Full Paper

Detection of Trace Phenol Based on Mesoporous Silica Derived Tyrosinase-Peroxidase Biosensor

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Abstract

A novel electrochemical biosensor for phenol based on immobilization of tyrosinase-peroxidase on mesoporous silica is described. The enhanced sensitivity of the tyrosinase-horseradish peroxidase based biosensor to phenol was observed on comparing with tyrosinase or horseradish peroxidase monoenzyme modified electrodes. Two enzymes retained their enzymatic activities for phenol determination without any mediator. The preparation conditions of the biosensor are discussed. Optimization of the experimental parameters was performed with regard to pH and operating potential. The phenol sensor exhibited a fast response of less than 10 seconds. The sensitivity of the biosensor for phenol was $14 \mu \text{A} \cdot \text{mM}^{-1} \cdot \text{cm}^{-2}$ with a linear range from $2 \times 10^{-7}$ to $2.3 \times 10^{-4} \text{M}$ and a detection limit of $4.1 \times 10^{-9} \text{M}$. The biosensor showed a good stability and reproducibility.

Keywords: Biosensors, Tyrosinase, Horseradish peroxidase, Phenol, Mesoporous silica

1. Introduction

Phenol is an important but toxic starting material in a broad range of chemical manufacturing processes. Soil and surface water of areas around formal production and processing plants are often contaminated by phenol with risk to ground water resources. Moreover, the quality of foods is also related to the presence of phenol substance in its composition mainly in beverages and juice [1]. Hence, a selective and sensitive detection of phenol is very important for controlling its concentration.

Many techniques have been used for monitoring phenols, such as colorimetry [2], gas chromatography [3], liquid chromatography [4] and capillary electrophoresis [5]. However, their demanding operations and low sensitivities limit their in situ applications. Electrochemical techniques possess many advantages such as good selectivity, relatively low cost of realization and storage, the potential for miniaturization and automation. Thus different electrochemical methods have been developed for phenol determinations. Amperometric biosensor for phenol based on tyrosinase has been considered to be simple, sensitive, effective and fast, and has proven to be an attractive approach for monitoring of phenolic compounds [6, 7]. Different immobilization methods have been developed to stabilize tyrosinase for biosensor preparation [8–11]. Tyrosinase is a copper-containing mono-oxygen oxidase that can catalyze the oxidation of phenol via catechol to o-quinone at the expense of molecular oxygen [12]. So most of the tyrosinase-based biosensors are to detect the electrochemical reduction signal of o-quinone product of enzyme reaction at low potential [13–16].

To increase the sensitivity of the biosensor, horseradish peroxidase (HRP) and tyrosinase have been coimmobilized on electrode surface [17–19]. HRP has a broad specificity for hydrogen donors such as hydroquinone and catechol [20], thus, it can lead to generation of benzoquinone (or o-quinone) from hydroquinone (or catechol) in the presence of hydrogen peroxide ($\text{H}_2\text{O}_2$). The coimmobilization of HRP and tyrosinase in poly(amphiphilic pyrrole) film [17] or bovine serum albumin (BSA) with glutaric dialdehyde [18] has been performed for flow injection analysis of phenol in presence of $\text{H}_2\text{O}_2$. Another tyrosinase-HRP peroxidase sensor by coimmobilizing HRP and tyrosinase in poly(carbamoylsulfonate) hydrogel [19] has also been reported.

Recently, a series of inorganic porous materials have been proven to be promising as the immobilization matrices [21–26]. The unique structural and catalytic properties of molecular sieves for structuring an electrochemical/electron transfer environment and retaining bioactivity of immobilized biomolecules have also attracted considerable attention [27]. As an immobilization matrix, molecular sieves can adhere protein molecules through physical or chemical action. We here used mesoporous molecular sieves, MCM-41, to structure a tyrosinase-horseradish peroxidase system for biosensor preparation. MCM-41 has a high surface area, controlled porosity and mechanical resistance, which make it more suitable for enzyme loading to get high sensitivity. Several papers have described the use of MCM-41 to immobilize proteins [28–33]. But up to now tyrosinase

immobilized on MCM-41 to detect phenol has not been reported. In this paper, a novel tyrosinase-horseradish peroxidase-based biosensor for phenol without addition of H$_2$O$_2$ was developed by coimmobilizing HRP and tyrosinase in MCM-41. The enzymes immobilized in the porous and hydrophilic matrix are much stable and exhibit good electrocatalytic behavior for sensitive detection of phenols. In comparison with immobilization of tyrosinase alone the additional HRP improved greatly the sensitivity for phenol detection.

2. Experimental

2.1. Materials and Reagents

Mushroom tyrosinase (from mushroom. EC.1.14.18.1, 2400 µg g$^{-1}$) and HRP (EC 1.11.1.7, RZ > 3.0, >250 µm g$^{-1}$) were from Sigma and used as received. Phenol was from Shanghai Regent Co. (China). Polyvinyl alcohol (PVA, average degree of polymerization, 1800 ± 100) was from Shanghai Laize Factory of Fine Chemicals Co. (China). All other chemicals were of analytical grade and used without further purification. 0.1 M phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of K$_2$HPO$_4$ and KH$_2$PO$_4$ and adjusting the pH with H$_2$PO$_4$ or NaOH. All the solutions were prepared with doubly distilled water.

2.2. Electrode Modification

MCM-41 was prepared following a recipe similar to that reported [34]. The specific surface area and pore volume obtained by the N$_2$ adsorption data and calculated by the BET method [35] and the pore diameter of the MCM-41 derived from the adsorption branch by the BJH method were listed in Table 1.

Thirty mg MCM-41 were dispersed in 10 mL water to obtain a suspension of MCM-41. The obtained suspension of 100 µL was then mixed with 5 µL 3% PVA solution of ethanol/water (V:V 1:1) to produce a MCM-41 colloid, which was used for the following work.

Glassy carbon electrode (GCE, 3 mm in diameter) was polished to a mirror-like finish with 0.3 and 0.05 µm alumina slurry (Beuhler, USA) followed by rinsing thoroughly with doubly distilled water. Then it was successively sonicated in acetone and doubly distilled water, and allowed to dry at room temperature. The real area of the pretreated GCE was determined by the slope of the plot of the anodic peak current of 1.0 mM K$_3$[Fe(CN)$_6$] in 0.1 M KCl vs. the square root of scan rate to be 0.092 cm$^2$. Two µL MCM-41 colloidal solution and 2 µL 3 mg mL$^{-1}$ tyrosinase or HRP were dropped on the pretreated GCE surface and allowed to dry under ambient conditions for 3 hours to produce tyrosinase/MCM-41/GCE or HRP/MCM-41/GCE, respectively. The coating of MCM-41 colloidal particles on the pretreated GCE did not obviously increase the real area of the electrode. The tyrosinase-HRP/MCM-41/GCE, were prepared by dropping 2 µL MCM-41 colloidal solution and then 2 µL tyrosinase-HRP mixture and allowed to dry under ambient conditions for 3 hours. The mixtures were obtained by dissolving 3 mg mL$^{-1}$ tyrosinase and different concentrations of HRP in 0.1 M pH 7.0 PBS. The preparation of multilayer modified electrode was performed by repeating the dropping-evaporation steps. When not in use the electrode was stored in 0.1 M pH 7.0 PBS at 4 °C.

Table 1. Pore Characterization of MCM-41. \( A_{\text{BET}} \), total specific surface area; \( V_{\text{total}} \), total mesopore volume; \( a_0 \), lattice parameter; \( D \), mesopore diameter; \( L \), wall thickness

<table>
<thead>
<tr>
<th>( A_{\text{BET}} ) (m$^2$ g$^{-1}$)</th>
<th>( V_{\text{total}} ) (cm$^3$ g$^{-1}$)</th>
<th>( a_0 ) (nm)</th>
<th>( D ) (nm)</th>
<th>( L ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>622</td>
<td>0.78</td>
<td>5.48</td>
<td>3.36</td>
<td>2.12</td>
</tr>
</tbody>
</table>

3. Results and Discussion

3.1. Electrochemical Response Enhanced by MCM-41

Figure 1 shows the cyclic voltammograms of HRP/GCE and HRP/MCM-41/GCE in air-free pH 7.0 PBS in the absence or presence of H$_2$O$_2$ or phenol. Upon addition of phenol to the solutions, little change is observed at HRP/GCE and HRP/MCM-41/GCE in air-free pH 7.0 PBS. The amperometric experiments were carried out by applying a potential of ~50 mV for phenol on a stirred cell at 20 ± 2°C. The amperometric response of the sensor was measured as the differences between total and residual currents.

\[
\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O} \quad (1)
\]

\[
\text{Compound I} + \text{phenol} \rightarrow \text{Compound II} + \text{Product} \quad (2)
\]

\[
\text{Compound II} + \text{phenol} \rightarrow \text{HRP} + \text{Product} + \text{H}_2\text{O} \quad (3)
\]

where compound I and compound II are two- and one-electron oxidation states of the native ferriperoxidase, respectively. The oxidized enzyme is reduced to its native
form in two steps (Eqs. 2 and 3). In each step the electron donor species, phenol, is oxidized [30]. Obviously, the presence of MCM-41 increased the reduction current by 3.2 times measured at $E_{1/2}$ 110 mV, which resulted from higher enzymatic activity of HRP immobilized on MCM-41/GCE than on GCE.

In air-saturated PBS, upon addition of 0.2 mM phenol, the cyclic voltammograms of tyrosinase/GCE, tyrosinase/MCM-41/GCE and tyrosinase-HRP/MCM-41/GCE showed dramatic change with a significant increase of the reduction current at the potential of about $-50$ mV (Fig. 2). Obviously, the enhanced reduction currents at the three electrodes were attributed to the efficient catalytic oxidation of phenol by $O_2$ to form $\alpha$-quinine in the presence of immobilized tyrosinase. The response of tyrosinase/GCE was 5.4 times smaller than that of tyrosinase/MCM-41/GCE at the phenol concentration of 0.2 mM (Fig. 2A and Fig. 2B). Thus, the MCM-41 played an important role in enhancing the enzymatic activity of tyrosinase, which was similar to the results at HRP/MCM-41/GCE (Fig. 1) and the biosensor based on tyrosinase modified graphite electrodes [9]. The increased cathodic current came from the reduction of $\alpha$-quinone, which was produced in the presence of dissolved $O_2$ by following enzymatic reactions [37, 38]:

phenol + tyrosinase ($O_2$) $\rightarrow$ catechol (4)

catechol + tyrosinase ($O_2$) $\rightarrow$ $\alpha$-quinone + $H_2O$ (5)

$\alpha$-quinone + $2H^+$ + $2e^- \rightarrow$ catechol (at electrode) (6)

At the same concentration of phenol the response of tyrosinase-HRP/MCM-41/GCE was about 2 times larger than that of tyrosinase/MCM-41/GCE (Fig. 2B and Fig. 2C), indicating tyrosinase-HRP possessed better electrocatalytic efficiency. The enhanced signal amplification to phenol resulted from the cooperative function of the two immobilized enzymes on the electrode surface. The catechol formed from the electrochemical reduction of $\alpha$-quinone could be oxidized by $H_2O_2$ with the catalysis of coimmobilized HRP, the $H_2O_2$ was produced from the reduction of dissolved $O_2$ [19]. The regeneration of $\alpha$-quinone resulted in the increase of reduction current. In air-saturated PBS containing 0.2 mM phenol, the addition of 0.1 mM $H_2O_2$ did not change the cyclic voltammogram of tyrosinase-HRP/MCM-41/GCE due to the presence of dissolved $O_2$. Thus following experiments and the detection of phenol were performed in air-saturated PBS without addition of $H_2O_2$.

Figure 3 shows the cyclic voltammograms of tyrosinase-HRP/MCM-41/GCE in 0.1 M pH 7.0 PBS containing different concentrations of phenol. With the increasing phenol concentration the reduction peak current increased linearly and the peak potential shifted to more negative potential, showing electrocatalytic character. When the concentration of phenol was over 0.25 mM, the reduction peak current trended to a constant value. This was typical of enzymatic reaction kinetics.

In absence of HRP, the catalytic response became quite small. The electrocatalytic response of the phenol sensor to 0.2 mM phenol increased with an increasing scan rate (inset A in Fig. 3). The peak current was proportional to the square root of the scan rate (inset B in Fig. 3), indicating that the overall electrocatalytic and enzymatic reaction process was controlled by the diffusion of phenol in solution, and the electrocatalytic reaction and enzymatic reaction at tyrosinase-HRP/MCM-41/GCE was quite fast.
3.2. Optimization of Tyrosinase-Horseradish Peroxidase Electrode Preparation

The performance of the tyrosinase-HRP electrode mainly depended on the amount of enzyme loaded on electrode surface, which was related to the concentration ratio of HRP to tyrosinase and the layer number of immobilized enzyme mixture.

The response of tyrosinase-HRP sensors usually originates from the successive reactions catalyzed by the enzymes. This work first optimized the ratio of HRP to tyrosinase by retaining tyrosinase concentration at 3 mg mL\(^{-1}\) and changing HRP concentration in the mixture to prepare the tyrosinase-HRP electrode. With the increasing concentration ratio of HRP to tyrosinase the catalytic current obtained at an applied potential of \(50 \text{ mV}\) increased and reached a constant value at the ratio of 1:3 (Fig. 4A). However, the catalytic response became quite small in absence of HRP. So here we used the ratio of 1:3 for HRP to tyrosinase to prepare the tyrosinase-HRP electrode.

The loading of enzymes and the deposition sequence of enzymes and MCM-41 on the electrode could be regulated precisely and easily based on the layer numbers of MCM-41 and enzymes. In this study, we dropped first MCM-41 film, and then HRP-tyrosinase mixture was cast on the film sequentially, to check the effect of enzyme layer number on amperometric response to phenol. As shown in Figure 4B, the electrocatalytical current increased with the increasing number of enzyme layers. The enzyme electrode gave the best performance at the enzyme layer number of 3. When the number was larger than 3, the response did not increase. So the phenol sensor based on tyrosinase-HRP/MCM-41/GCE was prepared by casting three layers of tyrosinase-HRP/MCM-41 on the GCE surface. This sensor was used throughout the following experiments.

3.3. Optimization of Detection Conditions

The pH dependence of the tyrosinase-HRP electrode was studied in air-saturated 0.1 M PBS containing 0.01 mM phenol. The current increased slightly as the pH changed from 5.5 to 7.0, following which a gradual decrease in the response was observed. All changes in voltammetric peak potentials and currents with pH were reversible. The optimum pH value for the best performance of the sensor was at 7.0. The reason for this was that the microenvironment of the enzyme did not change during the immobilization process.

The effect of the applied potential on the steady-state current of the sensor showed that the reduction of \(\text{o-quinone}\), the product of enzymatic Reactions 4 and 5, was already observed at around 50 mV, and the steady-state current increased rapidly as the applied potential moved negatively from 50 mV to \(50 \text{ mV}\), which was due to the increased driving force for the fast reduction of \(\text{o-quinone}\) at low potential. Then the steady-state current decreased slightly when the applied potential was more negative than \(50 \text{ mV}\). The maximum current occurred at \(50 \text{ mV}\), which was selected as the working potential for amperometric detection of phenol.

3.4. Amperometric Response of the Biosensor

Tyrosinase/GCE showed a much smaller response to phenol than both tyrosinase/MCM-41/GCE and tyrosinase-HRP/MCM-41/GCE at the same phenol concentration. Thus, MCM-41 played an important role in increasing the affinity of tyrosinase on phenol. Figure 5 illustrates typical current-time plots for tyrosinase/MCM-41/GCE and tyrosinase-HRP/MCM-41/GCE on successive step additions of 5.0 \(\mu\)L 0.2 mM phenol receptively to 5.0 mL air-saturated 0.1 M PBS.
pH 7.0 PBS at an applied potential of −50 mV. Tyrosinase-MCM-41/GCE in air-saturated PBS showed smaller responses to phenol than tyrosinase-HRP/MCM-41/GCE at the same concentration, displaying the tyrosinase-HRP sensor possessed much better response. The response of tyrosinase-HRP/MCM-41/GCE was almost 2 times higher than that at the tyrosinase/MCM-41/GCE. Therefore, tyrosinase-HRP sensor could be applied as a simple and efficient sensor for detecting phenols without the addition of H₂O₂. When an aliquot of phenol was added into the buffer solution, the reduction current rose steeply to reach a stable value, and achieved 95% of steady-state-current in less than 10 seconds. This was much faster than that of 50 seconds reported in the pure silica sol-gel matrix and 20 seconds in copolymer grafted silica sol-gel membrane [39, 40]. Compared to the sol-gel, this mesoporous matrix created a faster diffusion of substrate from bulk solution to the enzyme. The results demonstrated clearly that the electrocatalytic response was very fast.

The tyrosinase-HRP/MCM-41/GCE showed a linearly increased amperometric response to phenol ranging from 2 × 10⁻⁷ to 2.3 × 10⁻⁴ under the optimized experimental conditions (inset in Fig. 5). The correlation coefficient was 0.9995 (n = 40). It had a high sensitivity of 14 μA μM⁻¹ cm⁻², which was much higher than that of the copolymer grafted silica tyrosinase biosensor [39]. This illustrated that the MCM-41 matrix was more suitable for enzyme loading. The phenol sensor could reach a detection limit of 4.1 × 10⁻⁵ M at a signal-to-noise ratio of 3.

In order to observe the amplification of the tyrosinase-HRP and the matrix for enzyme immobilization, a series of amperometric detection parameters such as linear range, detection limit and sensitivity were examined using five different electrodes as outlined in Table 2. Figure 6 shows the calibration plots for phenol at these five electrodes under optimal conditions. Obviously the presence of MCM-41 increased greatly the sensitivity, widened the linear range and showed lower detection limit. Thus, the mesoporous silica, MCM-41, provided advantages for preparation of sensor with better analytical performance. The results obtained at tyrosinase-HRP/MCM-41/GCE indicated the tyrosinase-HRP system improved greatly the sensitivity of the phenol biosensor.

### 3.5. Amperometric Responses of the Sensor to other Phenolic Compounds

As other phenol biosensor reported, the proposed tyrosinase-HRP biosensor could response to several other phenolic compounds. The analytical performances of cat-

Table 2. Linear calibration parameters for HRP/GCE and HRP/MCM-41/GCE in presence of H₂O₂, tyrosinase/GCE, tyrosinase/MCM-41/GCE and tyrosinase-HRP/MCM-41/GCE.

<table>
<thead>
<tr>
<th>Electrodes</th>
<th>Linear range</th>
<th>Detection limit</th>
<th>RSD (%) (n)</th>
<th>Sensitivity (μA μM⁻¹ cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP/GCE in presence of H₂O₂</td>
<td>8 × 10⁻⁸–6.3 × 10⁻⁴ M</td>
<td>6.0 × 10⁻⁸ M</td>
<td>5.1 (15)</td>
<td>0.01</td>
</tr>
<tr>
<td>HRP/MCM-41/GCE in presence of H₂O₂</td>
<td>8 × 10⁻⁷–1.6 × 10⁻⁴ M</td>
<td>1.2 × 10⁻⁷ M</td>
<td>4.8 (17)</td>
<td>0.24</td>
</tr>
<tr>
<td>Tyrosinase/GCE</td>
<td>8 × 10⁻⁸–1.5 × 10⁻⁴ M</td>
<td>2.4 × 10⁻⁸ M</td>
<td>4.2 (36)</td>
<td>0.12</td>
</tr>
<tr>
<td>Tyrosinase/MCM-41/GCE</td>
<td>4 × 10⁻⁷–1.8 × 10⁻⁴ M</td>
<td>5.2 × 10⁻⁸ M</td>
<td>4.7 (38)</td>
<td>1.7</td>
</tr>
<tr>
<td>Tyrosinase-HRP/MCM-41/GCE</td>
<td>2 × 10⁻⁷–2.3 × 10⁻⁴ M</td>
<td>4.1 × 10⁻⁸ M</td>
<td>4.5 (40)</td>
<td>14</td>
</tr>
</tbody>
</table>

Fig. 5. Amperometric responses of tyrosinase/MCM-41/GCE (a) and tyrosinase-HRP/MCM-41/GCE (b) upon successive step additions of 5 μL 0.2 mM phenol to 5 mL air-saturated 0.1 M pH 7.0 PBS at −50 mV. Inset: Calibration plots for phenol at tyrosinase-HRP/MCM-41/GCE under optimal conditions.

Fig. 6. Calibration plots for phenol at HRP/GCE (a) and HRP/MCM-41/GCE in presence of H₂O₂ (b), tyrosinase/GCE (c), tyrosinase/MCM-41/GCE (d) and tyrosinase-HRP/MCM-41/GCE (e) under optimal conditions.
echol, phenol and p-cresol are listed in Table 3. Response characteristics varied with the different substitution group of phenolic compounds. The sensitivities of the biosensor for catechol, phenol and p-cresol were 45, 14 and 13 \( \mu \text{A} \text{mM}^{-1} \text{cm}^{-2} \), respectively.

3.6. Stability and Reproducibility of the Tyrosinase-HRP Sensor

The fabrication reproducibility of six electrodes, made independently, showed an acceptable reproducibility with a RSD of 5.1% for the currents determined at a phenol concentration of 0.2 mM. Thus, MCM-41 particles were very efficient for retaining the bioactivity of tyrosinase and HRP and preventing them from leaking out of the sensor.

In addition to good reproducibility, MCM-41 membrane imparted to the tyrosinase-HRP electrode a good long-term stability. The storage stability of phenol biosensor stored in 0.1 M pH 7.0 PBS or air at 4°C was examined by checking periodically their relative response currents (the ratios of the catalytic currents detected at different times to the initial current value) in 0.1 M pH 7.0 PBS containing 0.2 mM phenol. The sensors could retain 93% of initial activity to phenol within a storage period of 60 days in 0.1 M pH 7.0 PBS at 4°C (Fig. 7), while only 78% of activity to phenol was retained when stored in air at 4°C, respectively. After a storage period of 3 months in 0.1 M pH 7.0 PBS at 4°C the biosensor showed a 10% loss of activity for phenol. Thus, the biosensors stored in 0.1 M pH 7.0 PBS at 4°C showed a good stability when not in use.

4. Conclusions

This work developed a new tyrosinase-HRP electrode for phenol detection by immobilizing tyrosinase and HRP on a mesoporous matrix. The porous structure of MCM-41 results in a high catalytic activity and fast response rate of the immobilized tyrosinase-HRP. The enhanced sensitivity of the tyrosinase-HRP electrode to phenol is observed compared with tyrosinase and HRP monoenzyme electrodes. This matrix is very efficient for retaining the tyrosinase-HRP activity and preventing their leakage out of the film, which results in a long-term stability and good reproducibility of the sensor. MCM-41 provides an efficient strategy and a new promising platform for the study of the developments of biosensors.

5. Acknowledgements

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6. References


