Amperometric biosensors based on alumina nanoparticles-chitosan-horseradish peroxidase nanobiocomposites for the determination of phenolic compounds

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A horseradish peroxidase (HRP) biosensor based on alumina (Al_2O_3) nanoparticles-chitosan (CHIT) nanocomposites was developed for the detection of phenolic compounds. UV-Vis spectra and Fourier transform infrared spectra showed that HRP retained its original structure on the Al_2O_3/CHIT film. The surface morphologies of the composite films were characterized by scanning electron microscopy. Cyclic voltammetry and amperometry were used to study the proposed electrochemical biosensor. Optimization of the experimental parameters was performed with regard to pH, applied electrode potential and the concentration of hydrogen peroxide. The linear range, sensitivity and detection limit of the biosensor were investigated for eight phenolic compounds. In particular, the linearity of the biosensor for the detection of hydroquinone was obtained from 5×10^{-9} M to 7×10^{-5} M with a detection limit of 1 nM (based on the S/N = 3). The optimized biosensor for hydroquinone determination displayed a high sensitivity of 518.4 nA μM^{-1} with a response time of ~5 s.

Introduction

Phenolic compounds constitute a large group of organic pollutants, which are widely distributed throughout the environment. These phenolic compounds are used in a variety of industrial processes, *e.g.*, in the manufacture of plastics, paper, dyes, drugs, pesticides and antioxidants.[1,2] They are highly toxic to human (suspected carcinogens) and aquatic organisms. Therefore, the sensitive and rapid determination of phenolic compounds is very important.[3,4] In recent years, many analytical methods have been developed for detecting phenolic compounds, such as ultraviolet spectrophotometric analyses, gas chromatography, liquid chromatography and capillary electrophoresis.[5,6] However, electrochemical detection is more commonly used for measuring these compounds due to their advantages, such as low detection limit, wide linear response range, good stability and reproducibility.[7] Considering that the response of phenolic compounds at a traditional electrode is very poor and the bare electrode surface is easy to be fouled by insulating polymer films or by-products resulting from phenolic compounds oxidation or reduction,[8,9] some researchers are focusing on the use of nanostructured materials or chemically modified electrodes.[10,11] The electroanalytical techniques applied for the direct determination of phenolic compounds suffer a number of drawbacks due to the high overvoltage.[12] In this sense, amperometric enzyme-based biosensors offer an attractively alternative approach for monitoring these compounds.[13]

Among the amperometric enzyme biosensors, tyrosinase is the most widely used enzyme for the detection of phenolic compounds. However, tyrosinase biosensors are restricted to the monitoring of phenolic compounds having at least one free *ortho*-position.[14] A similar behavior is also observed for the laccase-based biosensors which give response to phenolic compounds having *para*- and *meta*-position free. The horseradish peroxidase (HRP) biosensor is proposed to be sensitive to a wider range of phenolic compounds compared with the above-mentioned biosensors.[15–17] The mechanism associated with the determination of phenolic compounds *via* HRP is different from those for the other two enzymes in electrochemical biosensors. Enzyme molecules are re-reduced by phenolic compounds after they are oxidized by oxygen (for tyrosinase and laccase) or hydrogen peroxide (for HRP) on the surface of the electrode.[18]

Chitosan (CHIT) is a polysaccharide consisting of the functional groups –OH and $–NH_2$, and possesses a rare combination of physicochemical properties such as low cost, chemical inertness, high mechanical strength, high hydrophilicity, good film-forming ability, biocompatibility, and no toxicity.[19–22] Therefore, CHIT is widely used for the immobilization of enzymes.[23]

The immobilization of enzyme is essential to the performance of biosensors. Up to now, HRP has been immobilized on various matrices such as surfactant, polymer, sol–gel and inorganic materials.[24] Among these matrices, inorganic materials are more attractive because of their regular structure, good mechanical, chemical and thermal stabilities.[25] Nano-Al_2O_3 has gained much research interest for a long time. In recent years, the research on the application of nanosized porous Al_2O_3 materials in electrochemical catalysis has been rather intense, which is mostly due to the nano-Al_2O_3 particles' special properties, such as electrochemical catalysis activation and large surface area.[26] In this work, HRP was immobilized in an Al_2O_3 nanoparticles matrix for the fabrication of the biosensor. The performance of the proposed HRP biosensor in the determination of phenolic compounds has been investigated in detail.

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Experimental

Reagents and apparatus

HRP (300 U mg^{-1}) was purchased from Dingguo Biological Technology Co., Ltd., (Beijing) and Al_2O_3 (40 nm) was obtained from Gona Powder Technology Co., Ltd., (Shanghai). CHIT/ K_2HPO_4, KH_2PO_4, hydroquinone and other phenolic compounds were from Sinopharm Chemical Reagent Co., Ltd., (Shanghai). All reagents were of analytical grade and doubly distilled water was used throughout. All experiments were performed in 0.1 M phosphate buffer solution (PBS) with different pH values adjusted with K_2HPO_4 and KH_2PO_4.

Electrochemical experiments were performed on CHI 660C electrochemical workstation (Shanghai) with a conventional three-electrode cell consisting of a saturated calomel reference electrode, a Pt foil counter electrode and a glassy carbon working electrode (Ø 3 mm). Cyclic voltammetry and amperometry were used to study and optimize the performance of the resulting electrochemical biosensor. Scanning electron microphotographs and transmission electron micrographs measurements were carried out on a scanning electron microscope (SEM, JEOL JSM-6700F) at 15 kV and transmission electron microscope (TEM, JEM 2010), respectively. UV-Vis absorbance spectra were performed with a UV-2501 spectrophotometer (Shimadzu Co., Japan). Fourier transform infrared (FTIR) spectra were carried out on AVATAR 370 Fourier transform infrared spectrometer (America). All measurements were carried out at room temperature.

Preparation of HRP/Al_2O_3/CHIT/GCE

0.5% (*w*/*v*) CHIT solution was prepared by dissolving appropriate amount of CHIT flakes into 0.1 M acetic acid with stirring for 2 h at room temperature until complete dissolution. The Al_2O_3 colloidal suspension (2 mg mL^{-1}) was prepared by dispersing nano-Al_2O_3 in 0.5% (*w*/*v*) CHIT solution with ultrasonication for 2 h. HRP was dissolved in doubly distilled water with a concentration of 8 mg mL^{-1}. Then, the stock solution of HRP (8 mg mL^{-1}) and Al_2O_3 colloid (2 mg mL^{-1}) were mixed together at a volume ratio of 1 : 1. The obtained suspension was stored in a refrigerator at 4 °C for use.

A glassy carbon electrode (GCE) was polished on chamois leather with 0.05 μm alumina slurry, then washed successively with HNO_3 (1 : 1, v/v), ethanol and doubly distilled water in an ultrasonic bath, and dried at room temperature. After that, 6 μL of the resulting HRP/Al_2O_3/CHIT suspension was dropped on the surface of GCE and dried in a refrigerator at 4 °C overnight to form a uniform structure. For comparison with HRP/Al_2O_3/CHIT/GCE, HRP/CHIT/GCE was fabricated with the similar procedure.

Results and discussion

Principle of HRP electrode for phenolic compounds detection

The response of HRP biosensors to phenolic compounds is based on the so-called double displacement or "ping-pong" mechanism in which two substrates, a peroxide (H_2O_2 in this case) and the given phenolic compounds are involved.[27,28] The enzymatic mechanism involved in HRP based biosensor for phenolic compounds detection can be expressed as Fig. 1, which consists of the oxidation of HRP by H_2O_2 followed with its reduction by phenolic compounds.[29] Compound I (oxidation state +5) comprising a ferryl species (Fe^{4+}=O) and a porphyrin radical cation, and compound II (oxidation state +4) which is formed by the first reduction of the porphyrin radical cation, are the corresponding enzyme intermediates.[18] The last reaction converts phenolic compounds to quinones or free radicals which are electroactive and can be electrochemically reduced on electrode surface.[4] The reduction current is proportional to the concentration of phenolic compounds in PBS (pH 6.0) if adequate concentration of H_2O_2 is achieved.

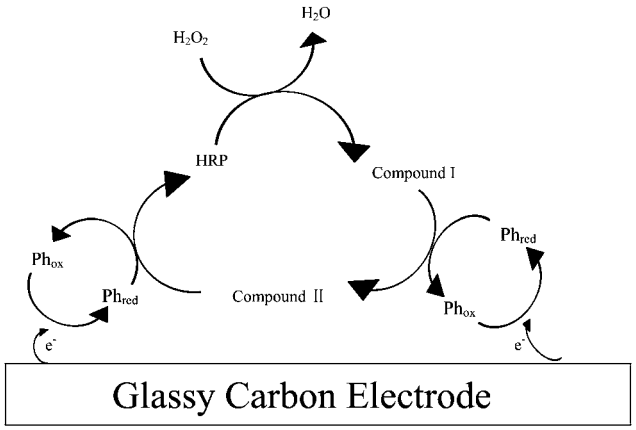

Fig. 1 Scheme of the reactions occurring at the surface of the HRP electrode for the determination of phenolic compounds. Ph_{ox} and Ph_{red} are the oxidized and reduced forms of the phenolic compounds, respectively.

Characterization of Al_2O_3, Al_2O_3/CHIT and HRP/Al_2O_3/CHIT

Fig. 2 shows TEM micrographs at different magnifications of Al_2O_3 nanoparticles (a, b) and SEM images of the electrode modified with Al_2O_3/CHIT (c) and HRP/Al_2O_3/CHIT (d). Fig. 2a and b demonstrate the rod-like morphology of Al_2O_3 nanoparticles, with lengths of about 30 nm and diameters of only a few nanometres. The surface morphologies of Al_2O_3/CHIT and HRP/Al_2O_3/CHIT composite films modified GCE were examined by SEM. As can be seen from Fig. 2c, the composite film of Al_2O_3/CHIT is uniform, indicating that Al_2O_3 nanoparticles are well dispersed in the CHIT. The SEM image of HRP/Al_2O_3/CHIT (d) confirms the presence of HRP which is adsorbed and immobilized onto the Al_2O_3 nanoparticles.

UV-Vis spectroscopy is a useful tool for conformational study of heme proteins. The position of the sensitive Soret absorption band of heme may provide information on the possible denaturation of heme proteins.[30] Fig. 3 shows UV-Vis spectra of HRP (curve a) and HRP/Al_2O_3/CHIT (curve b). The HRP exhibits an absorption peak at 403 nm, and the Soret band of HRP entrapped in the Al_2O_3/CHIT hybrid film is almost the same as that of HRP film. In the presence of Al_2O_3/CHIT, the absorption band at 403 nm shows no obvious change except the erect movement of the whole curve, which shows that the natural structure of HRP almost remains unchanged when it is

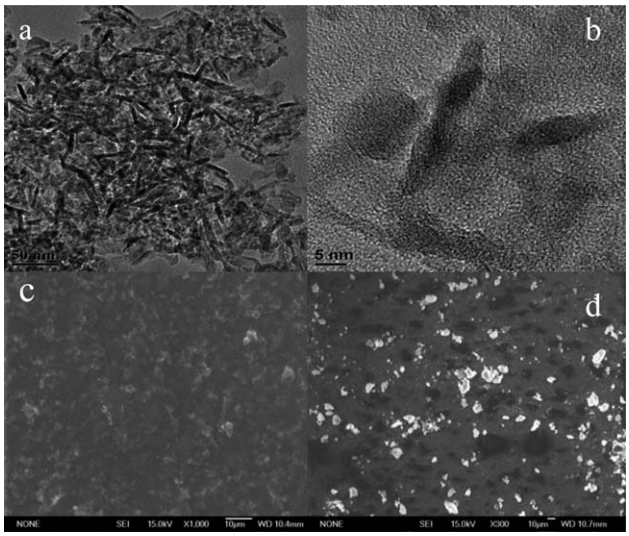

Fig. 2 TEM micrographs at different magnifications of Al_2O_3 nanoparticles (a, b) and SEM images of the electrode modified with Al_2O_3/CHIT (c) and HRP/Al_2O_3/CHIT (d).

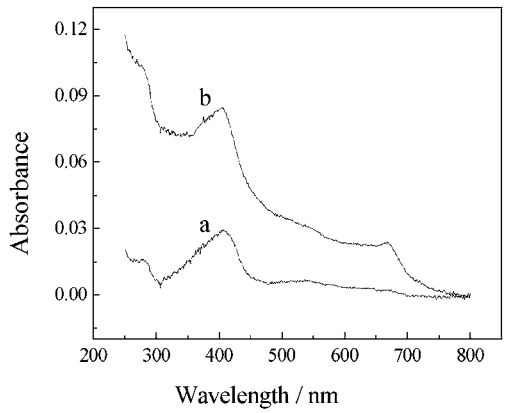

Fig. 3 UV-Vis absorption spectra of HRP (a) and HRP/Al_2O_3/CHIT (b).

immobilized in the Al_2O_3/CHIT film. So the composite system based on Al_2O_3/CHIT film has good biocompatibility for HRP.

Fig. 4 shows the FTIR spectra of HRP (a) and HRP/Al_2O_3/CHIT (b). The shapes of amides I (1700–1600 cm^{-1}) and amide II (1600–1500 cm^{-1}) infrared absorption bands of proteins provide detailed information on the secondary structure of the polypeptide chain.[31] It can be seen from Fig. 4 that the positions of the amide I and II bands of HRP and those of HRP in the Al_2O_3/CHIT film are nearly at the same place, suggesting that the HRP retains its native structure in HRP/Al_2O_3/CHIT.

Electrochemical properties of the biosensor

The electrochemical behaviors of HRP/Al_2O_3/CHIT/GCE and HRP/CHIT/GCE (inset) in various solutions were studied using cyclic voltammetry and the results are shown in Fig. 5. It can be seen from Fig. 5 that no obvious reduction peak is observed for the modified electrodes in 0.1 M PBS (pH 6.0) (curve a, a′) and in the presence of 50 μM H_2O_2 (curve b, b′). The reduction current

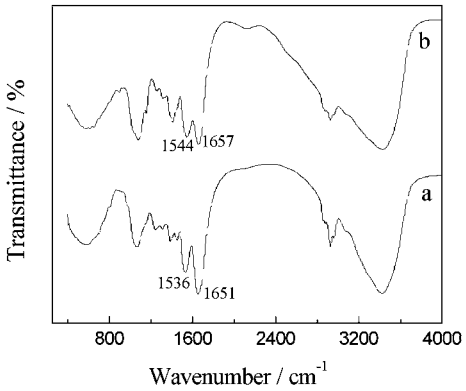

Fig. 4 FTIR spectra of HRP (a) and HRP/Al_2O_3/CHIT (b).

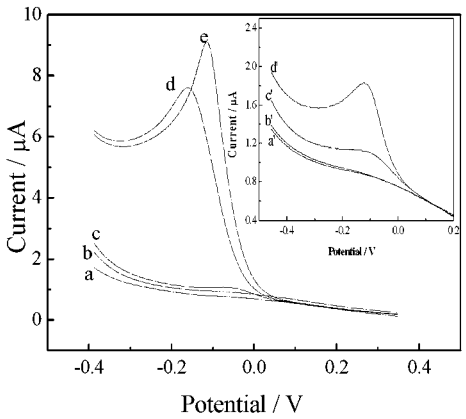

Fig. 5 Cyclic voltammograms of HRP/Al_2O_3/CHIT/GCE and HRP/CHIT/GCE (inset) recorded in the blank solution 0.1 M PBS (pH 6.0) (a, a′); in the presence of 50 μM H_2O_2 (b, b′); in the presence of 20 μM hydroquinone (c, c′); in the presence of 20 μM hydroquinone and 50 μM H_2O_2 (d, d′); in the presence of 40 μM hydroquinone and 50 μM H_2O_2 (e) at a scan rate of 0.1 V s^{-1}.

slightly increases while only hydroquinone is added (curve c, c′). Upon addition of 20 μM hydroquinone and 50 μM H_2O_2, a steep increase of reduction current is detected for HRP/Al_2O_3/CHIT/GCE (curve d), indicating that the presence of H_2O_2 is necessary for the detection of hydroquinone. However, the HRP/CHIT/GCE shows relatively low amperometric response (curve d′), indicating that the presence of Al_2O_3 nanoparticles not only facilitates electron transfer, but also remarkably improves the catalytic activity of the biosensor. The current response of HRP/Al_2O_3/CHIT/GCE increases as the concentration of hydroquinone is further increased (curve e), showing that hydroquinone is responsible for this enhanced current.

Influence of HRP amount on biosensor fabrication

The amount of the enzyme in the composite is a vital factor affecting the analytical sensitivity of the biosensor. The effect of HRP concentration on the biosensor response towards 50 μM hydroquinone is shown in Fig. 6. The current response increases as the HRP amount is increased and achieves a maximum value at 8.0 mg mL^{-1}. With HRP concentration from 8.0 to 10.0 mg mL^{-1}, the sensitivity decreases gradually. So, an optimum

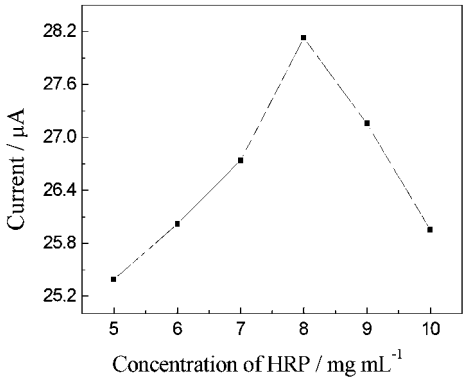

Fig. 6 Effect of HRP concentration on the biosensor response under constant hydroquinone concentration (50 μM) in 0.1 M PBS (pH 6.0) + 1500 μM H_2O_2, applied potential −0.25 V, at 25 °C.

loading of 8.0 mg mL^{-1} HRP was used in subsequent experiments.

Optimal parameters

The effect of the H_2O_2 concentration on the HRP/Al_2O_3/CHIT biosensor was studied in 0.1 M PBS (pH 6.0) containing 50 μM hydroquinone. It can be seen from Fig. 7 that the current response achieves a maximum value in the presence of 1500 μM H_2O_2, indicating that H_2O_2 concentration is very important in HRP reactions to produce good sensitivity and the presence of a high concentration of H_2O_2 can produce inhibition of the activity of HRP.[16]

The effect of pH on the biosensor response was also examined with 50 μM hydroquinone in 0.1 M PBS + 1500 μM H_2O_2 (Fig. 8). The maximum response of the biosensor occurs at pH 6.0 and the biosensor response decreases at pH > 6 or pH < 6. Therefore, 0.1 M PBS with pH 6.0 was selected for future experiments.

The effect of applied potential on the steady-state current of the biosensor is shown in Fig. 9. With the potential shifting from −0.1 to −0.25 V, the steady state current increases due to the increased driving force for the fast reduction of hydroquinone at the lower potential. The current approaches a maximum value at −0.25 V and then slowly increases. To avoid interferences and reduction of oxygen at high negative applied potential, −0.25 V was selected as the applied potential for amperometric measurement.

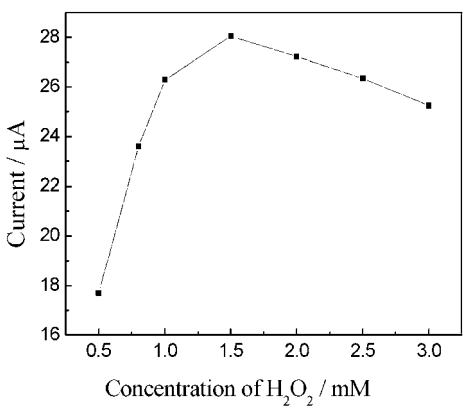

Fig. 7 Effect of the H_2O_2 concentration on the amperometric response of the biosensor to hydroquinone concentration (50 μM) in 0.1 M PBS (pH 6.0), applied potential −0.25 V, at 25 °C.

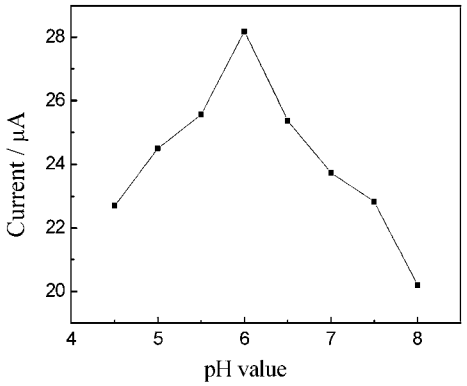

Fig. 8 Effect of pH on the amperometric response of the biosensor to hydroquinone concentration (50 μM) in 0.1 M PBS (pH 6.0) + 1500 μM H_2O_2, applied potential −0.25 V, at 25 °C.

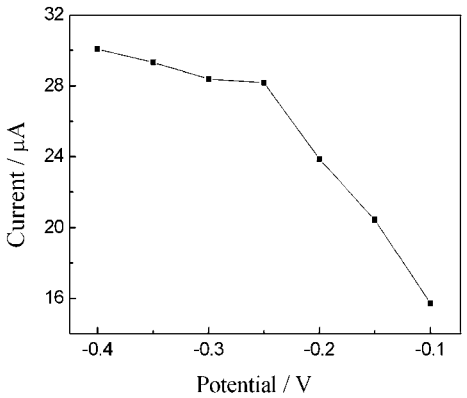

Fig. 9 Effect of applied potential on the amperometric response of the biosensor to hydroquinone concentration (50 μM) in 0.1 M PBS (pH 6.0) + 1500 μM H_2O_2, at 25 °C.

Electrocatalytic properties of HRP/Al_2O_3/CHIT/GCE

The electrocatalytic reduction of hydroquinone at the HRP/Al_2O_3/CHIT/GCE was investigated by amperometry. Upon addition of successive aliquots of hydroquinone to PBS (pH 6.0), a clearly defined reduction current proportional to the hydroquinone concentration was observed. The calibration curve under optimal conditions is shown in Fig. 10. The linear response range of the enzyme electrode to hydroquinone concentration is from 0.005 μM to 70 μM with a correlation coefficient of 0.9996 and a detection limit of 0.001 μM (based on the S/N = 3). The sensitivity is calculated to be 518.4 nA μM^{-1}. The biosensor achieves 95% of the steady-state current within 5 s.

Response characteristics of the biosensor to various phenolic compounds

Eight phenolic compounds were measured with the HRP/Al_2O_3/CHIT electrode. All phenolic compounds give fast responses (~5 s).

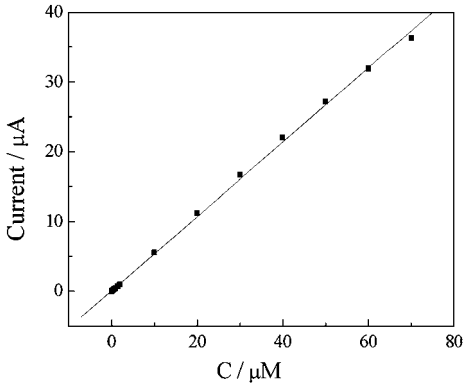

Fig. 10 Calibration curve between the catalytic current and the concentration of hydroquinone in the 0.1 M PBS (pH 6.0) + 1500 μM H_2O_2, applied potential −0.25 V, at 25 °C.

Table 1 The response characteristics of the HRP/Al_2O_3/CHIT bioelectrode to phenolic compounds

Phenolic compounds	Linear range/μM	Correlation coefficient	Sensitivity/ nA μM^{-1}	Detection limit/μM
Hydroquinone	0.005–70	0.999	518.4	0.001
Catechol	0.05–102	0.996	309.53	0.02
Resorcinol	0.5–62	0.995	36.53	0.06
Phenol	0.1–80	0.991	124.85	0.05
Acetaminophen	0.01–360	0.998	11.54	0.005
o-,*m*-,*p*-Nitrophenol		no response		

Table 1 presents the response characteristics of the HRP electrode including linear range, correlation coefficient, sensitivity and detection limit for different phenolic compounds. It is clear that response characteristics vary with the degree of substitution and position of substitution. The trend of the sensitivity is consistent with the ability of the electron-donor conjugation of the substituent. In this study, hydroquinone and catechol show the higher sensitivity because of strong ability of electron-donor conjugation of the substituent –OH. The presence of –OH structure conjugated with the aromatic ring facilitates the oxidation to the corresponding quinone. The free electron on the resulting quinone radical is then stabilized by the charge dissipation through the conjugated system. However, *m*-hydroxy of resorcinol group does not encourage the formation of the conjugation structure during resorcinol oxidation by HRP. Thus, the sensitivity is lower. Phenolic compounds with substituent NO_2 do not generate any noticeable signal upon the addition to PBS due to the electron withdrawing effect of the NO_2 group which makes them difficult to form free radical. The relative high sensitivity of hydroquinone is obtained with the HRP/Al_2O_3/CHIT biosensor (518.4 nA μM^{-1}), which is higher than those of HRP electrodes based on PAH-MWNTs (165 nA μM^{-1})[32] and carbon nanotube/polypyrrole nanobiocomposite film (8 nA μM^{-1}).[15] The sensitivity of catechol is 309.53 nA μM^{-1}, which is higher than those of polyphenol oxidase in nano-gold of 107 nA μM^{-1},[33] silica sol–gel of 59.6 nA μM^{-1},[34] and ZnO sol–gel of 166 nA μM^{-1}.[35] The high sensitivity at the HRP/Al_2O_3/CHIT based biosensor can be attributed to the biocompatible microenviroment around the enzyme.

Table 2 Recovery studies in real samples determined by the proposed biosensor[a,b]

Sample	Added hydroquinone/μM	Recovered hydroquinone[c]/μM	Recovery (%)
Tablet 1	10	9.88	98.8
Tablet 2	10	10.46	104.6
Green tea	10	10.008	100.08
River water	10	10.3	103

[a] Tablet 1: Paracetamol, Aminophenazone, Caffeine and Chlorphenamine Maleate Tablets. [b] Tablet 2: Vitamin C Yinqiao tablets. [c] An average of three determinations.

Reproducibility and stability of the biosensor

The reproducibility of the sensor was investigated at a hydroquinone concentration of 50 μM. The relative standard deviation (R.S.D.) with the same enzyme electrode is calculated to be 2.14% ($n = 7$). For the reproducibility of five enzyme electrodes, the R.S.D. is 5.09% at a hydroquinone concentration of 50 μM. When not in use, the HRP/Al_2O_3/CHIT bioelectrode is kept at 4 °C. The stability of the biosensor was also examined by intermittently measuring the current response to hydroquinone standard solution. The sensor retains 80% of its initial response to the reduction of hydroquinone after a month. These results demonstrate that Al_2O_3/CHIT hybrid film is suitable matrix for immobilization of HRP to retain its activity.

Application to sample analysis

The application of the biosensor to samples was investigated by the determination of the recoveries of hydroquinone in the samples which are "Aminophenazone, Caffeine and Chlorphenamine Maleate Tablets" (Shanghai Huashi Pharmaceutical Co., Ltd.), "Vitmin C Yinqiao tablets" (Shanghai Leiyunshang Pharmaceutical Co., Ltd.), "Green tea" (Unified Enterprise Investment Co., Ltd.) and "River water" (Shanghai University). The recovery tests for hydroquinone were performed by adding a known quantity of hydroquinone to sample solutions. The results from the sample recovery tests are summarized in Table 2, which shows satisfactory results.

Conclusions

An enzyme electrode based on the immobilization of HRP in the Al_2O_3/CHIT nanocomposite film was developed for the determination of phenolic compounds. The nanobiocomposite film provides a suitable microenvironment which could effectively retain native structure of HRP in the Al_2O_3/CHIT film. The developed biosensor exhibits fast amperometric response, high sensitivity and low detection limit for hydroquinone. This low cost Al_2O_3/CHIT composite is prospective for the development of other biosensors.

Acknowledgements

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