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Extradiol dioxygenase–SiO_2 sol–gel modified electrode for catechol and its derivatives detection

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ABSTRACT

A feasible and sensitive biosensor for catechol and its derivatives using 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC)-modified glassy carbon electrode was successfully constructed by polyvinyl alcohol-modified SiO_2 sol–gel method. The as-prepared biosensor was characterized by electrochemical impedance spectroscopy, and the surface topography of the film was imaged by atomic force microscope. Liquid chromatography–tandem mass spectrometry was applied to reveal the catalytic mechanism. BphC embedded in SiO_2 gel maintained its bioactivity well and exhibited excellent eletrocatalytical response to both catechol and some of its derivatives (such as 3-methylcatechol and 4-methylcatechol). The biosensor showed a linear amperometric response range between 0.002 mM and 0.8 mM catechol. And the sensitivity was 1.268 mA/(mM cm^2) with a detection limit of 0.428 μM for catechol (S/N = 3). Furthermore, the BphC biosensor exhibited perfect selectivity for catechol in the mixtures of catechol and phenol. It was suggested that this flexible protocol would open up a new avenue for designing other ring-cleavage enzyme biosensors, which could be widely used for monitoring various kinds of environmental pollutants.

1. Introduction

In recent years, there has been great emergence of interest in the development of sensitive and selective detection methods for phenolic compounds (Harnly et al., 2007). Of particular interest is the quantitative analysis of catechol, one of the most important targets produced by industrial and agricultural activities (Sun et al., 2000). It has been demonstrated that catechol and its derivatives are associated with the damage to physical health through dermal or respiratory tracks. In addition, as the important intermediates in the degradation of most aromatic compounds, catechol and its derivatives can ultimately accumulate in the environment, resulting in serious environmental problems. In this regard, it is highly desirable to develop sensitive and selective methods for the determination of catecholic compounds. Up to now, several methods have been established with good detection limit, such as high performance liquid chromatography (Mizukami et al., 2007), gas chromatography (Moldoveanu and Kiser, 2007), chemiluminescence (Liu and Cheng, 2005), fluorescence (Yuan et al., 2008) and electrochemical methods (Lin et al., 2009; Zhang et al., 2010). However, major drawbacks of current methods containing time-consuming, complicated pre-treatment processes, as well as poor selectivity restrict their further applications (Li et al., 2009b).

In contrast, the past years witnessed great progress in the development of various enzyme biosensors-based electrochemical methods for low-cost, sensitive, selective and convenient detection of targets. As reported, a great amount of enzyme biosensors utilizing tyrosinase (Adamski and Kochana, 2008; Ameer and Adeloju, 2009; Pérez López and Merkoçi, 2009), laccase (Tan et al., 2009), or horseradish peroxidase (HRP) (Orozco et al., 2009) as recognition unit have been developed for catecholic compounds. Nevertheless, most of these strategies still suffer from limitation with respect to selectivity or stability. In particular, it is difficult to obtain single electrochemical signal for catechol when in the mixture of other phenolic compounds owing to their similar electrochemical property (Li et al., 2009b), which will ultimately bring false positive results. For example, tyrosinase can effectively catalyze the conversion of phenol to catechol and the following formation of *ortho*-quinone (Portaccio et al., 2010; Sánchez-Ferrer et al., 1995; Wang et al., 2008). Thus, it is impossible to distinguish catechol in the presence of phenol. Laccase-based biosensors can give response to many phenolic and non-phenolic compounds with the reduction of oxygen (Solomon et al., 1996; Liu et al., 2006). Additional efforts of HRP-based biosensor also display poor selectivity to one kind of substrates due to its ability of catalyzing a large number of phenol derivatives (Courteix and Bergel, 1995; Kong et al., 2010; Liu et al., 2008). In a word, none of these hydroxylases show a specific

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selectivity in the detection of catecholic compounds. Therefore, it is necessary and significant to explore novel enzymes in realizing highly selective, stable and sensitive determination of catechol to meet the requirements in the fields of environmental monitoring, environmental risk assessment and biological toxicology.

As is well-known, the ring cleavage of catechol derivatives can be performed well by both intradiol dioxygenases (*ortho*-cleavage) and extradiol dioxygenases (*meta*-cleavage) (Khajamohiddin et al., 2008). Among them, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) is one of typical extradiol dioxygenase which can catalyze both of the oxygen atoms from O_2 incorporated to catecholic substrates, leading to ring-open. To our best knowledge, there have been no reports on the determination of catechol and its derivatives based on BphC-dependent biosensors so far. Moreover, many factors can affect the kinetics of various enzyme-catalytic reactions, especially those occurring on kinds of electrode surfaces such as pH, temperature, buffer, and toxic chemicals (Hsiao et al., 1996; Zou and Ju, 2004). Therefore, it is still a novel challenge to apply an extradiol dioxygenase to assembly a catechol biosensor in the electrochemical detection of dihydroxybenzenes.

In this study, we reported the first attempt to immobilize an extradiol dioxygenase in polyvinyl alcohol (PVA) modified SiO_2 sol–gel and demonstrated its application for electrochemical detection of catecholic compounds. It was observed that the resultant composites not only preserved the activity of the enzyme but also exhibited obvious electrochemical performance towards catecholics in terms of electrocatalytic behavior and chronoamperometric response. Furthermore, the BphC enzyme electrode demonstrated high selectivity to catechol even in the mixture of other phenolic compounds, which will potentially open new opportunities for the development and design of other extradiol dioxygenase-based electrochemical and optical methods in the future.

2. Experimental

2.1. Reagents and materials

Tetraethoxysilane (TEOS), polyvinyl alcohol (PVA), $NaH_2PO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}12H_2O$, phenol, catechol, 3-methylcatechol, 4-methylcatechol, $K_3[Fe(CN)_6]$, and $K_4[Fe(CN)_6]{\cdot}3H_2O$ were of analytical grade and used without further purification. Double distilled water was used throughout the experiments.

2.2. Apparatus

Electrochemical experiments were performed on a PARSTAT 2273 electrochemical workstation (PARSTAT 2273, Princeton applied research, USA) at room temperature. All experiments were carried out in a 40 mL reactive cell with saturated calomel electrode (SCE) as reference electrode, platinum plate electrode (10 mm × 10 mm × 0.1 mm) as auxiliary electrode and modified GCE as working electrode.

The morphologies of BphC–SiO_2 composites were characterized by atomic force microscopy (AFM, Agilent PicoPlus II). Liquid chromatography (LC) analysis was carried out by an Agilent (Santa Clara, CA, USA) Series 1200 HPLC system equipped with an Agilent ZORBAX C18 column (2.1 mm × 50 mm, particle size 1.8 μm). Tandem mass spectrometry analysis was performed on an Agilent 6410B triple-quadrupole mass spectrometer equipped with an electrospray ionization interface (ESI).

2.3. Preparation of BphC crude extracts

BphC crude extracts (587 1 U/mg protein) were obtained according to the methods described by our previous report (Li et al., 2009a). Briefly, BphC was overexpressed by *Escherichia coli* BL21 harboring pET-*bphC* constructed in our previous work. After accumulation culture, *E. coli* BL21 (pET-*bphC*) was harvested by centrifugation (8000 rpm, 5 min), washed twice, and suspended in phosphate buffer solution (PBS, 0.2 M, pH 8.0) prepared by mixing the stock solution of $NaH_2PO_4{\cdot}2H_2O$ and $Na_2HPO_4{\cdot}12H_2O$. Subsequently, the suspension was subject to ultrasonication for 30 min (225 W, 4 °C) and centrifugation for 20 min (22,000 rpm, 4 °C). The supernatant was taken as the crude extracts.

2.4. Synthesis of PVA modified SiO_2 sol–gel

SiO_2 sol solution was prepared by mixing 1.45 mL TEOS, 0.5 mL double distilled water and 50 μL HCl (0.1 M) in a centrifuge tube followed by ultrasonic treatment for 90 min (KQ 200DB). PVA modified SiO_2 sol–gel was obtained by mixing 5% PVA and SiO_2 sol solution (v:v = 8:1).

2.5. Electrode modification procedure

GCE was polished successively with 0.5–0.7 μm, 30–50 nm γ-Al_2O_3 powder and a polishing cloth, followed by ultra-sonication in 0.5 M H_2SO_4, absolute alcohol, and twice-distilled water, respectively. After that, GCE was chemically cleaned in 0.5 M H_2SO_4 solution by repeated potential scanning from −1.0 to +1.0 V until reproducible voltammograms were obtained. The cleaned GCE was dried with high-purity nitrogen steam before the next modification.

20 μL BphC crude extracts were mixed with 10 μL PVA modified SiO_2 sol solution thoroughly to form homogeneous sol–gel. BphC enzyme electrode was prepared by adding 20 μL of this mixture onto the surface of pretreated GCE and stored at 4 °C until use.

2.6. Electrochemical measurements

The cyclic voltammetry (CV) and chronoamperometry experiments were carried out in 10 mL quiescent PBS (0.2 M, pH 8.0). Sweep rate for CV experiments was selected as 100 mV/s. Electrochemical impedance spectroscopy (EIS) were performed in 0.1 M KCl solution containing 2 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) and the results were plotted in the form of complex plane diagrams (Nyquist plots) with a frequency ranging from 0.1 Hz to 100 kHz. The amplitude of the applied sine wave potential was 5 mV. All potentials were measured and reported versus the SCE.

2.7. Liquid chromatography–tandem mass spectrometry analysis

LC analysis was performed using 0.1% formic acid in MilliQ water as eluent A (65%) and methanol as eluent B (35%) under isocratic condition. The LC flow rate was 0.25 mL/min and the injection volume for LC system was selected as 2 μL.

Tandem MS analyses were operated in negative-ion (NI) mode with scan mode. The conditions for MS were as follows: drying gas temperature 300 °C, gas flow 1 L/min, capillary voltage 4.0 kV, nebulizer pressure 35 psi.

The data of LC–MS–MS analysis was collected and evaluated by Agilent MassHunter Qualitative analysis software.

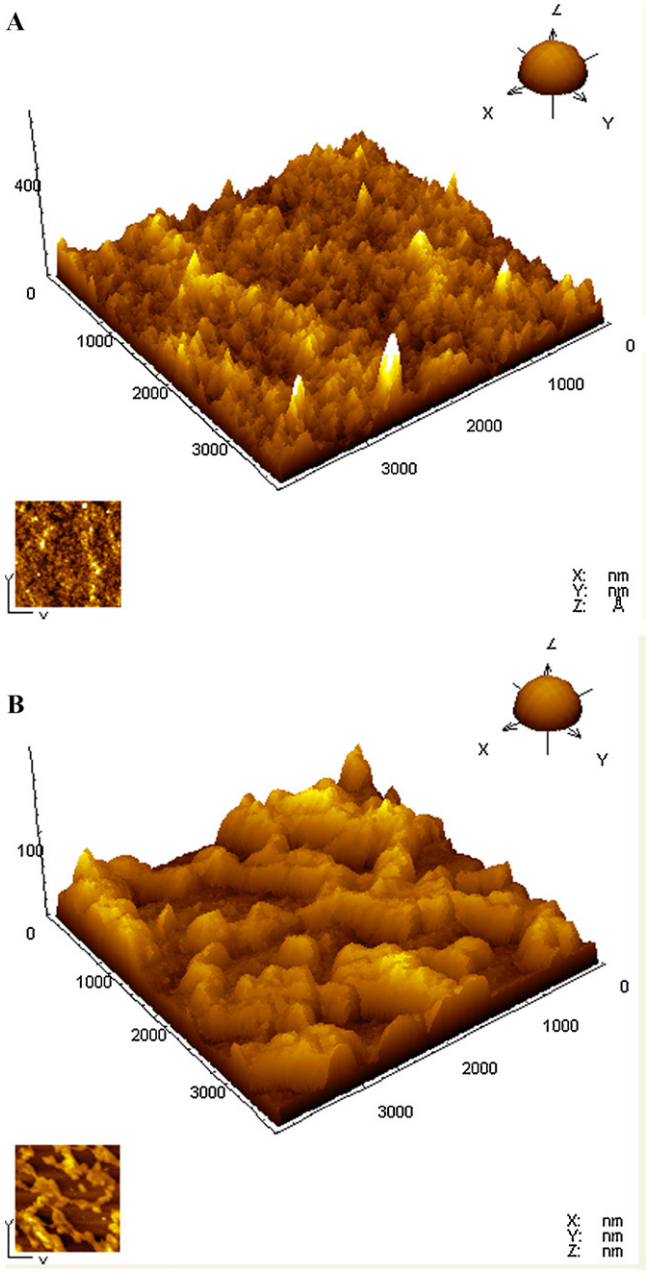


Fig. 1. AFM images of (A) PVA modified SiO_2 film and (B) PVA–SiO_2/BphC biological thin films.

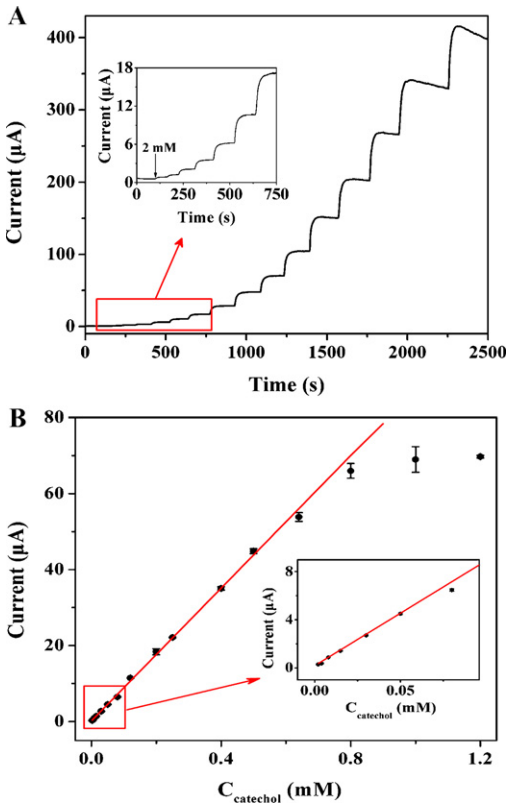


Fig. 2. (A) Amperometric responses of PVA–SiO_2–BphC modified GCE at 450 mV upon successive additions of 10 μL catechol (from 2 mM to 1.2 M). (B) Plots of catalytic current of PVA–SiO_2–BphC modified GCE versus catechol concentration.

3. Results and discussion

3.1. AFM characterization of the prepared thin films

In order to study the immobilization of BphC in PVA-modified SiO_2 sol–gel, AFM was performed to characterize the surface morphology and roughness of SiO_2 and BphC–SiO_2 composites on a freshly cleaved mica surface at room temperature. It was found that the surface of the PVA–SiO_2 thin film without the BphC was relatively smooth with a surface mean roughness of ca 30.111 Å and a root mean square (RMS) of ca 93.634 Å (Fig. 1(A)).

The structure of BphC from *Pseudomonas* sp. Strain KKS102 was reported to be a thick-walled cylinder with a diameter of ca 110 Å and a height of ca 93 Å (Senda et al., 1996), which indicated that the surface morphology of the PVA–SiO_2 thin film could be significantly influenced by the embedment of BphC macromolecules. As shown in Fig. 1(B), the surface morphology of PVA–SiO_2/BphC sol–gel films dramatically changed in contrast to the non-doped film. When BphC enzyme was embedded, the morphology presented obvious protuberance with clean outline, which looked like a relief sculpture. Moreover, the surface mean roughness increased to ca 156.705 Å with a root mean square (RMS) of 444.954 Å, which could be attributed to the presence of BphC macromolecules.

In order to demonstrate that the prepared PVA–SiO_2/BphC sol–gel film can effectively maintain the activity of BphC, enzyme activity assay of immobilized BphC was also performed. PVA–SiO_2 sol–gel mixed with or without BphC was coated onto the glass slides and dried overnight. It was clear that the color of the PVA–SiO_2/BphC film turned yellow when catechol was introduced by the sprinkle method, which was the typical chromogenic identification of catechol ring-cleavage (Zukowski et al., 1983). However, there was no color change in the black control without BphC (data not shown). Therefore, it was strongly suggested that the activity of BphC was well maintained in the sol–gel film.

3.2. Electrochemical characteristics of the BphC enzyme electrode

EIS was used to study the interface properties of modified electrode. In general, the semicircle portion at higher frequencies corresponded to the electron transfer limited process, while the linear part at lower frequencies corresponded with the diffusion process. Furthermore, the change in semicircle diameter was a result of the change in the electron transfer resistance, R_{et} (Chen et al., 2007). Fig. S1 (in supplementary materials) presented the Nyquist plots of impedance spectra for GCE with different modified steps. The lowest R_{et} on the bare GCE (curve a) indicated the fastest electron transfer between bare GCE and ferricyanide. It was observed that the semicircle diameters gradually increased

with the immobilization of PVA–SiO_2 sol–gel and PVA–SiO_2–BphC sol–gel. The R_{et} of PVA–SiO_2 modified GCE increased a little (curve b), which implied that the SiO_2 sol–gel modified by PVA possessed excellent conductivity. However, the R_{et} sharply increased after BphC was immobilized (curve c), which was very similar to that of polyphenol oxidase-based biosensor (Fan et al., 2007; Tan et al., 2010; Wang et al., 2009). The obvious increase in the interfacial resistance should be attributed to the hindrance of the macromolecular structure of BphC.

3.3. Electrochemical behaviors of the biosensor

The electrochemical behaviors of catechol on the PVA–SiO_2–BphC modified GCE were tested by cyclic voltammograms (CVs). Fig. S2(A) depicted CVs recorded for this enzyme electrode in 0.2 M, pH 8.0 PBS with 0, 0.1, 0.2 mM catechol, respectively. Without the addition of catechol in the electrolyte, only background current was observed (Fig. S2(A) curve a). As expected, the CVs changed dramatically when catechol was added (Fig. S2(A) curve b and c). It was obvious that there existed an increase of the oxidation current at about 0.4 V, and the corresponding fitting curves of peak current increased versus the concentration of catechol, indicating the good response of PVA–SiO_2–BphC modified GCE towards catechol.

As a control, CVs of catechol on the bare GCE and PVA–SiO_2 modified GCE (without enzyme) were also given in Fig. S2(B). As shown in curve a, a pair of redox peaks was observed at E_{pa} = 0.3 V, E_{pc} = −0.04 V, respectively, which suggested that the electrochemical behavior of catechol on the bare GCE was a reversible reaction (Rodriguez and Rivas, 2002). Compared with bare GCE, PVA–SiO_2 modified GCE exhibited the similar results with a pair of redox peaks shifting to E_{pa} = 0.36 V, E_{pc} = 0 V, respectively. This phenomenon was very different from that of ZnO sol–gel electrode without enzyme (Liu et al., 2005). This discrepancy may be attributed to the sensing properties of sol–gel materials, which can be strongly influenced by their operational and modified conditions, such as different sol–gel precursors, additives and so on (Tsai and Doong, 2007).

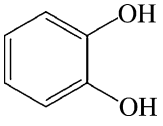
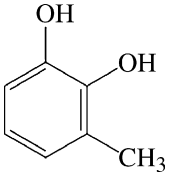
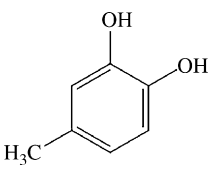
While for the PVA–SiO_2–BphC modified GCE, obviously, the oxidation peak shifted to a more positive potential and the reduction signal disappeared. As noted, after the CVs behavior of catechol on the BphC enzyme electrode was recorded, the enzyme film on the surface of modified GCE turned yellow, which was consistent with the catalytic reaction of catechol by free enzyme. Then the oxidation peak in Fig. S2(B) curve c could be deduced from the combined catalysis of electricity and enzyme. As is well-known, BphC is a key enzyme during the degradation of biphenyl, and it can be used as a ring-cleavage dioxygenase to convert 2,3-dihydroxybiphenyl into 2-hydroxy-6-oxo-6-phenyhexa-2,4-dienoic acid (HOPDA) (Ohtsubo et al., 2004). Moreover, it can also catalyze the ring-open of catechol and some of its derivatives by introducing two atoms of oxygen from O_2. It was presumed that the reaction product of catechol could be oxidized further, meanwhile, the oxidation products could not be reduced on the surface of the electrode, resulting in the disappearance of the reduction peak.

In order to get more information about the catalytic mechanism of the biosensor, the analysis of the reaction products of catechol by electro-catalysis was carried out by LC–MS/MS with an ESI source operated in the negative ion mode. As shown in Fig. S3(A), two major molecular ions at 176.9 $[M-H]^-$ and 194.9 $[M-H_3O]^-$ deduced to be aldohexonic acid were observed in products (Bruggink et al., 2010), which might be attributed to the oxidation of BphC-catechol reaction product, 2-hydroxy-6-oxohexa-2,4-dienoic acid. For comparison, the mass spectra for catechol standard sample revealed its $[M-H]^-$ molecular ion with mass to charge ratio of m/z 108.9 (Fig. S3(B)), clearly demonstrating that catechol was completely oxidized during the electro-catalysis.

3.4. Chronoamperometric response and calibration curve

Fig. 2(A) displayed a typical amperometric response of the BphC enzyme electrode upon addition of different concentrations (from 2 mM to 1.2 M) of catechol under stirring. It can be observed that the BphC biosensor responded rapidly to the increasing substrates and 95% steady-state current was obtained within 20 s. The plots (Fig. 2(B)) showed the dependence of the oxidation current on the concentration of catechol. The linear response range of the biosensor to the concentration of catechol was from 0.002 to 0.8 mM, and the linear regression equation was as follows: I (μA) = 84.0884$C_{catechol}$ (mM) + 0.5515 with a correlation coefficient 0.9978 (n = 14). The detection limit was 0.428 μM (S/N = 3), which was higher than that of MB-MCM-41/PVA/laccase modified Au electrode for catechol (0.331 μM) (Xu et al., 2009), but lower than that of gold nanoparticle-modified ultramicroelectrode arrays based on HRP (Orozco et al., 2009). Otherwise,

Table 1
Analytical parameters of the BphC biosensor for different catecholics.

Catecholics	Structures	Sensitivity (mA/(mM cm^2))	Linear range (mM)	R^2	Detection limit (μM)
Catechol		1.268	0.002–0.8	0.9978	0.428
3-Methylcatechol		0.926	0.002–0.25	0.9977	0.596
4-Methylcatechol		0.819	0.002–0.32	0.9949	0.758

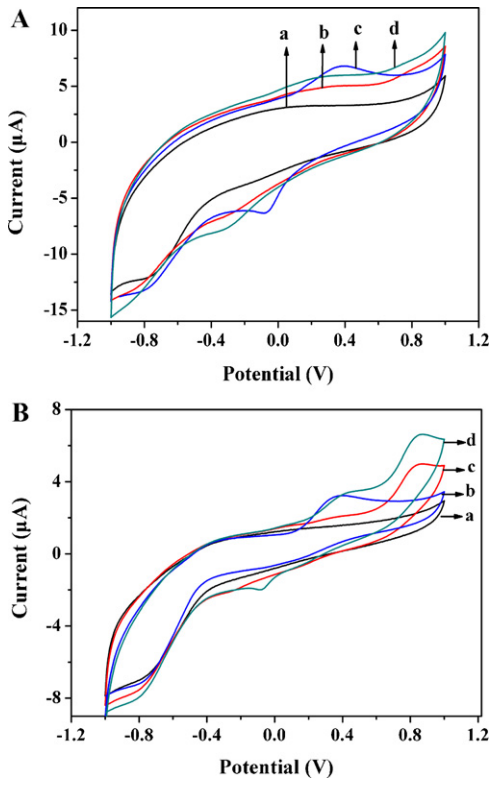


Fig. 3. (A) CVs of bare GCE in 0.2 M PBS of pH 8.0 with different concentrations of phenol and catechol. (a) Control, (b) 0.2 mM phenol, (c) 0.1 mM catechol, (d) 0.1 mM catechol and 0.2 mM phenol mixture. (B) CVs of PVA–SiO_2–BphC modified GCE in 0.2 M PBS of pH 8.0 with different concentrations of phenol and catechol. (a) Control, (b) 0.2 mM phenol, (c) 0.1 mM catechol, (d) 0.1 mM catechol and 0.2 mM phenol mixture.

the linear response range of PVA–SiO_2–BphC modified GCE was much wider compared with these two methods. Furthermore, BphC modified electrode exhibited much higher sensitivity of 1.268 mA/(mM cm^2).

The electrochemical behavior of several typical catecholic derivatives on the enzyme electrode was also investigated as shown in Table 1. The linear regression equation for 3-methylcatechol was I (μA) = 65.4559$C_{3\text{-methylcatechol}}$ (mM) + 0.6015 with correlation coefficient 0.9977 (n = 11). For 4-methylcatechol, the linear regression equation was I (μA) = 57.9252$C_{4\text{-methylcatechol}}$ (mM) + 0.7304 with a correlation coefficient 0.9949 (n = 12). The detection limits for 3-methylcatechol and 4-methylcatechol were 0.596 μM and 0.758 μM (S/N = 3), respectively. Therefore, it was concluded that the PVA–SiO_2–BphC modified GCE represented good eletrochemical response to catechol and some of its derivatives.

3.5. Selectivity of the biosensor

Fig. 3(A) displayed the CVs recording of single (curve b and c) and mixed components (d) on the bare GCE. It was obvious that only background current presented without any substrate in the PBS (Fig. 3(A) curve a). With the addition of 0.2 mM phenol, there was no obvious peak other than the larger current compared with background current (Fig. 3(A) curve b). Also, similar behavior was observed for mixed components of phenol and catechol (Fig. 3(A) curve d). The oxidation peak of catechol at about 0.3 V in curve c was not found in curve d, which was overlapped with the oxidation signal of phenol, and the corresponding reduction peak was also disturbed. Since the oxidation peaks overlapped seriously at the bare GCE, catechol could not be distinguished in the presence of phenol.

However, PVA–SiO_2–BphC modified GCE showed the distinct results compared with bare GCE. As shown in Fig. 3(B), an oxidation peak at 0.8 V appeared with the addition of phenol (Fig. 3(B) curve c). In addition, the corresponding oxidation peaks of catechol and phenol were separated well even in the mixture (Fig. 3(B) curve d). Meanwhile, the oxidation peak potential of catechol was still 0.4 V, and the electrochemical signal of phenol was enhanced. Liu et al. (2009) reported an electrochemical method based on a surfactant and a semi-derivative technique, which could separate catechol and hydroquinone completely by reduction peaks at a bare GCE. In our study, we demonstrated that catechol and phenol could be distinguished from each other by the oxidation peaks. Therefore, it was concluded that the PVA–SiO_2–BphC modified GCE possessed good selectivity for catechol.

4. Conclusions

A novel approach for electrochemical detection of catechol and some of its derivatives based on the immobilized BphC/PVA–SiO_2 film was successfully developed. The film not only kept good bioactivity of BphC but also presented excellent eletrocatalytical response to catechol and its derivatives. Compared with other enzyme-based catechol biosensor, this BphC biosensor exhibited good selectivity for catechol in the mixtures of catechol and phenol. Future experiments that further improve the sensitivity are being conducted. Moreover, it was suggested that this general design strategy could give a guide for employing available enzymes as sensitive elements to fabricate appropriate biosensors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.04.042.

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