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A novel tyrosinase biosensor based on hydroxyapatite–chitosan nanocomposite for the detection of phenolic compounds

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ABSTRACT

A novel tyrosinase biosensor based on hydroxyapatite nanoparticles (nano-HA)-chitosan nanocomposite has been developed for the detection of phenolic compounds. The uniform and size controlled nano-HA was synthesized by hydrothermal method, and its morphological characterization was examined by transmission electron microscope (TEM). Tyrosinase was then immobilized on a nano-HA-chitosan nanocomposite-modified gold electrode. Electrochemical impedance spectroscopy and cyclic voltammetry were used to characterize the sensing film. The prepared biosensor was applied to determine phenolic compounds by monitoring the reduction signal of the biocatalytically produced quinone species at −0.2 V (vs. saturated calomel electrode). The effects of the pH, temperature and applied potential on the biosensor performance were investigated, and experimental conditions were optimized. The biosensor exhibited a linear response to catechol over a wide concentration range from 10 nM to 7 μM, with a high sensitivity of 2.11×10^3 μA mM^{-1} cm^{-2}, and a limit of detection down to 5 nM (based on S/N = 3). The apparent Michaelis–Menten constants of the enzyme electrode were estimated to be 3.16, 1.31 and 3.52 μM for catechol, phenol and m-cresol, respectively. Moreover, the stability and reproducibility of this biosensor were evaluated with satisfactory results.

1. Introduction

Phenolic compounds are widely used in wood preservatives, textiles, herbicides and pesticides, and released into the ground and surface water. However, most of them are highly toxic. Therefore, the development of methods to identify and quantify phenolic compounds in various samples is of great importance for environment and human health. Several methods for the determination of phenolic compounds, including gas chromatography and spectrophotometry, have been proposed [1,2]. These methods offer good limits of detection (LODs) and wide working concentration ranges. However, they necessitate the use of sophisticated and relatively costly apparatus and require complicated pretreatment procedures, and are not suitable for in situ detection. Many research efforts have been focused on the development of simple and effective analytical methods for the determination of phenolic compounds. Electrochemical biosensor has been considered as the best choice for the in situ monitoring of phenolic compounds by virtue of its high sensitivity, simple instrumentation, low production cost and promising response speed [3–6]. Such biosensors are usually based on monitoring the reduction signal of the quinone species which is generated by the catalysis of tyrosinase in the presence of molecular oxygen [7]. Therefore, the effective immobilization of tyrosinase on the electrode surface is a key step in the development of tyrosinase biosensors for phenolic compounds.

Various supporting materials, such as 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenosulfonate [8], polymers [9], biopolymers [10], hydrogels [11,12], sol–gels [13,14] and clay [15], have been successfully utilized to immobilize tyrosinase on the electrode surface. Among them, inorganic nanoparticles have attracted much attention due to their excellent properties such as uniformity, good biocompatibility, large surface-to-volume-ratio and strong absorption abilities. Moreover, they could provide a desirable microenvironment to immobilize tyrosinase, and promote the direct electron transfer between the tyrosinase and underlying electrode [16,17].

Hydroxyapatite (HA, $Ca_5(PO_4)_3(OH)$), a main inorganic component of bone, has been extensively applied in biomedical implants, bone regeneration, drug delivery, protein separation and immunosensors owing to its excellent biocompatibility, bioactivity and multi-adsorbing sites [18–20]. Nanostructured HA particles with a higher surface area-to-volume-ratio would be more desirable than bulk HA particles for their application in many fields. For example, some studies have demonstrated that HA nanopar-

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ticles can be used for the preparation of gas sensors [21–23], and immobilization of enzymes [24]. However, the tendency to aggregation of pure HA-based materials limits their further applications, so it is necessary to disperse such nanomaterials in a suitable matrix to prevent aggregation. Chitosan is a kind of natural polysaccharide derived from chitin. It possesses good biocompatibility, biodegradability, excellent film-forming ability, low toxicity and anti-infectional activity [25]. These unique properties have prompted the wide application of chitosan in electrochemical biosensing platforms combined with metal nanoparticles [26], carbon nanotubes [27,28] and ionic liquids [29,30]. Moreover, chitosan can provide a biocompatible environment for enzyme immobilization because its hydrophilic primary amino groups are compatible with the biomolecules [31].

In the current study, nano-HA was prepared by hydrothermal method, and was then dispersed in the chitosan solution to form a nano-HA/chitosan matrix for the fabrication of an electrochemical biosensor for the detection of phenolic compounds. The large surface area-to-volume-ratio and other unique surface properties of the bio-nanocomposite film resulted in a high enzyme adsorption capacity with a high retention of the enzyme activity. The proposed biosensor exhibited a highly sensitive and fast response to phenolic compounds. And there is no need to use any other electron mediators.

2. Experimental

2.1. Reagents and apparatus

Tyrosinase (EC 1.14.18.1, 2870 U mg^{-1}, from mushroom) and chitosan (minimum 85% deacetylated, from crab shells) were supplied by Sigma (St. Louis, MO, USA). Catechol, phenol, and m-cresol were purchased from Shanghai Chemical Reagents (Shanghai, China). All other chemicals were of analytical grade and used without further purification, and doubly distilled water was used throughout the experiments. A 1/15 M phosphate buffer solution (PBS) prepared with KH_2PO_4 and Na_2HPO_4 was used as supporting electrolyte.

Cyclic voltammetric, amperometric measurements and electrochemical impedance spectroscopy (EIS) measurements were carried out on a CHI 760B electrochemical workstation (Shanghai, China). EIS was performed in a 0.1 M KCl solution containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture at a open-circuit potential of 0.24 V. The alternating voltage was set at 5 mV and the frequency range covered from 1 Hz to 100 kHz. A three-electrode cell (10 mL) was used with the modified Au electrode as the working electrode, a saturated calomel electrode (SCE) as reference electrode, and a platinum foil electrode as counter electrode. All potentials were measured and reported vs. the SCE, and all experiments were carried out at room temperature.

2.2. Preparation of nano-HA/chitosan/tyrosinase-modified Au electrode

A 2.0 wt% chitosan solution was prepared by dissolving chitosan in a 1.0-wt% acetic acid aqueous solution and then stirring for 3 h at room temperature. Nano-HA was prepared by hydrothermal method according to the literature [32,33]. The morphology of the Nano-HA was examined by TEM as shown in Fig. 1. A one-dimensional rod-like nanostructure was observed for the HA particles with the average width of 40 nm and length in the range of 110–260 nm. The nano-HA-chitosan suspension (4 mg mL^{-1}) was prepared by dispersing nano-HA into 2.0 wt% chitosan solution by ultrasonication for about 1 h. Tyrosinase was dissolved in 1/15 M PBS with a concentration of 40 mg mL^{-1}. The tyrosinase-HA (20 and 2 mg mL^{-1}) stock solution for modifying electrode is prepared by adding an equal volume of tyrosinase solution into HA nanoparticles sol.

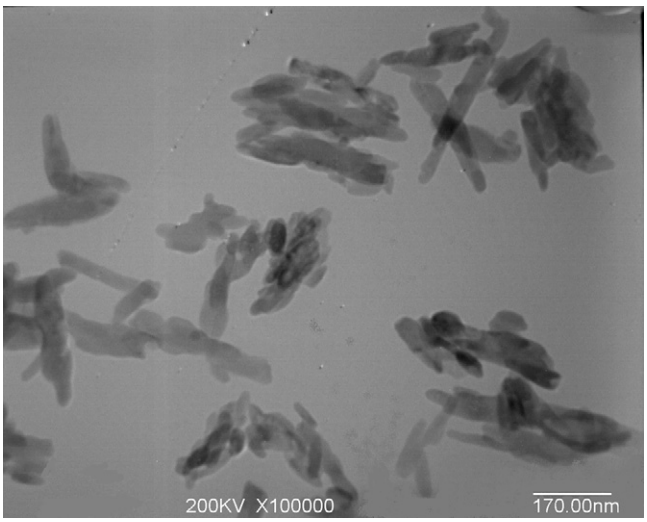

Fig. 1. Transmission electron micrographs of nano-HA.

Prior to modification, Au electrodes (polycrystalline gold rod, 99.99%, 2 mm diameter) were polished using alumina slurries with particle diameters 0.3 μm and 0.05 μm, respectively, and subsequently cleaned through ultrasonication in distilled water and ethanol, each for 5 min. Subsequently, the Au electrode was immersed in piranha solution (a bath of 7 parts H_2SO_4 to 3 parts 30% H_2O_2) for 20 min. The electrode was then rinsed with distilled water. After the electrode dried by N_2, 5 μL tyrosinase-nano-HA stock solution was dripped and spread out evenly on the freshly pretreated Au surface and dried in air. Finally, the enzyme electrode was immersed in 1/15 M PBS (pH 7.0) to wash out the non-immobilized enzyme from the electrode surface. Tyrosinase–chitosan-modified electrode and Nano-HA/chitosan-modified electrode without tyrosinase were prepared with the same procedures as described above.

3. Results and discussion

3.1. Characterization of tyrosinase-nano-HA-chitosan/Au electrode nanobiocomposite

EIS is a powerful tool for studying the interface properties of surface-modified electrodes. The curve of EIS present as Nyquist plot consists two parts: one part is the semicircle part corresponding to the electron transfer limited process and locating at the higher frequency. The electron-transfer resistance (Rct) can be gained by measuring its diameter; the other is the linear part affording the information about the diffusion process in solution and locating at the lower frequency. Fig. 2A displays the Nyquist plots of the EIS of the bare Au electrode (electrode a), the chitosan-modified Au electrode (electrode b), the nano-HA/chitosan-modified Au electrode (electrode c) and the nano-HA/chitosan/tyrosinase-modified Au electrode (electrode d). The Nyquist semicircle of the chitosan-modified Au electrode (curve b) increased dramatically compared with the bare Au electrode (curve a), which indicates that chitosan film was an obstacle making the electron transfer of interface more difficult. The result is consistent with that reported in literatures [34,35]. With nano-HA assembled on the chitosan film, the semicircle obviously decreased (curve c). The lower charge transfer resistance is probably due to the faster ion diffusion/migration in the chitosan/nano-HA film than in the chitosan film. The adsorp-

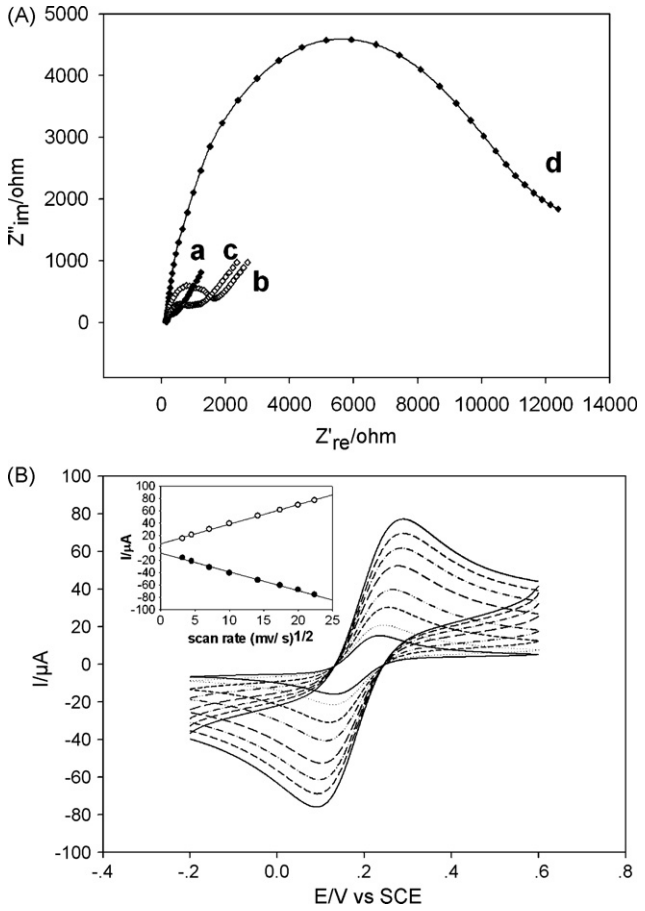

Fig. 2. (A) EIS of: (a) bare gold electrode; (b) chitosan-modified Au electrode; (c) nano-HA/chitosan-modified Au electrode; (d) nano-HA/chitosan/tyrosinase-modified Au electrode in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.1 M KCl. (B) CV curves of the nano-HA/chitosan/tyrosinase-modified Au electrode recorded in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.1 M KCl. at different scan rates (inner to outer): 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 V s^{-1}. Inset: plots of peak current vs. $v^{1/2}$.

tion of Tyrosinase (curve d) on the nano-HA made the semicircle increase again, indicating that Tyrosinase had been successfully immobilized.

Fig. 2B shows the CVs of the nano-HA/chitosan/tyrosinase-modified Au electrode recorded in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.1 M KCl at different scan rates. It is found that both the anodic and cathodic peak current clearly increase with increasing potential scan rate. Besides this, the redox peak currents are proportional to the square root of scan rate, $v^{1/2}$ (inset of Fig. 2B), indicating a diffusion electron-transfer process.

3.2. Electrochemical sensing capability of nano-HA/chitosan nanobiocomposite biosensor

The CVs for the enzyme sensors prepared with different schemes were comparably studied in the PBS (pH 7.0) in the (a) absence and (b) presence of catechol. As shown in Fig. 3A and B, both the sensors exhibit obvious reduction peaks in the absence of catechol. However, the cathodic (negative) current responses to catechol of the two modified electrodes were different. The nano-HA/chitosan/tyrosinase sensor showed a higher change of peak current (Fig. 3A) than that of chitosan/tyrosinase sensor (Fig. 3B), which might be ascribed to the enhanced electron transfer of the enzymatic reaction shown in Eqs. (1) and (2) in the presence of nano-HA [36–38]. The observed reduction peak was attributed to the direct reduction of quinone liberated from the enzyme-

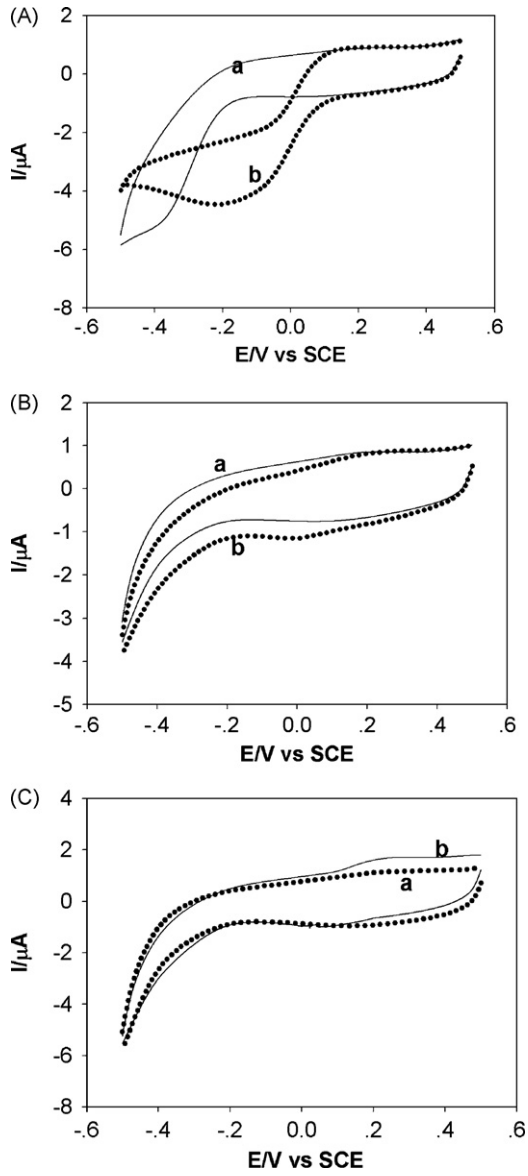

Fig. 3. Cyclic voltammograms of nano-HA/chitosan/tyrosinase-modified Au electrode (A), chitosan/tyrosinase-modified Au electrode (B) and nano-HA/chitosan-modified electrode (C) at a scan rate of 100 mV s^{-1} in 1/15 M PBS (pH 7.0) without (line a) and with 0.1 mM catechol (line b).

catalyzed reaction on the electrode surface.

$$\text{catechol} + \text{tyrosinase}(O_2) \rightarrow o\text{-quinone} + H_2O \quad (1)$$

$$o\text{-quinone} + 2H^+ + 2e^- \rightarrow \text{catechol(atelectrode)} \quad (2)$$

The electrochemical behavior of catechol at the nano-HA/chitosan-modified electrode without tyrosinase is shown in Fig. 3C. It can be seen that no reduction signal was observed at the electrode without tyrosinase. Additionally, using the proposed nano-HA/tyrosinase system, there was no need to use any electron mediators.

3.3. Optimization of detection variables

The pH value is one of the parameters that affect the response of Nano-HA/chitosan/tyrosinase-modified Au electrode to quinone species. Fig. 4A presents the pH dependence of the amperometric response of 20 μM catechol in the pH range 4.0–9.0. It can be seen that the current increased as the pH changed from 5.0 to 7.0,

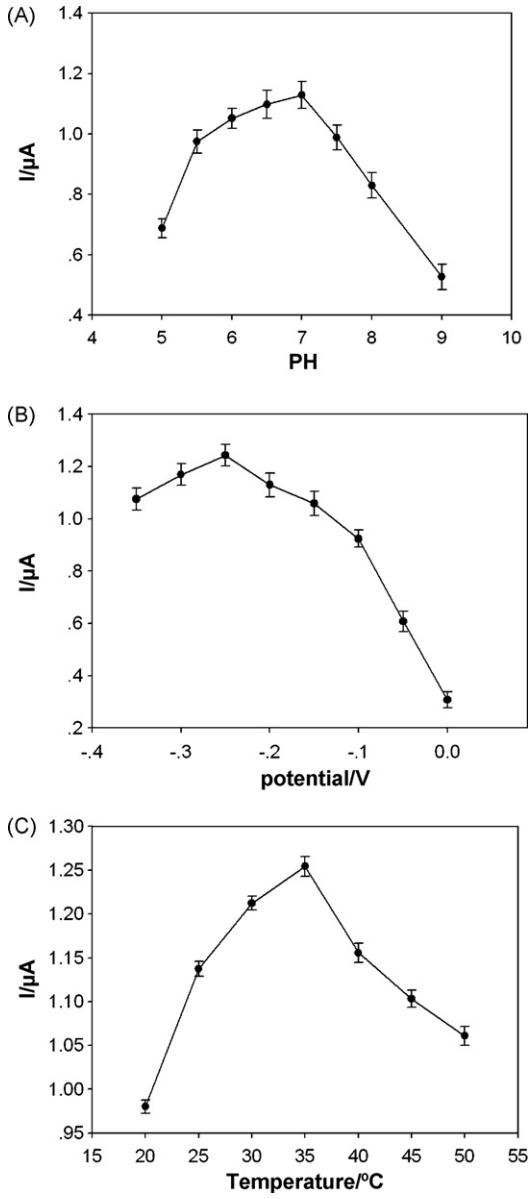

Fig. 4. Effects of pH (A), applied potential (B), and temperature (C) on amperometric response of the nano-HA/chitosan/tyrosinase-modified Au electrode to 20 μM catechol in 1/15 M pH 7.0 PBS.

following by a largely decrease in the pH range of 7.0–9.0. The maximum response was obtained at pH 7.0, which is consistent with other results of tyrosinase-based biosensors [39,40] and the optimum pH range of 5.0–8.0 [41] reported for the free tyrosinase. This indicated that the immobilization procedure did not alter the inherent properties of tyrosinase. Therefore, pH 7.0 PBS was used as the electrolyte in subsequent experiments.

Fig. 4B displays the dependence of applied potential on the amperometric response of the biosensor to 20 μM catechol. Although the maximum response current was observed at −0.25 V when the applied potential shifted from 0 to −0.35 V, an applied potential of −0.2 V was selected for the amperometric determination of catechol in the subsequent experiments. As such an applied potential could provide a low background current and minimize the interference from other co-existing species.

The effect of temperature on the amperometric response of catechol was also studied. The biosensor was immersed into the buffer solution at a given temperature for 10 min before amperometric

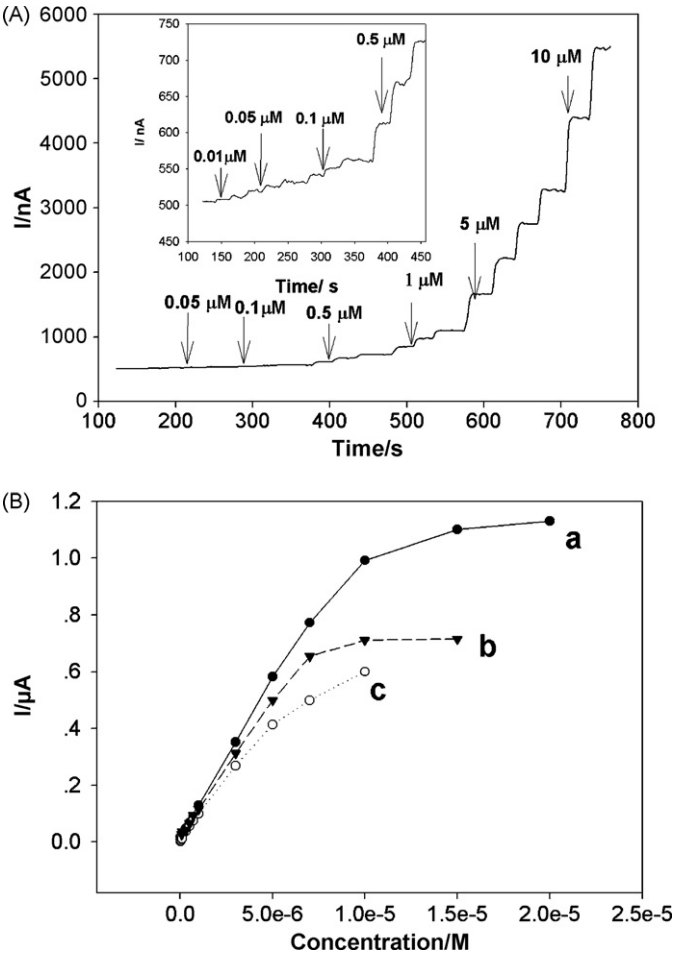

Fig. 5. (A) Typical current–time response curve of the biosensor upon successive additions of catechol with different concentrations. Upper inset: amplified response curve. Applied potential: −0.2 V. (B) The calibration curves between the reduction current and the concentration of different phenolic compounds. (a) Catechol, (b) phenol and (c) m-cresol, respectively.

detection, and then the response of the electrode was measured at this temperature. As shown in Fig. 4C, the electrochemical response increases with increasing temperature from 20 to 35 °C and then decreases as the temperature further increased. The sharp decrease of the response was due to the denaturation of tyrosinase at high temperatures. Although the response of the biosensor was greatest at 35 °C, for practical reasons it was suggested that room temperature be used to simplify the experimental procedure and prolong the useful lifetime of the biosensor given that most enzymes can be easily denatured at high temperature.

3.4. Amperometric sensing of phenolic compounds

The amperometric response of the enzyme electrode to successive additions of catechol was further evaluated under these optimized experimental conditions. Fig. 5A shows the typical current–time dynamic response of the nano-HA/chitosan/tyrosinase-modified Au electrode towards catechol. The electrode showed a rapid and sensitive bioelectrocatalytic response, reaching about 95% of the steady-state current within 8 s after each addition of catechol. Presumably, for the open structure of the nanocomposite matrix, small molecules can rapidly diffuse from the solution into the nano-HA modified membranes.

Fig. 5B showed the typical calibration curve of the tyronsinase electrode to catechol, m-cresol and phenol, respectively. The

Table 1
Response characteristics of the tyronsinase biosensor to phenolic compounds.

Phenolic compound	Linear range	Correlation coefficient	Sensitivity ($\mu A\,mM^{-1}\,cm^{-2}$)	Detection limit (nM)	K_m^{app} (μM)
Catechol	10 nM to 7.0 μM	0.999	2.11×10^3	5	3.16
Phenol	70 nM to 5.0 μM	0.999	1.77×10^3	10	1.31
m-Cresol	10 nM to 5.0 μM	0.998	2.05×10^3	5	3.52

Table 2
Comparison with other phenol biosensors based on tyrosinase.

Type of electrode	Sensitivity ($\mu A\,mM^{-1}\,cm^{-2}$)	Linear range	Detection limit (nM)	Stability	Reference
Poly3,4-ethylenedioxythiophene/tyrosinase electrode	608	–	5	Retains 30% of activity after 12 days	[46]
Colloidal gold nanoparticles/graphite-Teflon/tyrosinase	407.04	0.010–8.0 μM	20	39 Days	[47]
Tyrosinase/3-mercaptopropionic acid-modified Au electrodes	196.7	0.2–100 μM	88	5 Days	[48]
Organoclay-enzyme film electrodes	75	0.2–15 μM	–	–	[49]
Sol–gel immobilized tyrosinase electrode	208.83	1–60 μM	200	Retains 57% of activity after 2 weeks	[50]
Nafion/ZnO/tyrosinase films.	30.3	0.01–0.4 mM	4000	Retains 81.2% of activity after 20 days	[51]
Nano-HA/chitosan/tyrosinase-modified Au electrode	2110	10 nM to 7 μM	5	Retains 85% of activity after 1 month	This paper

Table 3
Recovery of tyrosinase biosensor.

Phenol added (μM)	Found[a] (μM)	Recovery (%)
0.052	0.0539	103.6
0.083	0.0877	105.7
0.365	0.362	99.3
0.85	0.822	96.7
1.39	1.33	95.8
2.85	2.864	100.5
4.55	4.23	92.9

[a] An average of three determinations.

corresponding response characteristics are shown in Table 1. The sensitivity in the linear calibration regions followed the order: catechol > phenol > m-cresol, which was consistent with the result reported by Zhang et al. [42]. The difference in sensitivity might depend on the tyrosinase catalytic selectivity for different phenolic compounds.

According to Lineweaver–Burk plots, the apparent Michaelis–Menten constants (K_m^{app}) were calculated to be 3.16, 1.31 and 3.52 μM for catechol, phenol and m-cresol, respectively. The low K_m^{app} values obtained demonstrated that the immobilized tyrosinase possessed high enzymatic activity and exhibited high affinity to phenolic compounds. For example, the value of K_m^{app} of the proposed biosensor for catechol was obviously lower than those reported for other biosensors based on (nanozeolite/PDDA)$_n$/ITO (24 μM) [43], Fe_3O_4 nanoparticles–chitosan nanocomposite (96.9 μM) [44] and Au nanoparticles–graphite–Teflon composite (6.6 μM) [45].

3.5. Repeatability, reproducibility and stability of the biosensor

The repeatability of the Nano-HA/chitosan/tyrosinase-modified Au electrode was examined by the detection of 0.8 μM catechol. A relative standard deviation (R.S.D.) value of 2.5% was obtained for 10 successive determinations, which indicated a good repeatability of the method. The fabrication reproducibility was also estimated with a series of 10 sensors constructed independently in the same way. The R.S.D. was 4.3% for the steady-state current to 0.8 μM catechol, which demonstrated the reliability of the fabrication procedure.

The long-term stability of the Nano-HA/chitosan/tyrosinase-modified Au electrode was explored. It was investigated through the response to 0.8 μM catechol at −0.2 V in 1/15 M PBS. When not in use, electrode was stored at 4 °C in a refrigerator. The data show that the sensitivity of the electrode remains relatively constant over the first 18 days, and decreased to about 85% of the original value after 1 month (used more than 80 times). Good long-term stability seems to result from the favorable microenvironment that maintains the tyrosinase activity and prevents the leakage of enzyme.

3.6. Comparison with other phenol biosensors based on tyrosinase

The analytical performances of the proposed biosensor were compared with other tyrosinase biosensors reported in the literatures. Characteristics such as sensitivity, linear response range, stability and the limit of detection of the biosensors were all summarized in Table 2. From Table 2 one can see that the proposed Nano-HA/chitosan/tyrosinase-modified Au electrode exhibited improved analytical performances in terms of linear range and limit of detection in comparison with other reported biosensors. All these results indicated that our developed electrochemical biosensor is an excellent candidate for the detection of phenol.

3.7. Selectivity and preliminarily analytical application of the biosensor

The selectivity of the nano-HA/chitosan/tyrosinase-modified Au electrode was investigated by detecting the amperometric response to 3 μM catechol in the presence of some possible interferents. The experimental results showed that 3 μM ascorbic acid, 30 μM uric acid, 30 μM caffeine, 50 μM H_2O_2 and 1000 μM glucose do not show significant interfering effect on the determination of catechol at a concentration of 3 μM. The good selectivity of this biosensor is largely attributed to the low working potential (−0.2 V).

The practical applications of the designed sensor were evaluated by determination of recovery of spiked phenol in real wastewater samples. The analytical results are shown in Table 3. One observed that the results obtained in real water samples show good results

with average recoveries from 92.9 to 105.7%, which confirmed that the proposed sensor was applicable for practical phenol detection.

4. Conclusion

In summary, a novel electrochemical tyrosinase biosensor based on a tyrosinase-nano-HA-chitosan bio-nanocomposite was developed for the determination of phenolic compounds. The bio-nanocomposite film provided a suitable microenvironment, which could effectively present large loading amount of enzyme and enhanced the direct electron transfer between the enzyme's active sites and the electrode. The biosensor exhibited good analytical performances to phenolic compounds in terms of sensitivity, low detection limit, reproducibility, and can be used for the accurate determination of several phenolic compounds without necessary of any extra electronic mediators. Its preliminarily analytical applications were also investigated with satisfying results.

Acknowledgments

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