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ORIGINAL PAPER

Acetone extracted propolis as a novel membrane and its application in phenol biosensors: the case of catechol

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Abstract A novel biosensor for catechol has been constructed by immobilizing polyphenol oxidase (PPO) into acetone-extracted propolis (AEP) composite modified with gold nanoparticles (GNPs) and attached to multiwalled carbon nanotube (MWCNTs) on a gold electrode surface. The propolis for AEP was obtained from honeybee colonies. Under the optimum conditions, this method could be successfully used for the amperometric determination of catechol within a concentration range of 1×10^{-6} to 5×10^{-4} M, with a detection limit of 8×10^{-7} M ($S/N = 3$). The effects of pH and operating potential are also explored to optimize the measurement conditions. The best response was obtained at pH 5, while an optimum ratio of signal-to-noise (S/N) was obtained at −20 mV (versus Ag/AgCl), which was selected as the applied potential for the amperometric measurements. All subsequent experiments were performed at pH 5. Cyclic voltammetry and electrochemical impedance spectroscopy was used to characterize the PPO/CNTs/GNPs/AEP/Au biosensor. The biosensor also exhibited good selectivity, stability, and reproducibility.

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Introduction

Phenolic compounds are very common substances in nature. These compounds are formed as a result of the biodegradation of natural compounds such as humic acid, tannins, and lignins. Phenolic compounds are widely used in industrial processes such as the manufacture of plastics, dyes, drugs, and antioxidants [1–3]. Therefore, these compounds are released into the environment by industrial wastes from such industries [4, 5]. Many of these compounds have toxic effects on animals and plants, resulting in an acute environmental problem [6]. Thus, the monitoring and control of these pollutants are of great importance towards protecting the environment, and hence there is an increasing demand for the selective and sensitive detection of phenol and its derivatives in water solutions [7].

Many analytical methods are available for the determination of phenolic compounds based on separation techniques including gas and liquid chromatography [8–10]. Although chromatographic methods are sensitive and specific, these methods suffer from complicated sample pretreatment, such as concentration and extraction steps that increase the risk of sample loss or contamination, and also from not being easily amenable for continuous monitoring and from the requirement of skilled operators [11]. There have been some reports about the application of spectrometric methods and chemometric tools for the resolution of phenolic compounds in mixtures [12–15]. In comparison, many reports exist in the literature describing the utilization of electrochemical techniques for the detection of phenolic

compounds [16]. One of the main objectives for electrochemical detection is the improvement in the versatility and scope of applications [17]. However, the electrochemical detection by the oxidation of phenols has some drawbacks, such as the poisoning of the electrode surfaces due to the accumulation of reaction by-products. Amperometric biosensors based on enzymes have been considered to be a promising method because of its effectiveness and simplicity [18–20]. In the last few years, research on phenol converting enzymes has resulted in technical applications for the analysis of environmentally and clinically important phenolic compounds by means of biosensors. In recent years, a number of papers on different aspects of phenol biosensors based on the amperometric approach have appeared, showing the broad interest of scientists worldwide [21–23].

Propolis (bee glue) is a resinous product found in beehives [24]. Propolis has attracted much attention in recent years and is being used in medicine and cosmetic products because of its antimicrobial, anti-oxidative, and anti-tumor activities [25–27]. Ethanol extract of propolis is the most common extract among other solvents [28]. Gas chromatography-mass spectroscopy technique and other detection techniques have shown that propolis is generally composed of about 45% resin, 35% wax, and 20% inert material [29]. The identification of the constituents, specifically "wax" that are insoluble in polar solvents such as water has been carried out with organic solvents [30]. Propolis has unusual combination properties, which includes excellent membrane-forming ability towards water, good adhesion, non-toxicity, high mechanical strength, especially good biocompatibility, and susceptibility to chemical modification due to the presence of a large number of reactive functional groups [31, 32].

The present study describes the first attempt to prepare acetone-extracted propolis (AEP) and its application in a propolis/poly phenol oxidase (PPO) enzyme layered film for electrode modification by the technique of electrostatic self-assembly and its usage in the determination of phenolic compounds as a highly sensitive and stable biosensor. Gold nanoparticle (GNP) attachment in multiwalled carbon nanotubes (MWCNTs) and onto AEP film can enhance the conductivity of the propolis film, and the subsequent graft attachment of the PPO to the propolis to form the PPO/MWCNTs/GNP layer on a gold surface electrode has been investigated as a phenol biosensor.

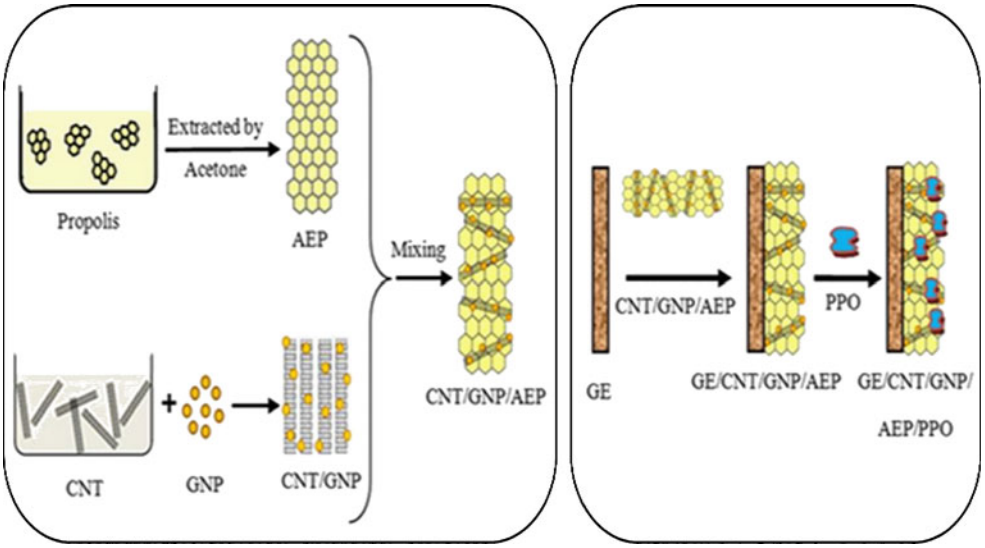
Experimental

Reagents and apparatus

Poly phenol oxidase enzyme (PPO) (Lot No.L.A045043 from mushroom, 20 units mg^{-1}) was purchased from AMANO-JAPAN. Hexane, dimethylformamide and acetone were obtained from Merck, Germany. Potassium hydroxide (KOH), sodium borohydride ($NaBH_4$), and catechol were purchased from Sigma–Aldrich. MWCNTs and pure gold metal were obtained from Gold Bazaar. The brown-colored propolis sample was obtained from colonies of honeybees from Urmia located in the northwestern part of Iran during the fall of 2009. Electrochemical experiments were performed by using an Autolab PGSTAT30 Potentiostat/Galvanostat equipped with a frequency response analyzer (FRA4.9) and controlled by General Purpose Electrochemical System (GPES4.9) software (Eco Chemie, Utrecht, Netherlands). A gold working electrode (2 mm diameter), a platinum wire counter electrode, a Ag/AgCl (3 M KCl) reference electrode, and a conventional three-electrode electrochemical cell were obtained from Azar Electrode Instruments.

Construction of the PPO/GNP/CNT/AEP/Au biosensor

The gold electrodes were polished before each experiment with 0.3 and 0.05 μm alumina powders in succession, rinsed thoroughly with double-distilled water between each polishing step, sonicated with acetone and double-distilled water, respectively, and then allowed to dry at room temperature. The preparation of the PPO/GNP/CNT/AEP/Au biosensor was as follows: First, an AEP solution (2 wt%) was prepared by dissolving propolis in 10 mL acetone with stirring for 1 h at room temperature until all the wax components were completely dissolved. The AEP solution was then filtered through a 0.45-μm Millex-HA syringe filter unit (Millipore) and stored in a refrigerator (4 °C) when not in use. MWCNTs were chemically functionalized by ultrasonic agitation in a mixture of sulfuric acid and nitric acid (3:1) for 4 h. The resulting CNTs were separated and washed repeatedly with distilled water by centrifugation until the pH was about 7. For the preparation of the CNT/GNP nanohybrid, first 0.01 g of MWCNT was mixed with 50 mL of 0.05% $HAuCl_4$ solution, and then 1 mL of 1% $NaBH_4$ was added to this resulting solution and stirred for 60 min and finally dried in a freeze-dryer for about 24 h. GNP/CNT/AEP films were prepared by mixing 2% AEP solution and GNP/CNT powder with stirring for 3 h to prepare a homogeneous dispersion of GNP/CNT; subsequently, 5 μL of this mixture was added onto the pretreated gold electrode and dried for 3 h in a vacuum desiccator at room temperature providing the GNP/CNT/AEP/Au electrode ready for use. For the preparation of PPO/GNP/CNT/AEP/Au biosensor, a defined amount of PPO solution was applied on the surface of the modified electrode, and the coating was then dried at 4 °C in a refrigerator and stored in 0.1 M phosphate buffer solution (PBS) at 4 °C (see Scheme 1).



Scheme 1 Schematic illustration of the process for the preparation of the phenol biosensor

Results and discussion

Characterization of PPO/GNP/CNT/AEP/Au biosensor

AEP is nearly nonconducting, and it thus hampers the transition of electrons in an enzyme assembly film. Thus, the improvement of the response current was lower than our expectation. MWCNTs, the graphitic allotropes of carbon, are by far the most widely used nano-materials for the fabrication of electrodes due to their semi-conducting behavior and high porosity and have been found to be suitable for application in enzyme electrodes as well. The present study shows that GNP attachment in MWCNTs and onto the AEP film can enhance the conductivity of the propolis film. The effect of the propolis film, added via a graft attachment with PPO/MWCNTs/GNP and onto a gold surface electrode, as a phenol biosensor was investigated. The modified electrode was subsequently functionalized via electrostatic self-assembly by immobilization of the PPO enzyme.

Cyclic voltammetry of hexacyanoferrate (HCF) was used to compare the conductivity of the modified and unmodified electrodes. Figure 1 shows cyclic voltammograms of different modified electrodes in a 3-mM HCF solution in PBS at pH 5. As can be seen from this figure, a couple of well-defined redox peaks are observed at the unmodified gold electrode. After insertion of the CNTs/AEP film onto the gold electrode surface, the redox peaks decreased dramatically. When dropping CNTs/GNPs/AEP film onto the gold electrode surface, there was a remarkable increase in current. This was due to the fact that CNT/GNP/AEP film possesses better conductivity and is more able to participate in efficient electron transfer than the CNTs/AEP film. However, this result indicates that electron conduction pathways between the electrode and the solution are formed due to a cooperative effect of CNT/GNP hybrids.

Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) is used in various areas of electrochemistry and materials research, such as the examination of passive layers (i.e., thickness, conductivity) to characterize organic and inorganic corrosion in protective layers. The EIS is an effective method for probing the features of surface-modified electrodes. The impedance spectra include a semicircular portion and a linear portion. The diameter of the semicircle at higher frequencies corresponds to the electron-transfer resistance (R_{et}), while the linear part at lower frequencies corresponds to the diffusion process.

Figure 2 shows the EIS of the gold electrode at different stages. It was observed that the EIS of the bare Au electrode displayed an almost straight line (Fig. 2a), which was characteristic of a mass diffusion limiting process. After the

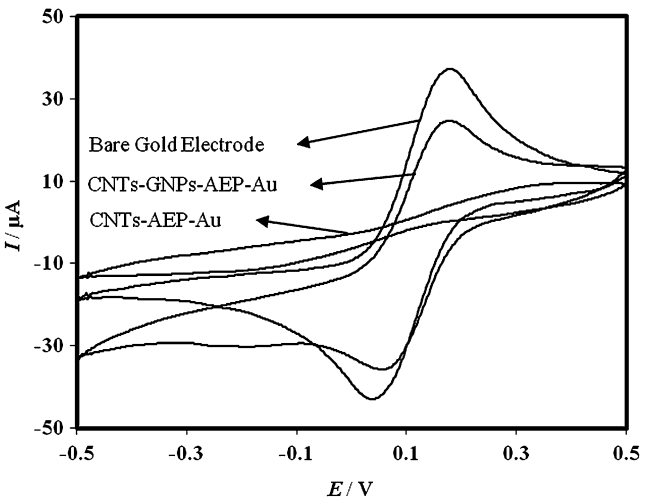


Fig. 1 Cyclic voltammograms of gold electrodes, modified with different films at a scan rate of 100 mVs^{-1} vs. Ag/AgCl in a 3-mM $K_3Fe(CN)_6$ solution in the presence of 0.1 M phosphate buffer at pH 5

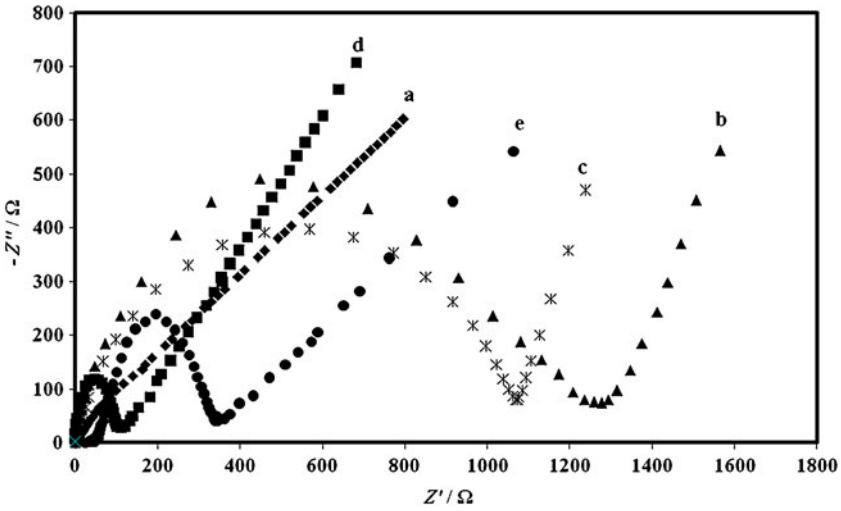


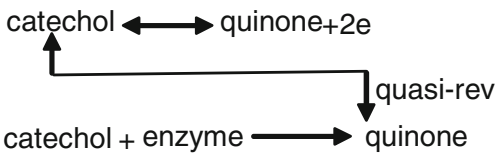
Fig. 2 EIS of *a* bare gold disk electrode, *b* AEP-Au electrode, *c* MWCNTs/AEP/Au electrode, *d* GNP/MWCNTs/AEP/Au electrode, and *e* PPO enzyme attached on GNP/MWCNTs/AEP/Au electrode in a 3-mM [Fe(CN)6] −4/−3 in PBS (0.1 M, pH 5.0). The frequency range was 1.0 × 10 − 2 to 1.0 × 105 Hz at 25 °C with signal amplitude of 5 mV

electrode was modified with AEP, the EIS showed a high electron-transfer resistance of about 1,300 Ω (Fig. 2b), implying that both MWCNTs and AEP reduced electron-transfer resistance to about 1,100 Ω (ΔR_{et} = 200 Ω) in comparison with Fig. 2b (as in Fig. 2c). After the Au electrode was covered with the GNP/MWCNT/AEP film, the diameter of the semicircle decreased, showing that the GNP/MWCNT/AEP film promoted the electron transfer at the electrochemical probe (Fig. 2d). This may be ascribed to the excellent conductivity of the GNP attachment in activated MWCNTs and the electrostatic force between $Fe(CN)_6^{4-/3-}$ and the AEP film at pH 5. Thus, GNP/MWCNTs/AEP possessed good electrical conductivity. However, after the PPO enzyme was adsorbed to the GNP/MWCNTs/AEP gold electrode, the electron-transfer resistance increased again (Fig. 2e). This increase is attributed to the non-conducting properties of the PPO enzyme, which presumably obstructs electron transfer at the electrochemical probe. The above result clearly confirms the success of the assembly of the electrode.

Electrochemical sensing capability of PPO/GNP/CNT/AEP/Au biosensor

The biosensor response including sensitivity, linear range, correlation coefficient, and limit of detection (LOD) for six phenolic compounds are shown in Table 1. As can be seen, the sensitivity in the linear range followed the sequence: catechol > phenol > *m*-cresol > *p*-Cresol. Hydroquinone and *o*-cresol did not give any response. The restricted activity of the biosensor is dependent on the hydrophobic characteristics of the immobilization matrix, with the hydrogen bonds in the GNP/CNT/AEP film desirable for holding the active configuration of the PPO enzyme [33].

Figure 3 shows cyclic voltammograms of PPO/GNP/CNT/AEP/Au biosensor in absence of catechol and in presence of 1×10^{-5} M catechol and also GNP/CNT/AEP/Au-modified electrode without PPO in presence of 1×10^{-5} M catechol. The catechol shows quasi-reversible peaks at the GNP/CNT/AEP/Au-modified electrode. On the basis of results obtained from this study, the electrode process may be described as follows: Eq. 1.



At the PPO/GNP/CNT/AEP/Au biosensor, anodic peak at the potential of 0.267 V vs. Ag/AgCl shows that an

Table 1 The response characteristics of the biosensor to phenolic compounds

Phenolic compound	Sensitivity(μA/μM)	Linear range (M)	R^a	LOD (M)
Catechol	0.15	1×10^{-6} to 5×10^{-4}	0.997	8.0×10^{-7}
Phenol	0.10	2.5×10^{-6} to 5×10^{-4}	0.997	1.1×10^{-6}
m-Cresol	0.02	1×10^{-5} to 4.5×10^{-4}	0.992	5.3×10^{-6}
p-Cresol	0.01	2×10^{-5} to 4.5×10^{-4}	0.996	8×10^{-6}
o-Cresol	0	–	0	–
Hydroquinone	0	–	0	–

[a] Correlation coefficient of the linear range

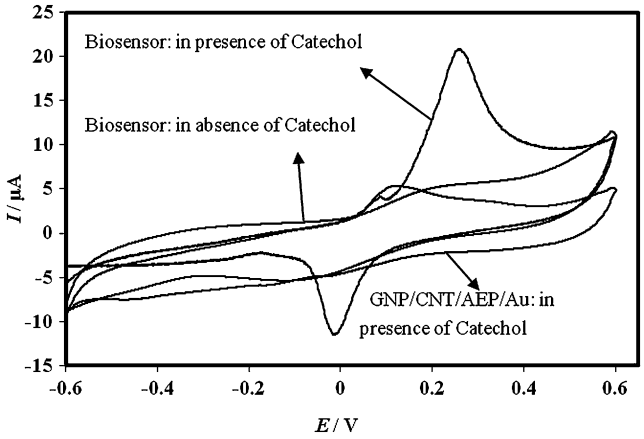


Fig. 3 Cyclic voltammetry response of PPO/GNP/CNT/AEP/Au-modified electrode and GNP/CNT/AEP/Au-modified electrode without PPO in absence and in presence of 1×10^{-5} M catechol. Scan rate 100 mV s^{-1} vs. Ag/AgCl in a phosphate buffer at pH 5

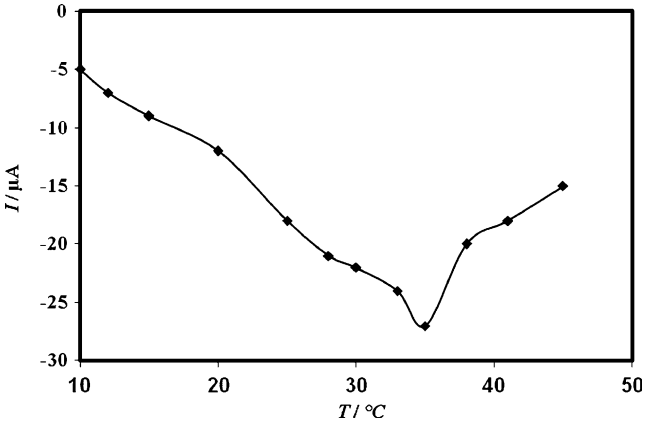


Fig. 5 The influence of temperature on the response of the PPO/GNP/CNT/AEP electrode in 0.1 M phosphate buffer solution containing 20 µM catechol with pH 5.0 and E_{app} = −20 mV

increase in the anodic peak current response and potential peak separation in presence of 1×10^{-5} M catechol appeared. This could be ascribed to the catalytic chemical oxidation of catechol by PPO on the electrode surface as compared with unmodified with PPO enzyme-modified electrode. These results indicate a very important point that the immobilization process retains the biological activity of PPO enzyme in the propolis film. For catechol, the sensor exhibited a fast response which resulted from the porous structure and high enzyme loading of the propolis matrix.

Effect of operational conditions on the biosensor response

Various operational conditions such as pH, operating potential, and temperature can affect the biosensor response. The influence of pH on the response of PPO/GNP/CNT/AEP-modified Au electrode was investigated to optimize the reaction conditions. The change of chronoamperometric current between the pH levels of 3–9 in 0.1 M phosphate buffer at a constant catechol concentration (0.1 mM) is shown in Fig. 4. As can be seen in Fig. 4, the maximum response was obtained at pH 5, which is close to the optimum pH (6–7) observed for the PPO enzyme [34]. The effect of operating potential on the response and the background current of the biosensor was studied, and an optimum ratio of signal-to-noise (*S*/*N*) was obtained at −20 mV (vs. Ag/AgCl), which was selected as the applied potential for the amperometric measurements. This low operating potential can minimize interferences from electroactive species.

Since the electrocatalytic activity of enzymes or proteins is strongly dependent on temperature, the effect of temperature on the biosensor response was studied and is shown in Fig. 5. The current response to 20 µM catechol increases with the increase in temperature from 10 to 35 °C but then decreases as the temperature increases further. The maximum response appears at about 35 °C. Moreover, with increasing temperature, the response time decreases because of the increase in

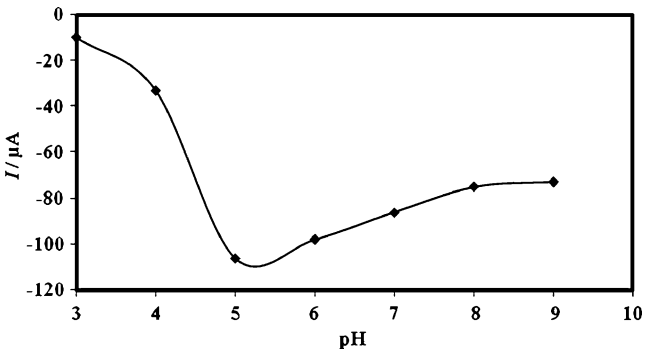


Fig. 4 The influence of pH on the PPO/GNP/CNT/AEP electrode response in 0.1 M phosphate buffer containing 0.1 mM catechol at 25 °C and Eapp = −20 mV

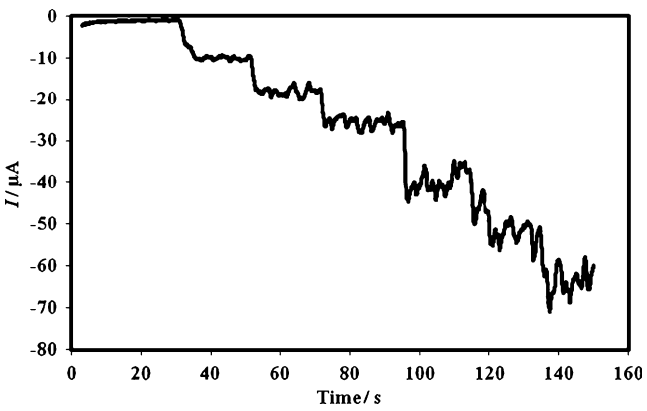


Fig. 6 Current–time recordings of the PPO/GNP/CNT/AEP-modified Au electrode with increasing catechol concentrations (initial concentration is 10 µM). The applied potential is −20 mV vs. Ag/AgCl in a phosphate buffer of pH 5

Table 2 Comparison of performances of proposed biosensor with those of various biosensors based on different matrices

Immobilization matrix	Linear range (μM)	LOD (μM)	Sensitivity (μA/μM)	Long-term stability	Reference
PPO–DAB–Ru-med complex	5 to 100	2.35	Nd	86% (21 days)	[36]
HRP-TiO_2 nanotube	0.8 to 130	0.2	0.19	92% (45 days)	[37]
MWNT-Nafion	1 to 23	0.22	0.14	81% (40 days)	[38]
PPO immobilized into PANI film	1.2 to 150	0.87	Nd	75% (30 days)	[39]
CNT/PPy/HRP nanobiocomposite	16 to 44	3.52	1	70% (30 days)	[40]
Fe_3O_4–chitosan nanocomposite	0.08 to 70	0.025	0.51	85% (30 days)	[41]
Agarose–Guar gum	40 to 800	6	0.01	90% (60 days)	[42]
Acetone extract propolis	1 to 500	0.8	0.15	80% (42 days)	This work

Nd not detected

activity of the enzyme at higher temperatures. Hence, room temperature was chosen for the experiments as the biosensor response was unstable at temperatures higher than 35 °C, with protein denaturation occurring with increased temperature [35], along with the AEP undergoing deformation with increasing temperature beyond 35 °C. In order to maintain the stability and reproducibility of the biosensor for a long time, we thus chose room temperature as the operating temperature in our experiments.

Amperometric sensing of phenolic compounds

The amperometric response of the enzyme electrode to successive additions of catechol was further evaluated. Figure 6 shows the typical current–time dynamic response of the PPO/GNP/CNT/AEP-modified Au electrode towards catechol. The electrode showed a rapid and sensitive response after each addition of catechol. Perhaps due to the open structure of the matrix, small molecules can rapidly diffuse from the solution into the PPO/GNP/CNT/AEP-modified gold electrode. The linear response of the biosensor was in the range of 1×10^{-6} to 5×10^{-4} M, with a detection limit of 8×10^{-7} M, and the best response was obtained at pH 5. All subsequent experiments were performed at pH 5. The stability of the biosensor was also evaluated.

The biosensor was tested for repetitive measurements, and calibration curves were constructed for catechol over a period of 2 h. The current responses were reproducible over the concentration range from 1×10^{-6} to 5×10^{-4} M (RSD = 4.3%, $n = 6$). The stability of the biosensor was considered as one of the key factors for the biosensor performance. After measurements, the biosensors were rinsed with phosphate buffer and stored at 4 °C in a refrigerator. It was found that the biosensor response maintained its initial value for 2 weeks but then decayed quickly to about 80% of its initial value after the end of 6 weeks (used more than 80 times). To the best of our knowledge, the PPO/GNP/CNT/AEP-modified Au electrode developed in this study is the first electrochemical biosensor that applied AEP as a membrane for catechol detection. Table 2 shows the performance of the proposed biosensor based on PPO/GNP/CNT/AEP/Au in comparison with other phenol biosensors based on a variety of matrices [36–43]. This fact indicates that the biocompatibility of PPO/GNP/CNT/AEP provides a very low detection limit, long linearity, high stability, and high sensitivity in comparison with some of the biosensors that need very expensive materials. By considering the other advantages of this biosensor such as the simplicity of preparation and relatively low cost, this type of biosensor can be potentially used for the detection of phenolic compounds for real samples.

Real sample analysis

A preliminary evaluation of the validity of the proposed PPO/GNP/CNT/AEP electrode and the recovery of phenolic content in tap water samples was performed. The phenols were estimated by the standard addition analysis of catechol. The results were compared with those obtained from high-performance liquid chromatography (HPLC), which is the

Table 3 Detection of catechol in real samples by the biosensor and HPLC method

Sample	Standard concentration of catechol (μM)	Determined by biosensor (μM)[a]	Determined by HPLC method (μM)[a]	Relative error (%)	Recovery (%)
1	0	0	0	–	–
2	10	9.75 ± 0.42	9.88 ± 0.59	−1.31	97.50
3	110	108.83 ± 40	107.36 ± 6.20	1.37	98.93
4	50	50.5 ± 1.94	50.08 ± 2.72	0.84	101.00

[a] Average of six determinations ± standard deviation

validated method of analysis for such samples. As can be seen from Table 3, the relative errors are within the limits of acceptability. Since the results determined by the PPO/GNP/CNT/AEP electrode are in satisfactory agreement with those given by the HPLC method for real samples, our reported method can provide a feasible alternative tool for such determinations. The AEP thus provides a good protection for the immobilized enzyme against possible inhibitors, and the recoveries are also satisfactory.

Conclusion

In this paper, we have reported an amperometric biosensor using AEP as a novel membrane. For the construction of PPO/GNP/CNT/AEP composite as an immobilization matrix, PPO was immobilized in GNP/CNT/AEP composite and used for the determination of phenolic compounds. Under the optimum conditions, this biosensor could be successfully applied for the amperometric determination of catechol over a concentration range of about two orders of magnitude (1×10^{-6} to 5×10^{-4} M).

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