

# A methylene blue-mediated enzyme electrode for the determination of trace mercury(II), mercury(I), methylmercury, and mercury–glutathione complex

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## Abstract

A methylene blue-mediated enzyme biosensor has been developed for the detection of inhibitors including mercury(II), mercury(I), methylmercury, and mercury–glutathione complex. The inhibition to horseradish peroxidase was apparently reversible and noncompetitive in the presence of HgCl<sub>2</sub> in less than 8 s and irreversibly inactivated when incubated with different concentrations of HgCl<sub>2</sub> for 1–8 min. The binding site of horseradish peroxidase with HgCl<sub>2</sub> probably was a cysteine residue –SH. Mercury compounds can be assayed amperometrically with the detection limits 0.1 ng ml<sup>-1</sup> Hg for HgCl<sub>2</sub> and methylmercury, 0.2 ng ml<sup>-1</sup> Hg for Hg<sub>2</sub>(NO<sub>3</sub>)<sub>2</sub> and 1.7 ng ml<sup>-1</sup> Hg for mercury–glutathione complex. Inactivation of the immobilized horseradish peroxidase was displayed in the AFM images of the enzyme membranes. © 2001 Published by Elsevier Science B.V.

**Keywords:** Mercury compounds; Peroxidase inhibition; Methylene blue; Mediated biosensor

## 1. Introduction

Rapid and sensitive measurements of mercury(II) and related compounds are required in various fields such as environment, food industry and medicine. Classical methods such as atomic absorption spectroscopy, inductively coupled plasma optical emission spectrometry, inductively coupled plasma mass spectrometry and their combination with chromatographic techniques are in wide use (Yuan et al., 1999a). These methods need sophisticated instrumentation, skilled personnel, complicated sample pretreatment and a long measuring period (Evtugyn et al., 1998). Therefore, electrochemical methods such as ion selective electrodes, polarography, and other voltammetry are also widely used due to their less complex instrumentation and shorter measuring period. However, more sensitive methods are ur-

gently needed in the analysis of environmental and clinical samples to measure trace amounts of mercury compounds usually in the range of ng ml<sup>-1</sup> or lower therein.

Biosensors provide rapid measurements without time-consuming purification or fractionation procedures for the analysis of heavy metal compounds. Several configurations have been described in the past including enzyme biosensors (Mattiasson et al., 1978; Ögren and Johansson, 1978; Danielsson et al., 1981; Gayet et al., 1993; Shekhovtsova and Chernetskaya, 1994; Amine et al., 1995; Preininger and Wolfbeis, 1996; Wong et al., 1997; Fennouth et al., 1998), whole cell biosensors (Evtugyn et al., 1998; Bontidean et al., 1998) and genetically modified biosensors (Watton et al., 1990; Salifonova et al., 1993; Tescione and Belfort, 1993; Virta et al., 1995; Klein et al., 1997; Evtugyn et al., 1998) for the determination of mercury(II) and related compounds. Among them, an inhibitor biosensor is far more sensitive than a substrate biosensor (Uo et al., 1992). The detection limit is much lower than the

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appropriate maximum permissible concentrations in environmental or clinical samples and detection limits typical for traditional analytical techniques, i.e. chromatography or spectrometry (Evtugyn et al., 1998; Bontidean et al., 1998).

Immobilized enzymes reported in the construction of inhibitor biosensors for mercury(II) and related compounds include L-lactate dehydrogenase (Gayet et al., 1993; Fennouth et al., 1998), glucose oxidase (Amine et al., 1995), pyruvate oxidases (Gayet et al., 1993), L-glycerophosphate oxidase (Gayet et al., 1993) and urease (Ögren and Johansson, 1978) in electrochemical biosensors with detection limits of  $\text{ng ml}^{-1}$  or  $\mu\text{g ml}^{-1}$  level, urease in thermal biosensors (Mattiasson et al., 1978; Danielsson et al., 1981) with a detection limit of  $0.2 \text{ ng ml}^{-1}$  and horseradish peroxidase (HRP) for determination of mercury at  $\text{pg ml}^{-1}$  level with solid supports of immobilized HRP and an indicator reaction of *o*-dianisidine, 3,3',5,5'-tetramethylbenzidine or *o*-phenylenediamine (Shekhovtsova and Chernetskaya, 1994). The detection limit for mercury chloride obtained using immobilized HRP was lower than other enzyme biosensors. Most biosensors are based on electrochemical transducers owing to the advantages they possess, e.g. simple user-friendly design, compatibility with standard commercial equipments, possibility of sensor miniaturization and automated measurements and a well-developed theory of the behavior of electrochemical biosensors (Evtugyn et al., 1998). However, it is difficult to use immobilized HRP electrochemically in the determination of inhibitors because of long response period and poor response sensitivity.

Methylene blue-mediated enzyme biosensor has been studied in our laboratory for the determination of  $\text{H}_2\text{O}_2$  using  $\beta$ -cyclodextrin polymer ( $\beta$ -CDP) as the immobilization matrix for HRP and the electron shuttles (Han et al., 1999; Yuan et al., 1999b). In this paper we studied the inhibition effects of the mercury species on the enzymatic activity of biosensors. In order to increase the sensitivity and shorten the time of the response, the methylene blue-mediated enzyme bioelectrode was employed in the measurement of mercury(II), mercury(I), methylmercury and mercury–glutathione complex, which is present in environmental samples or in the body of a patient taking mercury-containing medicines.

## 2. Experimental

### 2.1. Material

Peroxidase from horseradish (HRP) (EC 1.11.1.7,  $RZ \geq 3.0$ ,  $\geq 300 \text{ U mg}^{-1}$ , type IV) was purchased from Sigma. Mercuric chloride ( $\text{HgCl}_2$ ) and methylmercury chloride ( $\text{MeHgCl}$ ) were obtained from Ecoenvi-

ronmental Research Center, Chinese Academy of Sciences, and mercury cysteine complex ( $\text{HgCys}_2 \cdot \text{HCl} \cdot 1/2\text{H}_2\text{O}$ ), mercury glutathione ( $\text{HgG}_2$ ) from Chinese Academy of Chinese Medicine and Herbs. Mercurous nitrate was purchased from Taixing Reagent, China.

Britton–Robinson (B–R) buffer solutions were prepared by mixing the composite acid (2.71 ml of 85% (w/v)  $\text{H}_3\text{PO}_4$ , 2.40 g of glacial acetic acid and 2.47 g of boric acid in 1 l solution) and  $0.2 \text{ mol l}^{-1}$  sodium hydroxide.

The cross-linked polymer of  $\beta$ -cyclodextrin ( $\beta$ -CD) and epoxy chloropropane was synthesized according to the literature (Komiyama and Hirai, 1987) and milled in fine resin with a grain of 150 mesh. Methylene blue (MB) solution was stirred for 1 h at room temperature so that the aggregated dye reached equilibrium with its monomer form before it was applied in  $1.0 \times 10^{-3} \text{ mol l}^{-1}$  solution using phosphate buffer solution (pH 6.8, ionic strength 0.1) (Han et al., 1999). Hydrogen peroxide solution was freshly prepared by dilution of  $1.0 \times 10^{-3} \text{ mol l}^{-1}$  storage solution. B–R buffer solution (pH 6.8) served as the supporting electrolyte. Cellulose triacetate (average degree of polymerization 200–400) were obtained from Beijing Chemical Reagent Co. All reagents were of analytical reagent grade. All solutions were prepared with doubly distilled water.

### 2.2. Apparatus

Amperometric and cyclic voltammetric measurements were made with a JP-3A Electrochemical Analyzer (the Seventh Telecom of Shandong, China) equipped with an inhibitor biosensor as a working electrode. The reference electrode was Ag/AgCl (saturated KCl) and the auxiliary electrode was a platinum wire.

Atomic force microscopy (AFM) images were obtained with Benyuan 930B scanning probe microscope (Benyuan Co., China).

### 2.3. Construction of the inhibitor biosensor

Glassy carbon disc electrode (GCE, 4 mm in diameter) was polished in a rotating speed 1500 rpm with  $0.3 \mu\text{m Al}_2\text{O}_3$  powder. Then it was sonicated for a few minutes in 1:1 nitric acid, acetone and doubly distilled water successively, and dried in air prior to the immobilization of the enzyme HRP.

MB solution ( $1.0 \times 10^{-3} \text{ mol l}^{-1}$ ), B–R buffer solution and  $0.1 \text{ mol l}^{-1}$  NaCl were mixed with  $\beta$ -CDP, and the mixture was incubated at room temperature for 40 min. Then the inclusion compound of  $\beta$ -CDP and the mediator molecule was obtained. The inclusion compound was filtered and dried in air prior to use. Five microliters of  $40 \text{ g l}^{-1}$   $\beta$ -CDP-mediator colloid

was carefully and thoroughly mixed with 10  $\mu\text{l}$  of 2% cellulose triacetate in acetone. The mixture of 5.0 mg HRP and 5.0 mg BSA was dissolved into this colloid. Subsequently 5  $\mu\text{l}$  of 5% (w/v) glutaric dialdehyde was completely mixed with it as soon as possible. Then the mixture was spin-coated onto GCE at 3000 rpm and allowed drying under ambient condition for 1 h.

The prepared enzyme sensor was dipped in a  $1.0 \times 10^{-3} \text{ mol l}^{-1}$  MB solution for 4 min before every measurement and kept in  $0.1 \text{ mol l}^{-1}$  phosphate buffer (pH 6.8) at  $4^\circ\text{C}$  in a refrigerator for storage.

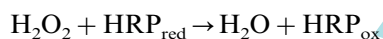
#### 2.4. Observation of AFM images

An operating condition of constant force mode was used in the observation of AFM images. The cantilever was 100  $\mu\text{m}$  long with a V-shaped standard silicon nitride tip coated with reflective gold (Model NP, Digital Instruments). The spring constant was  $0.12 \text{ N m}^{-1}$ . Constant force mode was used during the scanning. The reference current was set at 0.9 nA and the time required to obtain a single image was 50 s.

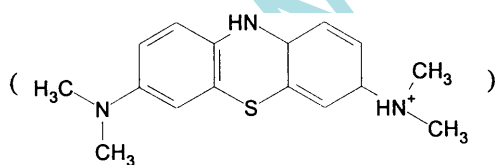
#### 2.5. Procedure

All experiments were conducted in a cell containing 5 ml B–R buffer solution (pH 6.8) as the supporting electrolyte at room temperature (approximately  $20^\circ\text{C}$ ). The tested solution was stirred with a magnetic bar at a constant rate. Measurements were performed at a constant operating potential  $-0.283 \text{ V}$  (vs. AgCl/AgCl, saturated KCl). Standards were injected into 5.00 ml of buffer solution contained in the cell.

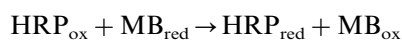
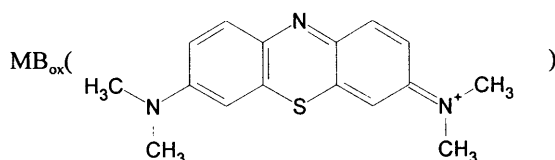
At first HRP expressed as  $\text{HRP}_{\text{red}}$  reduces  $\text{H}_2\text{O}_2$  molecules that touch the active sites of the enzyme into  $\text{H}_2\text{O}$ , and changes into its oxidation state,  $\text{HRP}_{\text{ox}}$ .  $\text{H}_2\text{O}_2$  molecules continuously diffuse to the sensor surface from the tested solution.



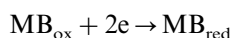
Then  $\text{HRP}_{\text{ox}}$  oxidizes methylene blue,  $\text{MB}_{\text{red}}$



to its oxidation state,  $\text{MB}_{\text{ox}}$



Subsequently, oxidized MB is reduced to regenerate  $\text{MB}_{\text{red}}$  to produce the cathodic catalytic current at the sensor,



The output current due to the reduction of  $\text{MB}_{\text{ox}}$  is correlated with both the concentration of hydrogen peroxide and HRP activity on the immobilized enzymatic membrane. At fixed concentrations of hydrogen peroxide and quantity of HRP immobilized in membrane, the decrease of the output current is correlated with the inhibitor concentration.

First, the three electrodes were immersed in a cell containing B–R buffer solution. The amperometric response was recorded. Then the resultant current–time curve after adding  $10.0 \text{ mmol l}^{-1} \text{ H}_2\text{O}_2$  was recorded. The highest value of the response current corresponded with HRP activity ( $v_1$ ). In the second step the three electrodes were immersed in the cell containing a known amount of mercury compound, the current–time curve was recorded with the same method as described above. The highest value of the response current corresponded with the remaining activity of HRP ( $v_2$ ). The degree of inhibition to the enzyme activity in the presence and absence of mercury compounds, can be calculated as follows:

$$I (\%) = \frac{100(v_1 - v_2)}{v_1}$$

The mediated enzyme biosensor after inhibition by mercury compounds was restored through ordinal washings of the immobilized HRP membrane with  $10 \text{ mmol l}^{-1}$  cysteine,  $10 \text{ mmol l}^{-1}$  ethylenediamine-*N,N*-tetraacetic acid (EDTA) and  $10 \text{ mmol l}^{-1}$  ammonium diethyldithiocarbamate (DDTC) in pH 6.8 of B–R buffer solution, respectively.

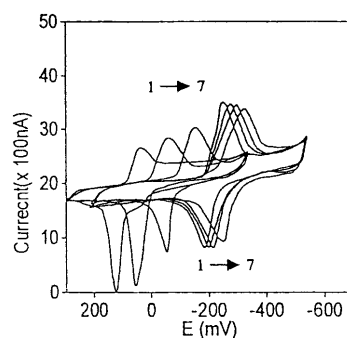


Fig. 1. Cyclic voltammograms of MB incorporated in  $\beta$ -CDP-modified membrane with potential scanning rate  $70 \text{ mV s}^{-1}$  in the BR buffer of different pHs (1, pH 1.54; 2, pH 2.54; 3, pH 4.70; 4, pH 6.06; 5, pH 6.96; 6, pH 7.64; 7, pH 9.56).

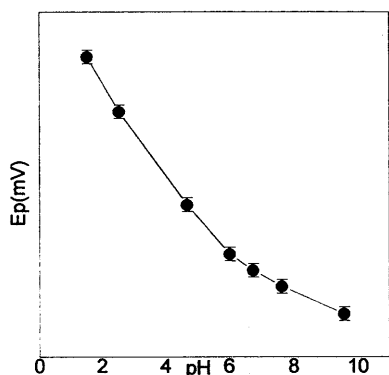


Fig. 2. Effect of pH on the peak potential of  $\beta$ -CDP-HRP-MB biosensor at a scan rate of  $70 \text{ mV s}^{-1}$  in the range of pH 1–9.6.

### 3. Results and discussion

#### 3.1. Electrochemical characteristic of the methylene blue-mediated HRP biosensor

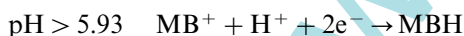
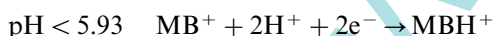
Cyclic voltammograms of MB incorporated in  $\beta$ -CDP-modified membrane in BR buffer solutions at different pHs are shown in Fig. 1. It is observed that pH markedly influences the peak potential of the  $\beta$ -CDP-HRP-MB biosensor. Fig. 2 shows the correlation between peak potential of adsorbed MB and pH of the solution. It gives a line with slopes of approximately  $60 \text{ mV}$  per pH unit from pH 1.8 to 6.0 and  $28 \text{ mV}$  per pH unit from pH 6.0 to 9.0 at a potential scan rate of  $70 \text{ mV s}^{-1}$ .

The exact inflection point obtained from Fig. 2 is at pH 5.93. The correlation equations between pH and  $E_p$  are accordingly result from Fig. 2,

$$E_p = 116.23 - 60.28\text{pH} \quad (\text{pH} < 5.93),$$

$$E_p = 75.38 - 27.96\text{pH} \quad (\text{pH} > 5.93).$$

Therefore, a reaction mechanism at the electrode surface is suggested as follows:



The peak height of MB immobilized at the biosensor significantly changed along with pH. This would influence the response increment in the presence of peroxide at different acidities. In the acidic buffer solution with  $\text{pH} < 5.93$ , the anodic peak at the cyclic voltammograms of MB was higher than the cathodic peak and difference between the anodic and cathodic peak height increased obviously with decreasing pH. It shows that the electro-reduction product of MB ( $\text{MB}_{\text{red}}$ ) is adsorbed weakly on GCE surface. While in the buffer solution with  $\text{pH} > 5.93$ , the anodic and the cathodic peak were approximately identical. It is manifested that

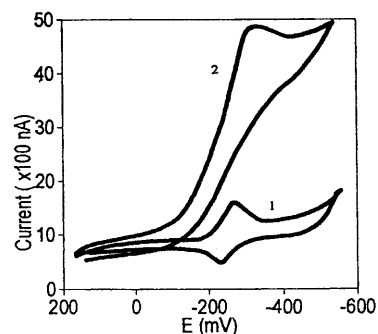


Fig. 3. Cyclic voltammograms of the mediated enzyme electrode at potential scanning rate  $70 \text{ mV s}^{-1}$  in B-R buffer solution at pH 6.8 in the presence of  $4.0 \text{ mmol l}^{-1} \text{ H}_2\text{O}_2$  (b) or absence (a).

no adsorption of  $\text{MB}_{\text{red}}$  happens at the GCE surface. As a result, the electrode reaction was a quasi-reversible controlled process in the buffer solution with  $\text{pH} > 5.93$ . It suggests the mediator can well be used to shuttle electrons between HRP and the GCE surface for the preparation of a mediator bioelectrode.

When  $\text{H}_2\text{O}_2$  was added to the solution, voltammetric behavior changed dramatically with an enhanced reduction current and a decreased oxidation current with MB as the mediator, showing that a catalytic reaction occurred at the electrode surface (Fig. 3). This demonstrates that MB incorporated in  $\beta$ -CD polymer effectively shuttles electrons between the redox center of HRP and GCE surface. Electron transfer via a mediator is more effective in the bioelectrocatalytic reduction of hydrogen peroxide at  $\beta$ -CDP-HRP-MB electrode than that directly between HRP and nude electrode surface, e.g. GCE or platinum. MB incorporated in  $\beta$ -CDP, as described in the literature (Han et al., 1999), increased the response sensitivity of MB and the stability of the immobilized enzyme membrane because of the specific supramolecular interaction. Based on the increase in sensitivity and shortening in response time, a MB-mediated HRP bioelectrode using  $\beta$ -CDP as the immobilization matrix can be used in the preparation of

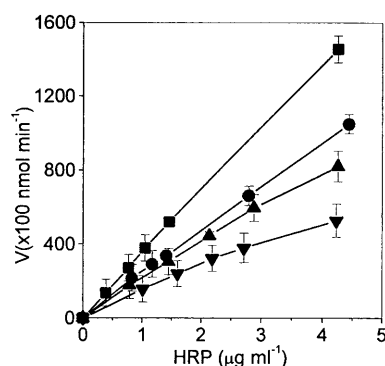


Fig. 4. The remaining activity of HRP in solution with MB- $\beta$ -CDP modified GCE with preincubation of  $\blacksquare$ ,  $\bullet$ ,  $18.5 \text{ ng ml}^{-1}$ ,  $\blacktriangle$ ,  $53 \text{ ng ml}^{-1}$ ,  $\blacktriangledown$ ,  $90 \text{ ng ml}^{-1}$  of  $\text{HgCl}_2$  in less than 8 s.

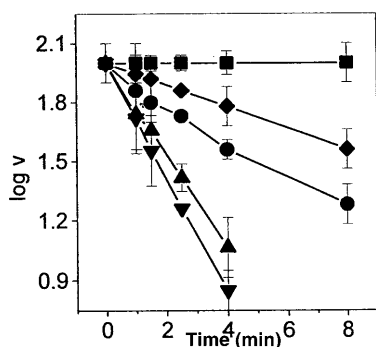


Fig. 5. Inactivation of HRP on electrode with increasing time from 1 to 8 min without  $\text{HgCl}_2$  (■) or with  $\text{HgCl}_2$ :  $2 \text{ ng ml}^{-1}$  (◆),  $3.1 \text{ ng ml}^{-1}$  (●),  $6.5 \text{ ng ml}^{-1}$  (▲),  $10 \text{ ng ml}^{-1}$  (▼).

an electrochemical inhibitor biosensor for mercury compounds.

### 3.2. Effect of mercury compounds on HRP in solution and immobilized HRP

#### 3.2.1. Reversible inhibition HRP by $\text{HgCl}_2$

The activity of HRP in solution was measured with MB- $\beta$ -CDP modified GCE after adding different concentrations of  $\text{HgCl}_2$  and then  $4 \text{ mmol l}^{-1} \text{ H}_2\text{O}_2$  into B-R solution containing HRP. The interval between the addition of  $\text{HgCl}_2$  and  $\text{H}_2\text{O}_2$  has to be controlled in less than 8 s. The remaining activity of HRP ( $v$ ) was plotted versus the concentration of HRP in the solution at different concentrations of  $\text{HgCl}_2$  (Fig. 4). It shows HRP inhibition by  $\text{HgCl}_2$  being apparently reversible in less than 8 s preincubation with  $\text{HgCl}_2$ .

The remaining activity of HRP in solution in the presence of various concentrations of  $\text{HgCl}_2$  and two concentrations of  $\text{H}_2\text{O}_2$  was measured in less than 8 s, without preincubation with  $\text{HgCl}_2$ . Dixon's plot deduced from the results indicates an apparent decrease in HRP activity in the presence of the mercury compound. HRP inhibition is also shown to be apparently reversible and noncompetitive.

For inhibitor determination, the inhibition resulted in the decay of the enzyme activity, which limited the times of consecutive measurements with the same biosensor. Reversible inhibition is thus probably better for application than irreversible inactivation considering the biosensor lifespan. However, the reversible inhibition to HRP happened in not more than 8 s, thus it is hard to accomplish the amperometric measurement of remaining activity so soon. Therefore, irreversible inhibition or inactivation has to be considered.

#### 3.2.2. Irreversible inactivation of HRP by $\text{HgCl}_2$

HRP immobilized on MB- $\beta$ -CDP modified GCE was incubated with different concentrations of  $\text{HgCl}_2$  for various times (from 1 to 30 min). Then the current–

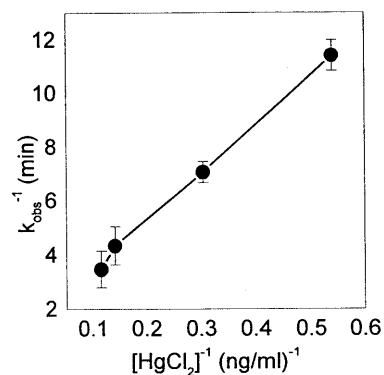


Fig. 6. Plot of  $1/k_{\text{obs}}$  against  $1/(\text{HgCl}_2)$ , with  $\text{HgCl}_2$  concentration for ( $\text{HgCl}_2$ ).

time curves were recorded. Remaining activity ( $v_i$ ) measured with incubation times from 1 to 8 min is demonstrated in Fig. 5. It shows that  $\log v$  is linear to incubation time within 8 min.

Suppose that the irreversible inactivation was first order versus enzyme concentration ( $E$ ), according to the literature (Fennouth et al., 1998),



$\ln v_i$  is linear to the incubation time:

$$\ln v_i = -k_{\text{obs}}t + \text{constant.}$$

The experimental slope is

$$k_{\text{obs}} = \frac{k_i[I]}{[I] + K_i}$$

where  $[I]$  is the inactivator concentration,  $K_i$  the equilibrium dissociation constant and  $k_i$  the rate constant of inactivation.

For  $[I] \ll K_i$

$$k_{\text{obs}} = \frac{k_i}{K_i[I]}$$

The  $1/k_{\text{obs}}$  plotted against  $1/(\text{HgCl}_2)$  obtained a straight line (Fig. 6), where ( $\text{HgCl}_2$ ) is the concentration

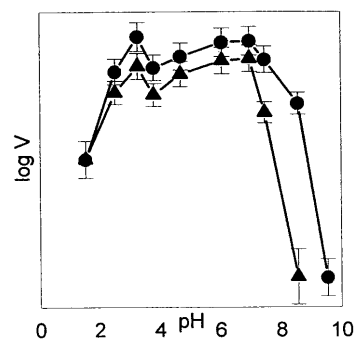


Fig. 7. pH curves of HRP activity without (●) or with  $10 \text{ ng ml}^{-1} \text{ HgCl}_2$  for 4 min (▲).  $\log v$  was measured as in Fig. 5, using B-R buffer solutions as described in Section 2.5.

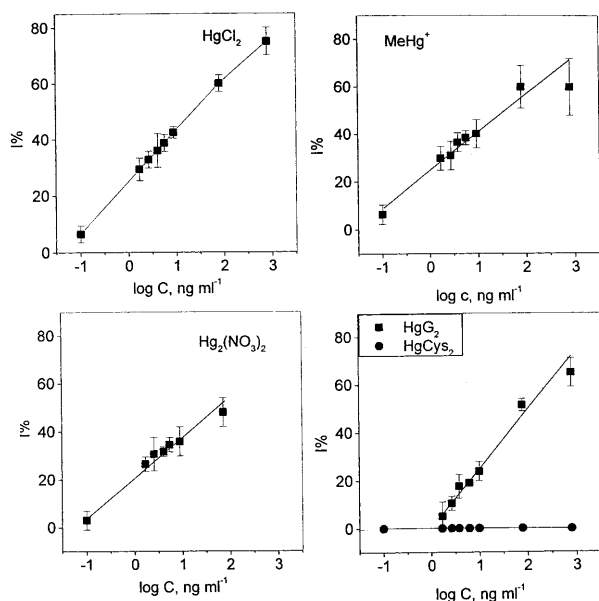


Fig. 8. Calibration curves of  $\text{HgCl}_2$ ,  $\text{MeHg}^+$ ,  $\text{Hg}_2(\text{NO}_3)_2$ ,  $\text{HgG}_2$  and  $\text{HgCys}_2$ .

of  $\text{HgCl}_2$ . The experimental results shown in Fig. 6 tallied with the above equations. The irreversible inactivation of HRP immobilized on a MB- $\beta$ -CDP modified GCE after incubation with different concentrations of  $\text{HgCl}_2$  for 1–8 min was related to the concentration of  $\text{HgCl}_2$ . The linear relation between  $1/k_{\text{obs}}$  and  $1/(\text{HgCl}_2)$  implied the possibility of the construction for the inhibitor biosensor of  $\text{HgCl}_2$ .

### 3.3. pH effect

The pH curves of HRP activity (Fig. 7) shows two  $pK_s$  characterizing residues of two amino acids involved the binding of  $\text{H}_2\text{O}_2$  in the active site. In the presence of  $\text{HgCl}_2$ , the  $pK_s$  of enzyme inactivation were found to be similar. These results are in favor of  $\text{HgCl}_2$  binding in the non-active site. The  $pK$  6.9 suggested that the active site is imidazolyl residue, which has a  $pK_a$  around 5.5–7.0. High response value of  $\text{H}_2\text{O}_2$  was obtained at pH 6.8. Therefore, it was selected as the working pH in the inhibitor biosensor for the ease of measurement with an amperometer.

### 3.4. Calibration curve

Irreversible inactivation of HRP can achieve more sensitive assay of mercury compounds than reversible inhibition. As a result, measurement was chosen according to the steps described in Section 2.5. Calibration curves for mercury compounds including  $\text{HgCl}_2$ ,  $\text{Hg}_2(\text{NO}_3)_2$ ,  $\text{MeHg}^+$  and  $\text{HgG}_2$  were obtained and are shown in Fig. 8.

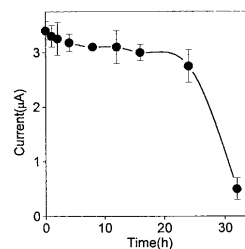


Fig. 9. Amperometric response for  $10 \text{ mmol l}^{-1} \text{H}_2\text{O}_2$  at different times alternated with three measurements for  $\text{HgCl}_2$ .

The detection limits were  $0.1 \text{ ng ml}^{-1} \text{Hg}$  for  $\text{HgCl}_2$  and  $\text{MeHg}^+$ ,  $0.2 \text{ ng ml}^{-1} \text{Hg}$  for  $\text{Hg}_2(\text{NO}_3)_2$  and  $1.7 \text{ ng ml}^{-1} \text{Hg}$  for  $\text{HgG}_2$ , respectively. No inhibition of HRP was observed in the presence of  $\text{HgCys}_2$ . Thus, the biosensor can not be used in the assay of  $\text{HgCys}_2$ . This fact implied that the binding site of HRP with  $\text{HgCl}_2$  probably was the  $-\text{SH}$  at a cysteine residue. The different inhibition effect between  $\text{HgG}_2$  and  $\text{HgCys}_2$  perhaps resulted from the difference at the spatial position of  $-\text{SH}$ . A detailed mechanism has not been realized up to now.

### 3.5. Restoration and stability

The activity of immobilized HRP was recovered by washing the biosensor, respectively, with  $10 \text{ mmol l}^{-1}$  cysteine,  $10 \text{ mmol l}^{-1}$  EDTA and  $10 \text{ mmol l}^{-1}$  DDTC in pH 6.8 of B-R buffer solution after each inhibitor measurement. Fig. 9 showed the effect on the amperometric response for  $10 \text{ mmol l}^{-1} \text{H}_2\text{O}_2$  after determination for inhibitor and above restoration procedures. It shows that the stability of the mediated enzymatic

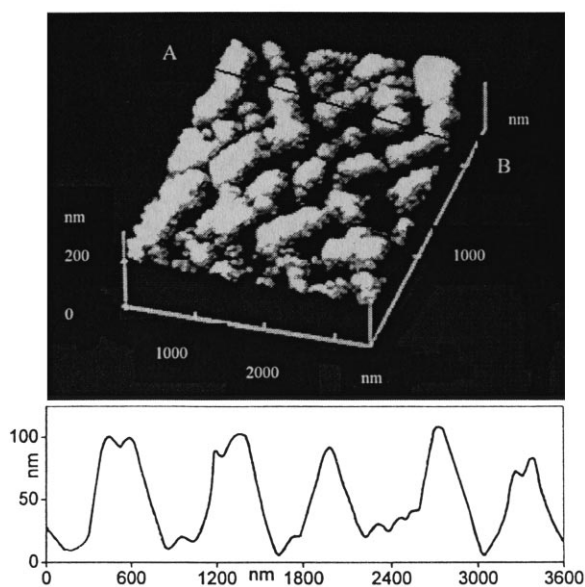


Fig. 10. AFM image of a cellulose triacetate membrane (a) and a cross line on the image from A to B (b).

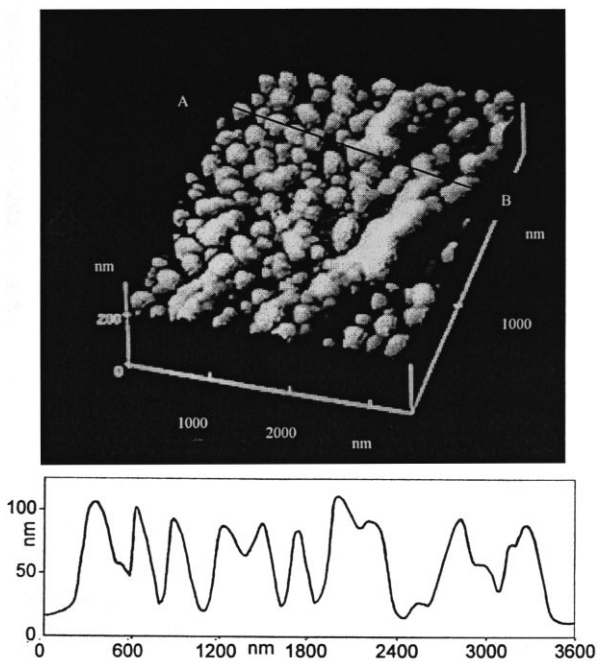


Fig. 11. AFM image of the immobilized HRP membrane of the inhibitor biosensor (a) and a cross line on the image from A to B (b).

membranes was not affected by exposure for at least 24 h to  $\text{HgCl}_2$  and 21 measurements of inhibitor when assays were alternated with the restoration procedure of enzymatic activity. Under storage conditions, the enzyme sensor was kept in  $0.1 \text{ mol l}^{-1}$  phosphate buffer (pH 6.8) at  $4^\circ\text{C}$  in a refrigerator. It was stable for more than 2 months, which is longer than the lifespan of other mercury biosensors reported in the literature (Mattiasson et al., 1978; Gayet et al., 1993).

### 3.6. AFM observation on HRP immobilization and HRP inactivation

An immobilized HRP membrane, the membrane inactivated by  $\text{HgCl}_2$  and the carrier membrane were observed by AFM in a contacted mode. AFM images offered the morphologies of the composite membranes. Fig. 10a, showed AFM image of the carrier membrane, a cellulose triacetate membrane. A porous structure was exhibited in Fig. 10a, with an average pore diameter of 600 nm (Fig. 10b). However, the immobilized enzyme membrane of the biosensor,  $\beta\text{-CDP-HRP-MB}$  wrapped with a cellulose triacetate membrane, showed a quite different appearance (Fig. 11a).  $\beta\text{-CDP-HRP-MB}$ , which was set in the cellulose triacetate membrane, formed pores with a diameter of about 300 nm as the active centers of the sensor (Fig. 11b). A few unfilled pores still existed in the composite membrane cellulose triacetate. Moreover, AFM image of the immobilized enzymatic membrane inactivated with  $\text{HgCl}_2$  solution showed smaller pores with a diameter of about 150 nm

due to the transfiguration of the enzymatic center (Fig. 12a and b).

## 4. Conclusion

In this paper we studied a methylene blue-mediated enzyme biosensor using  $\beta\text{-cyclodextrin}$  polymer ( $\beta\text{-CDP}$ ) as immobilization matrix for horseradish peroxidase in the detection of mercury(II), mercury(I), methylmercury, and mercury–glutathione. Cyclic voltammograms of MB incorporated in  $\beta\text{-CDP}$ -modified membrane in B–R buffer solutions of different pHs deduced the electrochemical process being a quasi-reversible controlled process in the buffer solution of  $\text{pH} > 5.93$ . It could effectively shuttle electrons between the enzyme's redox center and GCE surface. Results indicated an apparent decay in HRP catalytic activity in the presence of the mercury compounds by measuring the remaining activity of HRP in solution with the MB mediator electrode and immobilized on MB– $\beta\text{-CDP}$  modified GCE. HRP inhibition is apparently reversible and noncompetitive in less than 8 s and irreversibly inactivated in 1–8 min. Cysteine–Hg complex showing no inhibition to the biosensor, as well as the pH curves of HRP activity, indicate that the binding site of HRP with  $\text{HgCl}_2$  probably is the –SH of a cysteine residue.

The fabricated sensor exhibited the detection limits of  $0.1 \text{ ng ml}^{-1}$  Hg for  $\text{HgCl}_2$  and  $\text{MeHg}^+$ ,  $0.2 \text{ ng ml}^{-1}$  Hg for  $\text{Hg}_2(\text{NO}_3)_2$  and  $1.7 \text{ ng ml}^{-1}$  Hg for  $\text{HgG}_2$ , respectively. These limits are lower than that obtained

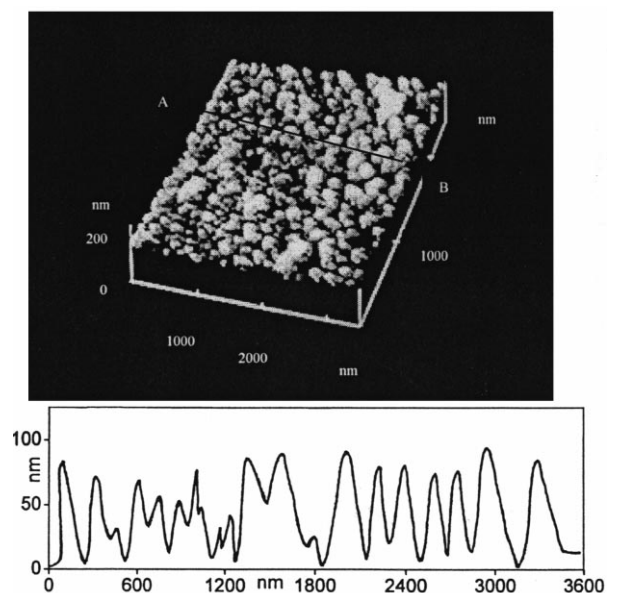


Fig. 12. AFM image of the inactivated immobilized HRP membrane (a) and a cross line on the image from A to B (b).

from L-lactate dehydrogenase ( $\text{Hg}^{2+}$  200 ng ml<sup>-1</sup>) (Gayet et al., 1993; Fennouth et al., 1998), glucose oxidase ( $\text{Hg}^{2+}$  1 ng ml<sup>-1</sup>, MeHg and EtHg 2–10 ng ml<sup>-1</sup>) (Amine et al., 1995), pyruvate oxidases ( $\text{Hg}^{2+}$  2 ng ml<sup>-1</sup>) (Gayet et al., 1993), L-glycerophosphate oxidase ( $\text{Hg}^{2+}$  approximately 800 ng ml<sup>-1</sup>) (Gayet et al., 1993) and urease ( $\text{Hg}^{2+}$  20 ng ml<sup>-1</sup>) (Ögren and Johansson, 1978). The biosensor did not respond to  $\text{HgCys}_2$ . It was stable after consecutive assays for more than 24 h and at storage conditions for more than 2 months. However, interference such as cyanides, other heavy metals, respiration poisons (Smit and Cass, 1990; Bogdanovskaya et al., 1994; Shekhovtsova and Chernetskaya, 1994; Adeyoju et al., 1995a,b; Wollenberger et al., 1994; Evtugyn et al., 1998) still need to be overcome before it turns into application.

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