic acid-2-phosphate trisodium salt (AAP) is obtained from DiBo Chemical Technology co., Ltd. (Shanghai, China). HA-1077 is afforded by EMD Millipore Corporation (Billerica, MA, USA). ITO conductive glass is purchased from Zhuhai Kaivo Electronic Components Co., Ltd. (Zhuhai, China, ITO coating 180 ± 25 nm, sheet resistance $< 15\ \Omega/cm^2$). The buffer solutions and instruments applied in this work are listed in Supporting Informations.

2.2. Synthesis of g-C_3N_4 and g-C_3N_4–AuNPs nanohybrids

In a typical experiment, 3 g of dicyandiamide (99%) is placed in a ceramic boat and then loaded into the central region of a horizontal tube furnace. The tube furnace is heated to 220 °C at a heating rate of 3 °C/min and maintained at 220 °C for 2 h, and then the reactant is further heated to 350 and 550 °C and maintained at these temperatures for 2 and 4 h, respectively. Afterwards, the reaction system is allowed to cool to room temperature naturally. The product is dispersed in double distilled deionized water under ultrasonication and centrifuged at 12,000 rpm for three times to remove soluble reactants and impurities, and then washed three times with ethanol to remove some organic impurities. After drying at 60 °C, the obtained yellow product is ground into powder for further use.

g-C_3N_4–AuNPs nanohybrids are prepared according to previous report with some major modifications (Chen et al., 2014) (see Supporting Informations)

2.3. Biosensor fabrication

See Supporting information.

2.4. Cell culture and lysate preparation

Human hepatoma cell line HepG-2 and human hepatic cell line L-02 are cultured and lysated according to our previous report (Yin et al., 2015). And the operation process was described in Supporting information.

For stimulation, HepG-2 cells are first cultured with the process as described above. Then those cells are moved into a serum-free medium and cultured with another 4 h before simulation. Subsequently, forskolin and IBMX (dissolved in DMSO) solutions are added into the medium to activate intracellular PKA. For