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Electrochemical biosensor based on enzyme substrate as a linker: Application for aldolase activity with pectin-thionine complex as recognization element and signal amplification probe

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ABSTRACT

A new strategy to fabricate electrochemical biosensor is reported based on the linkage of enzyme substrate, thereby an electrochemical method to detect aldolase activity is established using pectin-thionine complex (PTC) as recognization element and signal probe. The linkage effect of fructose-1,6-bisphosphate (FBP), the substrate of aldolase, can be achieved via its strong binding to magnetic nanoparticles (MNPs)/aminophenylboronic acid (APBA) and the formation of phosphoramidate bond derived from its reaction with *p*-phenylenediamine (PDA) on the surface of electrode. Aldolase can reversibly catalyze the substrates into the products which have no binding capacity with MNPs/APBA, resulting in the exposure of the corresponding binding sites and its subsequent recognization on signal probe. Meanwhile, signal amplification can be accomplished by using the firstly prepared PTC which can bind with MNPs/APBA, and accuracy can be strengthened through magnetic separation. With good precision and accuracy, the established sensor may be extended to other proteins with reversible catalyzed ability.

1. Introduction

For the establishment of electrochemical sensors, the linkers are essential and they can be classified into the several categories: (a) nanoparticles (Wu et al., 2013), (b) organic molecules (Ghanem et al., 2008), (c) DNA (Wang et al., 2015), and (d) polymers (Wang et al., 2011). Compared with these linkers, enzyme substrate has the following advantages for the construction of electrochemical sensors: (1) the usage of enzyme substrates can be avoided of the addition of other molecules, making the detection system simple, so as to ensure the analytical accuracy; (2) the number of enzyme substrates is directly related to the enzyme activity. In this paper, taking aldolase as an example, the biosensor has been fabricated based on enzyme substrate as a linker.

Aldolase is encoded by three different genes and highly expressed in the developing embryo and in adult muscle. High aldolase expression was found significantly associated with lymph node involvement (Chang, 2014) and a variety of malignant cancers including human lung squamous (Poschmann et al., 2009; Rho et al., 2009), renal cell, and hepatocellular carcinomas. Up to now, aldolase activity has been widely evaluated by testing the amount of nicotinamide adenine dinucleotide hydrogen (Flechner et al., 1999) via colorimetric method (Malcolm and Shepherd, 1972; Michelis and Gepstein, 2000; Roe et al., 1949). It is known that absorbance or optical density is easily influenced by various factors such as pH values of reaction solution and some colored substances. Therefore, it is of great importance to develop accurate and inexpensive method to assay the enzyme activity. As a simple and cost-effective technique (Swisher et al., 2013; Yixia et al., 2012), electrochemistry are accurate and sensitive. However, electrochemical method has not been established to determine aldolase activity.

Aldolase is responsible for catalyzing the reversible conversion of fructose-1,6-bisphosphate (FBP) to glyceraldehydes-3-phosphate and dihydroxyacetone phosphate (Du et al., 2014). As a specific substrate, FBP has greatly attracted our attention due to its interesting molecular structure containing 1,3-*cis*-diol and phosphate group. It is well known that the compounds like saccharides, having prearranged *cis*-diols with multiple chiral centers, can reversibly react with boronic acids to produce typically five- or six-

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membered esters (Shoji and Freund, 2002; Yum et al., 2011). Moreover, phosphate group can react with amino group to form phosphoramidate bond (Suginta et al., 2013). So the two groups, *cis*-diol and phosphate acid, provide the basis for FBP as a linker with the double recognition. Different from FBP, there is no *cis*-diol group in the molecular structures of two products, glyceraldehydes-3-phosphate and dihydroxyacetone phosphate. These structural characteristics contribute to the separation of substrate from the reaction mixture. Some interfering substances such as enzyme itself, enzyme buffer, and hydrolyzed product, may have great influence on the analytical accuracy, so it will be a good manner to separate the substrate after enzyme-catalyzed reversible reaction.

Magnetic nanoparticles (MNPs), with unique magnetic separation ability, can simplify the isolation procedure and speed up the assay process (Ma et al., 2013; Salamon et al., 2015). Meanwhile, MNPs with high surface to volume ratio and good biocompatibility, are capable of trapping a great deal of signal probes with *cis*-diol groups, so as to improve the analytical sensitivity (Zhao et al., 2006). As one kind of polysaccharides, pectin containing a great amount of *cis*-diol (Zhang et al., 2014a), can be loaded onto the surface of the functionalized MNPs (Wang et al., 2013; Yum et al., 2011; Zhang et al., 2015). Meanwhile, pectin containing carboxylic group, can covalently links with numerous electroactive compounds, to form the complex through the amide bonds.

In this work, enzyme substrate, FBP, has been utilized as a linker for the construction of electrochemical sensor for the analysis of aldolase activity with pectin/thionine complex (PTC) as recognization element and signal amplification probes. The sensitivity of the sensor can be enhanced by PTC and MNPs. Furthermore, magnetic separation can well improve the anti-interference ability.

2. Experimental

2.1. Materials and reagents

Trisodium citrate, sodium nitrate, *p*-phenylenediamine (PDA), 3-aminophenylboronic acid (APBA), thionine, pectin, 1-(3-dimethlaminopropyl)-3-ethylcarbodimide hydrochloride, and N-hydroxy succinimidepurum (NHS) were purchased from Sigma (Shanghai, China). FBP was obtained from Aladdin (Shanghai, China). Aldolase (EC 4.1.2.13, 10 U/mg, from rabbit muscle) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). $FeSO_4$ and $FeCl_3$ were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All buffers and aqueous solutions were prepared with ultrapure water which was purified with a Millipore Mill-Q water Purification system (Barnstead, USA) to a specific resistance of 18 MΩ cm.

2.2. Preparation of the functionalized magnetic nanoparticles

Firstly, Fe_3O_4 magnetic nanoparticles (MNPs) were synthesized by a co-precipitation method. In brief, a 5 mL iron solution containing $FeCl_2$ (40 mM), $FeCl_3$ (80 mM), and 0.4 M HCl was prepared. A 50 mL solution of 0.5 M NaOH was purged with nitrogen to remove oxygen and then heated to 80 °C. Under rapid stirring, the iron solution was added drop by drop, followed by the addition of sodium citrate (0.2 M) after 10 min later. The three carboxylate groups of sodium citrate have strong coordination affinity to iron ions, which favors the attachment of citrate groups on the surface of the MNPs and prevents them from aggregating (Liu et al., 2009; Nigam et al., 2011). After 30 min with continuous stirring, the reaction mixture was washed with water three times and isolated by magnetic decantation. The residue was finally made up to 50 mL with doubly distilled water to give MNPs.

For the immobilization of APBA on the surface of MNPs, EDC (0.22 M) and NHS (0.22 M) were added into 20 mL MNPs and ultrasonicated for 30 min. After magnetic separation to remove EDC and NHS, MNPs were dispersed in water followed by the addition of 2 mL ethanol containing 450 mg APBA. After continuous stirring for 3 h, the resulting mixture was separated by magnet and washed three times with ultrapure water and finally dispersed in 20 mL water to obtain APBA modified MNPs (MNPs/APBA).

2.3. Preparation of pectin/thionine complex (PTC)

1 mg/mL of pectin dispersion was prepared by ultrasonication for 10 min at room temperature. After that, 10 mM EDC and 10 mM NHS were added into the pectin dispersion for 30 min. Then, 1 mM thionine was added into the above solution, and the resulting mixture was stirred vigorously for 12 h. Subsequently, 5 mL NaOH (5%) was added into the mixture for 5 min. After centrifugation at 12,000 rpm for 20 min three times, the deposit was redissolved into 18 mL double-distilled water to give pectin/thionine complex (PTC) which was stored in a refrigerator at 4 °C.

2.4. Modification of gold electrode

Gold electrode was sequentially polished with emery paper (No. 3000 and 5000) and alumina slurry (1.0 and 0.3 μm), followed by ultrasonic cleaning in ethanol and ultrapure water. Subsequently, the electrode was cleaned in piranha solution (98% H_2SO_4: 30% H_2O_2=3:1) for 3 min. Afterward, the electrode was washed thoroughly with ultrapure water and dried under nitrogen gas. After that, the electrode was cleaned electrochemically to remove any remaining impurities with 0.5 M H_2SO_4 (Zhang et al., 2014b). Finally, the electrode was dried by purging with nitrogen.

The treated electrodes were firstly modified with PDA by *in situ* diazonium reduction experiments (Lyskawa and Bélanger, 2006; Zhang et al., 2014b). 5 mM PDA was diazotated in an aqueous solution of 500 mM HCl and 5 mM $NaNO_2$. Then the electrode was dipped in the diazonium solution, and cyclic voltammetric experiment was conducted from 0 to −600 mV at a scan rate of 100 mV/s. After the formation of phenyl ring layer, the electrode was fully rinsed with ultrapure water to remove unbound molecules to give PDA modified gold electrode (PDA/AuE).

2.5. Aldolase activity assay

Aldolase activity assay was performed using the following procedure. The aldolase was firstly dissolved in buffer solution (2.5 M $(NH_4)_2SO_4$, 0.01 M Tris, 0.001 M EDTA, pH 7.5) at different concentrations (0.1–1.25 U/L). After that, 100 μL enzyme solution was added into 100 μL FBP solution (0.3 mM) and the resulting mixture was allowed to stand at 25 °C for 30 min. Then, 200 μL MNPs/APBA was added to the mixture at room temperature, followed by magnetic separation after 3 h later, to give MNPs/APBA/FBP. Subsequently, 0.4 M EDC and 0.1 M NHS was added into the mixture to activate phosphate group and then separated by using magnet after 30 min. Finally, the PDA/AuE was dipped in the mixture containing 100 μL MNPs/APBA/FBP and 100 μL PTC for 4 h. After washing thoroughly using ultrapure water, the electrode was used for electrochemical measurements.

2.6. Electrochemical measurements

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed with a conventional three electrode cell at the room temperature using a CHI 660C electrochemical