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Journal of Electroanalytical Chemistry 540 (2003) 61–67
Journal of Electroanalytical Chemistry
www.elsevier.com/locate/jelechem

Renewable phenol biosensor based on a tyrosinase-colloidal gold modified carbon paste electrode

Songqin Liu, Jiuhong Yu, Huangxian Ju *

The State Key Laboratory of Coordination Chemistry, Department of Chemistry, Institute of Chemical Biology, Nanjing University. Nanjing 210093, People's Republic of China

Received 21 June 2002; received in revised form 25 September 2002; accepted 19 October 2002

Abstract

A novel renewable tyrosinase-based biosensor was developed for the detection of phenol by immobilizing tyrosinase on a colloidal gold modified carbon paste electrode. Anionic colloidal gold was beneficial to the immobilization of tyrosinase and to the retention of its bioactivity to a large extent. The biosensor showed a sensitive electrochemical response to the reduction of the oxidation product of phenol by dissolved O_2 in the presence of immobilized tyrosinase. The effects of pH, operating potential and the volume of the colloidal gold solution for sensor preparation on the amperometric response were explored for optimum analytical performance. The best performing biosensor exhibited a fast response (less than 5 s), a high sensitivity (12.3 μA cm^{-2} μM^{-1}) and good storage stability for monitoring phenol. The linear range spanned the concentration of phenol from 4 to 48 μM with a correlation coefficient of 0.9973 ($n = 12$) and a detection limit of 6.1 nM at 3σ. The response showed Michaelis–Menten behavior at larger phenol concentrations. The K_M^{app} value of immobilized tyrosinase on colloidal gold was calculated to be (53.6 ± 3.2) μM using phenol as the substrate.

Keywords: Tyrosinase; Biosensor; Phenol; Carbon paste electrode; Colloidal gold

1. Introduction

Phenols and substituted phenols are important contaminants in medical, food and environmental matrices [1]. In view of their high toxicity, reliable analytical procedures are required for sensitivity determination at low level in various matrices. Many techniques are now commonly used for monitoring phenols, such as colorimetry, gas chromatography, liquid chromatography, and capillary electrophoresis [2–5]. Electrochemical methods have been widely used for measuring these compounds due to their advantages such as good selectivity in the presence of phenol oxidases, relatively low cost of realization and storage, the potential for miniaturization and automation [6,7]. Numerous biosensors have been proposed for the detection of phenolic compounds based on primarily the phenol oxidase, tyrosinase [6–14].

Mushroom tyrosinase is the best-known dicopper oxidase [15] and has industrial applications such as its use in biosensors for oxygen and phenols [16]. It catalyzes the hydroxylation of monophenols to form *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinines, using molecular oxygen [16,17]:

$$\text{Phenol} + \text{tyrosinase } (O_2) \rightarrow \text{catechol} \quad (1)$$

$$\text{Catechol} + \text{tyrosinase } (O_2) \rightarrow o\text{-quinone} + H_2O \quad (2)$$

o-Quinones can be electrochemically reduced to *o*-diphenols with a low overpotential by the following equation without any electron transfer mediator

$$o\text{-quinone} + 2H^+ + 2e^- \rightarrow \text{catechol} \quad (3)$$

So, the detection of phenols relies on monitoring the liberation of the quinone products or the consumption of the oxygen cofactor.

* Corresponding author. Tel.: +86-25-359-3593; fax: +86-25-3593693.
E-mail address: hxju@nju.edu.cn (H. Ju).

0022-0728/02/$ - see front matter
PII: S0022-0728(02)01276-7

Recently reagentless biosensors based on the immobilization of tyrosinase in carbon paste electrodes have been widely reported [7,11–13,18]. At these electrodes, amplification of the overall process is obtained, leading to high sensitivity. Marko-Varga et al. [7] examined the influence of various silicone and paraffin oils on the response of tyrosinase immobilized in carbon paste electrode. The viscosity of the binder liquid had a significant effect on the performance of the tyrosinase modified carbon paste electrode [13]. Wang et al. [12] demonstrated that short-chain hydrocarbon binders provided a favorable environment for the activity of tyrosinase and thus improved the sensitivity of the biosensors. However, the short-chain hydrocarbon pasting liquid possessed a low hydrophobic character, which possibly led to low structural rigidity and hence short-term stability of the carbon paste electrode in the flow system. The incorporation of solid and chemical additives to carbon paste is another way to improve the sensitivity and operational stability of biosensors based on carbon paste electrodes [19]. The presence of additives in a tyrosinase modified carbon paste electrode might alter the electrochemical properties of the paste due to the change of the micro surface and its polarity, thus resulting in an improvement of enzyme stabilization, selectivity and sensitivity, and eventually to an enhanced performance of the biosensors.

Solid particles have previously been introduced into enzyme modified carbon paste [20]. Lutz et al. [19] investigated the modification of a tyrosinase modified carbon paste with different chemicals as well as solid particles. Recently there has been an increasing interest in the electrochemical behavior and application of nanomaterials [21]. It has been demonstrated that colloidal gold, a well-known nanomaterial, can help proteins to retain their biological activity upon adsorption [22,23]. Therefore, it has been used for the study of direct electron transfer of redox proteins [24,25] and the preparation of biosensors [25–27]. To the best of our knowledge, however, no study on the electron transfer and the immobilization of tyrosinase on colloidal gold for preparation of amperometric biosensors has been reported. Our previous work indicated that the hydrogen peroxide sensor based on the immobilization of cytochrome *c* or horseradish peroxidase on a colloidal gold modified carbon paste electrode was of good long-term stability and high sensitivity [24,26]. In this paper we develop a novel renewable tyrosinase-based biosensor for detection of phenol. The tyrosinase incorporated in a colloidal gold modified carbon paste electrode retains its bioactivity to a large extent and exhibits a fast response as well as high sensitivity to phenol, which is about 4.25 times higher than that without the presence of colloidal gold.

2. Experimental

2.1. Reagents

Mushroom tyrosinase (from mushroom, EC.1.14.18.1), noted activity of 2400 units mg^{-1} of solid, was purchased from Sigma and used as received. 3-Hydroxytyramine hydrochloride was purchased from Acros Organics. $AuCl_3HCl{\cdot}4H_2O$ (Au > 48%) was obtained from Aldrich. Carbon graphite powder (< 325 mesh, Johnson Matthey) and paraffin oil (from Fluka) were used for the preparation of the carbon paste. All of the other chemicals were analytical grade and were used without further purification. Colloidal gold was prepared by adding 0.5 ml of 1% Na_3-citrate solution to a boiling 50 ml solution of 0.01% $HAuCl_4$ [22,23]. Prior to use, $HAuCl_4$ and Na_3-citrate aqueous solutions were filtered through a 22 μm microporous membrane filter and all glassware used in this procedure was cleaned in freshly prepared 3:1 HNO_3–HCl and then rinsed thoroughly in twice-distilled water. The mixture was maintained at the boiling point for 15 min and stirred for another 15 min after removal of the heating source to produce 24-nm diameter colloidal Au particles [25]. The preparation was stored in a brown glass bottle at 4 °C. Phosphate buffer solutions (PBSs) (0.1 M) with various pHs were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or NaOH. All solutions were prepared with doubly distilled water.

2.2. Electrode preparation

The tyrosinase-colloidal gold modified carbon paste electrodes (Tyr-Au-CPEs) were prepared according to the literature [24]. Briefly, 10 mg of pretreated graphite powder was mixed thoroughly with 30 μl 24 nm colloidal gold solution. After evaporation of water in a desiccator for 3 h, 3.6 μl paraffin oil and 3.5 mg tyrosinase (or only 3.6 μl paraffin oil for control) were added to the mixture. A portion of the resulting paste was put into a plastic syringe tube with an inner diameter of 0.50 mm, little by little to form a Tyr-Au-CPE (or Au-CPE). The tyrosinase modified carbon paste electrode (Try-CPE) was prepared by mixing thoroughly 10 mg of pretreated graphite powder with 3.6 μl paraffin oil and 3.5 mg tyrosinase (or only 3.6 μl paraffin oil for CPE) and then putting a portion of the mixture into the plastic syringe tube. Electrical contact to the paste was established by inserting a copper wire down the plastic syringe tube and into the back of the mixture. The carbon paste electrode was stored at 4 °C. After the electrode tip was gently rubbed on fine paper to produce a flat surface, the following experiments were carried out.

2.3. Electrochemical measurements

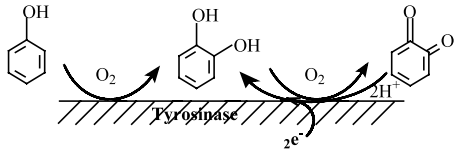
Electrochemical experiments were performed with a BAS-100B electrochemical analyzer connected a PA-1 preamplifier (Bioanalytical Systems, USA). A three-electrode cell equipped with a platinum wire counter electrode, a saturated calomel reference electrode (SCE), and a Tyr-Au-CPE (or Au-CPE, Tyr-CPE) as the working electrode, was used for all electrochemical experiments. The real area of the working electrode was determined to be 1.9×10^{-3} cm^2 from the slope of the plot of the anodic peak current of 1.0 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl versus the square root of scan rate. All experiments were performed at room temperature (18 ± 2 °C) in 0.1 M PBS as the background electrolyte. Amperometric experiments were performed at an applied potential of -150 mV (vs. SCE) by successive addition of 10 μl of 2.0×10^{-3} M phenol to 5 ml buffer solution kept under stirring with a magnetic stirrer. Before each measurement of the phenol signal, the system was put in open circuit for 1 min.

3. Results and discussion

3.1. Electrochemical response enhanced by the colloidal gold in Tyr-Au-CPE

Fig. 1 shows the cyclic voltammograms of air-saturated pH 7.0 PBS in the absence and presence of phenol at CPE, Au-CPE, Tyr-CPE and Tyr-Au-CPE. It is obvious that the enzyme modified electrodes show low background currents. Upon addition of phenol to the electrochemical cell, the cyclic voltammograms change dramatically with a significant increase of the reduction current at Tyr-CPE and Tyr-Au-CPE, while no change is observable at both CPE and Au-CPE even at concentrations of phenol up to 4.0×10^{-4} M. Obviously, the enhanced reduction current at Tyr-Au-CPE is attributed to the efficient catalytic reactions of phenol to form *o*-quinine by immobilized tyrosinase in air-saturated solution. The electrode process can be expressed as Scheme 1 [28,29]. The carbon microparticle is an important factor for enhancing the electron transfer between *o*-quinone and the electrode surface.

Although the cyclic voltammogram at Tyr-CPE shows the catalytic reactions to form *o*-quinone on the electrode surface, its response is 1.9 times smaller than that at Tyr-Au-CPE (Fig. 1). At a phenol concentration of 5.0×10^{-5} M, the response is 3.87 times smaller than that at Tyr-Au-CPE (Fig. 2). The presence of colloidal



Scheme 1.

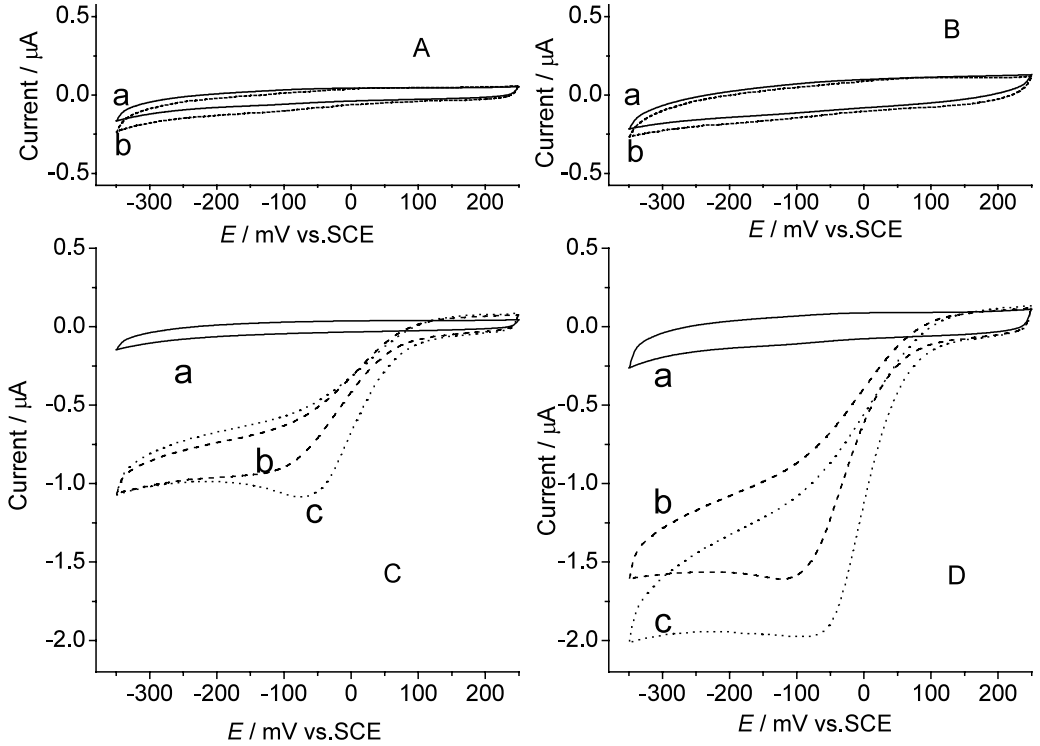


Fig. 1. Cyclic voltammograms of CPE (A), Au-CPE (B), Tyr-CPE (C) and Tyr-Au-CPE (D) before (a) and after addition of 1.0×10^{-4} (b) and 2.0×10^{-4} M (c) phenol to air-saturated pH 7.0–0.1 M PBS at 50 mV s^{-1}.

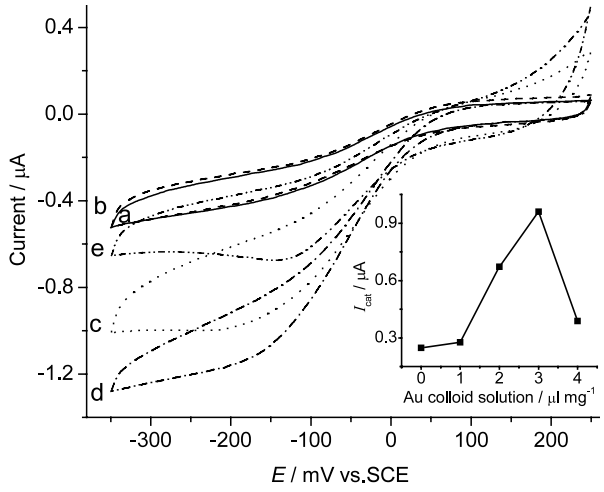


Fig. 2. Cyclic voltammograms of carbon paste electrodes prepared with 0.35 mg tyrosinase and 0 (a), 1.0 (b), 2.0 (c), 3.0 (d) and 4.0 μl (e) colloidal gold solution added to each mg of carbon graphite powder in air-saturated pH 7.0 PBS containing 5.0×10^{-5} M phenol. Inset: plot of catalytic current at −150 mV vs. the volume of the colloidal gold solution.

gold results in a sigmoidal-shaped response curve (Fig. 1D). Curve d in Fig. 2 also gives a constant Faraday current in the potential range from −200 to −350 mV. Thus, the colloidal gold nanoparticles play an important role in enhancing the enzyme catalytic sites accessible to substrate molecules. The immobilization of enzyme in the presence of anionic coagulants has the advantages of retaining it in the reactor and protecting it from inactivation by reaction with substrate [30,31]. The tyrosinase adsorbed on the surface of colloidal gold nanoparticles with negative charges is in a micro-environment similar to its native environment, so it retains its bioactivity to a large extent and makes the enzyme catalytic sites easily accessible to substrate molecules, resulting in high sensitivity and saturation of these sites at low substrate concentrations [14].

3.2. Influence of Au colloid content for preparation of Tyr-Au-CPE

Fig. 2 shows the cyclic voltammograms of Tyr-Au-CPEs, prepared with different volumes of gold colloid solution mixed with each mg of carbon powder, in pH 7.0 air-saturated PBS containing 5.0×10^{-5} M phenol. The reduction current gradually increases with an increasing volume of Au colloid solution, indicating that the colloidal gold nanoparticles make the enzyme catalytic sites closer to the substrate molecules. When the volume is greater than 3 μl, the reduction current decreases with increasing Au colloid content. This phenomenon is attributed to the increase in the resistance and double layer capacitance of the electrode (comparing curves d and e in Fig. 2) due to the decrease of the ratio of carbon sensing sites in the paste. The amperometric responses of the biosensors (Fig. 3) also exhibit the same results as the cyclic voltammetric responses. A higher sensitivity and wider linear range occur when a volume of 3 μl Au colloid solution mixed with each mg of carbon powder is used. The maximum catalytic response occurred at a gold colloid volume of 3 μl per mg carbon powder.

3.3. Effect of solution pH on the amperometric response of biosensors

The effect of solution pH on the amperometric response of Tyr-Au-CPE is shown in Fig. 4. Obviously, the amperometric response increases and then decreases with increasing pH value. When the pH > 7.4 the decrease of the amperometric response is due to the involvement of protons in the reduction reaction of *o*-quinone and the hydroxylation of phenol catalyzed by the enzyme to form *o*-diphenol. At low pH the increase in amperometric response with an increasing pH is attributed to the increase of the enzyme activity. The response is also related to the charge of the electrode surface, colloidal gold nanoparticles and immobilized tyrosinase, the diffusion of phenol to the electrode surface, the transport of phenol to the immobilized enzyme and catechol back to the enzyme, and the transport of *o*-quinone to the electrode. With this list of complex variables, the response shows an optimal pH range between 6.6 and 7.0 with a maximum sensitivity and the widest linear response range at pH 7.0 at −0.15 V.

3.4. Amperometric response for the enzyme electrode

The potential dependence of the amperometric signal and background current of the sensor was tested in the potential range from 0.0 to −0.4 V versus SCE. The

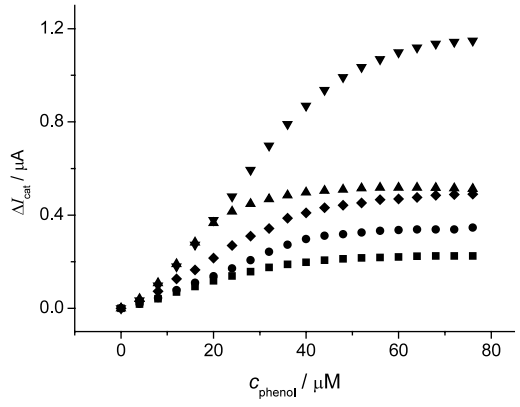


Fig. 3. Amperometric response of carbon paste electrodes prepared with 0.35 mg tyrosinase and 0 (■), 1.0 (●), 2.0 (▲), 3.0 (▼) and 4.0 μl (◆) colloidal gold solution added to each mg of carbon graphite powder by successive addition of phenol to air-saturated pH 7.0 PBS under stirring at −0.15 V.

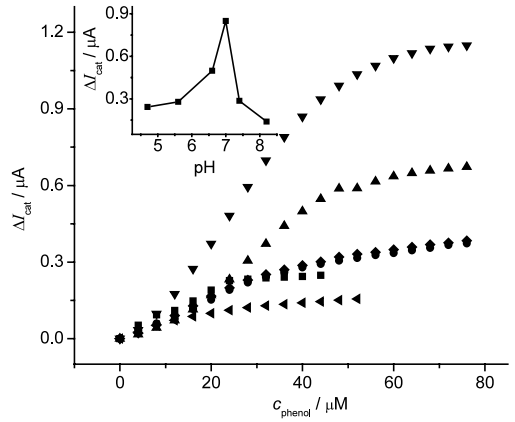


Fig. 4. Amperometric response of Tyr-Au-CPE by successive addition of phenol to air-saturated PBS of pH 4.7 (■), 5.6 (●), 6.6 (▲), 7.0 (▼), 7.4 (◆) and 8.2 (◀) under stirring at −0.15 V. Inset: plot of catalytic current vs. pH for 40 μM of phenol.

highest ratio of signal-to-background current was achieved at −0.15 V. The background current was caused by the reduction of dissolved oxygen. When the applied potential was more negative than −0.15 V a higher signal current was obtained, but the background current increased simultaneously. So, a working potential of −0.15 V was preferred for the amperometric determinations.

Fig. 5 illustrates a typical current-time plot for the sensor on successive additions of phenol to air-saturated pH 7.0–0.1 M PBS under stirring. Upon addition of successive aliquots of phenol to PBS, a clearly defined reduction current proportional to the phenol concentration is observed. The response occurring immediately after the addition of phenol is attributed to the reduction of *o*-quinone. The sensor response achieves 95% of steady-state-current in less than 5 s. A steady-state baseline current is reached typically after 30 s, which is

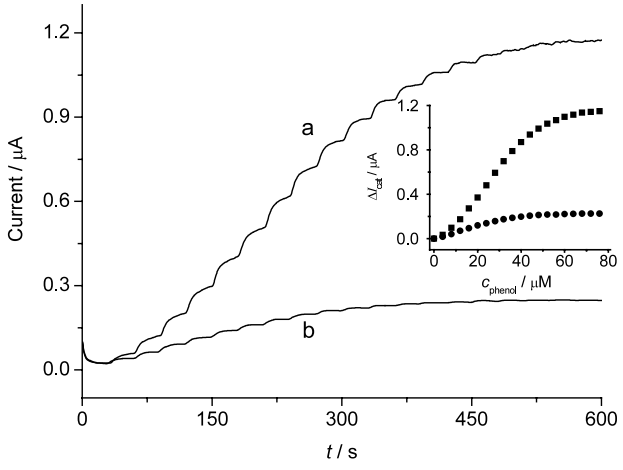


Fig. 5. Typical steady-state current–time response curves for increasing phenol concentration in 4.0×10^{-6} M steps at Tyr-Au-CPE (a) and Tyr-CPE (b) at −0.15 V. Inset shows the calibration curves at Tyr-Au-CPE (■) and Tyr-CPE (●).

faster than that of 1–2 min reported at a tyrosinase-Al_2O_3-sol–gel modified electrode [8]. Such a short response time further proves that the presence of colloidal gold is promising for the construction of biosensors.

The calibration curves of the enzyme electrodes in the phenol concentration range from 4.0 to 80 μM under the optimal conditions are shown in Fig. 5. The linear range of Tyr-Au-CPE spans the concentration of phenol from 4.0 to 48 μM with a correlation coefficient of 0.9973 ($n = 12$), while it is from 4.0 to 36 μM for Tyr-CPE. The sensitivity to phenol is 12.25 and 2.88 μA cm^{-2} μM^{-1} at Tyr-Au-CPE and Tyr-CPE, respectively. The limit of detection for phenol, defined as the amount required to give a signal of three times the standard deviation of the noise signal, is 6.1 nM at Tyr-Au-CPE. The presence of colloidal gold results in an increase of sensitivity by a factor of about 4.25. The sensitivity of 12.25 μA cm^{-2} μM^{-1} is greater than the valves of 0.23 μA cm^{-2} μM^{-1} of a tyrosinase-Nafion® based graphite electrode [6], 2.5 μA cm^{-2} μM^{-1} of a tyrosinase modified carbon paste electrode [7], 1.80 μA cm^{-2} μM^{-1} of a tyrosinase-Al_2O_3-sol–gel modified electrode [8], 5.74 μA cm^{-2} μM^{-1} of a biosensor fabricated from Gwent graphite ink [14], and 9.6 nA cm^{-2} μM^{-1} of a tyrosinase modified carbon paste electrode [18]. The improvement in sensitivity results from three factors. One is that colloidal gold allows the enzyme bioactivity to be retained to a large extent after the entrapping procedure (as shown in Figs. 1 and 2). The other is the decrease of the resistance of the carbon paste electrode from 2427 to 1297 Ω upon the addition of colloidal gold into the carbon paste. The resistance values were determined from ac impedance spectroscopy using the PGSTAT30/FRA2 system (Autolab, The Netherlands). The third factor is that colloidal gold gives the enzyme molecules more freedom in orientation, thus reducing the insulating property of the enzyme shell and allowing access to the substrate [23].

When the concentration of phenol is higher than 48 μM, the amperometric responses of both Tyr-Au-CPE and Tyr-CPE exhibit characteristics of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_M^{app}) of tyrosinase immobilized in Au-CPE is calculated to be (53.6 ± 3.2) μM according to the Lineweaver–Burk equation [32] when using phenol as substrate, while the value of tyrosinase immobilized in CPE is (71.2 ± 3.2) μM. The K_M^{app} values are markedly lower than those observed for the free enzyme in solution which was estimated to be 700 μM using phenol as the substrate [33] and 240 μM using catechol as the substrate [34]. This behavior may be due to some electroenzymatic recycling phenomenon which gives rise to the decrease of the apparent Michaelis constant of the entrapped enzyme [35]. The K_M^{app} values are also lower than the values of 133, 168, and 245 μM found for

tyrosinase immobilized in carbon paste electrodes with different binders [12]. Although such values reflect the interplay between numerous factors, including diffusional constraints of the substrate, the oxygen (cofactor) solubility and the enzyme reactivity in the pasting liquid, the action of colloidal gold is an important factor. The colloidal gold adsorbed on the surface of carbon particles gives the enzyme molecule more freedom in orientation, resulting in a higher affinity of tyrosinase to its substrate.

3.5. Amperometric response of the enzyme electrode to dopamine and catechol

The responses of Tyr-Au-CPE to other phenol derivative compounds, dopamine and catechol, obtained at −0.15 V, are shown in Fig. 6. Upon addition of successive aliquots of dopamine or catechol to air-saturated PBS, the amperometric response increases and shows a Michaelis–Menten mechanism at high concentrations. The upper limit of the linear range is 16 μM for dopamine and 32 μM for catechol, which is smaller than 48 μM for phenol. The values of K_M^{app} are calculated to be (13.0±0.3) and (48.5±1.6) μM for Tyr-Au-CPE using dopamine and catechol as substrates, respectively.

3.6. Stability of enzyme electrode

When Tyr-Au-CPE is stored in a refrigerator at 4 °C for a period of 2 weeks, no obvious decrease in the response to phenol is observable. After a storage period of 20 days, it retains 90% of its initial current response, while the Tyr-CPE retains 72% of its initial current response. Thus, colloidal gold nanoparticles are very efficient for maintaining the activity of tyrosinase. After the sensors are used successively for 16 h or cyclically swept for 300 cycles, the response of Tyr-Au-CPE to phenol remains at a constant value. Longer-term use results in a decrease of the current response. The surface can be renewed by gentle rubbing on a fine paper. The current response of the renewed surface is examined at a phenol concentration of 10 μM in air-saturated pH 7.0 PBS. The relative standard deviation is 3.2% for six successive renewals. Thus the method to renew the enzyme surface is rapid, easy and reproducible. The fabrication reproducibility is estimated for six electrodes made independently using the response of 10 μM phenol in air-saturated pH 7.0 PBS. The relative standard deviation is calculated to be 8.6%, indicating good fabrication reproducibility.

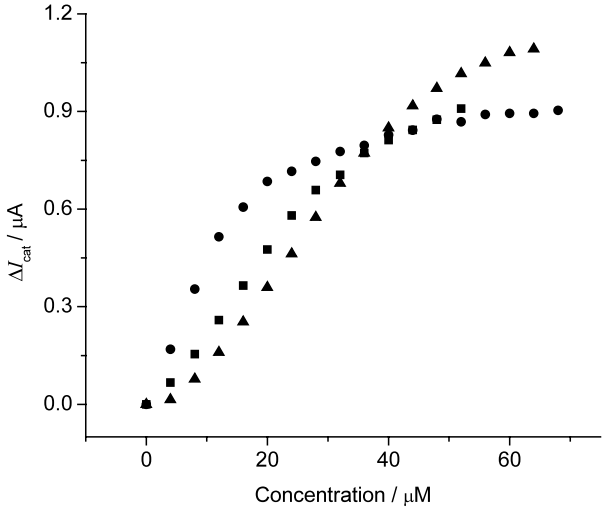


Fig. 6. Calibration curves of Tyr-Au-CPE upon successive addition of phenol (▲), catechol (■) and dopamine (●) to air-saturated pH 7.0 PBS at −0.15 V.

4. Conclusions

It is demonstrated that colloidal gold nanoparticles mixed in a carbon paste to prepare a tyrosinase electrode have the advantages of maintaining enzyme bioactivity in the reactor, making the enzyme catalytic sites close and easily accessible to the substrate molecules. These advantages lead to significant improvement of the stability, affinity and response sensitivity of Tyr-Au-CPE to phenol in air-saturated pH 7.0 phosphate buffer. The enzyme electrode possesses a fast response rate and can be conveniently renewed with a simple polishing step.

Acknowledgements

Financial support is acknowledged from the National Natural Science Foundation of China (29835110 and 29975013), the Science Foundation of Jiangsu (BS2001063), the Specialized Research Fund for the Doctoral Program of Higher Education from the Ministry of Education of China (200028403) and the State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry.

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