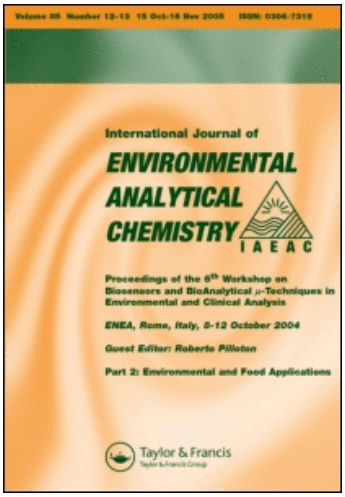

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Detection of phenylhydrazine based on lectin-glycoenzyme multilayer-film modified biosensor

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A novel inhibition-based amperometric biosensor of horseradish peroxidase (HRP) for phenylhydrazine detection is described. The multilayer-film modified sensor is prepared by a layer-by-layer deposition of concanavalin A (Con A) and HRP on glassy carbon electrode (GCE), which is forced by the bioaffinity between Con A and mannose residues on the surface of HRP molecule. Hydroquinone is chosen as electron mediator in the system. The performance of the sensor and the optimum experimental conditions are studied. The lower detection limit of the inhibition-based sensor for phenylhydrazine is 1.7×10^{-6} M, and the linear response range is divided into two parts, one of which is 1.7×10^{-6} to 1.07×10^{-5} M, and the other 1.07×10^{-5} to 6.98×10^{-5} M. The HRP sensor can be reactivated after inhibition and remains more than 91% activity after half a month of frequent running. Interference from phenol, aniline, hydroxylamine, Hg(II), Cd(II) and Bi(III) is found to be minimal, while a high concentration of hydrazine anhydrous, methylhydrazine and 1,1-dimethylhydrazine may interfere with the determination of trace phenylhydrazine. The sensor has been used in phenylhydrazine determination in water of Xiangjiang River.

Keywords: Phenylhydrazine; Horseradish peroxidase; Concanavalin A; Multilayer films; Inhibition

1. Introduction

The hydrazines constitute an important class of xenobiotic agents occurring in natural organisms, industrial settings, and medical therapeutics. Agents with a hydrazine functionality can be metabolized to radical intermediates which have toxic effects, such as carcinogenesis and haemolysis [1, 2]. Phenylhydrazine is one of the most widely distributed organic pollutants, which may irritate the eyes, the skin and the trachea, and may produce a rapid haemolysis, resulting in kidney impairment, liver impairment and total anaemia. It is produced and used in the manufacture of rocket propellant, dyes,

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pesticides and pharmaceuticals, and discharged into the environment through various waste streams. Furthermore, it is also ingested in considerable quantitites by the human population in edible mushrooms and tobacco [3]. Therefore, it is important to determine phenylhydrazine concentrations in the environment.

Heretofore, previously reported analytical methods for phenylhydrazine in water include titration [4, 5], spectrophotometry [6, 7] and several other classic techniques. Murty *et al*. used photometry and photometric titrations with cacotheline as a reagent to determine phenylhydrazine with a detection limit of 4.63×10^{-6} M [4]. Channu *et al*. applied 10-[3′-[*N*-bis(hydroxyethyl)amino]propyl]-2-chlorophenoxazine (BPCP) as a redox indicator and *N*-bromosuccinimide as an oxidant in the titration of phenylhydrazine in the concentration range of 0.0125–0.0025 M [5]. Hasan reported a spectrometric analytical method for measurement of phenylhydrazine on the basis of reduction of Cu(II) to Cu(I) by phenylhydrazine with a detection limit of 9.25×10^{-8} M, but the method was not specific for Cu(II) and could be reduced by other reducing substances [6]. Afkhami *et al*. determined phenylhydrazine based on its inhibition effect on the reaction between bromate and hydrochloric acid, which was monitored spectrophotometrically with a detection limit of 1.85×10^{-7} M [7]. However, all of these analytical methods call for expensive equipment and complicated sample pretreatment. None of them is, at present, suitable for 'on the spot' control in risk areas, nor for the continuous monitoring of phenylhydrazine levels during industrial operation. In recent years, a number of inhibition-based enzyme sensors for environmental analysis have been developed with a fast response, remarkably high sensitivity, good selectivity and low cost, which can overcome the shortage of classic analysis methods [8–12]. We have reported that a glucose oxidase biosensor can be used for detection of trace chromium(VI) in environmental samples [13].

Horseradish peroxidase (HRP) is a glycoprotein containing a single protohemin in its active site [14]. It carries out one-electron oxidation on a number of aromatic substances and single two-electron catalysis on H_2O_2 as substrate. It can bind a range of substances structurally related to phenols and aromatic amines [15, 16]. Enzyme-inhibitor studies using phenylhydrazine have revealed that the phenyl group could be incorporated into enzyme molecules at the haem periphery [15, 16]. Koga *et al*. reported that when the present concentration of phenylhydrazine reached 1 mM, the relative activity of HRP declined to zero [17]. Therefore, HRP can be a most sensitive enzyme selected for the phenylhydrazine inhibition test.

Another crucial issue is the immobilization of HRP onto the surface of a sensor with a good performance, minimizing the loss of HRP activity in sensor fabrication through partial denaturation or steric hindrance. Among various immobilization strategies, the lectin-saccharide system is a fresh protocol for enzyme immobilization, which can construct enzyme multilayer films through the specific complexation between concanavalin A (Con A) and mannose-labelled enzymes [18]. In this work, since HRP is a native glycoprotein, it can be assembled by Con A into multilayer films without any chemical modification [19, 20], and the preparation of HRP multilayer films for biosensor becomes much more convenient, minimizing the deterioration of HRP activity. In this paper, hydroquinone is selected as the electron mediator in the redox conversion, whose effect on the electrode performance is precisely analysed.

Water from Xiangjiang River is chosen as a matrix sample to test the recovery ratios of phenylhydrazine, holding great practical value. Xiangjiang River is the main receptacle for industrial and municipal wastewater in Hunan Province, China. The volume

of wastewater mostly without any treatment discharged into Xiangjiang River basin accounts for 45.6% of the total volume discharged from the whole province [21]. At the same time, it is the main water resource in Changsha City, Hunan Province. The local municipal government has attached great importance to the pollution control in the river. The results of the recovery test were found to be good.

2. Experimental

2.1 *Materials*

Lyophilized horseradish peroxidase (HRP, EC 1.11.1.7, $A > 250$ U/mg) was from Shanghai Biochemical Reagents (Shanghai, China). Concanavalin A (Con A) was obtained from Sigma. All the other chemicals used in this work were of analytical-regent grade. Double-distilled water was used throughout. The phosphate buffer solutions with 0.07 M KH_2PO_4 and 0.07 M Na_2HPO_4 were used in this work. An HRP solution (60 mg/mL) and a Con A solution (250 μg/mL) were obtained by dissolving HRP and Con A in phosphate buffer (pH 7.38), respectively. An H_2O_2 stock solution (0.1 M) was obtained by dilution of 30% H_2O_2 in water. A hydroquinone aqueous solution (0.1 M) was prepared daily. The stock solutions of phenylhydrazine hydrochloride, hydroxylamine hydrochloride, phenol, aniline, hydrazine anhydrous, methylhydrazine and 1,1-dimethylhydrazine (0.1 M) were prepared daily using phosphate buffer (pH 6.81) as solvent, and the working standard solutions of 0.01 M and 1 mM were prepared by successive dilution in phosphate buffer (pH 6.81). $Hg(NO_3)_2$, $Cd(NO_3)_2$ and $Bi(NO_3)_3$ were dissolved in 0.3% HNO_3 to prepare stock solutions of 0.01 M, 0.01 M and 1 mM, respectively.

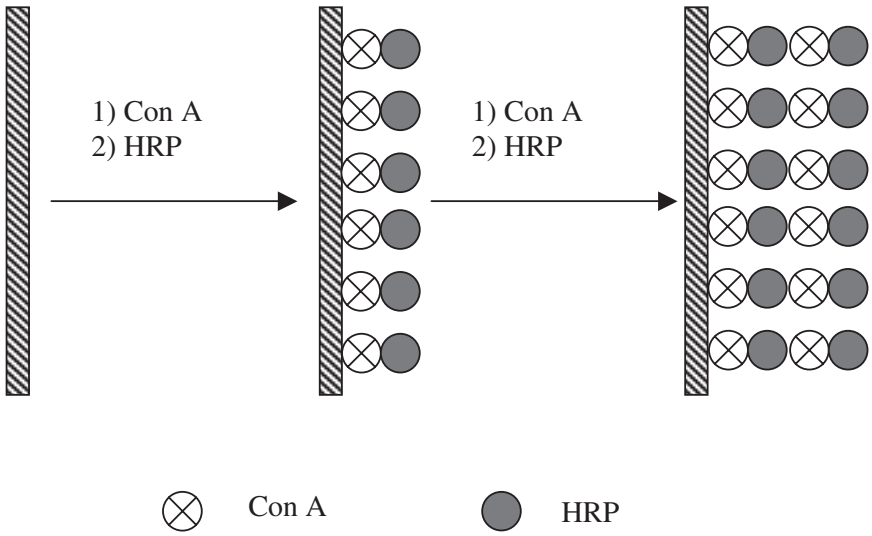
Water from Xiangjiang River was collected 1 m below the surface in the middle of the river in the reach of Changsha City. Then, the pH of river water was adjusted to 6.81 by phosphate buffer.

2.2 *Apparatus*

Electrochemical measurements were carried out on a VMP2 Multichannel Potentiostat (Princeton Applied Research, USA) and Model XJP-821 polarographic analyser (Jiangsu Electroanalytical Instruments, China). The three-electrode system used in this work consists of a glassy carbon electrode (GCE, 3 mm diameter) as the working electrode of interest, a saturated calomel electrode (SCE) as the reference electrode and a Pt foil auxiliary electrode. All work was done at room temperature (25°C) unless otherwise mentioned.

2.3 *HRP sensor fabrication*

The enzyme sensors were prepared by covering the GCE surface with Con A-enzyme films. The GCE was polished thoroughly with 0.5 μm alumina paste, sonicated in 1:1 (v/v) nitric acid, acetone and water successively, and rinsed with water before use. Then, the GCE was immersed in a Con A solution (250 μg/mL) for 30 min, rinsed with phosphate buffer (pH 7.38) to remove the weak adsorptive, and dried in the air. A 5 μL aliquot of the HRP solution was pipetted onto the electrode surface and allowed



Scheme 1. Construction of enzyme multilayers films on GCE using Con A and enzyme.

to dry in the air, and then the electrode was rinsed with phosphate buffer (pH 7.38). The fabrication steps were repeated to prepare the multilayer films. The procedure for constructing the Con A-enzyme multilayer film is shown in Scheme 1 [20]. When not in use, the multilayer enzyme electrode was rinsed with phosphate buffer (pH 7.38), and kept at 4°C in a refrigerator.

2.4 *Measurement procedure*

The cyclic voltammetry was performed between −1.0 and +0.6 V vs SCE at 100 mV/s to estimate the catalytic activity of the HRP electrode. Then, the chronoamperometry was carried out to calibrate the H_2O_2 concentration using HRP four-layer modified GCE in 10 mL of phosphate buffer (pH 6.81) containing 1 mM hydroquinone at −0.14 V vs SCE. These conditions were found to be the optimum conditions for the HRP electrode.

The inhibition study was performed under the optimum conditions for the best performance of the HRP electrode [22]. The sensor was dipped in phosphate buffer (pH 6.81) containing 0.5 mM H_2O_2: this value was selected as it represented the maximum biosensor response to H_2O_2 in the linear range. After the reduction current at −0.14 V reached a steady state (I_s) within 1 min, aliquots of standard phenylhydrazine solutions were subsequently added, decreasing the current as a function of phenylhydrazine concentration. The percentage of inhibition was calculated as $100 \times (I_s - I_i) / (I_s - I_0)$, where I_0 was the initial background current, and I_i was the steady-state current after addition of a determinate amount of phenylhydrazine. Several organic compounds structurally related to phenylhydrazine and inorganic ions as interferents were measured under the same conditions as phenylhydrazine calibration. After each inhibition, the working electrode was soaked in phosphate buffer (pH 7.38) for 30 min to be reactivated. Then, 10 mL of water from Xiangjiang River (pH 6.81) was used as the blank solution to measure the recovery ratios

of phenylhydrazine concentration added under the same conditions as phenylhydrazine calibration. Each calibration experiment was done three times to obtain the mean value.

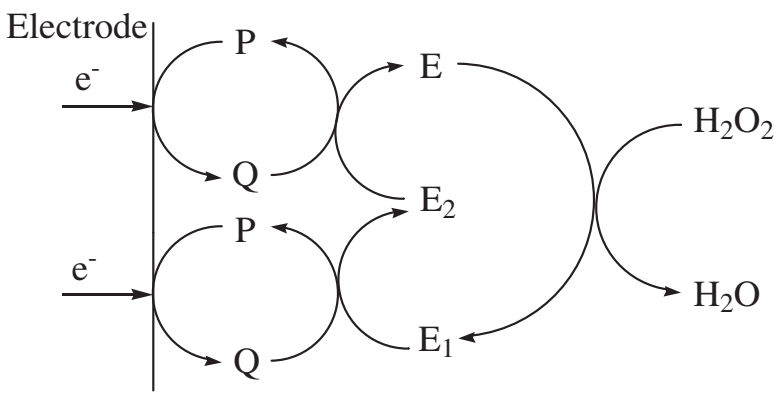
3. Results and discussion

3.1 *Studies of the redox mechanism and the effect of hydroquinone concentration*

The complete redox process including electron mediation on the working electrode is shown in Scheme 2 [23]. E is the native ferriperoxidase (Fe^{III}), E_1, oxyferryl π-cation radical haem compound I ($[Fe^{IV}{=}O]^{\bullet+}$, iron formal oxidation state: +V), E_2, the oxyferryl compound II ($[Fe^{IV}{=}O]$, iron formal oxidation state: +IV), Q, hydroquinone, and P, the hydroquinone cation radical. The first step is a rapid oxygen atom transfer from hydrogen peroxide to the ferric porphyrin (Fe^{III}) of HRP (formally a $2e^-$ oxidation) to form E_1 and water. In the second step, the porphyrin radical cation of E_1 is reduced by the one-electron donor Q to yield E_2 and P. In the last step, the enzyme is converted back to its native resting state, E, by a subsequent one-electron/two-proton reduction of E_2 by Q to give a second equivalent of P and water, and P is reduced on the working electrode to yield detectable electrochemical signals.

Figure 1 shows the shape of cyclic voltammetric curves of HRP three-layer modified GCE at different scan rates. There is a linear relation between the reduction peak currents and the square roots of scan rate, indicating that the catalytic current is diffusion-controlled, and confirming that hydroquinone does not adsorb on the working electrode surface. In the absence of electron mediator, no cathodic or anodic peak is observed in cyclic voltammetric studies of the HRP electrode, indicating the inefficiency of the direct electron transfer between the enzyme and electrode surface (figure 2). With the increase in hydroquinone concentration, the cathodic peak current increased, confirming that hydroquinine effectively shuttles electrons from GCE surface to HRP.

In order to obtain the optimum hydroquinone concentration in H_2O_2 calibration, the changes in reduction peak current with addition of H_2O_2 in the working electrolyte



Scheme 2. Schematic diagram of the redox reactions on the working electrode.

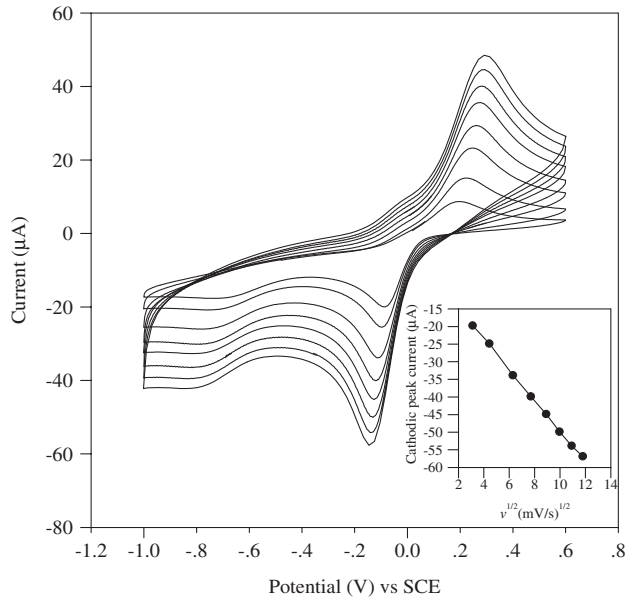


Figure 1. Cyclic voltammetric curves of HRP three-layer modified GCE in phosphate buffer (pH 6.98) containing 1 mM H_2O_2 and 1 mM hydroquinone at various scan rates (from inner to outer curves: 10, 20, 40, 60, 80, 100, 120 and 140 mV/s). Inset: plot of cathodic peak current vs the square root of scan rate ($v^{1/2}$).

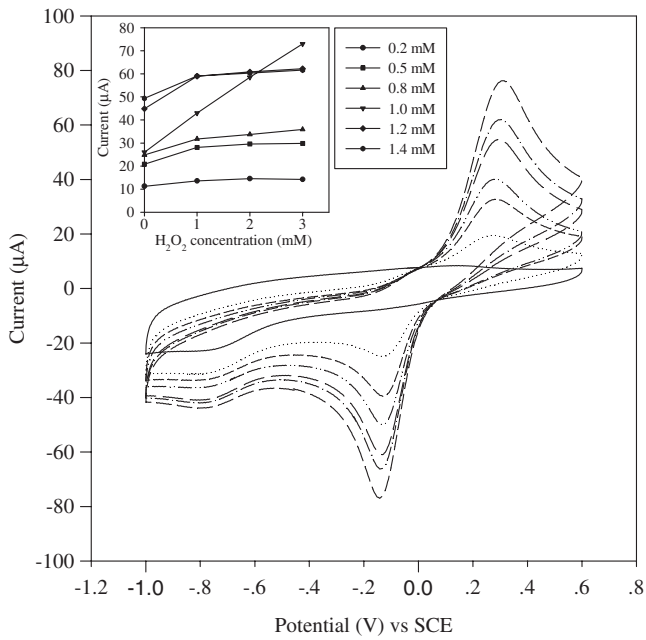


Figure 2. Cyclic voltammetric curves of HRP three-layer modified GCE in phosphate buffer (pH 6.98) containing 1 mM H_2O_2 and various hydroquinone concentrations at 100 mV/s (from inner to outer curves: 0, 0.4, 0.8, 1.0, 1.4, 1.6 and 2.0 mM hydroquinone). Inset: plot of the changes of absolute value of reduction peak current with addition of H_2O_2 in working electrolyte containing different hydroquinone concentrations.

containing different hydroquinone concentrations are also compared in figure 2. It is illustrated that the system is most sensitive for H_2O_2 detection while containing 1 mM hydroquinone.

3.2 *Performance of the Con A-HRP multilayer films*

Con A is a lectin protein containing four identical binding sites to α-D-mannose. Therefore, we can expect specific complexation between Con A and mannose residues intrinsically existing on the surface of HRP. This is the driving force of the formation of the multilayer films. Since Con A forms a monomolecular layer on the surface of GCE, the thickness of a layer of Con A should be the average molecular dimensions of Con A depending on the orientation of Con A molecule on the surface. Similarly, the thickness of a monomolecular layer of HRP should be the average molecular dimensions of HRP [19]. The molecular dimensions of Con A are reported to be $6.2 \times 8.6 \times 8.9$ nm [24], and the molecular dimensions of HRP are reported to be $4.0 \times 6.7 \times 11.7$ nm [25]. Therefore, the average thickness of each layer of Con A-HRP is calculated to be about 15.4 nm.

Figure 3 shows the cyclic voltammetric curves obtained on a bare GCE and HRP multilayer films modified GCEs in the presence of H_2O_2 and hydroquinone. For the bare GCE, there are obvious oxidation and reduction peaks of hydroquinone in the working electrolyte at $+0.28$ and -0.13 V vs SCE, respectively. For the HRP multilayer films' modified GCEs, the reduction peak is enhanced obviously as the number of HRP layers increases up to four and shifted in the negative potential direction, while the

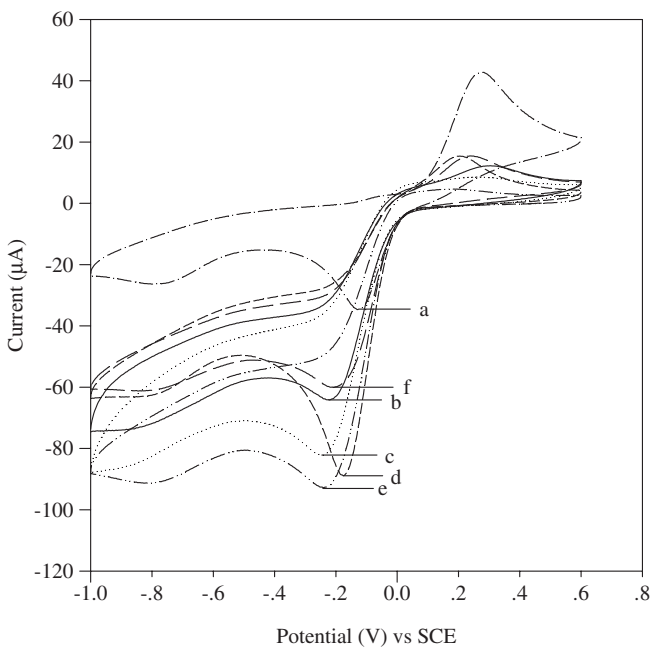


Figure 3. Cyclic voltammetric curves obtained on bare and modified GCEs in the presence of 5 mM H_2O_2 and 1 mM hydroquinone in phosphate buffer (pH 6.98) at 100 mV/s. Electrode: (a) unmodified GCE, (b) HRP monolayer, (c) two-layer, (d) three-layer, (e) four-layer, (f) five-layer modified GCE.

oxidation peak almost disappears. Such behaviour is a characteristic of an efficient catalysis enhanced with the increasing number of HRP layers. However, when the number of HRP layers reaches five, the reduction peak drops in that the modification membrane of GCE is too thick for electron conduction. The HRP four-layer modified GCE was found to be the most sensitive for H_2O_2 detection among all through the comparison of system sensitivity for H_2O_2 detection on HRP multilayer films modified GCEs.

3.3 *Determination of H_2O_2*

The pH effect on the sensitivity of the HRP electrode for H_2O_2 detection over the pH range of 6.47–7.48 in phosphate buffer containing 1 mM hydroquinone was investigated, showing that pH 6.81 was the optimum pH value in the H_2O_2 calibration experiment.

In the precise cyclic voltammetric studies of the HRP electrode in the working electrolyte shown in figure 4, it was found that the reduction peak increased and shifted in the negative potential direction, while the oxidation peak decreased upon raising the H_2O_2 concentration, indicating an efficient catalysis and a strong substrate consumption in the multilayer films. To detect minute H_2O_2 concentrations, the reduction peak potential of −0.14 V at low H_2O_2 concentrations is typically chosen as the applied potential in chronoamperometry.

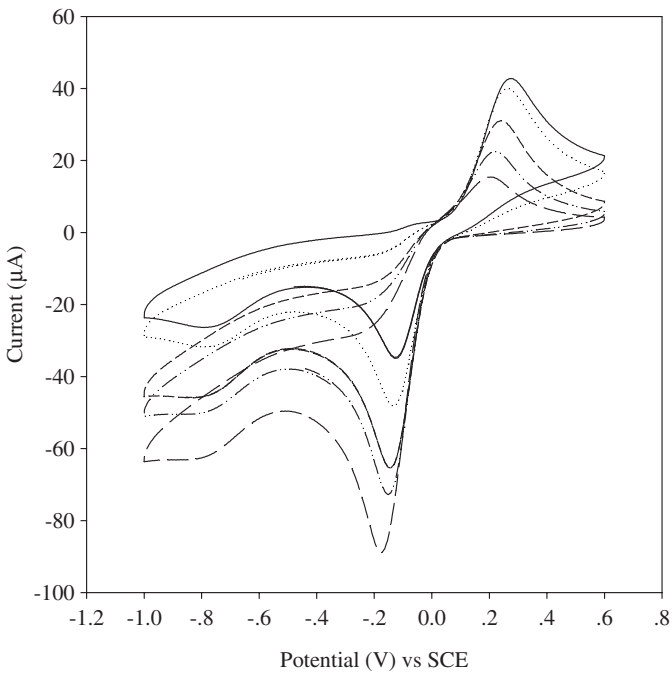


Figure 4. Cyclic voltammetric curves of HRP three-layer modified GCE in phosphate buffer (pH 6.98) containing 1 mM hydroquinone and various H_2O_2 concentrations at 100 mV/s (from upper to lower curves: 0, 1.0, 2.0, 3.0 and 4.0 mM H_2O_2).

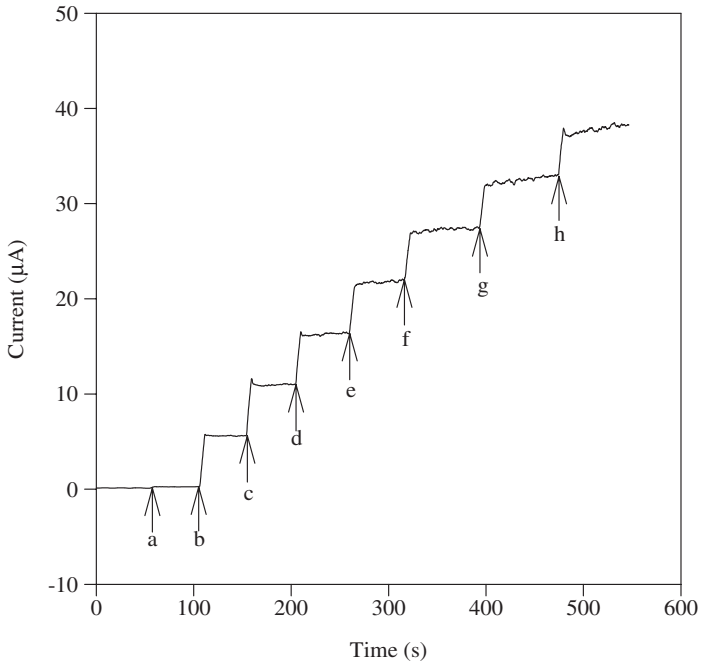


Figure 5. Current change on HRP four-layer modified GCE for successive additions of 1 mM hydroquinone (a), 0.1 mM H_2O_2 (b–h) to phosphate buffer (pH 6.98) measured by chronoamperometry at −0.2 V vs SCE.

Figure 5 shows the cathodic current change with the addition of H_2O_2 to phosphate buffer (pH 6.81) measured by chronoamperometry at a potential of −0.14 V. The change in current reaches a steady state in less than 1 min at low H_2O_2 concentrations due to the catalytic reaction.

A plot of the current change vs H_2O_2 concentration by chronoamperometry is shown in figure 6. The current change increases linearly with the H_2O_2 concentration in the range of 2×10^{-7} to 5.0×10^{-4} M. The corresponding regression equation is

$$\Delta I = 0.32 + 53.35C,$$

where ΔI is the current change (μA), C is the H_2O_2 concentration (mM), and the coefficient is 0.9986. The lower detection limit is 2×10^{-7} M. Five replicative determinations of a 1.0×10^{-5} M H_2O_2 solution give a standard deviation of 3.15% for a mean current change value of 0.771 μA.

3.4 *Comparison of HRP sensors with different immobilization methods*

Table 1 compares the Con A-HRP multilayer-film modified sensor and some other HRP sensors in the literature with different immobilization methods in H_2O_2 detection. It can be inferred that the analytical sensitivity of the sensor fabricated in this work is superior.

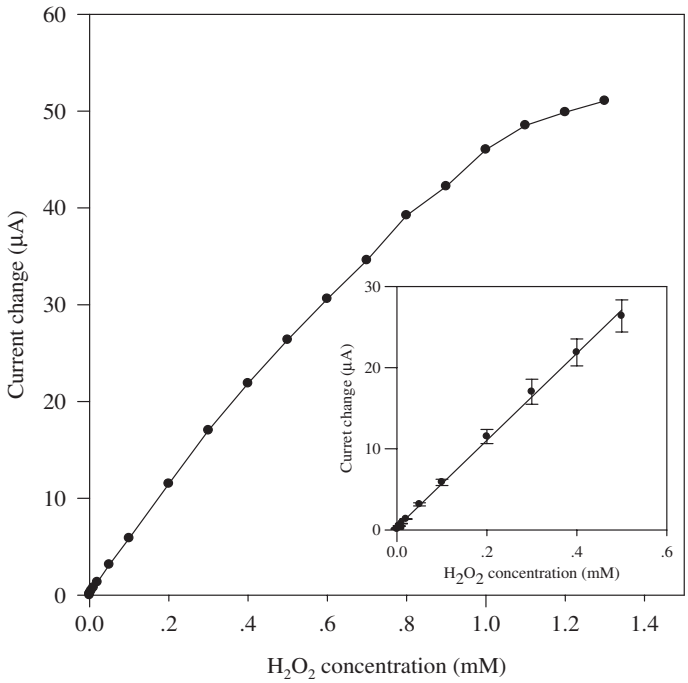


Figure 6. Calibration curves of HRP four-layer modified GCE in phosphate buffer (pH 6.81) containing 1 mM hydroquinone by chronoamperometry at −0.14 V vs SCE. Inset: linear regression of current change vs H_2O_2 concentration. The vertical bars designate the standard deviations for the mean of three replicative tests.

Table 1. Comparisons of HRP sensors with different immobilization methods in H_2O_2 detection.

Immobilization method	Linear range (M)	Lower detection limit (M)	Sensitivity ($A/M/cm^2$)	Reference
Con A-HRP multilayer-film modified GCE	2×10^{-7} to 5.0×10^{-4}	2×10^{-7}	0.758	This paper
Biotin-avidin/HRP monolayer modified screen-printed electrode	Not presented	2×10^{-7}	0.88	[26]
Nano-Au/HRP monolayer modified chitosan-entrapped carbon-paste electrode	1.22×10^{-5} to 2.43×10^{-3}	6.3×10^{-6}	0.013	[27]
HRP/kieselguhr/PVA modified pyrolytic graphite electrode	2.0×10^{-6} to 6.5×10^{-4}	1.0×10^{-6}	0.512	[28]

3.5 *Phenylhydrazine calibration by chronoamperometry*

According to the inhibitory effect of phenylhydrazine on the catalysis of HRP, H_2O_2 reduction will be reduced so that the response current will decline. In this work, the degree of inhibition of the HRP sensor has a linear relationship with the natural logarithm of phenylhydrazine concentration. Since the lower detection limit should

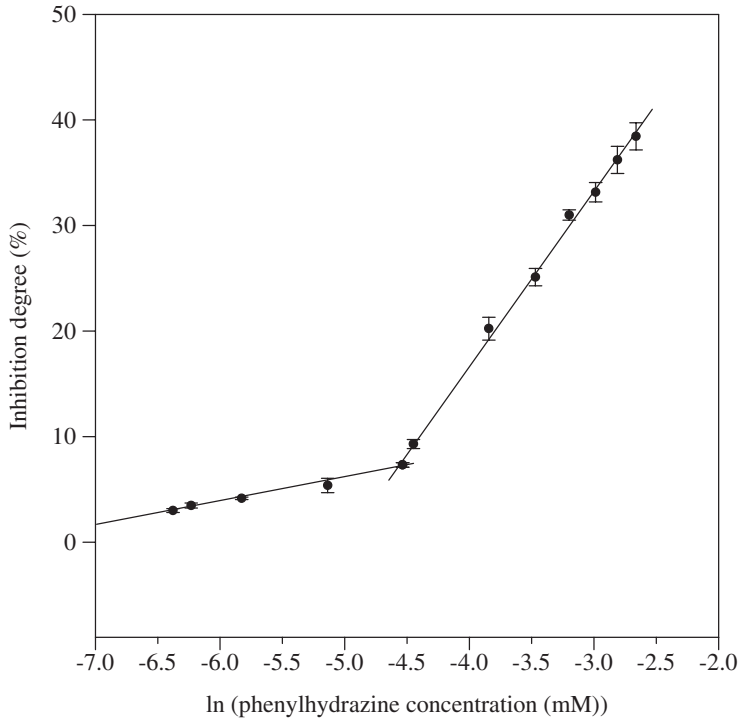


Figure 7. Linear regression of inhibition degree on HRP four-layer modified GCE vs the natural logarithm of phenylhydrazine concentration of 1.7×10^{-6} to 1.07×10^{-5} M and 1.07×10^{-5} to 6.98×10^{-5} M in phosphate buffer (pH 6.81) containing 0.5 mM H_2O_2 and 1 mM hydroquinone by chronoamperometry at −0.14 V vs SCE. The vertical bars designate the standard deviations for the mean of three replicative tests.

return the inhibitor concentration, which results in more than 3% of inhibition degree [29], here 1.7×10^{-6} M was chosen as the lower detection limit, which leads to 3% inhibition. The linear range can be divided into two parts shown in figure 7, one of which is 1.7×10^{-6} to 1.07×10^{-5} M, and the regression equation is

$$I\% = 17.292 + 2.2522C,$$

where $I\%$ is the inhibition degree, C is the natural logarithm of phenylhydrazine concentration (mM), and the coefficient is 0.9854.

The other is 1.07×10^{-5}–6.98×10^{-5} M, and the regression equation is

$$I\% = 82.82 + 16.519C,$$

with the coefficient of 0.9974. Since each of the calibrations is done three times, and the standard deviations of inhibition degree are no more than 1.3%, the stability and reproducibility of the inhibition electrode are guaranteed. It can be inferred that the sensitivity of the HRP sensor bears comparison with the reported classic analytical methods for phenylhydrazine.

Table 2. Interference effects of other structurally related compounds and inorganic ions under the same conditions as the calibration of phenylhydrazine.

Interferent	Concentration (mM)	Inhibition degree (%)	Phenylhydrazine concentration holding the same inhibition degree
Phenylhydrazine	0.07	38.44	0.0700
Hydroxylamine	0.07	2.05	0.0011
Phenol	0.07	Not detected	Not detected
Aniline	0.07	Not detected	Not detected
Hg(II)	0.07	Not detected	Not detected
Cd(II)	0.07	Not detected	Not detected
Bi(III)	0.07	1.33	0.0008
Hydrazine anhydrous	0.03	2.90	0.0017
	0.07	11.11	0.0130
Methylhydrazine	0.03	2.84	0.0016
	0.07	8.25	0.0109
1,1-Dimethylhydrazine	0.03	3.07	0.0018
	0.07	12.99	0.0145

3.6 *Interference*

As we mentioned earlier, the phenyl group could be incorporated into the HRP molecule, so all of the phenylhydrazine, phenol, and aniline could bind on the HRP sensor. However, it is found that only phenylhydrazine with a hydrazine functionality will inhibit the HRP activity, shown in table 2. Interference with phenylhydrazine detection from hydroxylamine, Hg(II), Cd(II) and Bi(III) is also found to be minimal. However, hydrazine anhydrous, methylhydrazine and 1,1-dimethylhydrazine at 0.07 mM lead to 11.11%, 8.25% and 12.99% inhibition of the enzyme sensor, respectively, which may interfere with the determination of trace phenylhydrazine in environmental samples; hence, pretreatment to remove these three compounds is needed. It can be seen, therefore, that hydrazine functionality may inhibit HRP activity, but the inhibition effect may be drastically intensified when the compound is incorporated into molecular HRP at the haem periphery from the fact that the inhibition effect of phenylhydrazine on the HRP sensor is remarkably beyond that of hydrazine anhydrous, methylhydrazine and 1,1-dimethylhydrazine.

3.7 *Reversibility and stability*

The reversibility and stability of the HRP multilayer films are necessary for a precise quantitative study of an enzyme sensor. In this work, the inhibition of the HRP sensor by phenylhydrazine and other interferents is reversible. The response time is usually less than 2 min, so no incubation is required. A typical inhibition assay is illustrated in figure 8. In chronoamperometry experiments, as the current response to H_2O_2 reaches a steady state, 0.02 mM phenylhydrazine is directly added into the working electrolyte, which induces a current decline. Then, after reactivation, soaking in phosphate buffer (pH 7.38) for 30 min, the reduction current on the electrode increases again in electrolyte containing the same H_2O_2 concentration, and the inhibition test is repeated.

The stability of the HRP sensor, gauged by following its amperometric responses to 0.5 mM H_2O_2 in phosphate buffer (pH 6.81), was found to be remarkably good,

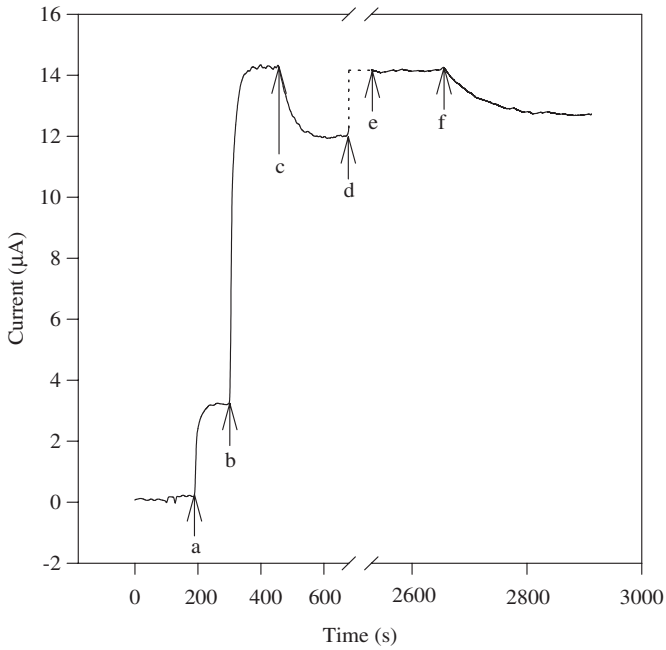


Figure 8. Chronoamperometry measurement of HRP two-layer modified GCE in phosphate buffer (pH 6.81) containing 1 mM hydroquinone at −0.14 V after the following stepwise changes: additions of 0.05 mM (a) and 0.20 mM (b) of H_2O_2 and 0.02 mM phenylhydrazine (c) into the electrolyte, immersing into phosphate buffer (pH 7.38) (d), again immersing into the electrolyte containing 0.25 mM H_2O_2 (e) and addition of 0.015 mM phenylhydrazine (f) into it.

Table 3. Results of the recovery test in water from Xiangjiang River as matrix.

Concentration added (mM)	Average concentration recovered (mM)	Recovery (%)
2.00×10^{-3}	$(2.13 \pm 0.40^{a}) \times 10^{-3}$	106.41
6.00×10^{-3}	$(6.22 \pm 0.32) \times 10^{-3}$	103.73
7.00×10^{-3}	$(6.78 \pm 0.51) \times 10^{-3}$	96.81
1.20×10^{-2}	$(1.10 \pm 0.20) \times 10^{-2}$	91.89
2.20×10^{-2}	$(2.09 \pm 0.27) \times 10^{-2}$	94.83

[a] Average values and standard deviations of three replicates.

with more than 91% of its original value remaining after at least 15 days of frequent running. During the 15 days, we performed inhibition tests on the HRP sensor 108 times.

3.8 *Recovery test in water from Xiangjiang River*

The environmental applicability of the HRP sensor was assessed by the determination of phenylhydrazine in water of Xiangjiang River. A certain amount of phenylhydrazine was added to river water to test the recovery ratios. The results are shown in table 3, with the average recovery ratio of 98.73%.

4. Conclusions

An inhibition-based enzyme sensor has been developed for the detection of phenylhydrazine on the basis of the inhibition of HRP. This HRP sensor is easily fabricated by the specific complexation between Con A and HRP to form multilayer films. Hydroquinone efficiently shuttles electrons between the HRP redox centre and the GCE surface. Optimized experimental conditions for the operation of the sensor have been studied. A superior sensitivity, stability and reusability are all achieved, with obvious advantages for phenylhydrazine inhibition studies. The performance of this sensor in H_2O_2 and phenylhydrazine detection is better than that of previous reports. Interference from phenol, aniline, hydroxylamine, Hg(II), Cd(II) and Bi(III) was found to be minimal, while high concentrations of anhydrous hydrazine, methylhydrazine and 1,1-dimethylhydrazine may interfere with the determination of trace phenylhydrazine; hence, a pretreatment to remove these three compounds is needed. The determination of phenylhydrazine in a real sample is in good agreement with the declared concentration. All of these observations clearly illustrate that this HRP sensor has great potential in further studies of environmental analysis in 'on the spot' monitoring for trace phenylhydrazine.

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