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Trans. Nonferrous Met. Soc. China 19(2009) 235−240
Transactions of Nonferrous Metals Society of China
www.tnmsc.cn

Determination of trace mercury in compost extract by inhibition based glucose oxidase biosensor

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Received 2 January 2008; accepted 31 March 2008

Abstract: A novel inhibition based biosensor of glucose oxidase(GOx) for environmental mercury detection was developed. An electropolymerized aniline membrane was prepared on a platinum electrode containing ferrocene as electron transfer mediator, on which GOx was cross-linked by glutaraldehyde. The response of the sensor was based on the current reduction in the electrochemical system by inhibition of mercury against GOx electrode. The detection limit of the inhibition-based sensor for mercury is 0.49 μg/L, and the linear response ranges are 0.49−783.21 μg/L and 783.21 μg/L−25.55 mg/L. The GOx membrane can be completely reactivated after inhibition, and remains 70% of the activity in more than one month. The sensor was used for mercury determination in compost extract with good results.

Key words: mercury; inhibition; glucose oxidase; biosensor; compost

1 Introduction

Mercury is a poisonous pollutant in the natural environment, and discharged from the industrial and domestic waste into the environment as inorganic mercury ions, transformed by biological process into other forms, causing serious contamination of the ecological system[1]. The conventional detection methods for mercury include cold-vapor atomic absorption spectrophotometry, potassium permanganate-potassium persulfate digestion-dithizone spectrophotometry, cold atomic fluorescent spectrophotometry, etc. These methods call for expensive equipment and complicated sample pretreatment which make them unsuitable for "on the spot" control. Growing attention is currently devoted to the study of biosensor in environmental analysis owing to its advantages, such as screening of various contaminants in environmental matrices, minimizing the sample pretreatment, reducing cost and time of analysis, and displaying sufficient sensitivity and selectivity[2−4]. Glucose oxidase(GOx) is an ideal enzyme for studies of inhibition due to its low cost, good stability and high specific activity, which can be potentially inhibited by some heavy metals[5−7]. With the addition of inhibitors into the electrolyte, the current response of the GOx sensor decreases in the presence of its substrate glucose. The detection limit can be as low as 10^{-8}−10^{-11} g/L. Hg^{2+} is a typical inhibitor of GOx, existing in acidic environment with low pH value, combining with some region in the active centre of GOx to inhibit the enzyme activity[8]. DONLAN et al[9] reported the development of an inhibition based GOx biosensor applied in flow injection analyses of Hg^{2+}. Limited by the adaptive pH values among 4−7, the lower detection limit could only be 2 mg/L. ALEXANDER and RECHNIZ[5] proposed an amperometric GOx biosensor with self-assembly of 2-amino-ethanethio on the gold electrode cross-linked with GOx and coated by polyvinyl-pyrrolidone(PVP) membrane, using sulphydryl as electron transfer media. Its lower detection limit was 0.2 μg/L. But the amperometric current response decreased due to the reaction between Hg^{2+} and sulphydryl on 2-amino-ethanethio, not the inhibition of enzyme[5].

In this work, the aim is to develop a novel

Foundation item: Project(50608029) supported by the National Natural Science Foundation of China; Project(2004AA649370) supported by the Hi-tech Research and Development Program of China; Project(2005CB724203) supported by the National Basic Research Program of China; Project(IRT0719) supported by the Program for Changjiang Scholars and Innovative Research Team in University of China
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DOI: 10.1016/S1003-6326(08)60258-7

amperometric inhibition based GOx sensor to determine trace Hg^{2+}. The immobilization of GOx integrated traditional electrochemical polymerization with self-assembly technique. Aniline was chosen to be electropolymerized on the electrode surface with a polymer membrane containing ferrocene as electron transfer media. GOx was cross-linked by glutaraldehyde(GA) on the polymer membrane. The polyaniline(PA) membrane could prevent the enzyme molecular from leaking out and hold back the penetration of some interferents in environmental samples, which added to the sensor's stability and selectivity[10]. The cross-linking by glutaraldehyde could stabilize the steric structure of enzyme molecular and avoided enzyme denaturalization under relatively severe conditions[11]. Therefore, the performance of this sensor in both glucose detection and Hg^{2+} detection was shown to be better compared with previous reports. Compost extract was chosen as a matrix to test the recovery ratios of Hg^{2+}.

2 Experimental

2.1 Materials

GOx was from Sigma, EC 1.1.3.4 from Aspergillus Niger, with activity of 196 units/mg. *β*-D-glucose was from ICN Biomedicals. Other chemicals were of analytical reagent grade. Double-distilled water was used throughout the work. The phosphate buffer solutions of 0.066 7 mol/L KH_2PO_4 and 0.066 7 mol/L Na_2HPO_4, and the acetate buffer solutions of 0.2 mol/L HAc, 0.2 mol/L NaAc and 1 mol/L HAc were used. A 0.5 mol/L glucose solution was obtained by dissolving *β*-D-glucose in phosphate buffer (pH 7.00). Hg^{2+} ions were dissolved in 0.3% HNO_3 to prepare stock solutions of 1 g/L. Then the working standard solutions of 50 mg/L and 1 mg/L were prepared by successive dilution of the stock solutions by acetate buffer (pH 2.55).

2.2 Apparatus

Cyclic voltammetric measurement and ampero-metric measurement were carried out on PAR 273 potentiostat/galvanostat and model 270 software (EG&G Princeton Applied Research, Princeton, NJ, USA), and Model XJP−821 polargraphic analyzer (Jiangsu Electroanalytical Instruments, Jiangsu, China). Agilent 3510 atomic absorption spectrophotometer (Agilent Technologies, Shanghai, China) was used for detection of heavy metals. The three-electrode system used in this work consisted of a Pt electrode (of 3.14×10^{-4} cm^2 planar area) as working electrode of interest, a saturated calomel electrode(SCE) as reference electrode and a Pt foil auxiliary electrode.

2.3 Compost extract preparation

The composting process has been introduced previously[12]. The components of compost were soil, straw, scraps and bran, and the water fraction was 51%. The soil was collected from 100 cm underground on the unfrequented hillside of Yuelu Mountain (Changsha, China), from which large organic scraps were removed. Then aerobic compost was managed for 40 d at 30℃ and 0.033 m^3/h of ventilation. 10 g compost sample was placed in a flask and 200 mL water was added in. The suspension was agitated on a mechanical vibrator at 200 r/min for 2 h. The supernatant was centrifuged at 10 000 r/min for 5 min, and then filtered to get the filtrate as the compost extract. The dosage of Hg^{2+} into each compost extract was controlled.

2.4 GOx sensor fabrication

The working Pt electrode with 200 μm in diameter was pretreated according to Ref.[13]. 5 mL phosphate buffer (pH 7.00) containing 0.2 mol/L aniline and 5 mL 12 mmol/L ferrocene (Fc) ethanolic solution were mixed into a water/ethanol (1:1 volume ratio) suspension[14]. Then cyclic voltammetry between 0 and 1.6 V at 10 mV/s was performed on the Pt electrode (vs SCE) in the mixture for 10 cycles. The resulting PA/Fc membrane was brown in color. The Pt/PA/Fc electrode was soaked in 2.5% (volume fraction) glutaraldehyde aqueous solution at 37℃ for 30 min, and then rinsed with water and dried in the air. 2 μL aliquot of the 60 mg/L GOx solution (equivalent to 23.52 unit of the enzyme) was pipetted onto the surface of electrode and dried in the air, and then rinsed with phosphate buffer (pH 7.00) to remove non-covalent adsorptive. The Pt/PA/ Fc/GA/GOx electrode was kept in phosphate buffer (pH 7.00) at 4 ℃ in refrigerator when it was not in use.

2.5 Measurement

The cyclic voltammetric scans were performed between 0 and 1.1 V at 10 mV/s (vs SCE) in phosphate buffer (pH 7.00) containing different concentrations of glucose. The peak voltage 0.7 V was typically chosen as the optimum voltage for amperometric measurement. For inhibition studies, aliquots of standard Hg^{2+} solutions were pipetted into buffer solutions containing glucose in amperometric measurement at 0.7 V, plotting the change in current as a function of Hg^{2+} concentration. The optimum pH and substrate concentration were found. After each inhibition, the working electrode was soaked in phosphate buffer (pH 7.00) to be reactivated for 8 min. The compost extract was analyzed by atomic absorption spectrophotometry to confirm that the background concentrations of Cr(total), Pb(Ⅱ), Cu(Ⅱ), Cd(Ⅱ) were

0, 20, 0 and 0 μg/L, respectively, according to the national standard[15−17]. Then a 10 mL compost extract was used as the blank solution to measure the recovery ratio of Hg^{2+} concentration added. Each calibration experiment was done three times to obtain the mean value.

3 Results and discussion

3.1 GOx biosensor characteristics

The pH value of the working solution is usually regarded as the most important impact factor in the performance of the enzyme electrode and its sensitivity for substrate and inhibitor. The pH range of an enzyme electrode often shifts from the pH limits of the dissolved enzyme activity for many reasons, such as the immobilization and the buffer solution[18]. As reported previously, the pH range of a dissolved GOx from Aspergillus Niger is 3.4−7.5, while the pH range of GOx immobilized on activated carbon is 2.5−9[19]. In this work, the solid immobilization of GOx and stabilization of its molecular structure may improve its pH adaptability, and make it still active even at low pH. The amperometric current changes against 1 mmol/L glucose in acetate buffer (pH 2.10−4.00) and phosphate buffer (pH 4.57−6.98) at 0.7 V were investigated. When pH was lower than 2.55, the current dropped due to the loss of GOx activity. In consideration of the existence of Hg^{2+} at low pH value, we chose acetate buffer (pH 2.55) for glucose detection.

Fig.1 shows the anodic current change with the addition of glucose to acetate buffer (pH 2.55) measured by amperometry at 0.7 V. The change in current reaches steady state in approximately 60 s due to the catalytic reaction. The current response to certain glucose concentration can remain a constant up to 30 min, which is an advantage for inhibition test. The plot of the current change vs glucose concentration in Fig.2 indicates that the lower detection limit is 0.01 mmol/L and the linear range is 0.1−2.3 mmol/L. The linear regression of current change as a function of glucose concentration is

$$\Delta I=0.144\ 5+2.312\ 2c \quad (1)$$

where ΔI is current change (nA), c is glucose concentration (mmol/L), and the mutuality coefficient is 0.999 3.

3.2 Inhibition study

Fig.3 shows the relationship between pH and inhibition degree by 2 mg/L Hg^{2+} with 1 mmol/L glucose. It is found that when pH value is higher than 2.55, the inhibition degree of GOx electrode rises as pH decreases; when pH value is lower than 2.55, the enzyme loses its activity while the inhibition degree drops as pH decreases. So pH 2.55 is chosen as the optimum pH of inhibition study. Fig.4 shows the relationship between

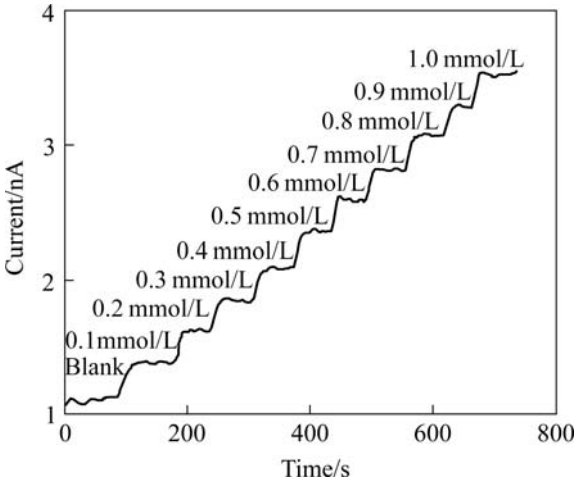

Fig.1 Current change vs addition of glucose to acetate buffer (pH 2.55) measured by amperometry at voltage of 0.7 V

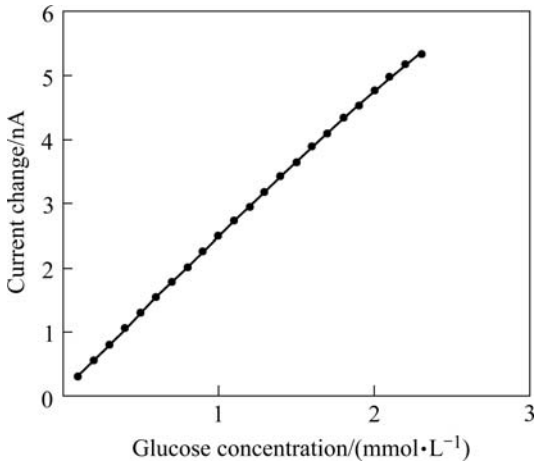

Fig.2 Glucose calibration plot by amperometric measurement at 0.7 V in acetate buffer (pH 2.55)

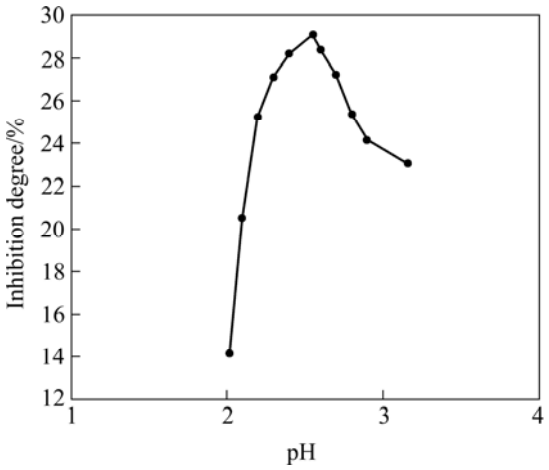

Fig.3 Plots of inhibition degree of 2 mg/L Hg^{2+} vs pH in acetate buffer at 0.7 V by amperometric measurement

inhibition degree of 2 mg/L Hg^{2+} and glucose concentration in acetate buffer (pH 2.55) at 0.7 V by amperometric measurement. The substrate will compete with inhibitor when the substrate concentration is high, so the increase of substrate concentration will lead to the decrease of inhibition of inhibitor on the enzyme[20]. Otherwise, when the substrate concentration is low, it is easy to be catalytically oxidized, and no obvious decrease of the response current can be observed with the addition of inhibitor. Therefore, we chose 1 mmol/L glucose as the optimum value for inhibition study.

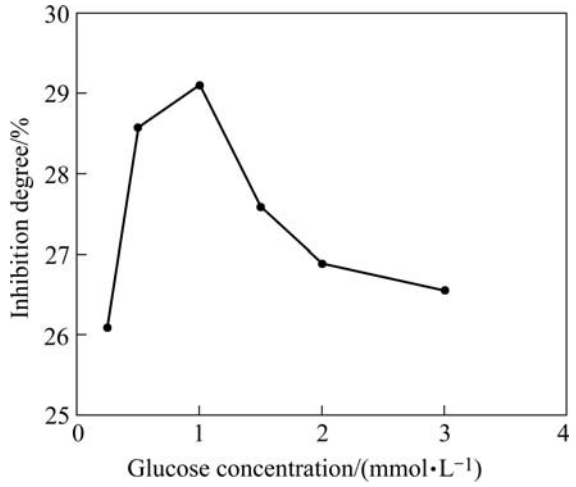

Fig.4 Plots of inhibition degree of 2 mg/L Hg^{2+} vs glucose concentration in acetate buffer (pH2.55) at 0.7 V by amperometric measurement

Certain concentration of Hg^{2+} is directly added into the working solution containing glucose in amperometric experiment at 0.7 V. The response current drops with the increase of Hg^{2+} concentration, indicating the increase of inhibition degree. The current change reaches steady state in less than 80 s, so no incubation is required in this work. Then after soaking in phosphate buffer (pH 7.00) for 8 min, the anodic current on the electrode increases again in electrolyte containing the same glucose concentration. In this work, the inhibition degree of GOx electrode has linear relationship with the natural logarithm of Hg^{2+} concentration, as shown in Fig.5. The lower detection limit should return the inhibitor concentration which results in at least 4%−6% of inhibition degree[18]. Here the concentration with inhibitionn degree of 6% is chosen as the lower detection limit, which is 0.49 μg/L. The linear range can be divided into two parts, one of which is 0.49−783.21 μg/L, and the regression equation is

$$I=8.412\,9+2.060\,2\rho \quad (2)$$

where I is the inhibition degree (%), ρ is the natural logarithm of Hg^{2+} concentration (μg/L), and the mutuality coefficient is 0.979 8.

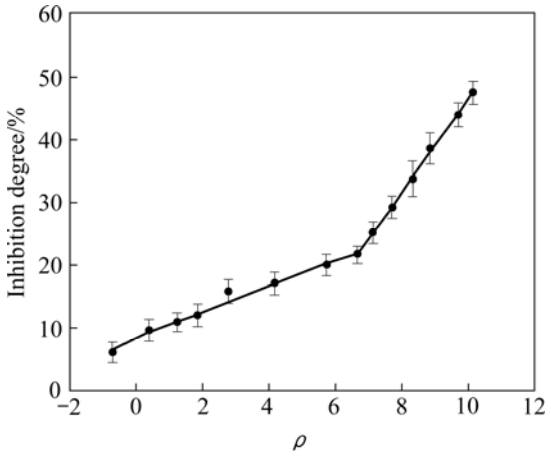

Fig.5 Linear regression of inhibition degree vs natural logarithm of Hg^{2+} concentration in acetate buffer (pH 2.55) (Vertical bars designate standard deviations for means of 3 replicative tests)

The other is 783.21 μg/L−25.55 mg/L, the mutuality coefficient is 0. 998 9, and the regression equation is

$$I=-28.048\,4+7.453\,8\rho \quad (3)$$

Since each of the calibration was done three times, and the standard deviations of inhibition degree are not more than 2.84%, the stability and reproducibility of the inhibition based sensor are guaranteed.

3.3 Reversibility and stability

The inhibition of GOx electrode requires no incubation, and after each inhibition, it doesn't need any antidote such as EDTA or dimercaptopropanol, but only an 8 min soaking in phosphate buffer (pH 7.00) is enough for its complete reactivation. Moreover, Fig.4 shows that the inhibition degree of GOx electrode distinctly declines as glucose concentration increases, which implicates that the Hg^{2+} inhibition is alleviated by addition of substrate. Considering the aforementioned stabilization of the enzyme steric structure, we presumed that the inhibition action by these heavy metals is not based on the irreversible reactions with some amino acid residues such as sulphur groups in the enzyme so as to break its steric structure[21−22], but based on the reversible absorption on some negative regions of flavin adenine dinucleotide (FAD) in the redox center of Gox [23−24]. From these facts, it can be drawn that the inhibition assay in this study is reversible and competitive.

The stability of the sensor was also investigated. Fig.6 shows the change of its response to 1 mmol/L glucose in acetate buffer (pH 2.55) with inhibition times, which shows that the activity of the enzyme sensor is not influenced by the increase of inhibition times.

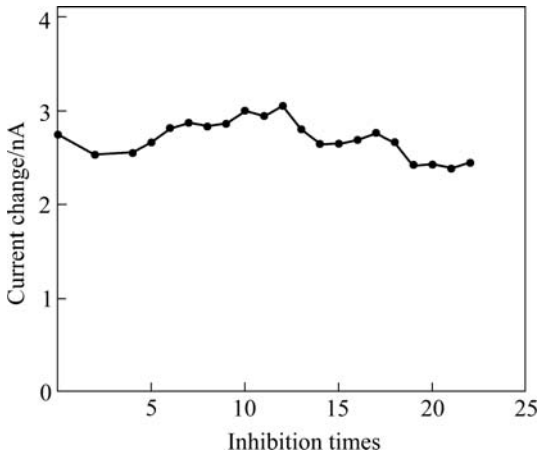

Fig.6 Sensor response to 1 mmol/L glucose in acetate buffer solution (pH2.55) with inhibition times by amperometry at 0.7 V

3.4 Selectivity

Selectivity is an important factor in the performance of an inhibition based enzyme sensor. In this experiment, four other heavy metals as interferents were measured under the same conditions as calibration of Hg^{2+}. From the results listed in Table 1, the interference to Hg^{2+} detection from these metal ions is found to be minimal.

Table 1 Interference results of Cr^{3+}, Pb^{2+}, Cu^{2+} and Cd^{2+} under same conditions as calibration of Hg^{2+}

Interferent	Concentration/ ($\mu g \cdot L^{-1}$)	Inhibition degree/%	Hg^{2+} concentration when holding same inhibition degree/ ($\mu g \cdot L^{-1}$)
Cr^{3+}	500	9.53	1.72
Pb^{2+}	500	4.97	no
Cu^{2+}	500	1.56	no
Cd^{2+}	500	6.17	no

3.5 Application

Certain amount of Hg^{2+} was added to compost extract, and was tested by this sensor. The results are listed in Table 2, with the average recovery ratio of 101.9%.

Table 2 Results of recovery test in compost extract as matrices

Concentration added/($\mu g \cdot L^{-1}$)	Average concentration recovered/($\mu g \cdot L^{-1}$)	Recovery/%
3	2.68±0.45	89.3
8×10^{1}	$(7.20\pm2.37)\times10^{1}$	90.0
3×10^{2}	$(3.25\pm1.02)\times10^{2}$	108.3
1.25×10^{3}	$(1.39\pm0.21)\times10^{3}$	111.2
5×10^{3}	$(5.53\pm1.78)\times10^{3}$	110.6

4 Conclusions

1) An inhibition based enzyme sensor for the detection of Hg^{2+} has been developed based on the inhibition of GOx. The fabrication of this sensor is low-cost, simple and fast. The adaptability of the sensor at low pH in glucose determination is an advantage over other enzyme sensors for heavy metal inhibition studies.

2) Good sensitivity, reversibility, stability and selectivity were all obtained. The determination of Hg^{2+} in a real compost sample is in good agreement with the declared concentration, indicating the potential for further application in "on the spot" environmental monitoring for trace Hg^{2+}.

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(Edited by LI Xiang-qian)