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Amplified Voltammetric Detection of Tyrosinase and Its Activity with Dopamine-Gold Nanoparticles as Redox Probes

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Dopamine (DA) is an electrochemically active compound. In this work, we reported an electrochemical affinity biosensor for the detection of tyrosinase activity with DA-modified gold nanoparticles (DA-AuNPs) as redox probes. Hydrophilic boronic acids are well known to form tight diol-boronate ester covalent bonds with members of the diol family. In the sensing platform, monophenols of tyrosine residues immobilized onto gold electrode were catalytically oxidized by tyrosinase to o-diphenols. The resultant o-diphenols captured 4-mercaptophenylboronic acid (MBA)-capped AuNPs (MBA-AuNPs) through the formation of tight covalent bonds between o-diphenols and boronic acids of MBA-AuNPs. Electrochemically active DA-AuNPs were then attached by the anchored MBA-AuNPs via the interaction of MBA with DA, which facilities the amplified voltammetric detection. The current increased with the increase of tyrosinase concentration. The well-defined redox waves and high sensitivity resulting from dual-amplification of MBA-AuNPs and DA-AuNPs demonstrated that this method would be valuable for probing the activity of enzymes by generating a diol-containing substrate, and designing new types of electrochemical biosensor for sensitive detection of diol derivatives.

Keywords: Electrochemical affinity biosensor; Tyrosinase; Gold nanoparticles; Dopamine; Phenylboronic acid

1. INTRODUCTION

Tyrosinase is a copper-containing enzyme present in plant and animal tissues that catalyzes the hydroxylation of monophenols and the oxidation of o-diphenols to o-quinols utilizing molecular oxygen (O_2) [1-3]. It is the rate limiting enzyme for controlling the production of melanin and its inhibitors have been a great concern solely for the past decades [4]. Also, analysis of tyrosinase levels in melanoma cancer cells revealed increased amounts of tyrosinase, and the enzyme is considered as

an indicative marker for this type of malignant cells [5,6]. Currently used methods for the routine detection of tyrosinase and its activity include high performance liquid chromatography, electrophoresis, isotope assay, spectrophotometric assay, and enzyme-linked immunosorbent assay (ELISA) [7-9]. Among them, ELISA has the best sensitivity, selectivity, and versatility. However, a typical sandwiched ELISA requires 1 ~ 2 days and the use of a relatively expensive enzyme-linked antibody. Therefore, simple and sensitive method for detection of tyrosinase and its activity is in demand.

Electrochemical technique is simple, fast and inexpensive to implement [10]. Among types of electrochemical sensors, sandwich-type affinity biosensor is one of attractive tools and has received considerable attention because it is easy and economical to mass production, and it achieves excellent detection limit with small analyte volume. The increasing demand for detection of ultralow amount of analytes is pushing the enhancement of detection sensitivity by selecting different signal amplification strategy. At present, gold nanoparticles (AuNPs) coated with biological recognition elements and redox tags have been widely used for the molecular recognition and signal amplification [11-14]. In particular, the antibody-coated AuNPs, relying on the specific antigen–antibody, have been extensively applied in the recognition of antigen. For the signal output and amplification, antibody-coated AuNPs were usually loaded with a large number of thionine (Th) or ferrocene (Fc). However, the utilization of the antibody-based biosensors might be hindered, especially in resource-poor setting areas such as undeveloped countries, by their high cost and relatively poor stability. Moreover, the loading of a large number of positively charged Th or hydrophobic Fc will decrease the dispersion and solubility of AuNPs.

Dopamine (DA), one of members of the catechol family, is hydrophilic and easily oxidized electrochemically at electrodes. DA was also suggested as an electron donor that could quench or sensitize quantum dots (QDs) [15-18]. Investigations indicated that DA-QDs conjugates show a pair of well-defined waves and function as charge-transfer coupled pH sensors [17,18]. Hydrophilic boronic acids are well known to form tight diol-boronate ester covalent bond with members of the diol family, such as catechol derivatives, glucose and glycoproteins [19-24], and boronic acid-capped nanoparticles have been used in the enrichment and detection of diol-containing biomolecules [20,25,26].

In this work, we developed a dual-amplified electrochemical sensor for the detection of tyrosinase and its activity using 4-mercaptophenylboronic acid (MBA)-capped AuNPs (MBA-AuNPs) and dopamine (DA)-capped AuNPs (DA-AuNPs). The system was formed by biocatalytical oxidation of tyrosine monolayer-modified gold electrode by tyrosinase to the respective o-diphenol monolayer, followed by the attachment of MBA-AuNPs and DA-AuNPs. Because a MBA-AuNP can capture more than one DA-AuNP and each DA-AuNP contains a large number of electrochemically active DA molecules [20,27], the analytical method based on this concept will be sensitive. Moreover, this biosensor obviates the need of expensive and less stable antibody-modified gold nanoparticles conjugates for the recognition of analytes and the need of positively charged Th or hydrophobic Fc for signal output.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Tyrosine methyl ester (TME), tyrosinase, dithiobis(succini-midylpropionate) (DSP), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), MBA and 3-mercaptopropionic acid (MPA) were obtained from Sigma–Aldrich. Dopamine hydrochloride was purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). Kojic acid was obtained from Aladdin Reagent Inc. (Shanghai, China). 4,4'-Dithiobis(phenylboronic acid) (DTPA) was prepared by the oxidation of MBA with $CuSO_4$ in 50% ethanol solution (pH 2). The resultant light yellow precipitation was dried in vacuum chamber and characterized by LCT Premier XE mass spectrometer. The exact mass of DTPA losing one proton was determined to be 305.0270. All aqueous solutions were prepared with a Millipore system (Simplicity Plus, Millipore Corp.), and saturated with N_2.

2.2 Preparation of MBA-AuNPs and DA-AuNPs

The preparation and characterization of DA-AuNPs and MBA-AuNPs have been reported by us previously [27]. Brief, the stock solution of DA-DSP was diluted with deionized water to 500 μM. To avoid the oxidation of DA-DSP, 1 mM Na_2SO_3 was added into the DA-DSP solution. The mixed solution of DA–DSP (1.4 μM) and citrated-stabilized AuNPs (3.2 nM) was then stirred at room temperature for 2 h to produce the DA-AuNPs. The MBA-AuNPs were prepared by mixing 500 nM MBA and 3.2 nM AuNPs for 2 h. Note that trace Fe^{3+}, Cu^{2+} and Al^{3+} can induce the aggregation of DA-AuNPs [28]. Therefore, DA-AuNPs suspension was stored in a clean environment at 4 °C.

2.3 Preparation of TME modified electrode

The gold disk electrodes were polished with diamond pastes down to 3 μm and alumina pastes down to 0.3 μm, and then sonicated in ethanol and water. The MPA self-assembled monolayers (SAMs) were formed by immersing the cleaned gold electrodes in a solution of 10 mmol L^{-1} MPA in the darkness for 12 hours. This step was followed by washing the electrode thoroughly with water. For the preparation of TME-modified electrode, the MPA-covered electrode was soaked in a HEPES buffer solution (10 mmol L^{-1}, pH 7.4) containing 1 mmol L^{-1} TME and 10 mmol L^{-1} EDC for 2 h [6,29]. This was followed by casting ethanolamine (100 mmol L^{-1}) onto the chips to block the EDC-activated unreacted sites for 5 min.

2.4 Detection of tyrosinase and its activity

For the detection of tyrosinase, 10 μL of PBS (pH 6.5) comprising a given concentration of tyrosinase was cast onto the electrode surface, followed by the treatment with 1 mmol L^{-1} citrate acid for 10 min [30]. Again, the electrode was rinsed with water to rid any non-specifically adsorbed substance. To attach DA-AuNPs, the electrode was first allowed to react with 10 μL of MBA-AuNPs

suspension containing 0.1 mM Na_2SO_3 for 30 min, and then exposed to 10 μL of DA-AuNPs suspension for 20 min. After the electrode had been rinsed with water, voltammetric determination in phosphate-buffered saline solution (PBS buffer, 10 mmol L^{-1}, pH 7.4) containing 50 mmol L^{-1} Na_2SO_4 was performed on a DY2013 electrochemical workstation (Digi-Ivy, Inc., Austin, TX) using a homemade plastic electrochemical cell. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively.

3. RESULTS AND DISCUSSION

3.1 *Principle of the amplified voltammetric detection with DA-AuNPs*

The schematic representation of the dual-amplified electrochemical detection of tyrosinase via oxidation of DA tags on the AuNPs is illustrated in Fig. 1. Previous studies have demonstrated that tyrosinase could catalyze the hydroxylation of tyrosine/tyramine residues modified on the surface of Au, Pt, or CdSe/ZnS substrates [6,30,31]. In this work, TME was immobilized onto the MPA SAMs through the standard amine coupling reaction. The monophenols anchored on the electrode was catalytically oxidized by tyrosinase to o-diphenols in the presence of O_2 [6,30]. The o-diphenols could be further oxidized by tyrosinase and O_2 to o-quinols. In order to ensure that all of surface of the monolayer is in the o-diphenols state, the electrode was treated with citric acid reducing any generated o-quinols to o-diphenols [30]. MBA-AuNPs were captured by o-diphenols via the formation of diol-boronate ester, which was followed by the attachment of DA-AuNPs in the same way. Since each gold nanoparticle was capped with a large number of DA molecules, the electrochemical signals were greatly amplified.

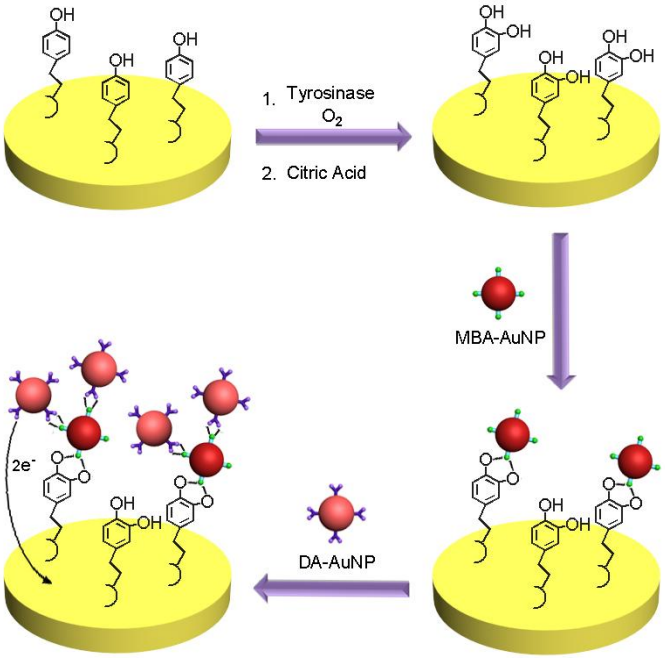

Figure 1. Schematic illustration of the strategy of tyrosinase detection using MBA-AuNPs and DA-AuNPs.

3.2 Feasibility of the amplified voltammetric detection

As shown in Fig. 2, no redox peak was observed at TME-covered electrode (black curve). After the surface was treated with tyrosinase, a couple of small voltammetric peaks was observed (red curve), which was attributed to the oxidation/reduction of o-diphenols generated from the hydroxylation of TME. To amplify the voltammetric signals and to achieve a low detection level for tyrosinase, the o-diphenols were derivatized with MBA-AuNPs followed by the attachment of DA-AuNPs. As shown by the blue curve, well-defined redox waves with high signal intensity were obtained. The redox peaks with E_{pa} = 0.173 V and E_{pc} = 0.135 V were attributed to the oxidation/reduction of the DA moieties of DA-AuNPs [27]. It is worth to illustrate that the oxidation potential of DA-boronate ester shifts positively to 0.619 V [22]. So scanning the electrode potential within the range of –0.1 ~ 0.4 V did not caused the loss of anchored MBA-AuNPs/DA-AuNPs. The controlled experiment was conducted in the absence of MBA-AuNPs (cyan curve). The voltammetric peak currents dropped to a background level, indicating that the attachment of DA-AuNPs is dependent on the anchored MBA-AuNPs. To verify the amplification effect of MBA-AuNPs, we conducted the experiment with 4,4'-dithiobis(phenylboronic acid) (DTPA) instead of MBA-AuNPs and found that the current was markedly lower with DTPA as cross-linkers (green curve) than that with MBA-AuNPs. The results indicated that the electrochemical signal was amplified by MBA-AuNPs. Additionally, we investigated the effect of oxidation time of tyrosinase and found that the oxidation current increased with reaction time. It began to level off beyond 30 min (Fig. 3), indicating that the tyrosinase-catalyzed oxidation is completed within 30 min.

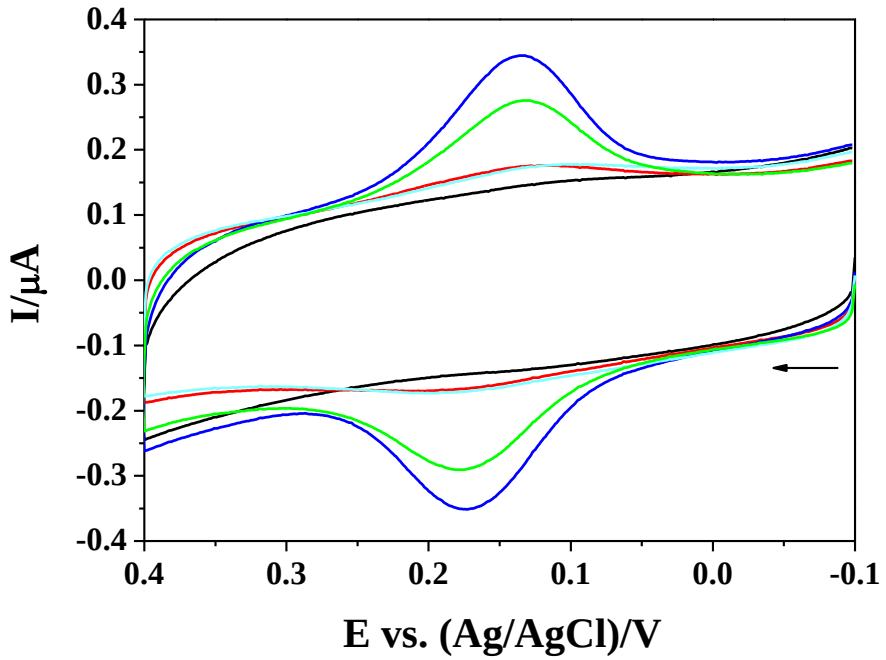

Figure 2. Cyclic voltammograms (CVs) acquired at TME-covered electrodes before (black curve) and after treatment with tyrosinase (red curve), tyrosinase/MBA-AuNPs/DA-AuNPs (blue curve), tyrosinase/DA-AuNPs (cyan curve) and tyrosinase/DTPA/DA-AuNPs (green curve), respectively. The concentration of tyrosinase was 0.2 units. The arrow indicates the scan direction and the scan rate was 100 mV/s.

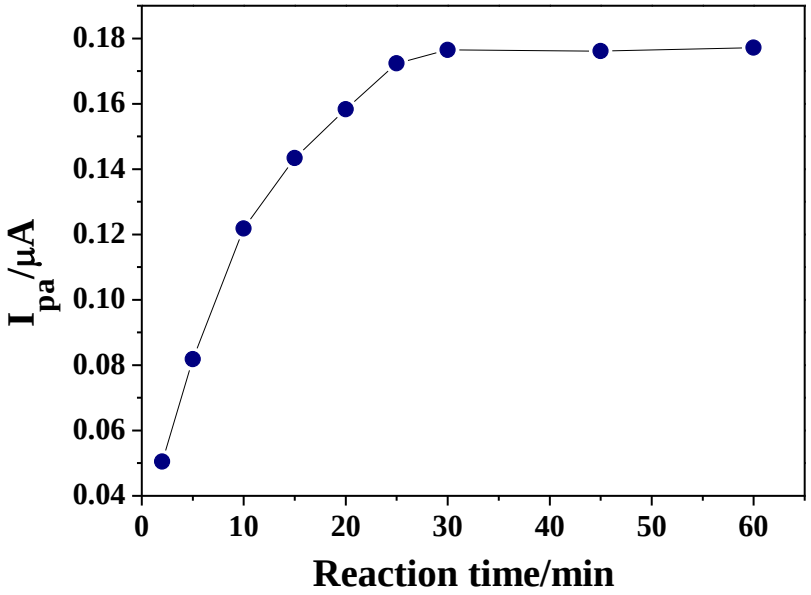

Figure 3. Time-dependence of TME oxidation by tyrosinase. The other experimental conditions are the same as those in Fig. 2.

3.3 Sensitivity to tyrosinase

Another electrochemical technique, differential pulse voltammetry, can decrease the background charging currents and in turn increase the detection sensitivity. Therefore, we evaluated the sensitivity and dynamic ranges of the proposed method using differential pulse voltammetry (Fig. 4A).

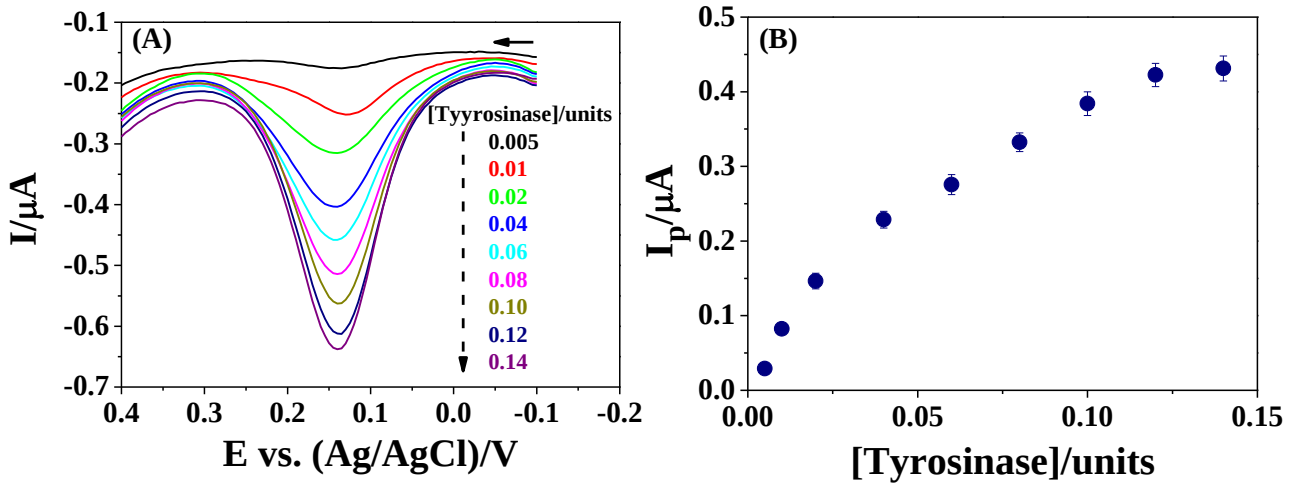

Figure 4. (A) DPVs collected at TME-covered electrodes after treatment with different concentrations of tyrosinase followed by attachment of MBA-AuNPs and DA-AuNPs. Pulse height: 50 mV; pulse width: 50 ms. (B) Plots of peak current (I_p) versus tyrosinase concentration. The relative standard deviations (RSDs) from three replicates were shown as the error bars.

As the increase of tyrosinase concentration, the content of tryrosinase-synthesized o-diphenols on electrode is higher, resulting in the increase of peak current (I_p) (Fig. 4B). The detection limit (3σ) of the method was determined to be 0.001 units (n = 11), which translates to ~ 0.01 μg mL^{-1} of the enzyme in the sample [32]. This value is at least one order of magnitude lower than those reported so far [6,30,32].

3.4 Assay of tyrosinase activity

Tyrosinase catalyzes the first two steps in mammalian melanogenesis and is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing [4]. Researchers have being encouraged to seek new potent tyrosinase inhibitors for use in foods and cosmetics because neither hyperpigmentation in human skin nor enzymatic browning in fruit is desirable. To demonstrate the amenability of our method to screen tyrosinase inhibitors, kojic acid, the most intensively studied inhibitor of tyrosinase [4], was tested. As shown in Fig. 5, a pair of well-defined redox waves was observed in the absence of kojic acid (black curve). The currents decreased and finally almost dropped to the background level with the increase of kojic acid concentration (red, green, and blue curves), indicating that the activity of tyrosinase was inhibited by kojic acid. These experiments demonstrated that the proposed method was applicable for screening tyrosinase inhibitors.

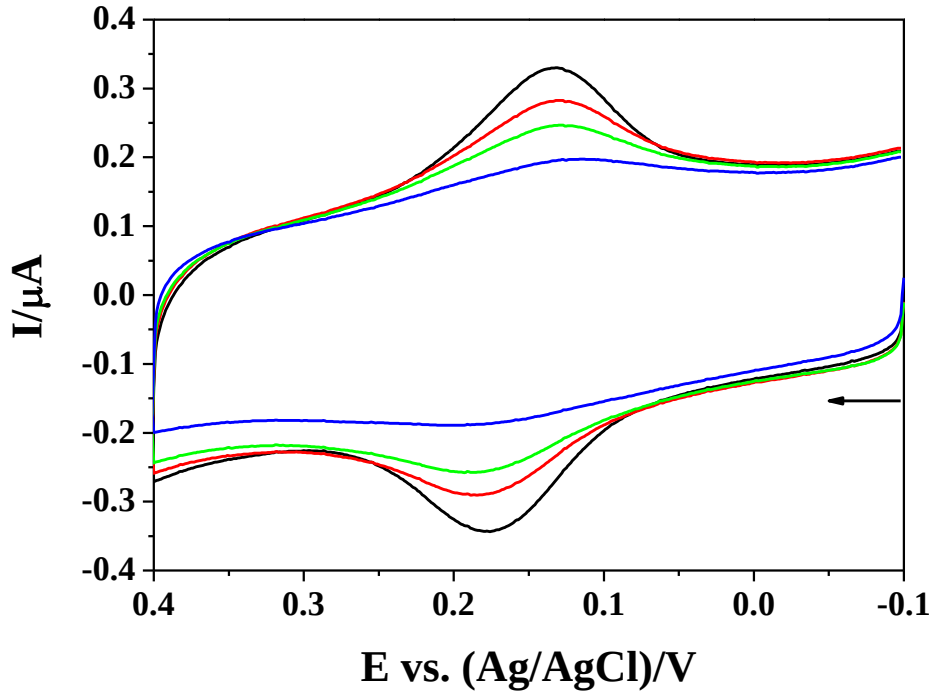

Figure 5. CVs acquired at TME-covered electrodes after treatment with the mixtures of 0.12 units tyrosinase and 0 (black curve), 0.1 (red curve), 0.2 (green curve) and 0.5 (blue curve) μmol L^{-1} kojic acid followed by the attachment of MBA-AuNPs and DA-AuNPs. The other experimental conditions are the same as those in Fig. 2.

4. CONCLUSIONS

In summary, we developed an electrochemical affinity biosensor for the detection of tyrosinase activity with DA-AuNPs as redox probes. Tyrosinase catalyzed the anchored monophenols to o-diphenols and the resultant o-diphenols monolayer was derivatized with MBA-AuNPs for the attachment of electroactive DA-AuNPs. The electrochemical signals were amplified by MBA-AuNPs and DA-AuNPs. The detection limit of the proposed method is at least one order of magnitude lower than those reported so far. We believe that this work would be valuable for the amplified voltammetric detection of diol derivatives and other enzymes, such as tyrosine kinase, alkaline phosphatase and protease.

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