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Ferrocene-functionalized SWCNT for electrochemical detection of T4 polynucleotide kinase activity

Yonghong Wang, Xiaoxiao He*, Kemin Wang*, Xiaoqi Ni, Jing Su, Zhifeng Chen

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Biology, College of Chemistry and Chemical Engineering, Hunan University, Key Laboratory for Bio-Nanotechnology and Molecule Engineering of Hunan Province, Changsha 410082, PR China

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ABSTRACT

A novel electrochemical strategy for monitoring the activity and inhibition of T4 polynucleotide kinase (PNK) is developed by use of titanium ion (Ti^{4+}) mediated signal transition coupled with signal amplification of single wall carbon nanotubes (SWCNTs). In this method, a DNA containing 5′-hydroxyl group is self-assembled onto the gold electrode and used as substrate for PNK. The biofunctionalized SWCNTs with anchor DNA and ferrocene are chosen as the signal indicator by virtue of the intrinsic 5′-phosphate end of anchor DNA and the high loading of ferrocene for electrochemical signal generation and amplification. The 5′-hydroxyl group of the substrate DNA on the electrode is phosphorylated by T4 PNK in the presence of ATP, and the resulting 5′-phosphoryl end product can be linked with the signal indicator by Ti^{4+}. The redox ferrocene group on the SWCNTs is grafted to the electrode and generates the electrochemical signal, the intensity of which is proportional to the activity of T4 PNK. This assay can measure activity of T4 PNK down to 0.01 U mL^{-1}. The developed method is a potentially useful tool in researching the interactions between proteins and nucleic acids and provides a diversified platform for a kinase activity assay.

1. Introduction

Since T4 polynucleotide kinase (PNK) was initially discovered in protein extracts of *Escherichia coli* bacteria infected with T-even phage (Richardson, 1965; Novogrodsky and Hurwitz, 1966), it has become one of the most frequently used enzymes in molecular biology. It has the capacity to phosphorylate DNA at 5′-OH termini by catalyzing the transfer of the γ-phosphate residue of ATP to nucleic acids and oligonucleotides, and it can act as a DNA 3′-phosphatase. Acceptable substrates include double- and single-stranded DNA, RNA, and individual 3′-phosphate nucleotide bases. T4 PNK plays an important role in detection of DNA adducts (Lee et al., 1995; Phillips and Arlt, 2007; El Atifi et al., 2003) or oligonucleotides (Frauendorf et al., 2003; Galburt et al., 2002), nucleic acid metabolism, and repair of DNA lesions (Chappell et al., 2002; Rasouli-Nia et al., 2004; Karimi-Busheri et al., 2007).

There have been several methods for the detection of phosphorylation and the assay of activity of DNA kinase, including radical isotope ^{32}P-labeling, polyacrylamide gel electrophoresis (PAGE), autoradiography and fluorescence (Meijer et al., 2002; Amitsur et al., 1987; Karimi-Busheri et al., 1999; Song and Zhao, 2009). However, these approaches were time-consuming, laborious, complex or costly. Furthermore, they required radio labeling for sensitive detection. Therefore, other assay method possessing the advantages of convenience, fast response and high sensitivity need to be explored to investigate the phosphorylation of nucleic acids. Molecular beacon is a stem-loop DNA oligonucleotide, which carries a fluorophore and a quencher at the 3′- and 5′-ends. They have been widely applied in studying DNA protein interactions, investigating genetic disease, and detecting nucleic acids in solution and living cells because of the advantages of excellent specificity, high sensitivity, and well reproducibility (Zhen et al., 2010; Wu et al., 2011). We have developed a fluorescence assay using molecular beacon DNA probes to investigate the phosphorylation process of nucleic acids by T4 PNK (Tang et al., 2005). The assay was based on the ligation enzyme-coupled reaction. Thus, the reaction mixture contained the two enzymes and two oligonucleotides in addition to the probe. Song and Zhao described a novel method for real-time monitoring of the activity and kinetics of T4 PNK by use of a singly fluorophore-labeled molecular beacon DNA probe coupled with λ exonuclease cleavage (Song and Zhao, 2009). However, this method needed label with molecular beacon and an additional λ exonuclease enzyme.

Compared to other methods such as radioactive, fluorescence and PAGE systems, electrochemical biosensors have attracted considerable interest in phosphorylation assay because of their

* Corresponding authors. Tel.: +86 731 88821566; fax: +86 731 88821566.
E-mail addresses: xiaoxiaohe@hnu.cn (X. He), kmwang@hnu.cn (K. Wang).

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simplicity, rapid response, cost-effective, sensitive and potential ability for real-time and on-site analysis (Chiku et al., 2010; Wang et al., 2010). In these years, a lot of electrochemical biosensors have been designed for the detection of protein kinase activity by measuring the current and charge responses of redox probes conjugated during the phosphorylation processes (Kerman and Kraatz, 2009; Kerman et al., 2008; Wang et al., 2011). To improve the sensitivity of the biosensors, several methods have been developed by exploiting signal amplification strategies (Li et al., 2007; Liu et al., 2006; Du et al., 2009). At present, various nanomaterials such as nanotubes, nanoparticles, and nanowires have been used successfully for ultrasensitive bioassays in connection with optical and electrochemical transducers. Especially, the application of carbon nanotubes in the biosensor fabrication is one of the most popular techniques based on their numerous signal amplifications and excellent electrocatalytic effects and have been widely used for the electrochemical detection of small molecules, protein. They are also attractive alternatives to the detection of kinase activity.

Since metal ion (such as Fe^{3+}, Ga^{3+}, Ti^{4+} and Zr^{4+}) has been proven as an effective capture agent for selective enrichment of phosphorylated peptides, a large number of publications have reported the application of metal ions (Feng et al., 2007; Ibanez et al., 2007; Tan et al., 2008; Blacken et al., 2007). Among these techniques, Ti^{4+} has been demonstrated to have the highest specificity and efficiency for isolation of phosphopeptides (Zhou et al., 2008).

In this work, we designed a sensitive electrochemical assay for detection of T4 PNK activity based on DNA wrapped aminoferrocene (Fc)-single wall carbon nanotube (SWCNT) and Ti^{4+}. The Ti^{4+} could selectively bind phosphorylated DNA over nonphosphorylated DNA, attributable to the specific binding of the terminal phosphate in preference to much weaker binding of the phosphodiester backbone. The anchor DNA wrapped Fc-SWCNT bioconjugate was chosen as the signal indicator due to the virtue of the intrinsic 5′-phosphate end of anchor DNA and the high loading of ferrocene for electrochemical signal generation and amplification. The method was successfully applied to monitor the activity of T4 PNK. Effects of external factors on this system were also investigated. To the best of our knowledge, it is the first time for DNA phosphorylation analysis based on electrochemical methods using Fc-SWCNT as a sensitive signal transduction probe. This strategy provides a simple, sensitive, selective, and universal platform for kinase activity assay and inhibitor screening.

2. Experimental

2.1. Chemicals and materials

All synthetic oligonucleotides were purchased from Sangon Inc. (Shanghai, China). The sequences of oligonucleotides used in this work were as follows: **Substrate DNA**: 3′-HS-GTGTGTG-TGTGTGTGTGTGT-5′; **Anchor DNA**: 5′-HPO_4-GTGTGTGTGTGTGT-GTGTGTGTGTGTGTGTGTGTGTGT-3′. T4 PNK and ATP were purchased from New England Biolabs (NEB, U.K.). Fc was obtained from TCI (shanghai, China). $Ti(SO_4)_2$ were purchased from Sinopharm chemical reagent Co. Ltd. (Shanghai, China). Single-walled carbon nanotubes (purity >90%, diameter <2 nm, length 5–15 μm) were purchased from Nanoport. Co. Ltd. (Shenzhen, China). 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 6-mercapto-1-hexanol (MCH) were from Sigma–Aldrich (St. Louis, USA). All other chemicals were obtained from Reagent & Glass Apparatus Corporation of Changsha and were used without further purification. All solutions were prepared and diluted using ultrapure water (18.2 MΩ cm) from the Millipore Milli-Q system.

2.2. Preparation of DNA/Fc-SWCNT bioconjugates

Here, SWCNTs were used as efficient carriers to load a large amount of electroactive species and anchor DNA as reporters for amplifying the detection of T4 PNK activity. To obtain DNA/Fc-SWCNT bioconjugates, the Fc-SWCNTs were first prepared as the following steps. Briefly, 20 mg SWCNTs were sonicated in an acidic mixture (H_2SO_4:HNO_3 = 3:1) for 24 h. The resulting dispersion was washed with ultrapure water and centrifuged until pH 7.0. This procedure was known to produce SWCNTs which can be easily suspended, sorted, and modified with other molecules. As a result, shortened carbon nanotubes functionalized with —COOH groups were obtained. Next, the shortened SWCNTs (10 mg) were sonicated for 1 h, followed by addition of the solution containing 20 mM EDC and 50 mM NHS for 2 h. After the activated SWCNTs were thoroughly rinsed with deionized water, it was immersed immediately into a mixture of 1 mL of 200 μg/L Fc solution. The reaction mixture was allowed to stir overnight. The resulting precipitates were then received and thoroughly washed with ultrapure water and centrifuged (12,000 rpm for 20 min) to remove any un-reacted Fc.

It has been reported that single-stranded DNA (ssDNA) can wrap around SWCNTs to form a DNA-SWCNT complex and effectively disperse SWCNT into aqueous solution (Napier et al., 2005; Zheng and Diner, 2004), which has been used for construction of a highly sensitive sensor for target molecules. Then the DNA/Fc-SWCNT bioconjugates could be easily prepared by wrapping the single-stranded phosphate-DNA on the Fc-SWCNTs. In brief, 10 μM anchor DNA were added to 0.1 mg/mL Fc-SWCNTs and then shaking for 2 h. Then the DNA/Fc-SWCNT bioconjugates were thoroughly washed with 0.1 M TBS (100 mM Tris–HCl, 300 mM KNO_3, pH 7.5) three times and resuspended in 500 μL TBS for further usage. And the characterization of DNA/Fc-SWCNT bioconjugates was shown in the supporting information (Figs. S1–S3).

2.3. Substrate DNA self-assembled on gold electrode

The gold electrode (2 mm diameter) was first pretreated with freshly made piranha solution (H_2SO_4:H_2O_2 = 7:3, v/v) for 5 min and then rinsed with water. The gold electrode was then polished sequentially with 0.3 and 0.05 μm alumina powder followed by ultrasonic cleaning with ethanol, and ultrapure water for 5 min each. After that, the electrode was scanned in 0.1 M H_2SO_4 between −0.2 and 1.55 V at 100 mV/s until a reproducible cyclic voltammogram (CV) was obtained. After being dried with nitrogen, the electrode was immediately used for substrate DNA immobilization. 10 μL of 5 μM substrate DNA was dropped on the pre-cleaned Au electrode surface and incubated for 2 h in 100% humidity. After the incubation step, the electrode was washed with 0.01 M Tris–HCl buffer solution (pH 7.5) to remove the nonspecific adsorption. Then the unmodified region of the electrode was blocked by immersing the electrode into 1 mM MCH for 15 min and then rinsing the surface with ultrapure water.

2.4. DNA phosphorylation at gold electrode and Ti^{4+} mediated linkage between substrate DNA and DNA/Fc-SWCNT bioconjugates

The substrate DNA self-assembled gold electrode was dunked into 100 μL different concentrations of active T4 PNK (50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.6) for 1 h and then washed twice with TBS. The phosphorylated DNA electrode was then treated with 5 μL 0.5 mM Ti^{4+} at room temperature for 10 min and was washed successively in 2 × TBS, 0.1% SDS (sodium dodecylsulfate) (5 min), 1 × TBS (5 min), and 0.2 × TBS (2 times, 5 min), followed by dipping twice in water. After being dried with nitrogen, 10 μL of diluted DNA/Fc-SWCNT bioconjugates was dropped onto the electrode surface and incubated at room temperature for 30 min. Then