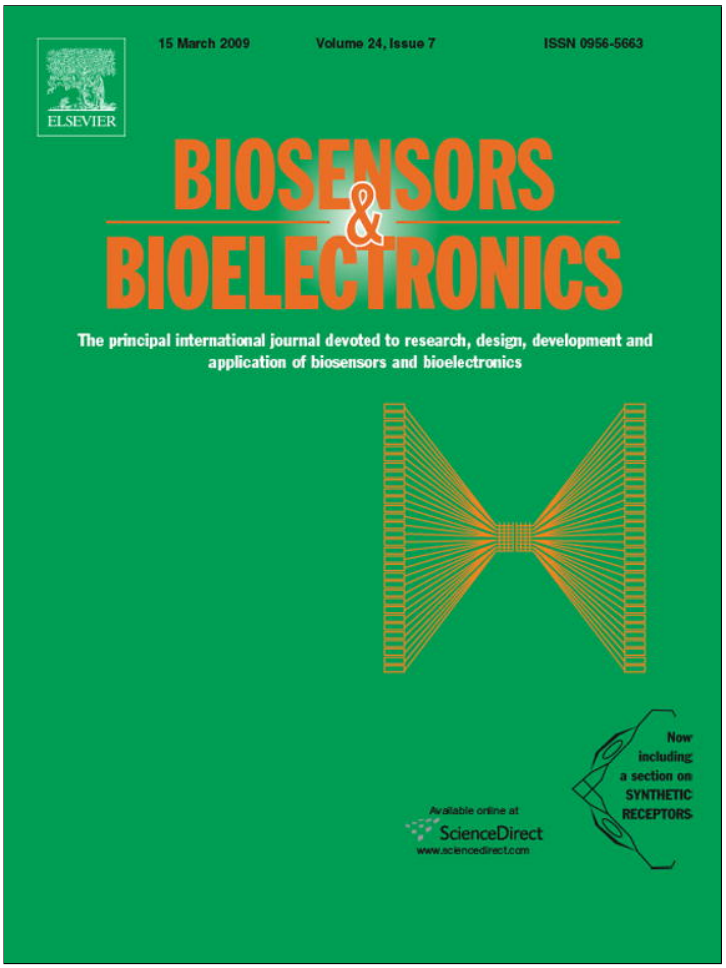

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Highly sensitive amperometric biosensors for phenols based on polyaniline–ionic liquid–carbon nanofiber composite

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ABSTRACT

A novel polyaniline–ionic liquid–carbon nanofiber (PANI–IL–CNF) composite was greenly prepared by *in situ* one-step electropolymerization of aniline in the presence of IL and CNF for fabrication of amperometric biosensors. The scanning electron micrographs confirmed that the PANI uniformly grew along with the structure of CNF and the PANI–IL–CNF composite film showed a fibrillar morphology with the diameter of around 95 nm. A phenol biosensor was constructed by immobilizing tyrosinase on the surface of the composite modified glassy carbon electrode via the cross-linking step with glutaraldehyde. The biosensor exhibited a wide linear response to catechol ranging from 4.0×10^{-10} to 2.1×10^{-6} M with a high sensitivity of 296 ± 4 A M^{-1} cm^{-2}, a limit of detection down to 0.1 nM at the signal to noise ratio of 3 and applied potential of −0.05 V. According to the Arrhenius equation, the activation energy for enzymatic reaction was calculated to be 38.8 kJ mol^{-1} using catechol as the substrate. The apparent Michaelis–Menten constants of the enzyme electrode were estimated to be 1.44, 1.33, 1.16, 0.65 μM for catechol, *p*-cresol, phenol, *m*-cresol, respectively. The functionalization of CNF with PANI in IL provided good biocompatible platform for biosensing and biocatalysis.

1. Introduction

Phenolic compounds are highly toxic. They are widely used in wood preservatives, textiles, herbicides and pesticides, and released into the ground and surface water. Therefore, the identification and quantification of these compounds are of great importance in environment monitoring. Electrochemical techniques, especially amperometric biosensors, have been considered as the best candidates for the *in situ* detection of phenolic compounds due to the high sensitivity, simplicity, and easy to miniaturization (Brown et al., 1994; Wang et al., 2002; Dai et al., 2005; Hervás Pérez et al., 2006; Han et al., 2007; Njagi and Andreescu, 2007; Wang et al., 2008). Many amperometric biosensors for phenols have been presented (Sánchez-Ferrer et al., 1995; Yu et al., 2003; Serra et al., 2005; Han et al., 2007; Lei et al., 2007; Kochana et al., 2008). These biosensors are commonly based on monitoring the reduction signal of their enzymatic oxidation products, *o*-quinones, by molecular oxygen in presence of tyrosinase Thus, the effective immobilization of enzyme on the electrode surface is a crucial step in the fabrication of these biosensors. Various materials such as inorganic mesoporous materials (Dai et al., 2005), sol–gels (Wang et al., 2000; Yu et al., 2003), clays (Shan et al., 2003), nanomaterials (Campuzano et al., 2003; Njagi and Andreescu, 2007), polymers (Xue and Shen, 2002; Mailley et al., 2003; Hervás Pérez et al., 2006), and magnetic nanoparticles (Wang et al., 2008) have been exploited to immobilize tyrosinase on electrode surface. However, the preparation of some materials is relatively complicated. The bad biocompatibity and low capacity for immobization of tyrosinase also result in inefficient performance of some biosensors for the detection of phenolic compounds. Therefore, searching for green and biocompatible supports with high capacity to immobilize tyrosinase is of considerable interest for the detection of phenols.

Polyaniline (PANI) as a conducting polymer has showed many practical applications such as electrocatalysis, electrochromic devices and biosensors due to its high conductivity, good redox reversibility. In order to further improve its conductivity for preparation of amperometric biosensors, a PANI–carbon nanotube (PANI–CNT) composite has been synthesized by a chemical polymerization to form core/shell structure via effective site-selective interactions between the quinoid ring of the PANI and CNT. This composite shows more active sites and larger accessible area for faradic reaction than pure PANI (Cochet et al., 2001; Wei et al., 2003; Luo et al., 2006; Ma et al., 2006; Wu and Lin, 2006; Zhang et al., 2006; Yan et al., 2007; Zhu et al., 2008). The presence of CNT can facilitate the charge transfer, and thus improves the

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sensitivity of resulting biosensors. This work prepared a novel PANI-nanocomposite, polyaniline–ionic liquid–carbon nanofiber (PANI–IL–CNF) composite, by *in situ* one-step electropolymerization of aniline in the presence of IL and CNF.

Compared with CNT, CNF has intrinsic advantages such as lower-cost mass production (Cui et al., 2004), better mechanical stability (Jang et al., 2005), easier surface functionalization, and more edge sites on the outer wall (Kim and Lee, 2004). The acid-treated CNF as a biocompatible electron conductor has been used to construct an amperometric biosensor for dihydronicotinamide adenine dinucleotide with low oxidation overpotential (Wu et al., 2007) and a sensitive impedance sensor for cells (Hao et al., 2007). Due to the synergistic combination of unique characteristics of PANI and CNF, PANI–CNF composite has also been prepared for fabrication of electronic supercapacitor by vapor deposition polymerization method (Jang et al., 2005). This work further facilitated the electronic properties by entrapping IL in the composite for preparation of sensitive amperometric biosensors.

Ionic liquids, as green solvents, have been applied in synthesis, catalysis and liquid–liquid extractions (Gutowski et al., 2003) due to negligible vapor pressure, low toxicity, outstanding chemical and thermal stabilities. More recently, ionic liquids have gained increasing interest in the area of electrochemistry for their wide potential window and extremely high ionic conductivity as supporting electrolyte (Xiong et al., 2007). Furthermore, they have been incorporated to conventional materials, such as chitosan (Lu et al., 2006), carbon nanotubes (Liu et al., 2007), polymer (Mu, 2007; Kim et al., 2008), and Nafion (Chen et al., 2007) for improving conductivity and promoting the electron transfer (Ding et al., 2007; Xi et al., 2008). The newly designed PANI–IL–CNF composite showed a fibrillar morphology, which was beneficial to the loading of enzyme, and thus improved the capacity for immobization of enzyme. Using tyrosinase (Tyr) as a model, the enzyme could be immobilized on the surface of this nanocomposite via the cross-linking step with glutaraldehyde. The combination of the advantages of PANI, IL and CNF with the beneficial morphology for enzyme loading made the proposed biosensors for various phenolic compounds have good performance, such as high sensitivity, wide linear range and excellent stability. The *in situ* one-step electropolymerization provided a green method for preparation of amperometric biosensors.

2. Experimental

2.1. Materials

CNF was a gift from WPI (Sarasota, USA). Tyrosinase (EC 1.14.18.1, 5370 U mg^{-1} from mushroom) was purchased from Sigma. Ionic liquid, 1-ethyl-3-methylimidazolium ethyl sulfate (EMIES), was prepared by alkylation of 1-methylimidazole with diethyl sulfate in toluene as an inert solvent according to the procedures reported by Holbrey et al. (2002). Aniline was distilled under reduced pressure before use. Other reagents were of analytical reagent grade and were used without further purification. All aqueous solutions were prepared with twice-distilled water. The buffer for assay was 0.1 M phosphate buffer saline (PBS) prepared by mixing stock-standard solution of K_2HPO_4 and KH_2PO_4. Phenolic solutions in 0.1 M PBS were prepared daily.

2.2. Apparatus

Electrochemical measurements were performed on a CHI 812B electrochemical analyzer (Co., CHI, USA) with a conventional three-electrode system. Glassy carbon electrode (GCE) (diameter 3 mm) was used as working electrode, saturated calomel electrode (SCE) as reference electrode, and a Pt foil (4.0 mm × 4.0 mm) or a platinum wire was used as auxiliary electrode for the synthesis of the composite films or assay of phenolic compounds. Scanning electron microscopic (SEM) images of different films formed on indiumetin oxide (ITO) slides were obtained on a Hitachi S-4800 scanning electron microscope (Japan). The pH values of the solutions were determined using a Sartorius PB-10 pH-meter (Germany). All measurements were carried out in a thermostated cell at 25 °C.

2.3. Preparation of Tyr/PANI–IL–CNF/GCE

40 mg CNF was dispersed in 60 ml 30% HNO_3, and the resultant mixture was refluxed for 24 h at 140 °C (Wu et al., 2007). The resulting suspension was centrifuged and the precipitate was washed thoroughly with water until the pH value was about 7.0. The black solid was collected and dried in a vacuum at 80 °C. 20 mg of the treated-acid CNF and 1.2 ml EMIES were added successively into 2.8 ml aqueous solution containing 0.2 M aniline and 2.0 M H_2SO_4 followed with sonication at room temperature for 1 h to obtain a well-dispersed CNF solution. Tyrosinase solution was prepared by dissolving 4 mg of tyrosinase in 1.0 ml of 0.1 M pH 7.0 PBS.

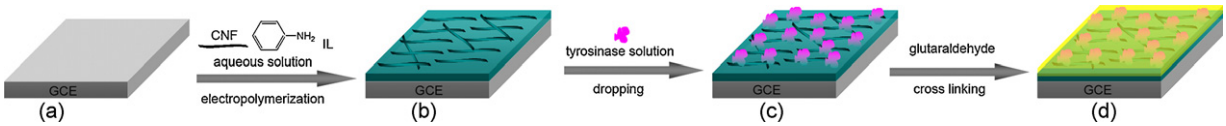
The preparation of the Try/PANI–IL–CNF modified electrode is schematically presented in Scheme 1. The GCE was firstly polished successively with 0.3- and 0.05-μm alumina slurry (Beuhler) followed by rinsing thoroughly with doubly distilled water and successive sonication in 1:1 nitric acid, acetone and doubly distilled water to obtain a pretreated GCE (Scheme 1a). The GCE was then used for preparation of the PANI–IL–CNF composite film by cyclic scanning between −0.2 and 0.9 V in 2.0 M H_2SO_4 solution containing aniline, CNF and EMIES at 60 mV s^{-1} for an optimum cyclic number. After electropolymerization, the green PANI–IL–CNF composite film could be observed on GCE surface (Scheme 1b). Next, a 10.0 μl aliquot of the tyrosinase solution was dropped on the nanocomposite film modified GCE (Scheme 1c) and dried in a silica gel desiccator for 30 min. The modified electrode was finally hanged in a glutaraldehyde vapor-saturated vessel for the cross-link of tyrosinase to the composite film at room temperature for 15 min to obtain a Tyr/PANI–IL–CNF-based biosensor (Scheme 1d).

The PANI, PANI–IL and PANI–CNF films and the biosensors based on Try/PANI, Try/PANI–IL and Try/PANI–CNF were prepared with the same electrochemical conditions and method as those for preparation of PANI–IL–CNF nanocomposite film and Tyr/PANI–IL–CNF-based biosensor. When not in use, the biosensor was stored in 0.1 M pH 7.0 PBS at 4 °C.

3. Results and discussion

3.1. Characterization of PANI, PANI–IL, PANI–IL–CNF and Try/PANI–IL–CNF films

Fig. 1 shows the typical SEM images of PANI (a), PANI–IL (b), PANI–IL–CNF (c) and Tyr/PANI–IL–CNF (d) films *in situ* formed by one-step electropolymerization. The fiber-like PANI nanostructures tend to link into network rather than bundles (Fig. 1a). In comparison with the PANI film, the mushy PANI–IL film (Fig. 1b) looks more uniform and is strongly adhered to the surface of ITO slide. The distinguishable PANI–IL nanofiber shows smaller diameter than PANI nanofiber, leading to larger surface area, which is favorable to mass transport during the redox process (Mu, 2007). The mushy configuration of PANI–IL reveals that a part of IL is adsorbed on the outer surface of the PANI due to the high viscosity of EMIES. The PANI–IL–CNF composite film (Fig. 1c) shows the fibrillar morphology with the diameter of around 95 nm, which is larger than 30–50 nm of the pristine CNF (Wu et al., 2007). This



Scheme 1. Preparation of Try/PANI–IL–CNF-based biosensor.

result indicates that during polymerization carbon nanofiber acts as molecular template, thereby guiding the growth of PANI on the surface of the CNF (Wei et al., 2003). As seen on the SEM image of the Tyr/PANI–IL–CNF composite film (Fig. 1d), the tyrosinase particles are distributed regularly throughout the composite film. The aggregates of the enzyme molecules exhibited an island-like image, which may facilitate the specific reaction between the substrate and the enzyme, resulting in a good amperometric response of the enzyme electrode (Li et al., 2006; Zhou and Zhi, 2006; Wang et al., 2008).

3.2. Optimization conditions for biosensor fabrication

Fig. 2 displays the amperometric responses of different modified electrodes to 0.3 μM catechol. The amperometric response of Try/PANI–IL modified electrode (Fig. 2b) is 5 times higher than that of Try/PANI modified electrode (Fig. 2a), which can be contributed to the promoted electron transfer (Ding et al., 2007; Mu, 2007; Xi et al., 2008) due to the presence of IL. Owing to the unique characteristics of CNF (Jang et al., 2005), the Try/PANI–CNF modified electrode (Fig. 2c) also shows larger amperometric response than that of Try/PANI modified electrode. Significantly, the Try/PANI–IL–CNF modified electrode (Fig. 2d) exhibited 12 times higher amperometric response than that of Try/PANI modified electrode, which benefits from the combined enhancement effect of three components.

The thickness of the formed film or the cyclic number for the electropolymerization of PANI–IL–CNF composite was one important parameter affecting the biosensor performance. As shown in Fig. 3a, the amperometric response of resulting biosensor to 0.3 μM catechol increased with the increasing cyclic number and trended to a stable response after the ninth cyclic number. The increase could be attributed to the formation of uniform film for loading of enzyme. Therefore, nine cyclic scan was selected to *in situ* prepare the PANI–IL–CNF composite for fabrication of biosensor.

3.3. Optimization of detection variables

The effect of pH on the amperometric response of the biosensor to 0.3 μM catechol was shown in Fig. 3b. The current increased slightly as the pH changed from 5.0 to 7.0, following with a largely decrease in the pH range of 7.0–8.0. The maximum response was obtained at pH 7.0, which is consistent with other results of tyrosinase-based biosensors (Wang et al., 2000; Yu et al., 2003) and

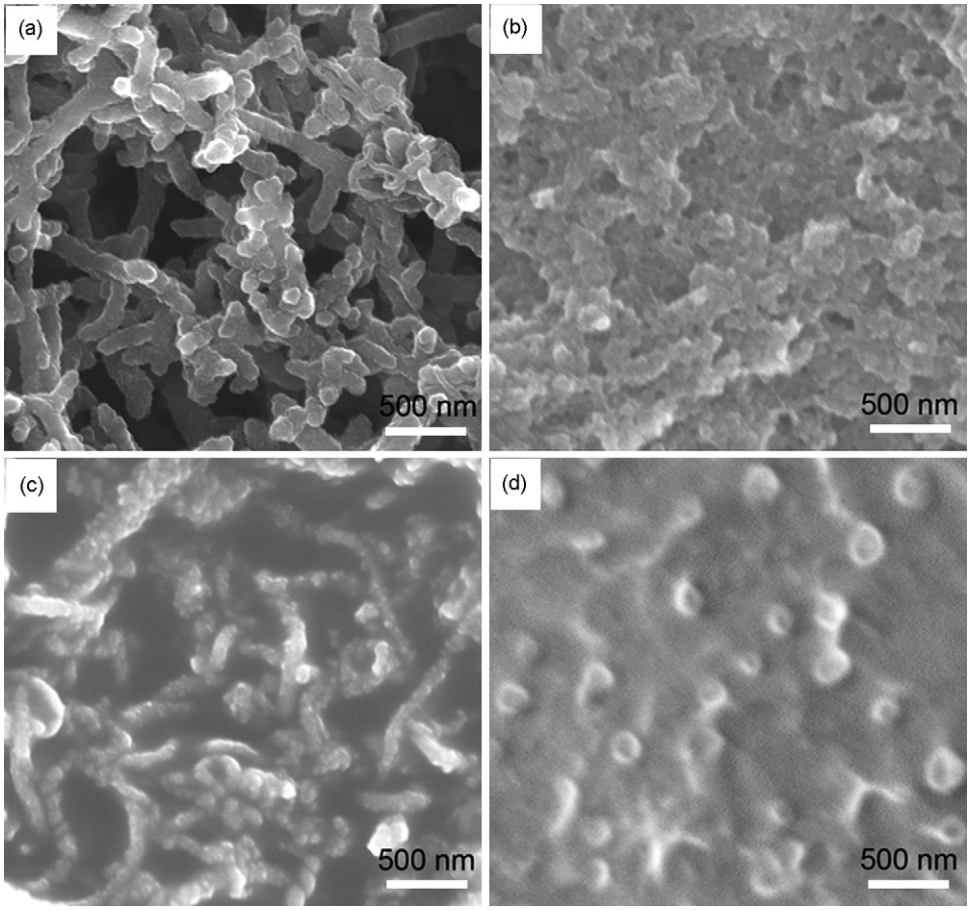


Fig. 1. SEM images of PANI (a), PANI–IL (b), PANI–IL–CNF (c) and Try/PANI–IL–CNF (d) films.

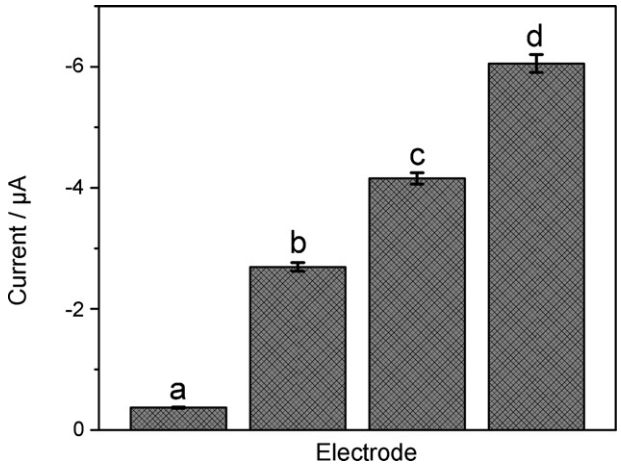


Fig. 2. The amperometric responses of Try/PANI (a), Try/PANI–IL (b), Try/PANI–CNF (c) and Try/PANI–IL–CNF (d) modified electrodes to 0.3 μM catechol in 0.1 M pH 7.0 PBS at −0.05 V.

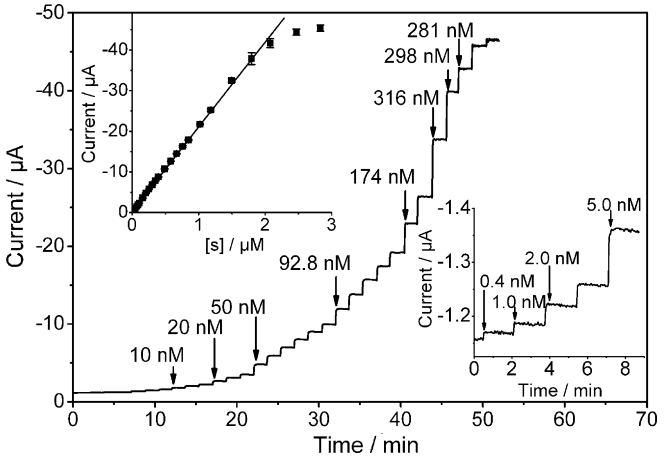


Fig. 4. Typical current–time response curve of the biosensor upon successive additions of catechol with different concentrations. Upper inset: linear calibration curve. Lower inset: amplified response curve. Applied potential: −0.05 V.

the optimum pH range of 5.0–8.0 (Barman, 1985) reported for the free tyrosinase. This indicated that the immobilization procedure did not alter the optimum pH of tyrosinase. Therefore, pH 7.0 PBS was used as the electrolyte in further work.

Fig. 3c displays the dependence of applied potential on the amperometric response of the biosensor to 0.3 μM catechol. The maximum response was observed at −0.05 V, and more negative potentials led to a decrease of the response. The decrease of the response at more negative potentials was due to the decrease in the tyrosinase enzymatic rate resulting from the oxygen depletion in the vicinity of the tyrosinase immobilized at the electrode surface, which was verified by the increase of the background current with the negative shifting of the potential in absence of substrate. Therefore, −0.05 V was an optimal applied potential.

The effect of temperature on the amperometric response of the biosensor to 0.3 μM catechol was shown in Fig. 3d. With an increasing temperature from 8 to 35 °C the response increased, afterwards the response decreased as the temperature further increased. The sharp decrease of the response was due to the denaturation of tyrosinase at high temperatures. In the temperature range of 8–35 °C the plot of ln i versus T^{-1} showed a straight line (r = 0.97). According to the Arrhenius equation, the apparent

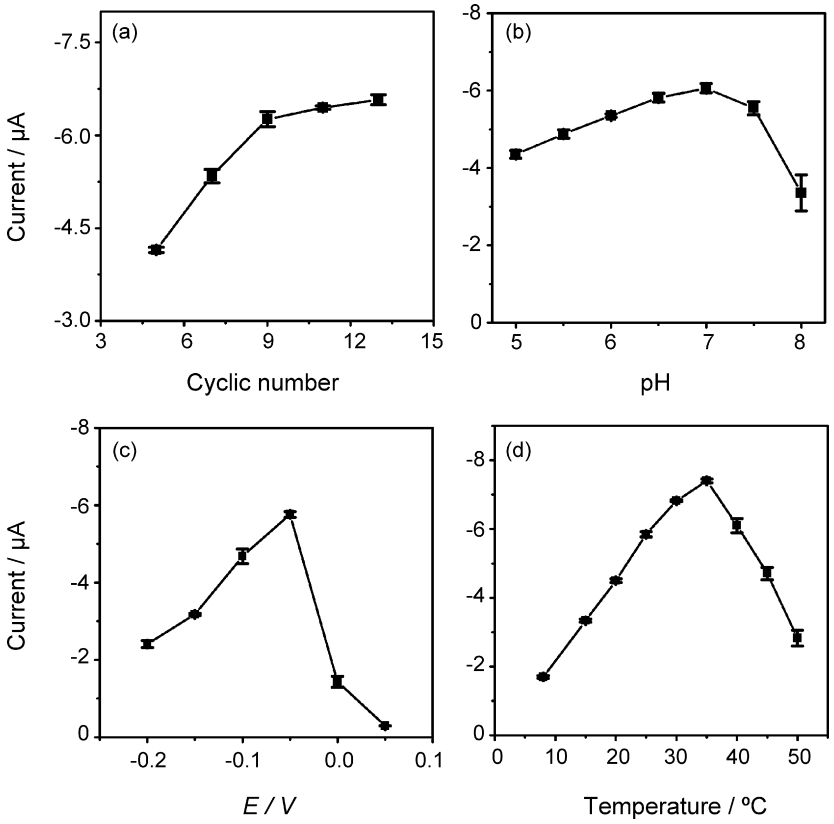


Fig. 3. Effects of the cyclic number for electropolymerization (a), pH (b), applied potential (c) and temperature (d) on amperometric response of resulting Try/PANI–IL–CNF film modified electrode to 0.3 μM catechol in 0.1 M pH 7.0 PBS. When one parameter changed other parameters were at their optimal values.

Table 1
Response characteristics of the Tyr/PANI–IL–CNF modified electrode to various phenolic compounds (average values ± S.D. were obtained with three biosensors).

Phenolic compound	Linear range (M)	Correlation coefficient	Sensitivity ($A\,M^{-1}\,cm^{-2}$)	Detection limit (nM)	K_M^{app} (μM)
Catechol	4.0×10^{-10}–2.1×10^{-6}	0.999	296 ± 4	0.1	1.44 ± 0.05
p-Cresol	4.0×10^{-10}–2.0×10^{-6}	0.999	262 ± 3	0.1	1.33 ± 0.03
Phenol	4.0×10^{-10}–1.9×10^{-6}	0.997	253 ± 4	0.1	1.16 ± 0.05
m-Cresol	1.0×10^{-9}–6.6×10^{-6}	0.997	47.1 ± 1	0.5	0.65 ± 0.01

activation energy (E_a) of the enzyme catalytic reaction, calculated from the slope of the straight line, was 38.8 kJ mol^{-1}, which is similar to that (35 kJ mol^{-1}) of the laponite-tyrosinase electrode (Shan et al., 2003). For a simple experimental procedure and long lifetime of the biosensor, further measurements were performed at room temperature (25 °C).

3.4. Amperometric sensing of phenolic compounds

The steady-state amperometric responses of the Tyr/PANI–IL–CNF electrode to different phenolic compounds were determined by the successive addition of phenolic compounds into 12 ml PBS under the optimum conditions. The enzyme electrode exhibited a rapid response to the change of phenolic compound concentration. After the addition of phenolic compound, the cathodic current immediately increased and reached 95% of steady-state current within 10 s.

Fig. 4 shows a typical current–time plot for the amperometric sensor on the successive additions of different volumes of 1.2×10^{-5} M catechol to 12 ml PBS. The linear range spanned the 4-order concentration of catechol from 4.0×10^{-10} to 2.1×10^{-6} M with a correlation coefficient of 0.999, and a detection limit of 0.1 nM, at which the amperometric response and the noise were 4.2 and 1.3 nA (S/N ~ 0.3), respectively. Its sensitivity was $296\pm4\,A\,M^{-1}\,cm^{-2}$, which was much higher than those of tyrosinase biosensor based on polyaniline–polyacrylonitrile composite ($2.03\,A\,M^{-1}\,cm^{-2}$) (Xue and Shen, 2002), electrogenerated polypyrrole/carbon paste ($4.7\,A\,M^{-1}\,cm^{-2}$) (Mailley et al., 2003), polypyrrole–alginate conjunct ($0.350\,A\,M^{-1}\,cm^{-2}$) (Abu-Rabeah et al., 2005), layered double hydroxides (LDH) ($7.81\,A\,M^{-1}\,cm^{-2}$) (Shan et al., 2003), graphite–Teflon composite in presence of gold nanoparticles ($10.6\,A\,M^{-1}\,cm^{-2}$) (Carralero et al., 2006) and Fe_3O_4 nanoparticles–chitosan composite ($22.84\,A\,M^{-1}\,cm^{-2}$) (Wang et al., 2008). The high sensitivity of the Tyr/PANI–IL–CNF biosensor could be attributed to the combination of the advantages of three components. Although a biosensor based on tyrosinase–chitosan–$Fe(CN)_6^{4-}$ showed a higher sensitivity to phenol ($2123\,A\,M^{-1}\,cm^{-2}$) (Wang et al., 2002), its linear response ranging from 1.0×10^{-10} to 2.3×10^{-8} M was much narrower than that of the proposed biosensor, furthermore, the potential leakage of $Fe(CN)_6^{4-}$ from chitosan film would affect its stability.

The Tyr/PANI–IL–CNF modified electrode exhibited highly sensitive amperometric responses to the analogs of phenolic compounds such as catechol, *p*-cresol, phenol and *m*-cresol. The response characteristics including the linear range, correlation coefficient, sensitivity and detection limit were listed in Table 1. The sensitivity in the linear calibration regions followed the order: catechol > *p*-cresol > phenol > *m*-cresol, which was consistent with the result reported by Han et al. (2007) and Shan et al. (2003). According to Lineweaver–Burk plots, the apparent Michaelis–Menten constants (K_M^{app}) were calculated to be 1.44, 1.33, 1.16 and 0.65 μM for catechol, *p*-cresol, phenol and *m*-cresol, respectively. The value of K_M^{app} for catechol of the proposed biosensor was obviously lower than those reported for other biosensors based on chitosan/LDH (130 μM) (Han et al., 2007), Fe_3O_4 nanoparticles–chitosan nanocomposite (96.9 μM) (Wang et al., 2008), gold nanoparticles–graphite–Teflon composite (6.6 μM) (Carralero et al., 2006) and free enzyme in solution (300 μM) (Espín et al., 2000). The low K_M^{app} value indicated the tyrosinase immobilized on the surface of PANI–IL–CNF composite had better affinity to phenolic compounds.

3.5. Interference tests

The amperometric response of the Tyr/PANI–IL–CNF modified electrode to 0.3 μM catechol was not affected by additions of 3 μM ascorbic acid, 30 μM uric acid and 30 μM caffeine. That may be due to the use of a low operating potential (−0.05 V). So, the Tyr/PANI–IL–CNF electrode exhibited good selectivity to phenolic compounds detection. Considering the good responses of the biosensor to all analogs of phenolic compounds, the biosensor could be coupled with a separation step for simultaneous detection of these compounds.

3.6. Repeatability, reproducibility and stability of the biosensor

The repeatability of the same Tyr/PANI–IL–CNF modified electrode was examined at the catechol concentrations of 5×10^{-9} and 5×10^{-7} M, and the relative standard deviations for six determinations were 3.7 and 1.0%, respectively. In addition, the relative standard deviation of current signals for measurement of 0.3 μM catechol at six independently prepared biosensors was 3.0%, which proved good reproducibility of the biosensor preparation.

The long-term stability of the Try/PANI–IL–CNF electrode was investigated in two ways. The enzyme electrode retained 94% of its original response after 40 days storage in 0.1 M pH 7.0 PBS at 4 °C. At the same storage condition, the enzyme electrode retained 94% of its initial activity after 25 days and decreased to 70% after 40 days when using once per 5 days.

4. Conclusions

A biocompatible PANI–IL–CNF composite has been successfully prepared by *in situ* one-step electropolymerization of aniline on CNF in the presence of IL. During polymerization carbon nanofiber acts as molecular template, thereby guiding the growth of PANI on its surface, and IL is adsorbed on the outer surface of the PANI due to the high viscosity of EMIES, which greatly enhances the adhesion of the nanocomposite on support surface and the electronic properties of the resulting film. By cross-linking tyrosinse to the nanocomposite with glutaraldehyde, a phenol biosensor is further constructed. The combination of the advantages of three components makes the immobilized tyrosinase have good affinity to phenolic compounds and the designed biosensor have excellent performance for amperometric biosensing of phenolic compounds. The biosensor shows fast response, wide linear range, high sensitivity, low detection limit, good reproducibility, and accepted long-term stability. The functionalization of CNF with PANI and IL has a great potential for multiple applications in electrically active devices.

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