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Immobilized Laccase on Activated Poly(Vinyl Alcohol) Microspheres For Enzyme Thermistor Application

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Abstract Poly(vinyl alcohol) (PVA) microspheres were prepared by inverse suspension crosslinked method, with glutaraldehyde as a crosslinking agent. PVA microspheres activated with aldehyde groups were employed for *Trametes versicolor* laccase immobilization. Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy were used to characterize the activated PVA microspheres and PVA microspheres with immobilized laccase (Lac/PVA microspheres), which show that laccase was successfully immobilized on the PVA microspheres. The optimum pH and temperature coupling conditions for the immobilized laccase were determined to be 3.3 and 30 °C, respectively. Residual activity was also investigated by soaking the immobilized laccase in organic solvents at different concentrations, proving it chemically stable. Immobilized laccase exhibited good storage stability at 4 °C. The enzyme biosensor showed good performance in 2,2-azinobis(3-ethylthiazoline-6-sulfonate) and bisphenol A, with concentration ranges of 2 to 8 mM and 0.05 to 0.25 mM, respectively. Therefore, PVA microspheres may have high potential as support for enzyme thermistor applications.

Keywords Laccase · Poly(vinyl alcohol) · Microspheres · Immobilization · Enzyme thermistor

Introduction

Laccase (EC 1.10.3.2) is a multi-copper protein that can directly reduce oxygen to water under ambient conditions while oxidizing a variety of aromatic compounds. Laccase has drawn

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significant attention for its potential applications in the field of biosensors because of its broad specificity for substrate [1], [2].

The thermometric biosensor, i.e., enzyme thermistor (ET), has been developed to monitor enzyme reactions based on changes in the reaction system enthalpy [3], [4]. Immobilization of specific enzymes on an appropriate support is a promising method for the manufacture of thermometric biosensors [5]. Previous studies indicated that the specific enzyme is immobilized on controlled pore glass by crosslinking with glutaraldehyde [6], [7], on ceramic hydroxyapatite by non-covalent adsorption [8], and entrapped on the surface of reticulated vitreous carbon cylinder using sol–gel as a binder [9]. However, current supports have the following disadvantages: (1) inorganic carriers without active groups need further functionalization to immobilize the enzyme efficiently [10]; (2) enzyme immobilization at the solid/liquid interface is easily affected by factors such as pH value, temperature, surface tension of the medium, and nature of inorganic support [11]; and (3) enzyme entrapment in gel can limit enzyme activity for the diffusion of large molecules and susceptibility of ET [12]. Thus, new supports are badly needed in this area.

Poly(vinyl alcohol) (PVA)-based materials for biosensor applications are well-known entrapment enzymes within the PVA matrix [13]. However, the addition of enzymes in the PVA preparation process can inevitably cause activity loss. Furthermore, immobilization of enzymes entrapped in the PVA matrix can cause significant mass transfer resistances, e.g., hindrance of substrate diffusion to immobilized enzymes and metabolites leaving the matrix. The inverse suspension crosslinked method (ISCM) is used to prepare PVA microspheres because of its easy manipulation, low cost, and controllable particle size [14]. As a novel biosensor support, PVA microspheres with large external surface areas and functional groups can be applied for enzyme immobilization by covalent grafting method. PVA microspheres can be modified for immobilization with different activation methods; they have been discussed in previous studies on enzyme immobilization [15] [16]. To the best of our knowledge, the PVA-based material immobilization approach has not yet been reported in combination with thermometric sensing using an ET.

This study aims to develop an effective support for ET application. The specific objectives of this study are as follows: (1) prepare PVA microspheres for laccase immobilization; (2) discuss the immobilization efficiency and properties of the immobilized laccase; and (iii) determine the thermometric response of the immobilized laccase.

Experimental Section

Materials

PVA with a polymerization degree of 1,750 and glutaraldehyde (GA; analytical grade) were purchased from Beijing Chemical Reagent Company (China). Ethanol and petroleum ether (analytical grade) and chemically purified liquid paraffin were purchased from Tianjin Chemical Reagent Company (China). Laccase (≥10 U/mg) from fungus *Trametes versicolor*, 2,2-azinobis(3-ethylthiazoline-6-sulfonate) (ABTS), and Bisphenol A (BPA) were purchased from Sigma–Aldrich (Shanghai, China). Purified water was supplied by a Milli-Q water purification system (Millipore, Bedford, MA, USA). HPLC-grade methanol was obtained from Fisher Scientific (NJ, USA).

Preparation of PVA Microspheres

ISCM was used to prepare the PVA microspheres. The preparation was conducted in a 250-mL three-necked round bottom flask fitted with a condenser. Briefly, 3 g PVA was dissolved in

30 mL water and sufficiently blended with a mechanical stirrer in a boiling water bath for 1 h. After the mixture cooled to 50 °C, 60 mL liquid paraffin was added. The mixed solution was stirred at 500 rpm for 30 min to form the microspheres. Subsequently, 1 mL (1 M) HCl and 2 mL GA (50 %) was added to the contents of the three-necked round bottom flask and stirred for 2 h at 500 rpm. Microspheres were recovered by centrifugation at 10,000 rpm, washed three times with petroleum ether, and then vacuum-dried for 48 h.

Immobilization of Laccase

PVA microspheres (1 g) were suspended in 10 mL HAC–NaAC buffer (pH 4.5) containing 2.0 g L^{-1} laccase at 4 °C for 24 h. The immobilized laccase was used directly for activity measurement after separation and extensive washing with water.

Characterization

Fourier transform infrared spectroscopy (FTIR) reflection absorption spectra were recorded using a Nicolet 750 FTIR spectrometer.

X-ray photoelectron spectroscopy (XPS) data were obtained with an AXIS-Ultra instrument from Kratos Analytical using monochromatic Al K radiation (225 W, 15 mA, 15 kV) and low-energy electron flooding for charge compensation.

Scanning electron microscopy (SEM) was performed with an FEI QUANTA 200 F microscope with a tungsten electron source and an accelerating voltage of 0.5 to 15 kV.

Brunauer–Emmett–Teller (BET) surface analysis was obtained using a QuantaChrome Autosorb-1 analyzer. All samples were degassed under vacuum at 90 °C for more than 5 h. Specific surface areas were determined from the linear part of the BET plot.

UV-vis measurements were carried out using a DR5000 spectrophotometer (HACH, USA).

Determination of Laccase Activity

The activity of free and immobilized laccase was spectrophotometrically determined in a reaction medium containing 1 mM ABTS as substrate in HAC-NaAC buffer (pH 4.5) at room temperature in the absorbance at 420 nm. A suitable amount of free and immobilized laccase was added to the substrate solution and immediately stirred. The absorbance of the supernatant was determined using a UV–vis spectrophotometer after 5 min. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute.

The activities of the free and immobilized laccase were determined by measuring their activities after immersion in different buffer solutions at 30 °C for 6 h to determine the resistance to pH changes. Free and immobilized laccases were incubated in HAC-NaAC buffer (pH 4.5) at temperatures ranging from 10 to 70 °C for 6 h to measure the effect of temperature on laccase activity. Aliquots of the enzyme solutions were taken for activity assay at room temperature. Each measurement was performed in triplicate.

The stability of the enzymes in methanol, ethanol, dimethylsulfoxide (DMSO), and acetone at different concentrations (20, 40, 60, and 80 %*v*/*v*) were measured by incubating free and immobilized laccase in 1 mL solutions at 30 °C for 24 h. Initial and final laccase activities were measured at room temperature. Each measurement was performed in triplicate.

Storage stabilities of the free and immobilized laccase were investigated at room temperature and 4 °C. Free laccase preparation was stored as a HAC–NaAC buffer solution (pH 4.5), whereas immobilized laccase was stored as solid particles in a closed glass tube. Residual activities were measured during their 100 days of storage.

Application in Enzyme Thermistor System

The ET system was produced by Omic Bioscience, Wuhan, China. The schematic setup of the ET biosensor for Lac/PVA application is presented in Fig. 1. The biosensor consists of a peristaltic pump, injection valve, sample loop (35 μL), enzyme thermistor, and Wheatstone bridge equipped with a chopper-stabilized amplifier and data recorder. The buffer was HAC–NaAC (pH 4.5).

Results and Discussion

Synthesis of Lac/PVA Microspheres

The synthesis of Lac/PVA microspheres is shown schematically in Fig. 2.

The morphologies of the PVA microspheres (Fig. 2) and the small size of the microspheres (~30 μm in diameter) provided a high surface area (10.2 $m^2\ g^{-1}$), thereby increasing the density of the binding sites for enzyme immobilization within a given volume [17]. In addition, higher-reactive surface groups are preferable for immobilizing an enzyme, which can easily preserve the greater advantage of laccase. Each aldehyde group (–CHO) reacted with two hydroxyl groups (–OH) on the PVA chains; thus, each GA molecule consumed four PVA hydroxyl groups. However, some side reactions can be expected, e.g., some GA molecules may only react with two hydroxyl groups. The residual aldehyde groups on PVA microspheres can react with nucleophiles (such as the amino, thiol, carboxyl, and phenolic groups of tyrosines) of the laccases. The mechanism of activation of glutaraldehyde may be explained by the reaction of the two aldehyde groups from activating agent with two hydroxyl groups from PVA (inter- or intra-crosslinking) and reaction of one aldehyde group with hydroxyl group (activation) leading to the formation of reactive group that reacts with amine groups of the enzyme molecule (Schiff's bases formation) [18].

The FTIR spectrum of PVA microsphere (Fig. 3a) shows the absorption peaks at 3,459 cm^{-1} (O–H stretching) for the –OH group, 1,647 cm^{-1} (C–O stretching) for the –C=O group, and 1,441 cm^{-1} (C–O–H in-plane bending) for the –OH group. Figure 3b illustrates the effect of laccase on PVA microsphere chemical structure. There is a new absorption peak at 1,593 cm^{-1} (Fig. 3b) for the C=N group which formed between laccase and PVA microsphere during immobilization, indicating that laccase was immobilized on PVA microsphere. The C=O band peak shifted to lower wavenumber values (i.e., C=O stretching at 1,578 cm^{-1}) when laccase

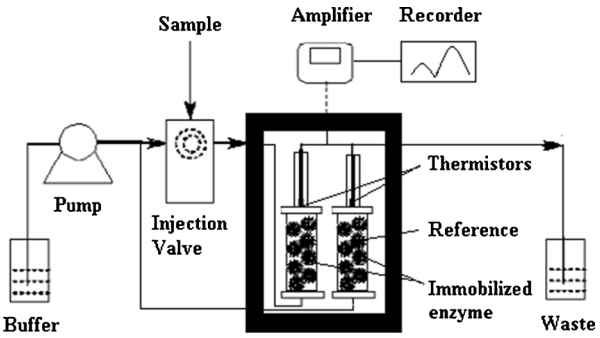

Fig. 1 Scheme of the experimental arrangement of the enzyme thermistor system

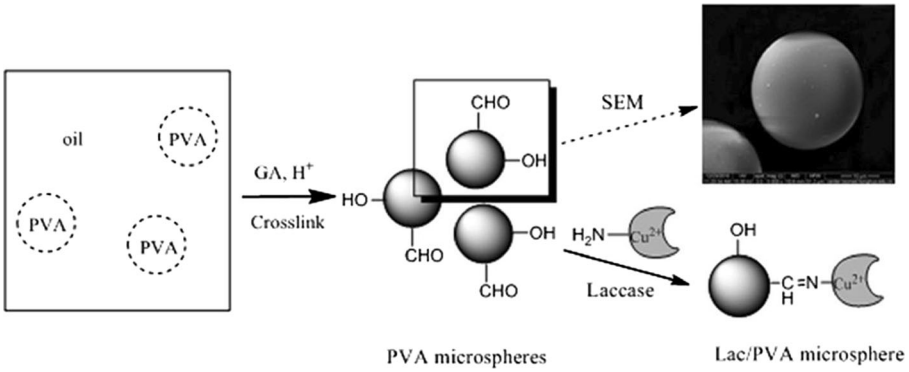

Fig. 2 Schematic of the inverse suspension crosslinked method for the preparation of Lac/PVA microspheres

was immobilized on PVA microsphere. The shifts were attributed to weak C–O bonds as a result of the drainage of electrons from oxygen atoms when metal ions (Cu^{2+}/Cu^{+}) were coordinated. The new absorption peak at 650 cm^{-1} (Fig. 3b) is possibly due to the Cu^{2+}/Cu^{+} in laccase.

XPS spectra of the PVA microspheres and Lac/PVA microspheres were recorded for further confirmation (Fig. 4). The peak assignments were carried out based on the electron densities of the specific components. The XPS spectra in Fig. 4b show that the small peak at 398.0 eV is analogous to the band in N 1 s from laccase. The peak at 532.5 and 285.6 eV (Fig. 4b) is attributed to O 1 s, and C 1 s for the protein in laccase.

Properties of the Immobilized Laccase

The immobilized amounts of laccase on PVA microspheres were based on the method of Zhu et al. [19]. The free laccase concentrations in the supernatant can be measured by ABTS UV–vis spectrophotoscopy when the equilibrium mixtures of the free laccase and Lac/PVA suspensions are separated. Subsequently, the immobilized amount of laccase can be calculated. Figure 5a shows the immobilized amounts of laccase on the PVA microsphere with different laccase concentrations at 4 °C. The immobilized amount of laccase increases with increase in the laccase concentration in 24 h. Maximum immobilized enzyme amount was reached up to 2 g enzyme L^{-1}. Therefore, the immobilized amounts of laccase on PVA microspheres can

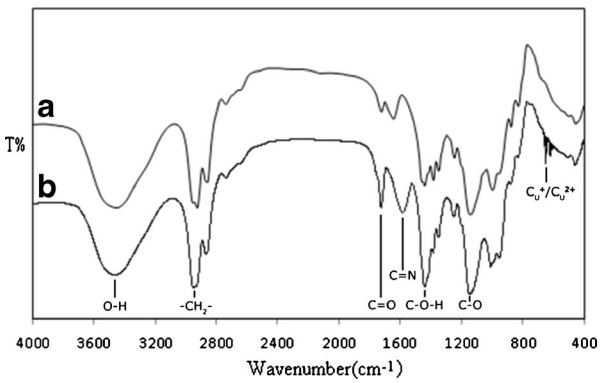

Fig. 3 FTIR spectra of **a** the PVA microspheres and **b** the Lac/PVA microspheres

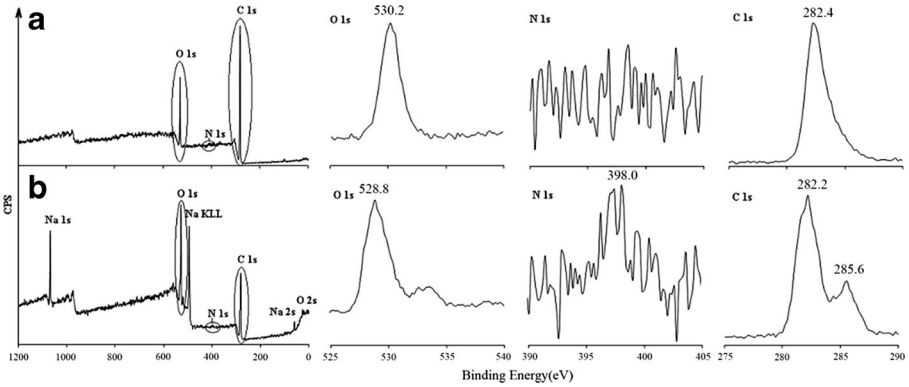

Fig. 4 XPS spectra of **a** the PVA microspheres and **b** the Lac/PVA microspheres

reach 1,540 U g^{-1}. Figure 5b shows the immobilized amounts of laccase on the microspheres as a function of time when laccase concentration is 2.0 g L^{-1}. The immobilized amounts of laccase can reach to equilibrium after 12 h. Based on the response of the maximum

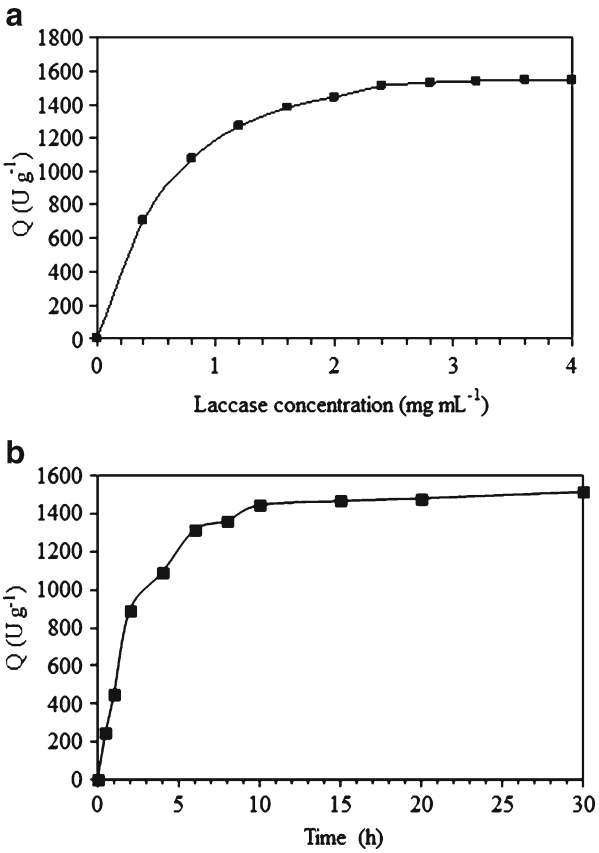

Fig. 5 Immobilized amounts of laccase on the PVA microspheres with **a** different laccase concentrations at 4 °C and **b** function of time when laccase concentration is 2.0 g L^{-1}

immobilized amounts, 2.0 g L^{-1} laccase concentration and 12 h were selected for immobilized conditions.

The activity of laccase immobilized on PVA microsphere was tested at different pH values and then compared with that of free laccase. Figure 6a shows that free and immobilized laccase exhibited maximal activity at pH 4.1 and pH 3.3, respectively. The immobilized laccase

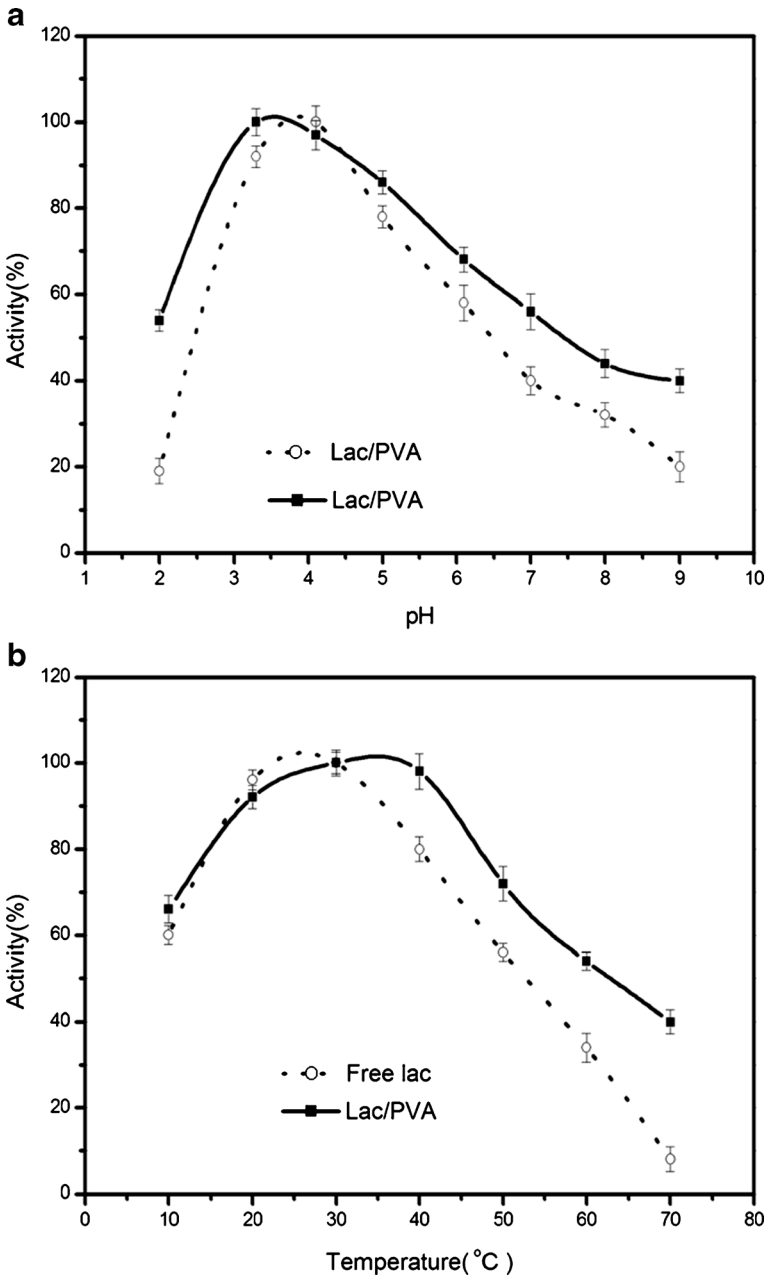

Fig. 6 Effect of **a** pH and **b** temperature on free and immobilized laccase (Lac/PVA) activities

Table 1 Residual activity after 24 h in organic solvents

Solvent	Laccase	Laccase activities				
		0 %	20 %	40 %	60 %	80 %
Methanol	Free	100	92.3±2.2	67.6±3.5	25.1±2.8	3.5±4.2
	Immobilized	100	98.5±3.7	91.7±4.1	76.4±5.4	65.2±5.2
Ethanol	Free	100	94.7±4.3	75.6±3.6	48.9±6.1	29.1±5.4
	Immobilized	100	99.1±3.3	93.3±4.5	89.4±5.1	78.9±5.5
DMSO	Free	100	81.5±2.1	59.1±4.5	18.6±2.8	–
	Immobilized	100	92.7±3.1	72.3±2.2	20.2±4.8	1.7±2.9
Acetone	Free	100	84.4±2.5	43.4±1.9	11.5±1.8	–
	Immobilized	100	89.3±2.3	64.1±2.7	24.6±1.9	7.8±3.2

showed higher resistance to changes in the pH value of the medium. Moreover, laccase coupling to other support via GA yielded high activity [17].

Temperature has two major effects on laccase activity, namely, the effect on enzymatic kinetics and the effect on protein structure. Figure 6b shows that the apparent optimum temperature for free laccase was approximately 30 °C, whereas Lac/PVA exhibited a significantly broader temperature profile. The laccases immobilized on PVA supports retain a higher activity at the range of 40 to 70 °C compared with free laccase. The increase in optimum temperature was caused by changes in the physical and chemical properties of laccase. The multipoint non-covalent bond formation between the PVA microsphere and laccase molecules can reduce conformational flexibility and may result in higher activation energy for the molecule to reorganize the proper conformation for the binding to substrate [20], [21]. Thus, immobilized laccase on PVA microsphere can be effectively used where high temperatures are required.

Enzymatic activity in the presence of organic solvents was primarily determined by the interactions between water and the enzyme [22]. Aleksey Zaks et al. [22] demonstrated that the enzymatic activity greatly increased upon an increase in the water content in the solvents, which confirmed that our research was reasonable and accurate. In this study, the highest stability for the free and immobilized laccase using ethanol as an organic solvent is observed (Table 1).

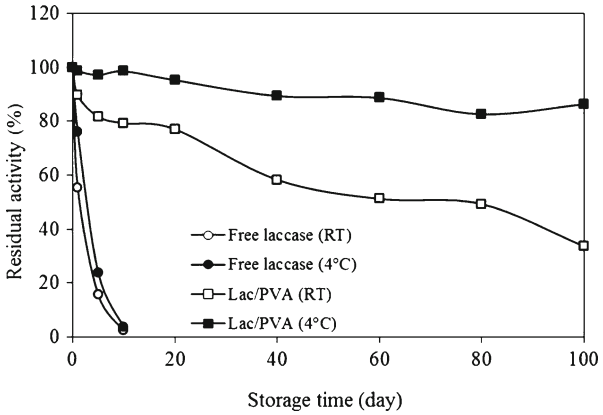

Fig. 7 Storage stability of free and immobilized laccase at 4 °C and room temperature (RT)

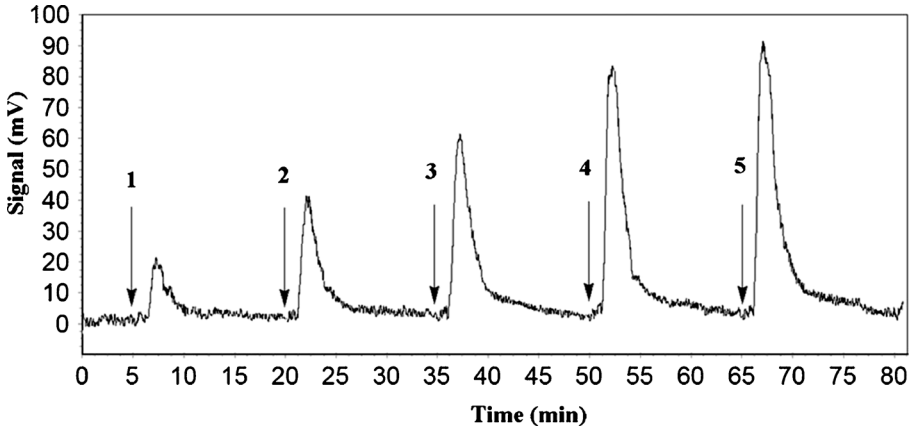

Fig. 8 Calibration plot for ABTS using the ET biosensor. The recording represents injections of 2 (1), 4 (*2*), 6 (*3*), 8 (*4*), and 10 (*5*) mM solution of ABTS

Storage stability of the free and immobilized laccase is shown in Fig. 7. The stability of Lac/PVA was greater than that of free laccase, both at room temperature and at 4 °C. Free laccase completely lost its initial activity at the end of 10 days, both at room temperature and at 4 °C. However, Lac/PVA retained 33.7 % of its initial activity at room temperature and 86.3 % of its initial activity at 4 °C at the end of 100 days. A remarkable difference in the activity retentions with storage time was observed. Thus, laccase immobilized onto the PVA microsphere showed high stability, whereas free laccase lost most of its initial activity early. Tastan et al. [2] reported that immobilized laccase on polymer-grafted polytetrafluoroethylene membranes using two different techniques retained about 40 % of their initial activities at the end of 6 and 20 days, respectively. Compared with our storage stability results, PVA microsphere has higher storage stability.

Biosensor Detection

ABTS was analyzed using an ET biosensor using immobilized laccase. Standard samples were prepared by dissolving ABTS in HAC–NaAC buffer (pH 4.5). A recording of the

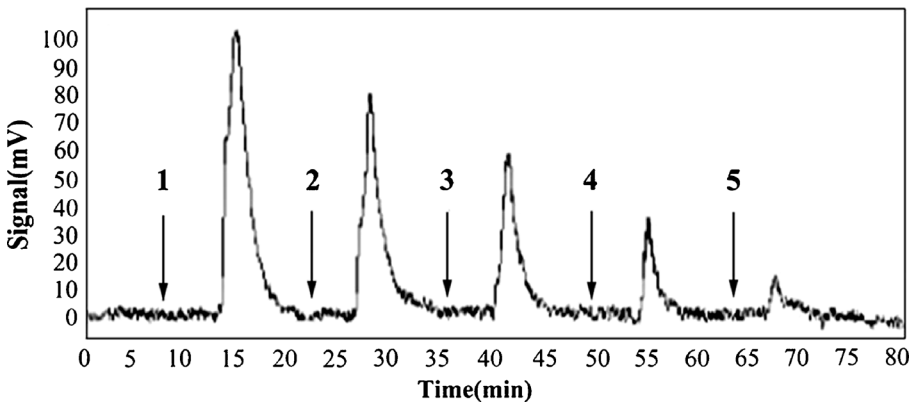

Fig. 9 Calibration plot for BPA determination using the ET biosensor

measurement standards was used to construct the calibration curve depicted in Fig. 8. The ET biosensor showed an excellent dynamic range of 2 to 8 mM for ABTS (R^2=0.995).

BPA is an important endocrine-disrupting chemical that has harmful effects. The applicability of the ET biosensor using immobilized laccase was assessed by BPA in a water–ethanol (20 % ethanol) mixture. The calibration plot for BPA using the ET biosensor is shown in Fig. 9, where good linear relationship was obtained in the range 0.05 to 0.25 mM (R^2=0.995). The results in this study suggest that the presented system is robust and suitable for BPA determination in organic wastewater.

Conclusions

Based on the above results, the performance of the immobilized laccase described in this study was satisfactory. Laccase immobilization on aldehyde-activated PVA microspheres is simple and easily scalable. The optimal immobilization conditions for laccase on PVA microspheres were determined. Immobilization of the laccase on the surface of the activated PVA microspheres leads to increased pH value and temperature resistance and improved storage stability of laccase. The improved stability of immobilized laccase can be a potential advantage in ET biosensors. To our best knowledge, this study is the first report on immobilization of laccase on PVA microspheres.

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