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Amperometric Phenol Biosensor Based on a New Immobilization Matrix: Polypyrrole Nanotubes Derived from Methyl Orange as Dopant

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A novel polypyrrole nanotubes (PPy-NTs) derived from methyl orange (MO) was used as matrix to construct a highly responsive phenol biosensor. The phenol sensor based on polyphenol oxidase could be easily obtained by casting the bio-composite via the cross-linking in the presence of glutaraldehyde on glassy carbon electrode surface. PPy-NTs matrix has features of special three-dimensional structure, biocompatible properties and high surface area, and the features above resulted in high enzyme immobilization and the enzyme buried in PPy-NTs retained its activity to a large extent. This biosensor exhibited a series of better performance such as high sensitivity (2981 $mA \cdot M^{-1} \cdot cm^{-2}$), good affinity to its substrate (the apparent Michaelis-Menten constant was 0.12 mM) and remarkable long-term stability (it retained 88% of the original activity after 30 days) and acceptable repeatability. The detection limit of the biosensor was 1.22 nM. Furthermore, the optimization of biosensor preparation and effects of experimental variables, such as pH, temperature and potential of the sensor were discussed.

Keywords: Electrochemical biosensor; Polypyrrole nanotubes; Phenols

1. INTRODUCTION

Phenolic compounds are widely used in the chemical industry and agriculture, and are discharging into the environment. These chemicals can be easily adsorbed by organism through skins and mucous membranes. They are accumulated in the bodies, because of that it is hard for phenolic compounds to be excreted during the metabolic processes. Therefore, the detection of phenolic compounds is of great importance in actual production. At present, the traditional detection methods of phenols are based on chromatography [1, 2] and spectrophotometer [3, 4]. These methods are lack of

monitoring instrument and high cost. With the development of biosensors, researchers began to apply enzyme-based biosensors to environmental monitoring. Amperometric biosensor based on polyphenol oxidase (PPO) has been recognized as a promising tool for the detection of phenols owing to its effectivity as well as simplified device [5-8]. A simple and reliable method of immobilizing enzymes has been sought for a long time. Creating a microenvironment (allows the immobilization of biochemical compounds on the sensor surface), making the enzyme to maintain high activity and long-term stability became critical challenge.

PPO is a tetramer, which contains four copper per molecule, two aromatic compounds and oxygen of binding sites[9, 10]. PPO is classified as a bio-catalyst and a protein that can form two or more different homo-oligomers, but in the course of this change it must come apart and change its shape to convert between different forms. PPO exists in the form of monomer, trimer, tetramer, octamer, dodecamer in general. Phenols were oxidized to 1,2-benzoquinone in the presence of oxygen catalytic by PPO [11, 12]. By the amperometry, the biological sensors can translate the current into the concentration of phenols. To prepare a biosensor, the chosen of carrier is very important. Numerous efforts have been devoted to this improvement by using surfactants [13-16], biopolymers [17-20], hydrogel polymers [18, 21-23], and nanoparticles [24, 25].

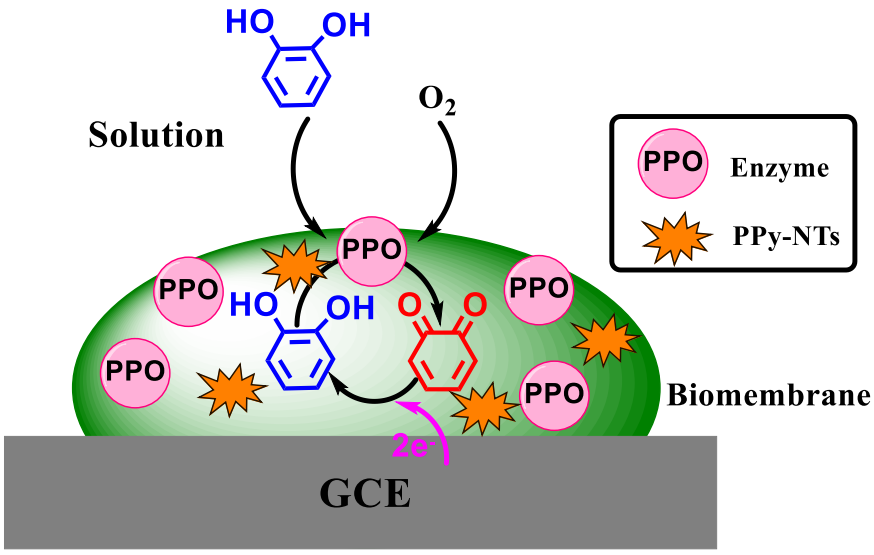

Figure 1. The mechanism of phenols oxidized by PPO

The biocompatible nanomaterials play a significant role in enzyme immobilization because of their unique advantages. The desirable microenvironments enhanced the electron transfers between electrodes and enzymes, and maximize the activity of the enzyme. Among the family of conducting polymers, PPy-NTs have potential applications in chemical sensors, solar cells [26], energy storage [27-29] and other applications [30] due to its electrical properties, environmental stability and biocompatibility. Yang et al [31] successfully synthesized PPy-NTs via a facile self-assembly process by adding a new cheap dopant, i.e., methyl orange (MO), which was an additional advantage with respect to conventional template-synthesis. MO promoted the conducting polymer to grow in a tubular

form and needed not be removed after the polymerization. The MO template automatically eliminated during the preparation process and most MO molecules were removed from the reaction medium during the water-washing process. This method was low cost and simple to operate and homogeneous nanotubes were obtained. The PPy-NTs would be a good immobilization matrix for enzymes.

In this study, a facile method of solution casting coupled with self-assembly was developed to successfully prepare PPy-NTs/PPO membrane on electrode surface. The PPy-NTs and PPO had a great performance on the electrochemical activity. PPy-NTs could prevent enzyme release from the film. Glutaraldehyde was used as a chemical cross-linking agent. The electrode was successfully applied to rapid and convenient detection of phenols, which was expected to be profitable for the monitoring of waste water and protection of environment.

2. EXPERIMENTAL

2.1. Materials

PPO from mushrooms (pI < 5.0) was purchased from Sigma-alorich with 2687 U/mg. Catechol was purchased from Sigma-alorich. Phosphate buffer (PB) solution was transferred to 6.0 with 0.1 M of KH_2PO_4 and K_2HPO_4 in total. Phenol, K_2HPO_4, and KH_2PO_4 were purchased from the Sinopharm Chemical Reagent Co., Ltd. *p*-chlorophenol and *p*-methylphenol were purchased from the China Shanghai Qingpu synthetic Reagent Factory. All other chemicals were used as received.

2.2. Apparatus

An electrochemical workstation (Model CHI660A by the CH Instruments) was used for amperometric measurements. Electrochemical workstation (Auto Lab, Nova 1.9, Metrohm, Utrecht, The Netherlands) was used for electrochemical impedance spectroscopy (EIS) (frequency range of 0.01-100000 Hz). A three-electrode system included reference electrode (saturated calomel electrode (SCE)), counter electrode (platinum sheet as a (10 × 10 mm^2)), working electrode (glassy carbon electrode (GCE) (Φ = 3 mm)). GCE was polished with 0.05 μm alumina particles on silk carefully and then rinsed with secondary distilled water and dried with nitrogen before use. The PPy-NTs/PPO film was formed on the surface of GCE. The diameter of the electrolytic cell was 23 mm, and 54 mm in height. Electrolyte solutions was PB solution (pH = 6, 10 mL) in the electrolytic cell. Adjustable micro-pipettes (Model YE16CAA0120343 was purchased from the Ningbo Experimental Instrument Co., Ltd) were used for the preparation and transfer of solution. The morphology features of the products were characterized by scanning electron microscopy (SEM) (S-4800 field emission Hitachi). The molecular structure of PPy-NTs and PPy-NTs/PPO were characterized by FT-IR spectrometer (Bruker Vector-22). Working electrodes were cleaned by an ultrasound machine (Model JT-410HT, the Shenzhen Jie Tuo ultrasound equipment Co., Ltd). All measurements were carried out in a constant temperature cell at 25 °C as well as the phosphate buffer solution.

2.3. Preparation of PPy-NTs

During the typical test, 105 μL (1.5 mM) of Pyrrole monomer was added in 30 mL of 5 mM MO (sodium 4-[4'-(dimethylamino) phenyldiazo] phenylsulfonate) ($(CH_3)_2NC_6H_4$-N=$NC_6H_4SO_3Na$) deionized water solution. Then 0.406 g (1.5 mM) of $FeCl_3 \cdot 6H_2O$ was added. A flocculent precipitate appeared immediately and the mixture was stirred at 25 °C for 24 h. The PPy-NTs precipitate was washed with ethanol/deionized water for several times until filtrate was neutral and colorless. After dried under a vacuum atmosphere (60 °C for 24 h), the PPy-NTs was obtained.

2.4. Prepare the electrode before use

Glassy carbon electrode (GCE) were polished using alumina on a polishing pad, rinsed with deionised water, ethanol and once again with deionised water. Electrodes were sonicated for 10 minutes in deionised water to remove any alumina particles and finally dried in N_2 flux. The polished electrode was placed in 0.5 M NaOH and 1 M H_2SO_4 solution and the electrochemical activation of the electrode was carried out by a constant current method at 0.025 mV/s for 50 circles. Making sure the electrode was clean enough. This electrode was used for the preparation of modified electrode.

2.5. Preparation of PPy-NTs/PPO electrode

GCE was used as a work electrode. PPO was prepared (2 mg/mL). PPy-NTs were ground in an agate mortar for 30 min before use and ensure that the granules were sufficiently small, then formulated into 2 mg/mL and 4 mg/mL solution, dispersed for 5 min in the ultrasound system. The PPy-NTs solution was diluted at the ratio set previously, and then mixed with PPO in an equal volume. Adjustable micro-pipettes was used for transfer of solution (a certain of volume, V_0) to drip on the surface of electrode. The coated electrodes were placed in a desiccator at room temperature for 30 minutes and then placed in a desiccator filled with vapor of saturated glutaraldehyde for 15 ~ 20 min. Droplets on the surface of the electrode were quick-drying and the PPy-NTs/PPO biosensor was obtained. PPy-NTs could absorb PPO because the surface was rough and porous, which could be used as a matrix, and the cross-linking effect made it difficult for the biofilm to fall off from GCE.

2.6. Amperometric analysis

The amperometric measurements of PPO substrates were performed under vigorous stirring in air-saturated phosphate. 10 μL of 1 mM catechol was added to 10 mL of 0.1 M PB solution by adjustable micro-pipettes with pH 6.0 every 100 s at -200 mV (vs. SCE). The current corresponding to the reduction of the enzymatically generated *o*-quinones was recorded and curve was obtained about current (*i*) and time (*t*).

3. RESULT AND DISCUSSION

3.1. Characterization of PPy-NTs/PPO Film.

SEM images were taken to know the morphology of the PPy-NTs and PPy-NTs/PPO membrane on the GCE. As shown in Figure 2a, the PPy-NTs nanotubes were obtained with MO as the dopant. The diameters were ~100 nm and the nanotubes were very homogeneous. Figure 2b showed that a large number of enzyme particles deposited on the surface of electrode. Morphology changed and showed a network-like structure. Large aggregation of particles was evenly distributed on the surface, indicating the successful deposition of enzymes.

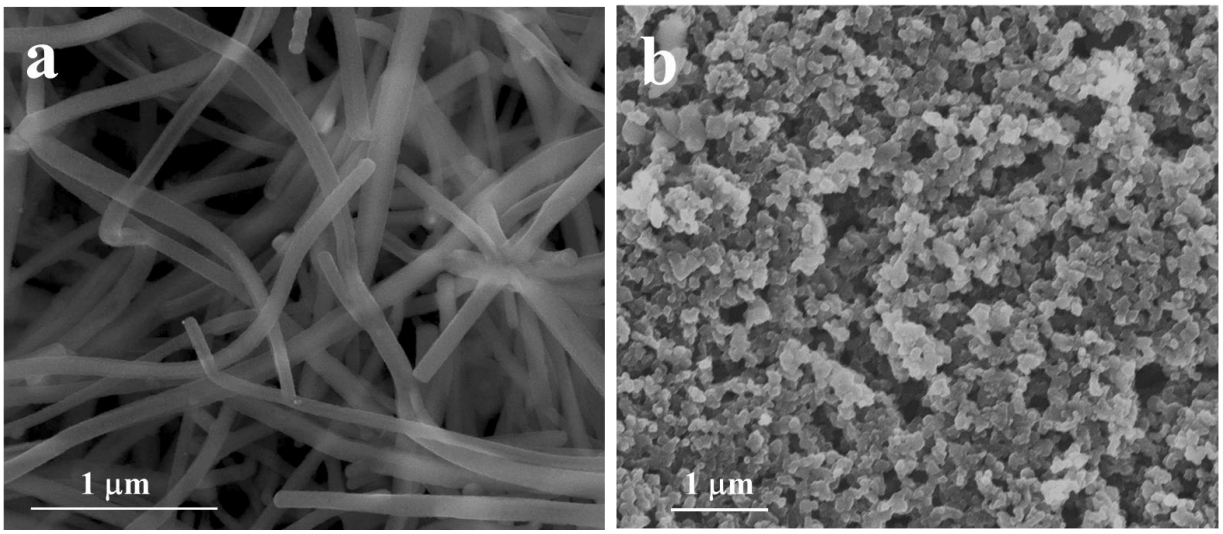

Figure 2. SEM images of a) PPy-NTs surface, b) PPy-NTs/PPO surface

PPy-NTs strongly interact with PPO under the action of glutaraldehyde, which lead to enzyme deposition. The subjects were researched by FT-IR spectra. Figure 3 showed the FTIR spectra of PPy-NTs (Figure 3a) and PPy-NTs/PPO (Figure 3b). The intensities of the bands at 1549 and 1373 cm^{-1} were characteristic of bending vibration peak of N-H (δ_{N-H}) and C-H (δ_{C-H}), respectively. The peaks at 3450 cm^{-1} could be assigned to the stretching vibration of N-H (δ_{N-H}) [32]. After PPy-NTs and PPO crosslinked in the presence of glutaraldehyde, there were changes in the FTIR spectra. The C=O stretching modes of amide band of the protein (PPO) could be observed at 1626 cm^{-1}. The peak at 1550 cm^{-1} in the PPy-NTs/PPO spectrum was about 12 cm^{-1} positively shifted to 1562 cm^{-1}. These results showed that there might be interactions between PPy-NTs and PPO [33]. The release of PPO from the PPy-NTs matrix could be prevented effectively due to molecular interaction between PPO and PPy-NTs. This indicated PPy-NTs were a good immobilization matrix for enzymes.

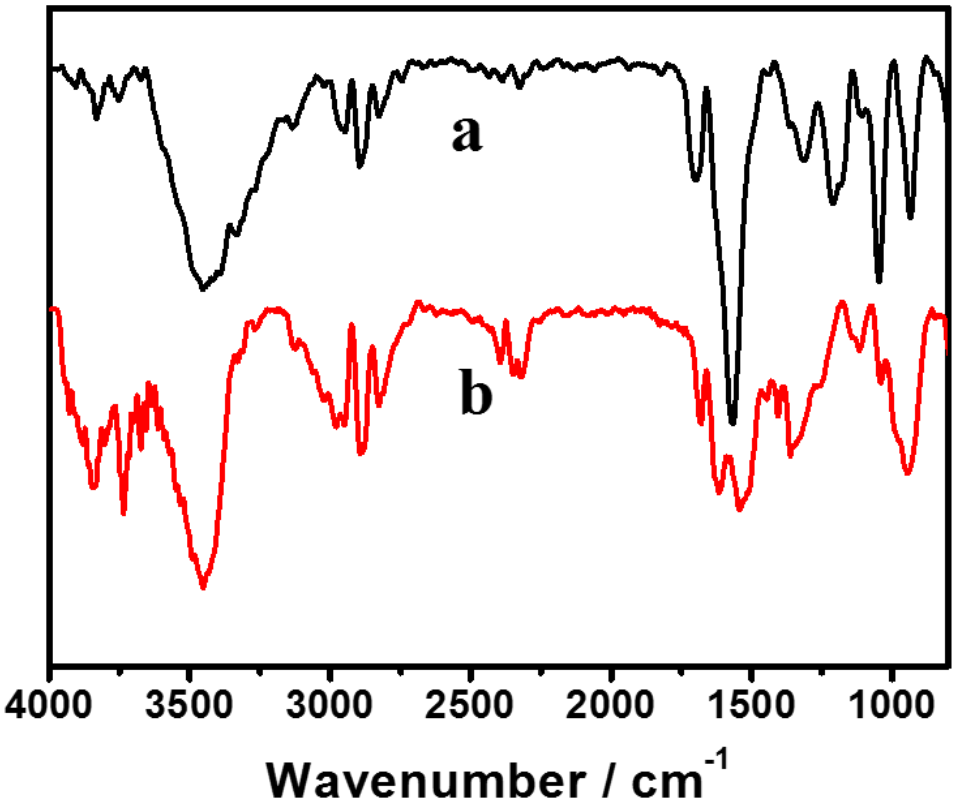

Figure 3. FT-IR spectra of a) PPy-NTs; b) PPy-NTs/PPO.

Interface properties of electrodes were investigated by EIS. The electron-transfer resistance (R_{ct}) was a pivotal parameter at the electrode surface. Figure 4 displayed the Nyquist polt of the PPy-NTs and PPy-NTs/PPO. The impedance of the biofilm electrodes was consisted of an incomplete semicircle and a straight line (Figure 4a). The semicircular impedance was caused by the interface between the composite membrane and the electrode. Figure 4c was the equivalent circuit diagram [34]. R_{ct} of PPy-NTs was very small (~220 Ω, Figure 4a), which was conducive to the transmission of electrons and redox reaction. It was observed that the R_{ct} was significantly increased (~2012 Ω, Figure 4b) when the PPO was fixed on the film. The increase in R_{ct} might have been caused by the obstruction of the macromolecule structure of PPO to the electron-transfer and it also confirmed the successful immobilization of PPO [33].

The cyclic voltammograms obtained from the PPO/PPy-NTs electrode biocomposite film (Figure 4b 2) at a potential between -0.6 and 0.7 V at a scan rate of 25 mV/s in PB solution (pH 6) and 10 μM of catechol was injected. Figure 4b revealed that voltammetric peak at -200 mV in the scanning potential range was obtained. The voltage was necessary for the chronoamperometric determinations, the potential of which was reported as the optimum operating potential for PPO based on biosensors. [35]

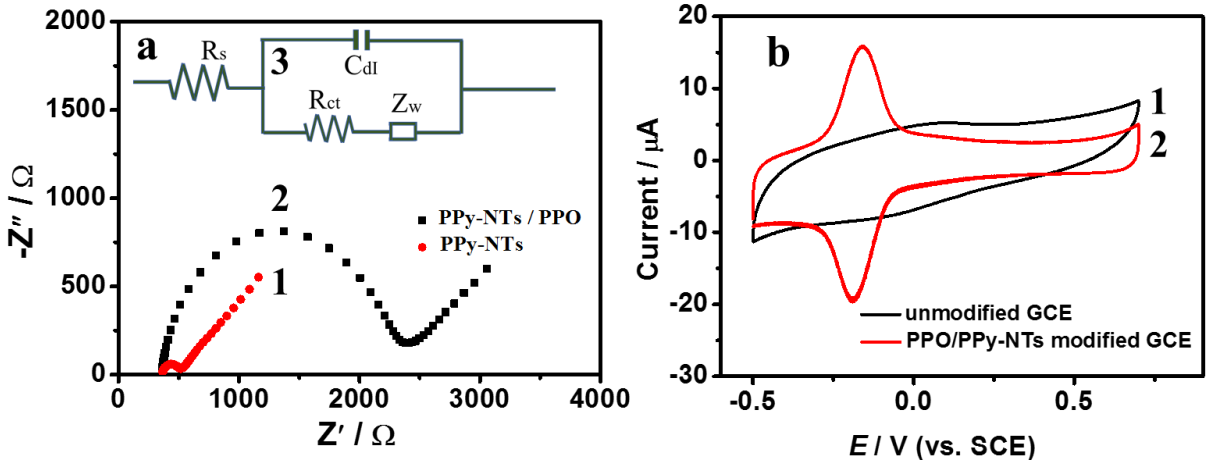

Figure 4. a) Nyquist plots of the EIS for 1) PPy-NTs, 2) PPy-NTs/PPO, 3) Equivalent circuit diagram; b) Cyclic voltammograms of 1) bare electrode, 2) PPO/PPy-NTs electrode in 0.1 M PB solution containing 10 μM of catechol at 25 °C with scan rate of 25 mV/s.

3.2. Optimization of the preparation conditions

An amperometry method has been recognized as an effective and easy way to study the performance of biosensors. Curve was obtained about current (*i*) and time (t).

PPy-NTs/PPO electrodes were tested at -200 mV (vs. SCE) in 0.1 M PB solution with pH 6.0 under vigorous stirring (500 r/min). 10 μL of 1 mM catechol was added every 100 s.

Response time and response current were characteristics of a biosensor which were influenced by the mass ratio in weight of PPy-NTs over PPO ($m_{\text{PPy-NTs}}$: m_{PPO}) and volume of the mixed solution (dropped on the electrode, V_0). Adding too much amount of PPy-NTs obstructed the electron transfer and PPO couldn't react timely, while adding little PPy-NTs could not load enough enzyme and the biofilm often fell off. As matrix material, too little PPy-NTs could not load enough enzyme. In addition, an important factor affecting the response time and current was the film thickness on the surface of electrodes, which was influenced by the volume (V_0) of membrane casting solution while dispensing. Too thick film causing by much casting solution went against the diffusion of catechol, which resulted in the longer response time. Contrary, too thin film causing by too little casting solution could not load enough enzyme and the biofilm often fell off. To obtain a higher response current to phenols, $m_{\text{PPy-NTs}}$: m_{PPO} and volume of the mixed solution (V_0) were optimized through design a series of experiments.

In this paper, 1 : 0.75 : 1 : 1, 1 : 1.25, 1 : 1.5, 1 : 1.75, 1 : 2 of $m_{\text{PPy-NTs}}$: m_{PPO} (V_0 = 7 μL) were evenly distributed on the surface of the GCE. The modified electrodes were placed in a desiccator at room temperature for 30 minutes. The water was mostly evaporated, and the electrodes were partially dried and then placed in a desiccator filled with vapor of saturated glutaraldehyde for 15 ~ 20 min. Prepared electrodes were put into 10 mL of 0.1 M PB solution (pH = 6.0) to test their electrochemical response at -200 mV (vs. SCE) with a temperature of 25 °C. All the response currents were compared with the highest one. The results in Figure 5a showed that $m_{\text{PPy-NTs}}$: m_{PPO} = 1 : 1.5 could achieve a higher electrochemical response.

The volume of mixed solution (V_0) with 3, 5, 7, 9, 11 μL ($m_{PPy\text{-}NTs}$: m_{PPO} = 1 : 1.5) was changed and evenly distributed on the surface of the GCE. Coated electrodes were put in air at room temperature for about 30 min. The most water was evaporated, and the samples were partially dried. Then they were placed in a desiccant containing filled with saturated glutaraldehyde vapor for 15 ~ 20 min. The prepared electrodes were put into 10 mL of 0.1 M PB solution (pH = 6.0), to test their electrochemical response at -200 mV (vs. SCE) with a temperature of 25 °C. All the response currents were compared with the highest one. The results (Figure 5b) showed that a higher electrochemical response was achieved when the volume of mixed solution was 7 μL.

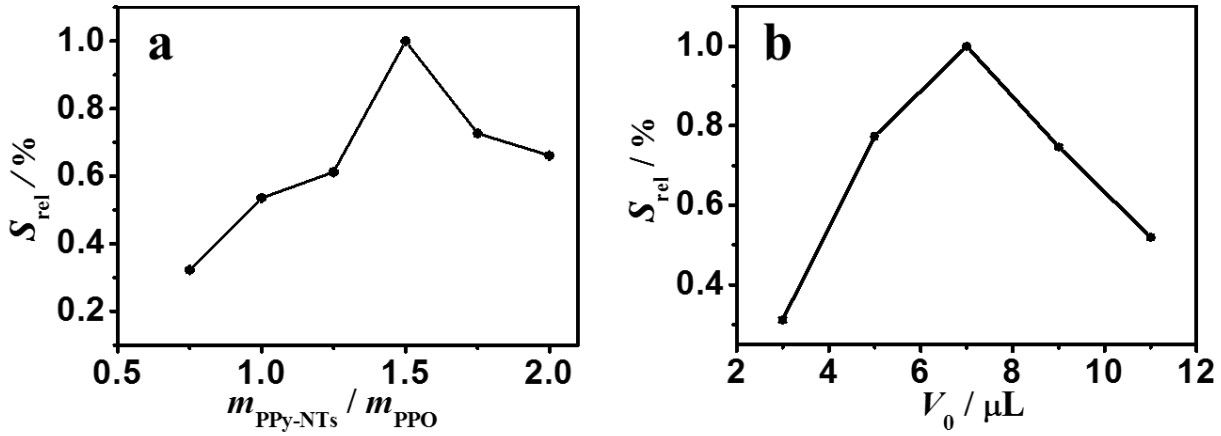

Figure 5. a) The influence of $m_{PPy\text{-}NTs}$: m_{PPO} on the response of PPy-NTs/PPO. b) The influence of V_o (volume of the film) to the response of PPy-NTs/PPO in 10 mL of 0.1 M PB solution of pH 6.0 containing 1mM catechol at 25 °C. Potential: -200 mV, S_{rel} was the percentage of i/i_0 (i_0 was the largest current, i was the current obtained in different condition).

3.3. Optimization of the conditions

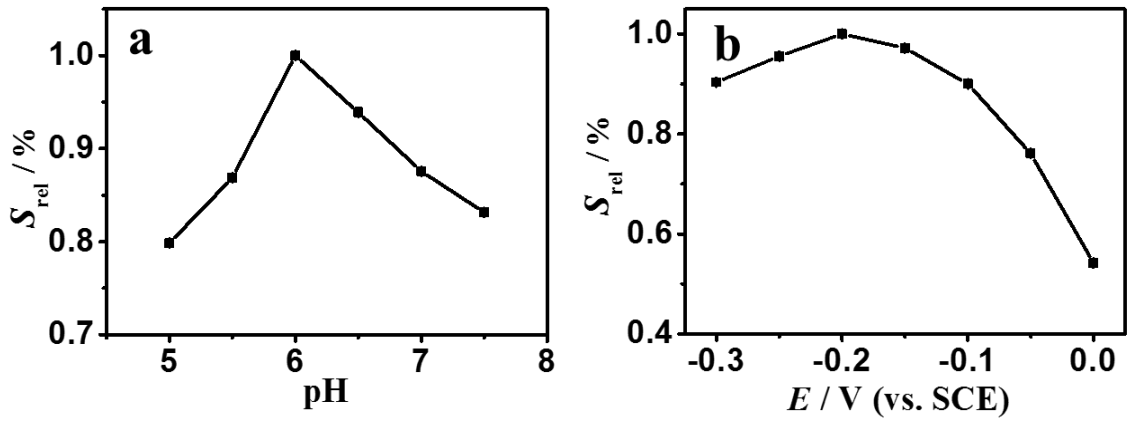

Figure 6. a) The influence of pH on the response of PPy-NTs/PPO. b) The influence of potential to the response of PPy-NTs/PPO in 10 mL of 0.1 M PB solution of pH 6.0 containing 1 mM catechol at 25 °C. Potential: -200 mV, S_{rel} was the percentage of i/i_0 (i_0 was the largest current, i was the current obtained in different condition).

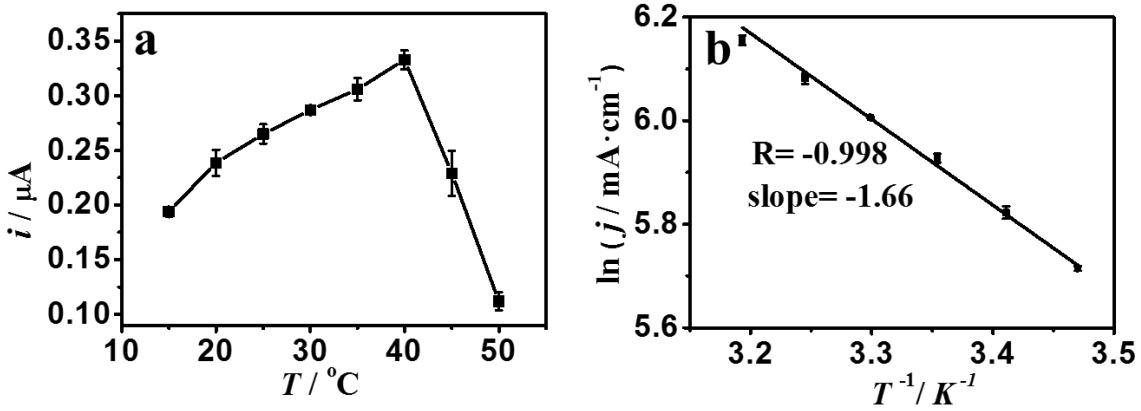

Figure 7. a) The influence of temperature on the response of PPy-NTs/PPO in 10 mL of 0.1 M PB solution of pH 6.0 containing 1 mM catechol. Potential: -200 mV. b) The linear calibration curve of ln *j* vs. T^{-1} (*j* was the current density).

To find the better test conditions, electrodes were prepared as noted above ($m_{PPy\text{-}NTs}$: m_{PPO} = 1 : 1.5, V_0 = 7 μL) and then electrochemical response at different pH, potential, and temperature was tested. All the response currents were compared with the highest one. When pH was 6.0 (Figure 6a), potential was -0.2 V (Figure 6b), and temperature was 40 °C (Figure 7a), the response current reached maximum value. But Gouzi [36] found that stability of PPO decreased at the temperature ranging from 30 °C to 35 °C. In this paper, 25 °C was chosen to test the characteristics of electrodes in following experiments.

For rate constant (*k*) was proportional to electrochemical response, the dependence of current response on temperature ranging from 20 °C to 40 °C could be recognized as an electrochemical formula of the Arrhenius relationship: ln*k* = -E_a/RT + lnA, ln*k* could replaced with ln*i*. The apparent activation energy (E_a) of the reaction could be obtained by the slope of the line and the E_a was 13.8 kJ/mol.

3.4 Electrocatalytic activity of PPy-NTs/PPO electrode on catechol

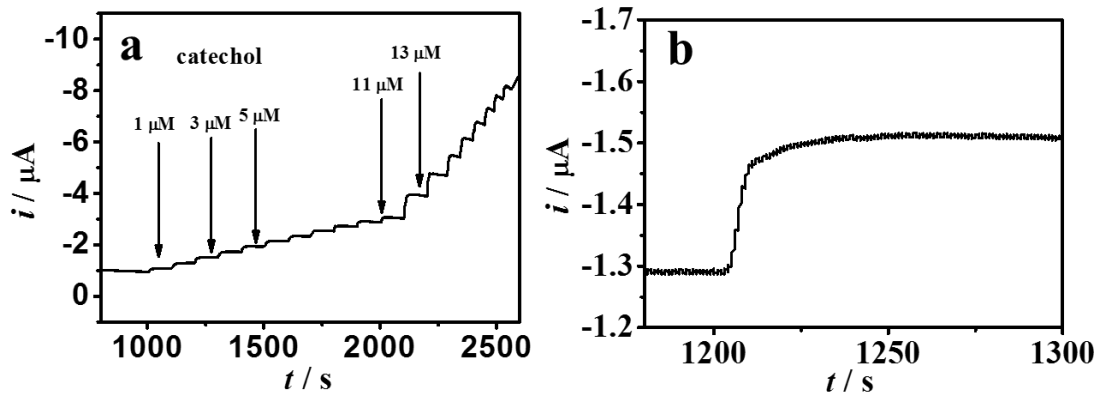

Figure 8. a) Curve of steady-state responses after injecting catechol into 10 mL of 0.1 M PB solution under fast stirring (500 r/min). Potential: -200 mV. b) Partial magnification of Figure 8

An amperometry method was used study the performance of biosensors. PPy-NTs/PPO electrodes were tested at potential of -0.2 V, in 0.1 M PB solution of pH 6.0 (Figure 8a). Response time was only 12 s (Figure 8b).

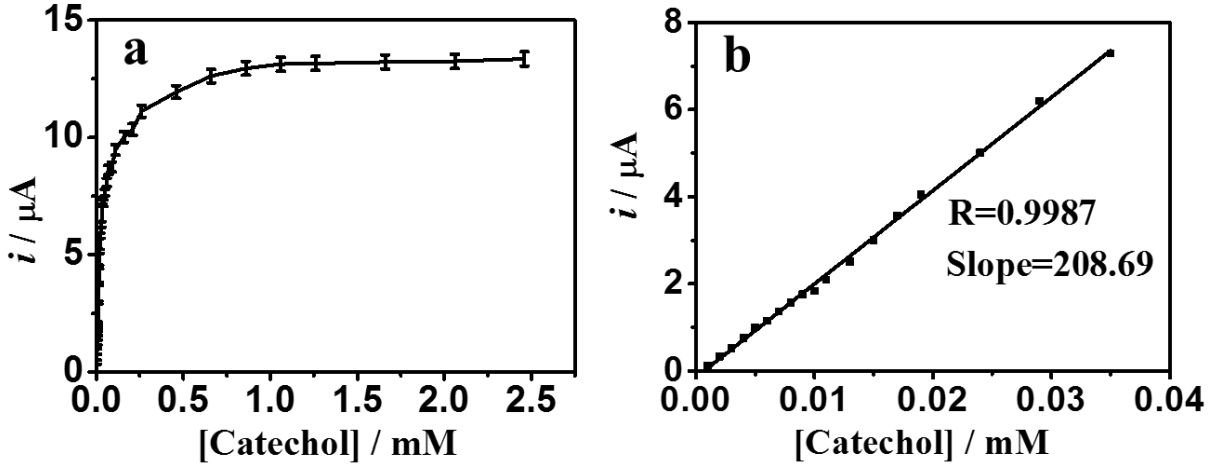

Figure 9. a) Steady-state current-time response of the PPy-NTs/PPO for increasing catechol concentrations in 10^{-8} M steps. b) calibration curve.

Table 1. Comparison of catechol detection using various modified electrodes

Electrode	Response time (s)	Linear range (μM)	Detection limit (μM)	Sensitivity ($mA \cdot M^{-1} \cdot cm^{-2}$)	References
PPy-NTs modified GCE	12	0.1 ~ 35	1.22×10^{-3}	2981	This work
CPE/PPO/β-CDEP/Ir-BMI·PF_6	--	0.13 ~ 2	0.079	--	[45]
Fe^{2+}-PPy modified ITO	50	6.7–72.6	2.03	2100	[37]
PPy/MWCNT modified GCE	10	3 ~ 50	0.671	--	[41]
Fe^{2+}/PPy modified ITO	80	4.5 ~ 107	0.7	330	[38]
Polyrrole-HRP modified GCE	< 20	9.3 ~ 83.7	8.4	--	[42]
HRP–ZrO_2 – PEI modified SPE	--	0.43 ~ 4.98	0.06	--	[44]
Au/PANI-cMWCNT/Basillus sp./GA	< 2	5 ~ 630	2.9	--	[47]
PPO/nano-$CaCO_3$ modified GCE	12	0.006 ~ 20	4.4×10^{-2}	474	[39]
ZnO-sol-gel modified GCE	15	0.1 ~ 50	5×10^{-2}	166	[48]
Ty/PO_4-PPy/Pt	--	4 ~ 80	0.57	106.9	[49]
Pt-Au-OSi@CS	--	0.06 ~ 90.98	0.02	1714.2	[40]
MIS/MWCNT-VTMS modified GCE	--	0.08 ~ 100	0.032	--	[50]

The analytical performances of the proposed biosensor were compared with those based on other immobilization matrices, as summarized in Table 1. The sensitivity was calculated to be 2981 $mA \cdot M^{-1} \cdot cm^{-2}$ via the slope of Figure 9b. It proved that the relative high sensitivity (2981 $mA \cdot M^{-1} \cdot cm^{-2}$) was higher than that PPO electrodes based on Fe^{2+}-PPy/ITO (2100 $mA \cdot M^{-1} \cdot cm^{-2}$) [37], Fe^{2+}/PPy modified ITO (330 $mA \cdot M^{-1} \cdot cm^{-2}$) [38], PPO/nano-$CaCO_3$ (474 $mA \cdot M^{-1} \cdot cm^{-2}$) [39], Pt-Au-OSi@CS

(1714.2 $mA \cdot M^{-1} \cdot cm^{-2}$) [40]. The detection limit of the concentration was 1.22 nM with S/N = 3, where S was a signal of current, and N was a noise and equal to 1×10^{-9} A. The detection limit was lower than electrodes based Fe^{2+}/PPy/ITO (0.7 μM) [38], PPy/MWCNT (0.671 μM) [41] and Polyrrole-HRP (8.4 μM) [42]. As displayed in Figure 9b, the linear range was 0.1 ~ 35 μM, and it was a relatively wide range compared to PPO electrodes based on PPy [43], HRP–ZrO_2–PEI (0.43 ~ 4.98 μM) [44], CPE + PPO/β-CDEP +Ir-BMI·PF_6 (0.13 ~ 2 μM) [45] and PPO/nano-$CaCO_3$ (0.006 ~ 20 μM) [39]. The Michaelis-Menten constant (K_M) evaluated from the Figure 9a was 0.12 mM, which was the concentration of catechol, when the i was half to the i_0 (the curve was near to a certain value with the increase of catechol). This value was lower than free enzyme (0.28 mM) [46]. Smaller K_M value demonstrated that phenol biosensor based on PPy-NTs possessed high enzymatic activity and high affinity to catechol was performed.

3.5 Electrocatalytic activity of PPy-NTs/PPO electrode on other phenols

Catechol, *p*-methylthiophenol, phenol, *p*-chlorophenol were also measured by the PPy-NTs/PPO electrode. Fast response (~12 s) were obtained for the four phenolic compounds. The response parameter of designed biosensor, including sensitivity, detection limit, linear range and the K_M value of the four compounds were listed in Table 1. The sensitivity decreased as following: *p*-chlorophenol > *p*-methylthiophenol > catechol > phenol. The unequal value in sensitivity between different compounds might due to the hindrance of molecular steric and hydrophobic characteristics of the matrix. The K_M value gave information of the enzyme-substrate kinetics for the PPy-NTs/PPO electrode. They were 0.12, 0.01, 0.08 and 0.006 mM for catechol, *p*-methylphenol, phenol, *p*-chlorophenol, respectively (Table 2). The linear range were 0.1 ~ 35, 0.1 ~ 50, 0.5 ~ 40, 0.1 ~ 5 μM for catechol, *p*-methylphenol, phenol, *p*-chlorophenol, respectively that read from calibration curves (calibration curves for *p*-methylphenol, phenol, *p*-chlorophenol were not shown).

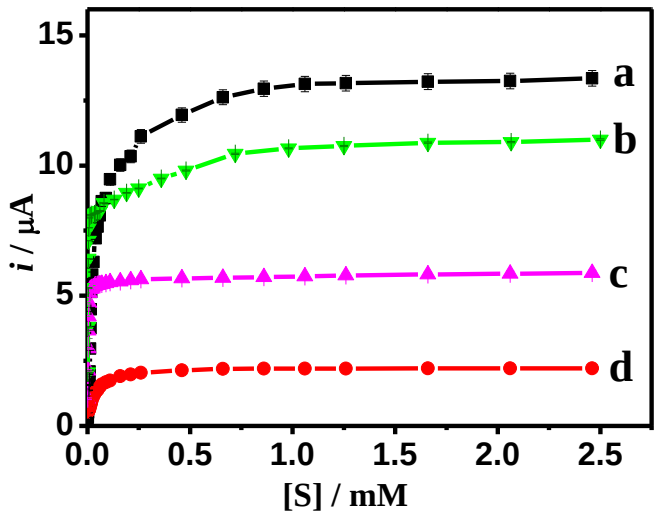

Figure 10. Steady-state current-time response of the PPy-NTs/PPO for increasing concentrations in 10^{-8} M steps. a) catechol, b) *p*-chlorophenol, c) *p*-methylthiophenol, and d) phenol into 10 mL of 0.1 M PB solution under fast stirring (500 r/min).

Table 2. The characteristics of the PPy-NTs/PPO biosensor to phenolic compounds

phenols	catechol	*p*-methylphenol	phenol	*p*-chlorophenol
Sensitivity ($mA \cdot M^{-1} \cdot cm^{-2}$)	2981	11392	330	20971
K_m (mmol/L)	0.12	0.01	0.08	0.006
Detection limit (nm)	1.22	0.23	14.4	4.18
Linear range (μM)	0.1 ~ 35	0.1 ~ 50	0.5 ~ 40	0.1 ~ 5

3.6. Real sample determination

To evaluate the validity of the developed sensor for catechol determination, we extended our study to the detection of low concentration of real sample. Drinking water, river water and milk were assayed in order to demonstrate the practical usage of the biosensor. Milk was first centrifuged to remove insoluble residue, drinking water and river water were used without any treatment. Three samples were evenly diluted by 100 times with PB solution and then they were used as the sample solution. 1 mM of catechol was prepared preparatorily which was used as the standard stock solution. This solution was then diluted quantitatively with diluent to obtain a series of standard solutions.Three samples solution were first spiked with dierent concentrations of catechol, 5, 10, 15 μM respectively, and then analyzed by our proposed electrochemical assay and the results were compared with the value detected by HPLC method. As shown in Table 3, the results obtained from the PPO/PPy-NTs sensor were in good agreement with those from the HPLC, and the recovery values were in the range of 96–104%, which suggested that the PPO/PPy-NTs sensor has a promising application in determination.

Table 3. Determination of catechol in various samples (n = 5)

Sample	Original (μM)	Added (μM)	Found at PPO/PPy-NTs (μM)	Found in HPLC (μM)	RSD (%)	Recovery (%)
Drinking	0	5	4.97	4.95	4.21	98.8
water	0	10	9.96	4.97	2.34	99.6
	0	15	15.03	14.98	3.81	100.02
River water	0	5	4.93	4.96	2.12	98.6
	0	10	10.11	10.08	3.22	1.011
	0	15	15.51	15.48	4.32	103.4
Milk	0	5	4.88	4.91	3.31	97.6
	0	10	9.64	9.89	4.11	96.4
	0	15	15.36	15.27	5.22	102.4

3.7 Long-term stability and reproducibility

The repeatability and long-term stability of biosensors were critical for the practical application. Six identical PPy-NTs/PPO electrodes were prepared under optimized conditions at the

same time. The experimental results showed that the relative standard deviation of response currents was 3.53% (10 μL 1 mM catechol was dripped into 10 mL of 0.1 M PB solution with pH 6.0; potential: -200 mV). The electrodes were stored in a 4 °C refrigerator and the response currents for 10 μM catechol were measured at an interval time. The response current (i) of the biofilm electrode remained up to 88% of the initial current (i_0) (0.26 μA) after 30 days (Figure 11). So PPy-NTs/PPO biofilm electrode had a good reproducibility and long-term stability.

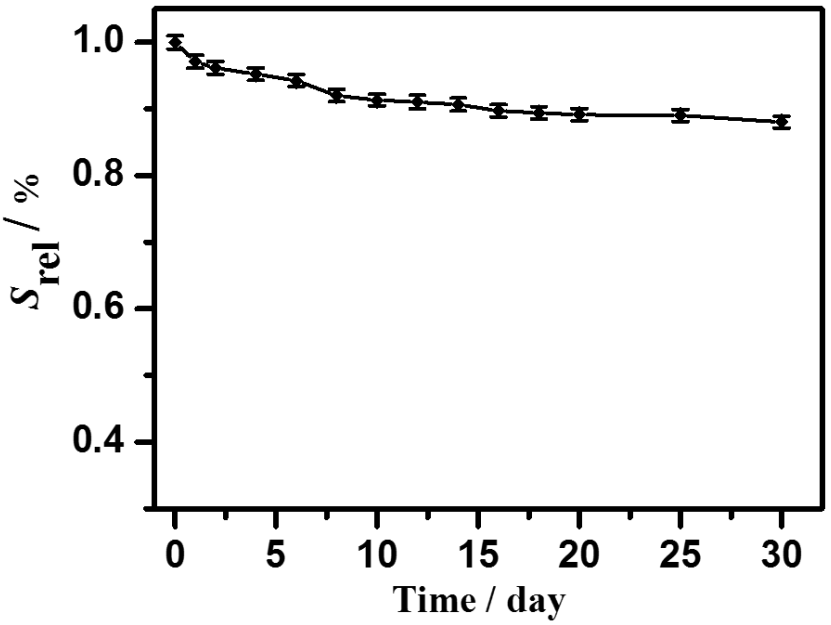

Figure 11. Long-term stability of PPy-NTs/PPO biosensor, S_{rel} was the percentage of i/i_0 (i_0 was the largest current, i was the current obtained in different conditions).

4. CONCLUSION

A facile method has been developed to construct PPO biosensor. PPy-NTs and PPO were simply mixed mechanically and dripped onto the electrodes. The resulted electrodes were put in a desiccant filled with saturated glutaraldehyde vapor for 15 ~ 20 min. This immobilization method provided an efficient entrapment of enzyme within the polymer film and reflects a long-term stability. The sensitivity was 2981 $mA \cdot M^{-1} \cdot cm^{-2}$. The limit of detection was 1.12 nM. The wider linear response range was 0.1 ~ 35 μM. The Michaelis constant (K_M) was 0.12 mM. This low cost and simple method of prepared biosensor was perspective for the development of sensor devices.

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