Full Paper

Pure Organic Phase Phenol Biosensor Based on Tyrosinase Entrapped in a Vapor Deposited Titania Sol-Gel Membrane

Jiuhong Yu, *Huangxian Ju**

Department of Chemistry, State Key Laboratory of Coordination Chemistry, Institute of Analytical Science, Nanjing University, Nanjing 210093, P. R. China
*e-mail: hxju@nju.edu.cn

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Abstract
A novel amperometric biosensor was constructed for the determination of phenols in pure organic phase. This biosensor was fabricated by immobilizing tyrosinase in a titania sol-gel membrane which was obtained with a vapor deposition method. This method was facile and avoided the calcination step needed in conventional titania sol-gel process. The titania sol-gel membrane could effectively retain the essential water layer around the enzyme molecule needed for maintaining its activity in organic phase. The experimental parameters such as solvent and operating potential were optimized. At -100 mV this biosensor showed a good amperometric response to phenols in pure chloroform without any mediator and rehydration of the enzyme. For catechol determination the sensor exhibited a fast response of less than 5 seconds. The sensitivity of different phenols was as follows: catechol > phenol > *p*-cresol. Additionally, the apparent Michaelis-Menten constants of the encapsulated tyrosinase to catechol, phenol and p-cresol were found to be 0.15 ± 0.003, 0.17 ± 0.008 and 0.21 ± 0.004 mM, respectively. The biosensor had also good reproducibility and stability. This work provided a promising platform for the construction of pure organic phase biosensors and the determination of substrates with poor water solubility.

Keywords: Pure organic phase biosensor, Titania sol-gel, Tyrosinase, Phenols, Vapor deposition

1. Introduction

Since the remarkable finding that an enzyme can maintain its biocatalytic activity in nonaqueous environments [1, 2], organic-phase enzyme electrodes used in nonaqueous media have attracted considerable interest in sensor construction. They possess distinct advantages, including better thermostability, elimination of microbial contamination, decrease of side reactions, and relative ease of enzyme immobilization based on their insolubility in organic solvents, thus one can monitor hydrophobic substrates and greatly expand the scope of biosensors toward many previously inaccessible analytes and toward many challenging samples matrices and hostile environments. In the absolute absence of water, an enzyme will lose its activity as biocatalyst, no response can be observed [2]. Thus the presence of a small amount of water plays an important role in retaining the enzymatic activity [3, 4]. The minimal amount of water required for biocatalytic activity is called the "essential water" layer.

In order to apply the enzyme electrodes to an organic solution, Hall and co-workers [2] dropped 2 μL PBS onto each side of the electrode to maintain the hydration of the polyphenol oxidase. Campanella et al [5] prepared a tyrosinase enzyme electrode by placing the enzyme solution over the head of the oxygen sensor by means of a Teflon O-ring with a simple dialysis membrance and used this electrode for phenol determinations. They found the sensor to be more efficient and long-lasting in water-saturated (4 days) than in pure chloroform (< 1 day). Thus, it is important to seek a suitable method or matrix for enzyme immobilization and providing essential water layer around the enzyme. Some materials such as poly(ester-sulfonic acid)polymer [6], polyhydroxyl cellulose [7,8] and silica sol-gel membranes [9 – 14] have been used as attractive matrices well suited for the immobilization of enzymes for the construction of organic-phase biosensors. Researchers have demonstrated that the silica sol-gel materials can retain the catalytic activities of enzymes to a large extent [15]. This inorganic silica sol-gel material is biocompatible, because it can be prepared under ambient conditions and exhibits tunable porosity, high thermal stability, chemical inertness and negligible swelling in nonaqueous solutions [16, 17]. In the sol-gel network enzymes can be temperately entrapped and easily accessed by substrates, thus the sol-gel material is particularly useful to immobilize enzymes for the fabrication of electrochemical biosensors [18]. Many attempts have been made to immobilize tyrosinase in silica sol-gel matrix for fabrication of amperometric sensors for phenols in nonaqueous phase [9, 11]. However, the silica sol-gel derived matrix is fragile and easily cracked and easy to desquamate from the electrode surface [16, 17]. Some surface-active agents have been employed to overcome this problem. It is obvious that surfactant is detrimental to the enzyme [19]. Meanwhile, silica sol-gel process is usually carried out in acidic conditions, which are also hostile to the

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activities of enzymes. Although some new sol-gel materials such as sol-gel vanadium pentaoxide [20] and alumina [21] have been developed to overcome the shortcomings caused by the silica sol-gel process for enzyme immobilization in biosensor constructions, they have not been used for preparation of organic-phase biosensors yet. In this work, we report on the first application of another sol-gel matrix, titania sol-gel, in preparation of organic-phase biosensors. Titania sol-gel can be prepared in neutral medium at ambient temperature with vapor deposition method and has been proved to be clean, homogeneous, porous, and suitable to high enzyme loading [22].

In our previous work this matrix has been used to immobilize tyrosinase on a glassy carbon electrode for preparation of phenol sensor [23]. Phenol in aqueous solution can diffuse to the immobilized enzyme with low mass transport barrier and be oxidized by dissolving oxygen to form a detectable product at -150 mV. This sensor here is further used for determinations of three phenol derivatives in the organic phase. The results demonstrate that the tyrosinase electrode is a good organic-phase biosensor for phenols and the titania sol-gel film can retain the essential water needed for maintaining the enzyme activity. Although the detection limit of 8.0×10^{-7} mol L^{-1} for phenol in pure chloroform is worse than that of 1.0×10^{-7} mol L^{-1} in aqueous phase [23], the immobilized tyrosinase shows slightly better affinity to phenol in pure chloroform. Different from some phenol biosensors [2, 5], no extra water is necessary for this sensor in organic phase. Under optimal conditions it shows a fast response, high sensitivity and long term stability. This work provides a new matrix for the construction of organic-phase biosensors.

2. Experimental

2.1. Reagents

Mushroom tyrosinase (from mushroom. EC.1.14.18.1), noted activity of 2400 units mg^{-1} of solid (Catalog No. T. 7755), was purchased from Sigma Chemical Co. (St. Louis. MO) and used as received. Titanium isopropoxide (Ti(*i*-PrO)$_4$) was obtained from Aldrich. Phenol, catechol, *p*-cresol, and chloroform were from Beijing Chemical Factory (Beijing, China). Tetrabutylammonium perchlorate (TBAP) was purchased form Fluka as the supporting electrolyte. All of the other chemicals were of analytical grade.

2.2. Preparation of Enzyme Electrode

Glassy carbon electrodes (diameter of 4 mm) were polished before each experiment with 1, 0.3 and 0.05 μm α-alumina powder respectively, rinsed thoroughly with doubly distilled water between each polishing step, sonicated in 1:1 nitric acid, acetone and doubly distilled water successively, and then allowed to dry at room temperature.

For preparation of organic-phase biosensors, a tyrosinase solution was first obtained by dissolving 4 mg tyrosinase in 1 mL 0.02 mol L^{-1} pH 7.0 phosphate buffer solution (PBS). Ten μL tyrosinase solution was dropped onto the surface of a glassy carbon electrode pretreated as above. The electrode was then suspended vertically above titanium isopropoxide in a sealed flask kept at a constant temperature of 25 °C for 6 hours. This resulted in absorption of saturate titanium isopropoxide vapor at 25 °C by the enzyme solution and slow formation of a titania sol-gel membrane through hydrolysis of titanium isopropoxide on the surface trapping the tyrosinase in the membrane to produce a tyrosinase/titania sol-gel modified electrode. As contrast a titania sol-gel modified electrode was prepared with the same procedure except for a substitute of the enzyme solution with 0.02 mol L^{-1} pH 7.0 PBS.

2.3. Apparatus

Electrochemical measurements were performed with a conventional three-electrode system with the enzyme electrode as working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference against which all potentials were measured. The electrodes were connected to a BAS-100B electrochemical analyzer (Bioanalytical System, USA). Cyclic voltammetric measurements were done in thermostated and unstirred electrochemical cell at 25 ± 0.2 °C. Amperometric experiments were carried out in thermostated and stirred cell at 25 ± 0.2 °C by applying a constant potential of -100 mV to the working electrode. The standard solutions of phenols were prepared with chloroform and the 0.05 mol L^{-1} TBAP chloroform solution was used as supporting electrolyte. Aliquots of phenol standard solution were successively added to the solution. Current-time data were recorded after a steady-state current had been achieved.

3. Results and Discussion

3.1. Cyclic Voltammetric Behavior of the Tyrosinase/Titania Sol-Gel Modified Electrode

The cyclic voltammograms of tyrosinase/titania sol-gel modified electrode in 0.05 mol L^{-1} TBAP chloroform solution are shown in Figure 1. In absence of phenols, both the enzyme electrode and the titania sol-gel modified electrode showed a low background without detectable signal. Upon addition of catechol to the TBAP chloroform solution, no obvious response was observable at the titania sol-gel modified electrode, while the cyclic voltammogram of the enzyme electrode gave a reduction current with the shape of catalytic wave and a maximum value occurring at the potential of -60 mV, which was attributed to the reduction of enzymatic reaction product, *o*-quinone. *o*-Quinone was formed from the oxidation of catechol by dissolved oxygen in presence of immobilized tyrosinase [21].

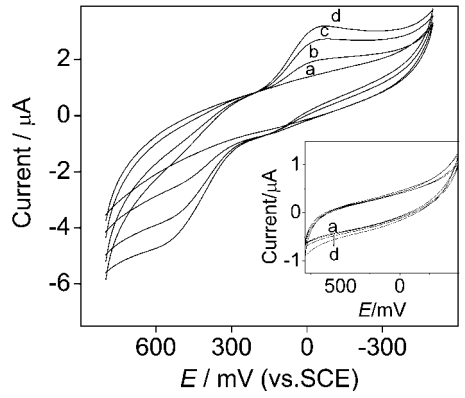

Fig. 1. Cyclic voltammograms of the tyrosinase/titania electrode in 0.05 mol L^{-1} TBAP chloroform solution without (a) and with 4.0×10^{-5} (b), 8.0×10^{-5} (c) and 1.0×10^{-4} mol L^{-1} (d) catechol at 50 mV s^{-1}. Inset: cyclic voltammograms of the titania electrode under the same conditions.

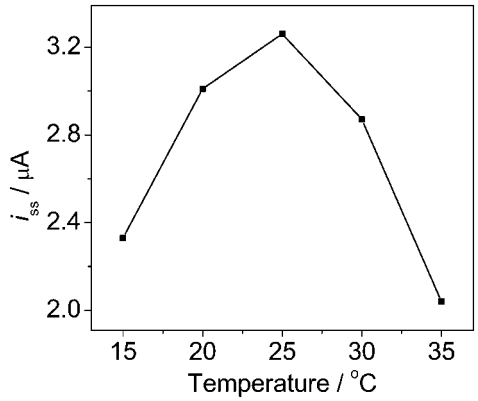

Fig. 2. Effect of temperature for preparation of sol-gel film the tyrosinase/titania electrode on its amperometric response in 0.05 mol L^{-1} TBAP chloroform solution containing 1.0×10^{-4} mol L^{-1} catechol at -100 mV.

The product of the electrochemical reduction was further oxidized to give one observed oxidation current.

The reduction peak current increased with an increasing catechol concentration in the test solution. The appearance of reduction current indicated that the tyrosinase immobilized in titania sol-gel film retained its biological activity in the organic phase. This proved the existence of bound and free water in the sol-gel network [8].

3.2. Optimization of Enzyme Electrode Preparation

Titanium isopropoxide is much more active to water than tetraethyl orthosilicate. In case of touching with water, a precipitate of titanium dioxide formed immediately. So the vapor deposition method was engaged to make the hydrolysis process slow down to form a sol-gel but not titanium dioxide powder. The performance of the enzyme electrode mainly depended on two aspects: preparation temperature and the amount of enzyme entrapped on the electrode surface.

Figure 2 shows the effect of the temperature for tyrosinase/titanium sol-gel preparation on the amperometric response. The enzyme electrode shows the best performance at the preparation temperature of 25 °C. Too high temperature makes the titanium isopropoxide vapor pressure too high and leads to a too rapid hydrolysis rate, which results in the formation of titanium powder but not a sol-gel. Thus the enzyme is not effectively entrapped. Low temperature results in a very slow gas deposition rate, which cannot catch up with the rate of water volatilization. This brings on a poor yield of the hydrolysis product of titania sol-gel. At the temperature of 25 °C, hydrolysis rate matches with water volatilization rate.

Figure 3 shows a dependence of the amperometric response of the enzyme electrode on the enzyme amount entrapped on the electrode surface. The response current increases with the increasing enzyme concentration in the solution dropped onto the surface of electrode. The curve shows a plateau at an enzyme concentration of 4 mg mL^{-1}. This indicates the enzyme loading capacity of titania sol-gel thin film has been saturated.

An additional factor is the pH of the enzyme solution dropped onto the electrode surface. When an enzyme works in organic phase, the organic solvent has no ability to alter the charge of enzyme molecules, so the enzyme shows a memory effect to the pH of the last aqueous solution to which it has been exposed [3]. The pH value of the last aqueous solution influences the ionization states of the charged groups of the enzyme. The charged groups of enzyme retain their existing ionizations from the last solution to which they have been exposed [3]. Our previous work demonstrated that the tyrosinase immobilized in vapor deposition derived titania sol-gel matrix retained its optimal activity at pH 7.0 in aqueous solution [23]. So we selected pH 7.0 PBS containing 4 mg mL^{-1} tyrosinase for enzyme electrode preparation.

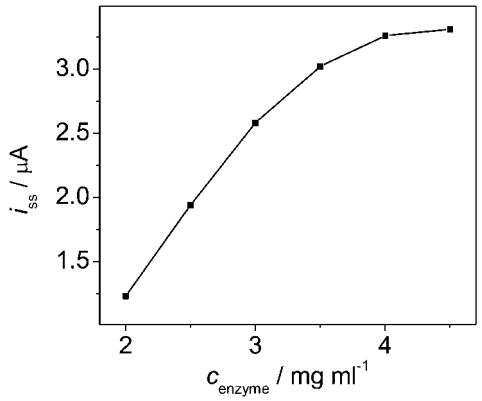

Fig. 3. Effect of enzyme concentration for preparation of the tyrosinase/titania electrode on its amperometric response in 0.05 mol L^{-1} TBAP chloroform solution containing 1.0×10^{-4} mol L^{-1} catechol at -100 mV.

3.3. Optimization of the Experimental Parameters

Water layer around the enzyme molecule is essential in retaining the enzyme activity. The choice of an appropriate solvent is very important for keeping the essential water around the enzyme. Sufficiently hydrophobic, water-immiscible organic solvents are usually desirable for enzymatic catalysis media because of their decreased ability to interact with the water closely associated with the enzyme surface. When water-miscible solvents are used, the enzyme loses its activity in most instances due to the distortion of the essential water layer by the solvent. The works reported by Dong [7] and Turner [4] discovered that less water is desorbed from enzyme molecule in solvent with $\log P > 2$ (P is the partition coefficient of a given solvent between n-octanol and water in a two-phase system). Therefore, the enzyme can keep the essential water and its bioactivity in hydrophobic solvents such as chloroform and chlorobenzene. Additionally, the solvent viscosity (η) and dielectric constant (ξ) influence the substrate diffusion. Low $1/\eta\xi$ value results in a low frictional force between the solvent and substrate and brings a fast diffusion of substrate [24]. In biosensor construction, we aim to a high sensitivity and fast response. Chloroform is a highly hydrophobic solvent with low $1/\eta\xi$ value and thus selected as the solvent in this work.

The dependence of the amperometric response of the sensor on the applied potential for amperometric determination of catechol is shown in Figure 4. The reduction of o-quinone was already observed at around 50 mV, and the steady-state current increased rapidly as the applied potential moved negatively from 50 mV to -100 mV, which was due to the increased driving force for the fast reduction of o-quinone at low potentials. The current approached a plateau at -100 mV, so this value was selected as the working potential. In comparison with the results reported [9], working potential for phenol reduction was at a more positive value. The low working potential made the sensor capable of minimizing possible interferences.

3.4. Amperometric Response of the Biosensor

Figure 5 illustrates a typical current-time plot for the sensor on successive additions of catechol at -100 mV. When an aliquot of catechol is added into the supporting solution, the reduction current rises steeply to reach a stable value. The enzyme electrode achieves 95% of steady-state-current in less than 5 seconds. The response rate is much faster than that of 18 seconds reported in the pure silica sol-gel matrix [9]. Compared to the element of silicon, titanium has a larger covalent radius, which results in larger sol-gel pore size. This is also conducive to a fast equilibrium of the substrate between the organic solvent and titania film. Both factors are favorable to create a faster diffusion of the substrate from bulk solution to the enzyme. This results in a faster response of the sensor. Such a short response time further proves that the vapor deposition derived titania sol-gel material is a promising matrix for the construction of organic-phase biosensors.

Figure 6 displays the calibration plots of the enzyme electrode for catechol, phenol and p-cresol in 0.05 mol L^{-1} TBAP chloroform solution under the optimal experimental conditions. The analytical performance including sensitivity, linear range, detection limit of the enzyme electrode to different substrates is listed in Table 1. The results illustrate that the titania sol-gel matrix is suitable for enzyme loading. The sensitivity trend is catechol > phenol > p-cresol. This is different from that of the tyrosinase/silica sol-gel electrode in aqueous solution [25]. This could result from the different hydrophobicity of the immobilization matrix and the altered substrate solubility in the organic solvent [26]. At high phenol concentrations, platform responses are observed, showing the characteristic of the Michaelis – Menten kinetic mechanism. The apparent Michaelis – Menten constants (K_M^{app}), a reflection of the enzymatic affinity, are calculated according to the Lineweaver – Burk equation [27] and also listed in Table 1. The K_M^{app} value is smaller than that of the free enzyme [28], indicative of a high affinity of tyrosinase entrapped in titania sol-gel matrix to phenols. This is due to the fact that catechol generated by the

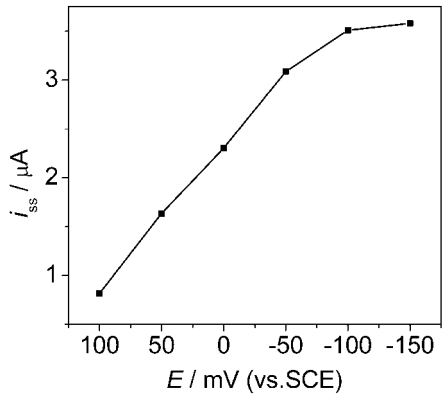

Fig. 4. Influence of applied potential on amperometric response of the sensor to 1.0×10^{-4} mol L^{-1} catechol in 0.05 mol L^{-1} TBAP chloroform solution.

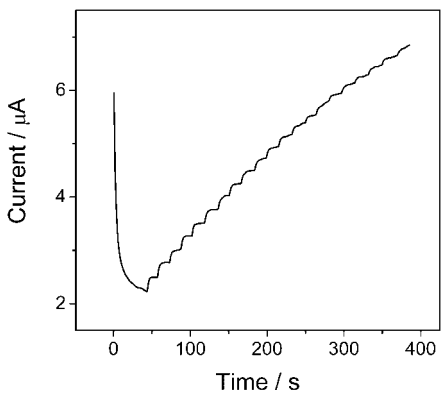

Fig. 5. Typical current-time response curve of the sensor upon successive additions of 2.0×10^{-5} mol L^{-1} catechol in 0.05 mol L^{-1} TBAP chloroform solution at -100 mV.

electrochemical reduction of enzymatically-produced *o*-quinone could enter into another enzymatic oxidation cycle, providing a local increase in substrate concentration and an amplification of the electrode response. Comparing the results for phenol determination with those reported previously [23], the linear range, sensitivity and detection limit in chloroform are worse than those in aqueous solution, which are due to the change in electrolyte solution. However, the K_M^{app} value of 0.17 mM is slightly lower than that of 0.29 mM in aqueous solution. Thus, the immobilized tyrosinase shows slightly better affinity to phenol in chloroform. The titania sol-gel can effectively retain the essential water layer around the enzyme molecule needed for maintaining its activity in organic phase.

3.5. Reproducibility and Stability of the Enzyme Electrode

The reproducibility of the organic phase sensor was explored at a catechol concentration of 1.0×10^{-4} mol L^{-1}. The mean steady-state response of one sensor is 3.5 μA with a relative standard deviation of 3.6% for nine determinations. The fabrication reproducibility of six sensors, independently constructed based on the same bare electrode, shows an acceptable reproducibility with a relative standard deviation of 4.2% for the steady-state current obtained at the catechol concentration of 1.0×10^{-4} mol L^{-1}.

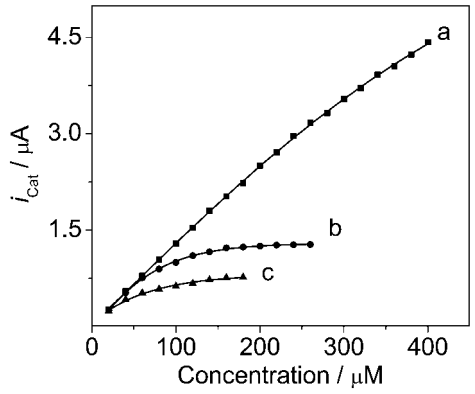

Fig. 6. Calibration plots of the tyrosinase electrode for catechol (a), phenol (b) and *p*-cresol (c) determinations in 0.05 mol L^{-1} TBAP chloroform solution at -100 mV.

The operational stability of the organic phase sensor was investigated by 100 consecutive measurements of its response to a catechol sample of 1.0×10^{-4} mol L^{-1} within a period of 5 h per day. After a week the sensor retained ca. 92% of its initial response. It was observed that the titania sol-gel enzyme film on the electrode surface did not swell in the organic solvent during continuous measurements. Additionally the titania sol-gel enzyme electrode exhibited good long-term stability. When not in use, the enzyme electrode was stored in the refrigerator at 4 °C, it retained ca. 87% of the initial current response after a 70-day storage. If its current response was detected once per 10 days, after the intermitted use over the 70-day period it retained ca. 82% of its initial current response. These results demonstrated titania sol-gel film was very efficient for retaining the activity of tyrosinase. Good long-term stability was attributed to three facts. The vapor deposition sol-gel method provided a mild immobilization process. This process did not involve any additive that resulted in chemical modification and fouling of the enzyme molecules, so the enzyme could maintain its biological activity to a large extent. The other was attributed to the large quantities of hydroxyl groups in the sol-gel film, which could form strong hydrogen bonds with tyrosinase and sol-gel cages for enzyme loading. The cages in the film had a rigidity and protective nature [10], which were beneficial for preventing the enzyme from leaking out of the titania sol-gel thin film. On the other hand, the bound and free water molecules in the titania sol-gel network could supply the essential water layer, thus retaining the enzymatic activity in the organic solvent.

4. Conclusions

This work develops a novel organic-phase biosensor for phenols by entrapping tyrosinase in titania sol-gel matrix with a vapor deposition method. This sol-gel method provides a mild process for immobilization of tyrosinase and a biocompatible microenvironment, which can supply the essential water layer needed for retaining enzymatic activity in the organic solvent. The porous structure of the titania sol-gel matrix leads to a low mass transport barrier to the substrates of tyrosinase, a good loading and high catalytic activity of the enzyme, and a fast response rate of the sensor. This film is very efficient for preventing leakage of the tyrosinase out of the film and retaining the essential water layer around the enzyme, which results in a long-term

Table 1. Analytical performance of the enzyme electrode for different substrate.

Substrate	Sensitivity [a] (μA $mmol^{-1}$ L cm^{-2})	Linear range (mol L^{-1})	*R* [b]	Detection limit (mol L^{-1})	K_m^{app} (mmol L^{-1})
Catechol	27.5	$2.0 \times 10^{-6} - 3.2 \times 10^{-4}$	0.999	6.4×10^{-7}	0.15 ± 0.003
Phenol	17.5	$1.8 \times 10^{-6} - 8.0 \times 10^{-5}$	0.984	8.0×10^{-7}	0.17 ± 0.008
p-Cresol	7.11	$2.4 \times 10^{-6} - 1.6 \times 10^{-4}$	0.987	1.6×10^{-6}	0.21 ± 0.004

[a] Slope of linear portion of calibration plot.
[b] Correlation coefficient of the linear range.

stability and good reproducibility of the sensor, demonstrating the titania sol-gel matrix is a suitable material for enzyme immobilization and the organic-phase biosensor preparation.

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