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# A $H_2O_2$ biosensor based on immobilization of horseradish peroxidase in electropolymerized methylene green film on GCE

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A novel $H_2O_2$ sensor is achieved by immobilizing horseradish peroxidase (HRP) in an electropolymerized methylene green (PMG) redox film. The electropolymerization of MG is carried out in a neutral phosphate buffer solution with $5 \times 10^{-5}$ M methylene green (MG) by using a two-step method. The polymer film only occurs on glassy carbon (electrodes and the reason for this is identified. The critical factor for the electropolymerization of MG lies in the preanodization on a glassy carbon) electrode, because a large amount of positive charges are accumulated and used to create the cation radicals form the polymer film. The formal potentials of PMG is pH dependent with a slope of 57 mV per pH unit between pH 6.0 and 8.0, which is close to the anticipated Nernstian value of 59 mV for a two-electron, two-proton process. The PMG itself and the PMG on the $H_2O_2$ sensor show electrochemical behaviour with a linear plot of peak current against scan rate in the range 20 to 100 mV $s^{-1}$.

Keywords: *biosensor*, *electropolymerization*, *methylene green*, *horseradish peroxidase*

## 1. Introduction

Recently, electrochemical methods have proved useful in the preparation of enzyme electrodes. Since the pioneering work of Foulds and Lowe [1], the immobilization of enzymes in an electropolymerized polymer film has been developed to prepare biosensors [2–10]. Most work in this area has been concentrated on use of electropolymerized conducting films, such as polypyrrole [1–5] and its derivatives [6] as well as polyaniline [7], and nonconducting polymers, such as polyphenol [8] and poly(*o*-phenylenediamine) [9, 10]. The technique based on immobilization of enzymes in electropolymerized films has proved to be well suited to the preparation of electrochemical biosensors owing to their simple and convenient fabrication and good reproducibility. However, among most of these biosensors, oxygen is the electron acceptor and the quantitative analysis is based on determination of $H_2O_2$ liberated by the enzymatic reaction. Therefore, they belong to the first generation of biosensors. In addition, little work has been done on the electrochemical codeposition of metal particles and glucose oxidase to prepare glucose sensors [11, 12].

In the past few years, much interest has arisen in the preparation, properties and application of electropolymerized redox mediators owing to their various chemical properties and their numerous possible applications in electrocatalysis and sensors [13–22]. For example, several phenazine, phenoxazine and phenothiazine derivatives have been filmed by electropolymerization and used as mediators [13–19]. Compared with the conventional methods of mediator immobilization, the redox dye-modified electrodes produced by electropolymerization with a three-dimensional distribution of mediators are preferable for the design of biosensors because the much larger catalytic response of polymer coatings than monolayers results from the volume effect, and hence the catalytic activity and sensitivity are expected to be improved. For instance, Karyakin *et al.* [13] reported that the long-term operational stability of poly(methylene blue) film was much higher than that of the adsorbed mediator and its catalytic activity was found to be at least ten times more active in NADH oxidation compared with the monolayer. High NADH oxidation currents were also obtained with electrodes modified by electropolymerization of 3,4-dihydroxybenzaldehyde [20].

The electropolymerized redox mediators work as artificial electron donors/acceptors in enzyme reactions on the one hand and are able to be electropolymerized on electrode surfaces so as to produce stable redox-active layers on the other hand. The catalytic oxidation of NADH [16–20]. In addition, platinum electrodes modified with a film of poly(azur (A) gives an electrocatalytic response to hemoglobin) [15]. An amperometric dehydrogenase biosensor based on poly(methylene blue) onto glassy carbon electrodes

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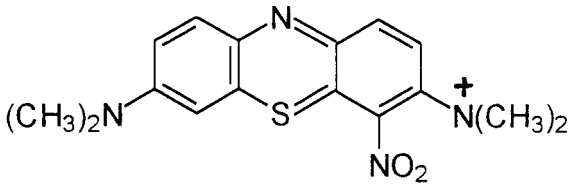
has been reported [13]. However, the immobilization of enzyme in the biosensor was by virtue of the formation of the enzyme-containing Nafion® membrane. As far as we know, no work on immobilization of enzymes in electropolymerized redox mediator films in biosensor fabrication has been reported.

In this paper we show a novel enzyme immobilization technique by incorporation of horseradish peroxidase into an electropolymerized methylene green redox polymer film; this is achieved only on glassy carbon electrodes from a neutral phosphate buffer solution using a two-step method.




Scheme 1. Structure of MG.

## 2. Experimental details

### 2.1. *Reagents*

Horseradish peroxidase (HRP) was obtained from sigma. Methylene green (MG, a cation dye, the structure is shown in Scheme 1) from Fluka. Hydrogen peroxide (30% w/v) was purchased from Shanghai Chemical Reagent Company, PRC. The concentration of more diluted hydrogen peroxide solutions prepared from this material was determined by titration with cerium(IV) to a ferroin endpoint [23]. All other chemicals were of analytical grade. All electrochemical experiments were carried out in 5 ml of 0.1 M phosphate buffer (pH 6.5, apart from the pH parameter experiments). All solutions were prepared using doubly distilled deionized water.

### 2.2. *Apparatus*

A three-electrode system was employed, with a platinum foil counter electrode, a reference electrode of silver/silver chloride in 3 M potassium chloride (Ag/AgCl) and a glassy carbon working electrode. The glassy carbon electrode (GCE, 3 mm dia.) was polished first with diamond paper, followed by 0.5 $\mu$m alumina paste, sonicated in distilled water to remove any residual alumina, and then further cleaned in 1 : 1 nitric acid, acetone and doubly distilled water successively. All potentials are reported against Ag/AgCl unless stated otherwise. Electrochemical measurements were carried out with a cyclic voltamperogragh (Scientific Equipment Company of Fudan University, Shanghai, China) in conjunction with a type 3086 X–Y recorder (Tokyo, Japan). All experiments concerning the redox reaction of horseradish peroxidase were carried out in a thermostated electrochemical cell at 25 °C. A Hewlett–Packard 8452A diode array spectrophotometer (US) was used for u.v.–vis. spectra measurements.

### 2.3. *Electropolymerization of MG and preparation of enzyme electrode*

The electropolymerization of MG was carried out in two steps: (i) preanodization under a constant potential of +1.4 V for 10 min and (ii) voltammetric cycling between −0.4 and +0.1 V at 100 mV $s^{-1}$. This procedure was employed to achieve the electropolymerization of thionine from neutral aqueous solution for the first time [24]; the process being carried out from acetonitrile solution or $H_2SO_4$ aqueous solution in the past. The electropolymerization of MG was carried out in an one-compartment electrochemical cell, containing $5 \times 10^{-5}$ M MG in 5 ml of 0.1 M phosphate buffer solution (pH 6.5). The $H_2O_2$ sensing biosensor was prepared by cyclic scanning in a mixed solution of $5 \times 10^{-5}$ M MG and 1000 U HRP in 1 ml of pH 6.5 phosphate buffer according to the two-step method. After the films of PMG and PMG with HRP were formed, the electrodes were rinsed thoroughly with doubly distilled water and dipped into the buffer solution to test their electrochemical behaviour and the electrocatalytic activity toward $H_2O_2$ reduction in the potential range −0.4 to +0.1 V. When not in use, the enzyme electrodes were stored in pH 6.5 phosphate buffer at 4 °C.

All solutions tested were thoroughly deoxygenated by bubbling pure nitrogen and a continuous flow of nitrogen was maintained over the solution during experiments.

## 3. Result and discussion

### 3.1. *Electropolymerization of MG and electrochemical responses of the PMG-modified GCE*

After a preanodization operation under a constant potential of +1.4 V had been carried out on a bare GCE in pH 6.5 phosphate buffer solution containing $5 \times 10^{-5}$ M MG, the cyclic voltammograms which indicate the 'progressive' nature during the formation course of PMG with consecutive cycles were recorded, as shown in Fig. 1A. It is observed that a pair of redox peaks appears around −0.25 V with successive scanning. With increase in scan number, the peak currents increase. At the same time the anodic peak potential shifts in the positive direction and the cathodic peak potential in the negative direction. These results indicate the increase in quantity of electroactive species on the electrode and hence a decrease in the rate electron transfer. The two steps described above in the preparation of the PMG-modified electrode are absolutely necessary. If the potential sweep range is confined within the range −0.4 to +0.1 V directly, without the pretreatment of oxidation at high positive voltage, a cyclic voltammogram corresponding to the redox reaction of MG monomer is observed (Fig. 1B), which is not well-defined. In this case, the cyclic sweep in this potential range results in no substantial film formation. The oxidation potential in the first step is the most important factor for

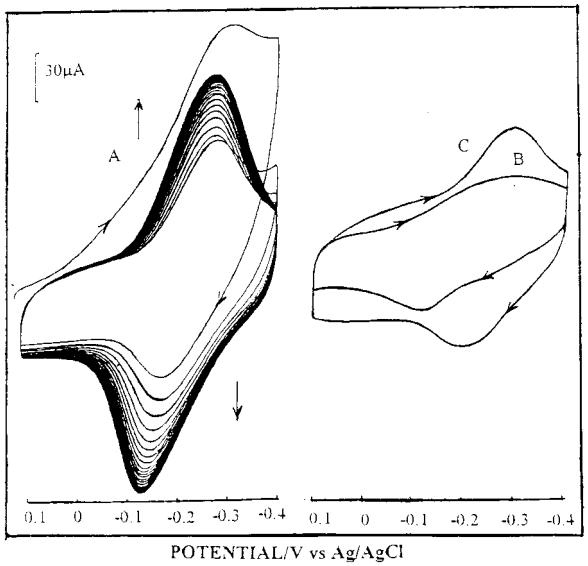



Fig. 1. Cyclic voltammograms of (A) progressive course of the PMG on a preanodized GCE at +1.4 V for 10 min in pH 6.5 phosphate buffer containing $5 \times 10^{-5}$ M MG, (B) an unpreanodized GCE in the same solution as (A), (C) PMG-modified GCE in pH 6.5 blank phosphate buffer; scan rate, 100 mV $s^{-1}$.

the electropolymerization of MG and should not be less than +1.2 V. It is considered to involve two main aspects. One is the surface activation of GCE by preanodization, the other is the formation of 'active species' for polymerization (e.g., radical cation) [21, 22]. On removal of the electrode from the dye-containing solution, a golden film was seen on the electrode surface. When the filmed electrode was washed and examined in background phosphate buffer (pH 6.5), the cyclic voltammogram (Fig. 1C) confirmed the presence of surface-attached electroactive material with a formal potential value of −240 mV.

Figure 2A shows the cyclic voltammograms of the PMG-modified GCE at various scan rates obtained in pH 6.5 phosphate buffer solution. The peak currents are linearly proportional to the scan rates (Fig. 2B), confirming a surface-confined redox couple. The values of surface coverage of PMG from the recorded voltammograms by integration of the cathodic current peaks, correspond to the range $4.38 \times 10^{-8}$ to $2.05 \times 10^{-9}$ mol $cm^{-2}$ which is dependent on the time and potential of preanodization.

The immobilization of MG by electropolymerization on a GCE surface is very firm and permanent. Treatment in an ultrasonic bath for 15 minutes in doubly distilled water does not affect the redox peaks of PMG. Only polishing again with alumina paste can remove the golden film completely. A long term ultrasonication operation has been reported as decreasing the redox peak of the poly(azur I) film [17]. A further successive potential scanning at 100 mV $s^{-1}$ for 1 h in pH 6.5 phosphate buffer can cause about a 10% decrease in current response.

The electrochemical behavior of PMG is affected by pH as are other polymers of dyes [17, 19]. The pH dependence of the formal potential is shown in Fig. 3. The anodic and cathodic peak potentials shift in the negative direction with increasing pH in phosphate buffer solution. The $E^{\circ\prime}$ decreases by 57 mV per pH unit between pH 6 and 8 for the immobilized PMG, which is close to the anticipated Nernstian value of 59 mV for a two-electron, two-proton process.

### 3.2. *Preanodization dependence of electropolymerization of MG*

As already mentioned, the preanodization process is critical for the electropolymerization of MG and should not be less than +1.2 V. In fact, this preanodization treatment can be carried out in either the background phosphate buffer or the buffer containing MG. Experiments reveal that the amount of electropolymerized MG increases with increasing preanodization potential and time. These results have been discussed in detail in another paper [24]. When the two-step method is applied to electropolymerize MG on a gold, platinum or a $SnO_2$ electrode, we can not obtain the PMG film on these substrates.

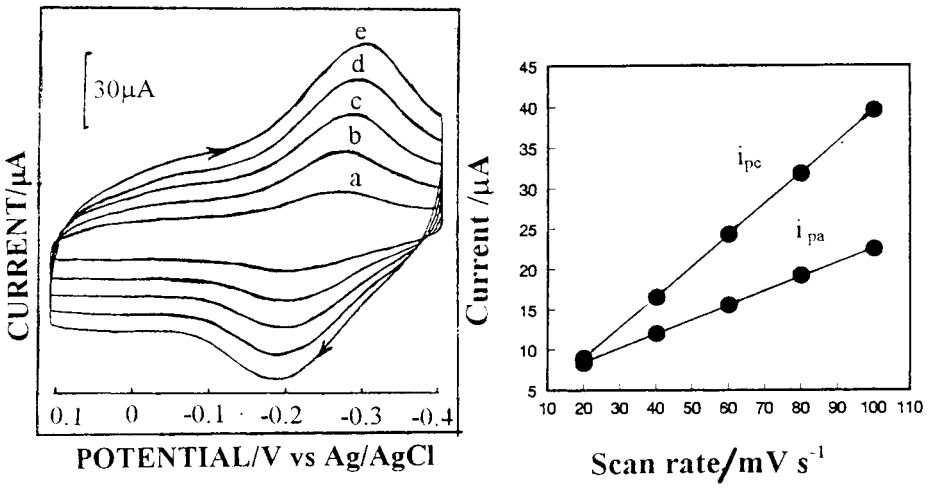



Fig. 2. (A) Cyclic voltammograms of a PMG-modified GCE in 0.1 M phosphate buffer solution (pH 6.5) at scan rates of (a) 20, (b) 40, (c) 60, (d) 80, (e) 100 mV $s^{-1}$. (B) Plot of anodic and cathodic peak current against scan rates.

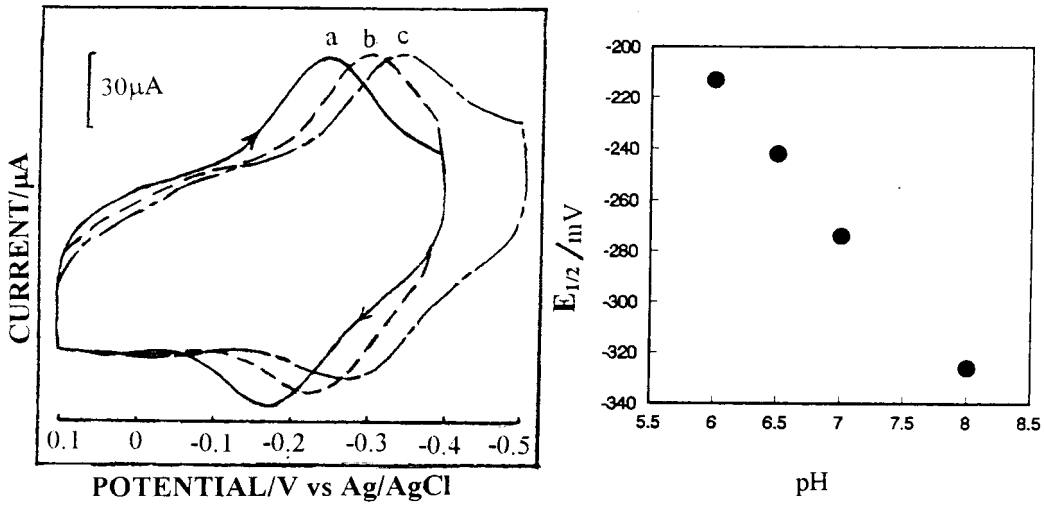



Fig. 3. (A) Cylic voltammograms of a PMG-modified GCE in 0.1 M phosphate buffer at pH value of (a) 6.0, (b) 7.0, (c) 8.0; scan rate 100 mV $s^{-1}$. (B) pH dependence of $E^{\circ\prime}$ of a PMG-modified GCE.

These experiments prompt consideration of the reasons for polymer film formation occurring only at a glassy carbon electrode. Schlereth and Karyakin have suggested that if the parent monomer has primary or secondary amino groups as ring substituents, the cation-radical species are formed relatively early (at about +800 mV) [25]. The mechanism is like that proposed by Bandreay and Archer [21]. In the case when the parent monomer contains two tertiary amino substituents, the cation–radical species are formed at about +1200 mV and an oxidation of at least one of the amino groups is required before the polymerization process can start [25].

The fact that the preanodization treatment can be performed in either the background phosphate buffer or the buffer containing MG, indicates that the preanodization itself does not involve the formation of the cation–radical species. However, if the potential sweep is confined directly within the region of −0.4 to +0.1 V, only an ill-defined cyclic voltammogram is observed (Fig. 1B) and no substantial film results in. Therefore, it is considered that the preanodization treatment is mainly used to activate the glassy carbon electrode.

To gain insight into the process that occurs during the electropolymerization of MG, we performed some experiments on the preanodization of glassy carbon electrodes at a high positive voltage and then a cycle between 0 and −1.0 V in supporting electrolyte solution alone, without the addition of the electroactive species. The cyclic voltammogram is shown in Fig. 4A. From the reductive segment of the first cycle, a cathodic peak is observed, which also appeared during electropolymerization of MG (Fig. 4B), while in the subsequent cycles, no further anodic or cathodic peaks are observed (Fig. 4A). These cathodic peaks in the first cycle after an oxidation treatment are similar to the cathodic scans following preanodization in Engstrom's work [26].

Engstrom [26] reported that cathodic scan yielded a peak after preanodization of GCEs and the cathodic peak was considered to originate from a reaction involving the electrode itself. When the charge passed during preanodization is compared with the charge passed in the resulting cathodic peak in the subsequent cathodic scan, the cathodic peak accounts for only 4.5% of the total charge passed during preanodization. The remaining charge passed during preanodization apparently goes into surface process not reduced in the cathodic peak or into the production of solution species, or both [26]. Furthermore, the large increases in the voltammetric peak heights for probe species, for example, electroactive cations, on the activated against polished GCE surfaces suggested that anodization of a GCE induced adsorption [27, 28]. Hence, when a preanodized electrode is immersed in a solution containing MG, MG molecules can be adsorbed on the electrode

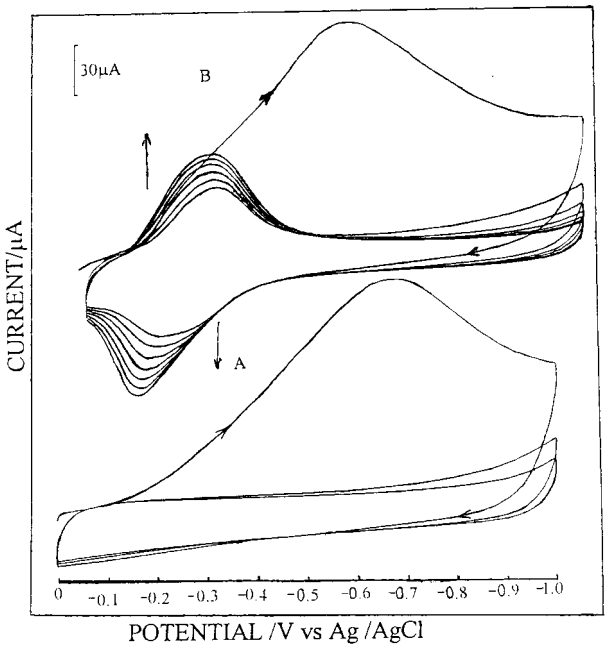



Fig. 4. Cyclic voltammograms following preanodization of GCE at +1.4 V for 10 min in a pH 6.5 phosphate buffer solution (A) without and (B) with MG; scan rate 100 mV $s^{-1}$.

surface and then oxidized into cation radicals by those accumulated charges during preanodization, Finally, these reactive cation radicals link through a nitrogen atom as a common linkage between monomer units in these kinds of polymer [25] with cycling between −0.4 and +0.1 V. The amount of electropolymerized MG on a glassy carbon electrode increases with increase in the accumulated positive charges. If MG molecules are adsorbed onto the electrode surface directly, without cycling between −0.4 and +0.1 V, the colour of the electrode surface is purple, not golden. That is why we emphasized above that the two steps are absolutely necessary for the electropolymerization of MG in this manner.

### 3.3. *Immobilization of HRP in the PMG film*

The fact that the GCE can be modified by the electropolymerization of MG monomer according to the two-step method under very gentle conditions suggests the immobilizing of enzymes by the PMG film. The enzyme electrode is fabricated by electrochemical polymerization of MG in the presence of HRP onto the GCE surface. HRP can be readily incorporated into the polymerized MG film during growth of the polymer. The electropolymerization of MG and the immobilization of HRP are carried out according to the two-step method used for PMG. The presence of HRP in the solution for the electropolymerization of MG does not result in a substantial effect. The GCE surface modified by the electropolymerization from the mixed solution of MG and HRP is also a golden, fairly even film.

Unlike PMG, a similar operation failed to effect the electropolymerization of thionine from a mixed solution of thionine and HRP in an attempt to fabricate an enzyme electrode. This may be attributed to the strong electron attracting group (–$NO_2$) in MG. Although both MG and thionine are cation dyes in neutral solution [25, 29], the MG molecule, with the strong electron-attracting–$NO_2$ functional group, is expected to have a stronger affinity with HRP than thionine, with its electron-donating pendant amine group. To understand the affinity action between MG and HRP, the u.v.–vis. spectra are recorded in pH 6.5 phosphate buffer with $1 \times 10^{-5}$ M MG and 50 U $ml^{-1}$ HRP, respectively, as well as mixed solution of MG and HRP. However, the spectrum of the mixed solution of MG and HRP is simply the addition of the spectra from MG and HRP alone, that is, the affinity action between HRP and MG is not so strong as to cause apparent changes in the u.v.–vis. spectra although the immobilization of HRP can be achieved by the electropolymerization of MG. The PMG-modified GCE surface with HRP is also a golden, fairly even film.

### 3.4. *Amperometric biosensoring of $H_2O_2$ through the $H_2O_2$ sensor*

In pH 6.5 phosphate buffer solution with no $H_2O_2$, the enzyme immobilized in the film of PMG does not contribute to the response and only the PMG on the $H_2O_2$ sensor shows electrochemical behaviour with a linear plot of peak current against scan rate within the range 20 to 100 mV $s^{-1}$. This is very similar to the behaviour of PMG without HRP (not shown here). No catalytic reduction current is found on the GCE modified by the PMG when $H_2O_2$ is added to the phosphate buffer, but on the electrode modified by the PMG film with HRP, a catalytic reduction wave is observed. Figure 5 shows the cyclic voltammograms of the $H_2O_2$ sensor in the absence (curve a) and the presence (curve b) of 1 mM $H_2O_2$ in the pH 6.5 phosphate buffer solution at a scan rate of 60 mV $s^{-1}$. Without $H_2O_2$, in the pH 6.5 phosphate solution, the cyclic voltammogram shows the reduction of PMG at approximately −0.28 V. When $H_2O_2$ is added, the voltammetric behaviour changes with an increase in the reduction current and a decrease in the oxidation current, which indicates that a catalytic reaction occurs on the $H_2O_2$ sensor. This demonstrates that PMG cannot only immobilize the horseradish peroxidase but also effectively shuttle electrons between the redox centre of the enzyme and the base electrode. The process of electron transfer between $H_2O_2$ and the $H_2O_2$ sensor can be illustrated as follows.

At first HRP reduces the $H_2O_2$ diffusing from the solution:

$$H_2O_2 + HRP_{(red)} \longrightarrow H_2O + HRP_{(OX)}$$

Then the oxidized HRP oxidizes PMGH to $PMG^+$:

$$HRP_{(OX)} + PMGH \longrightarrow HRP_{(red)} + PMG^+$$

This overall rereduction reaction of $HRP_{(OX)}$ includes two separate steps [30, 31]:

$$HRP(I) + PMGH \longrightarrow HRP(II) + PMG\bullet \quad (1)$$

$$HRP(II) + PMG\bullet \longrightarrow HRP_{(red)} + PMG^+ \quad (2)$$

Where one electron is donated at a time, HRP(I) is $HRP_{(OX)}$ and PMG• represented the free radical

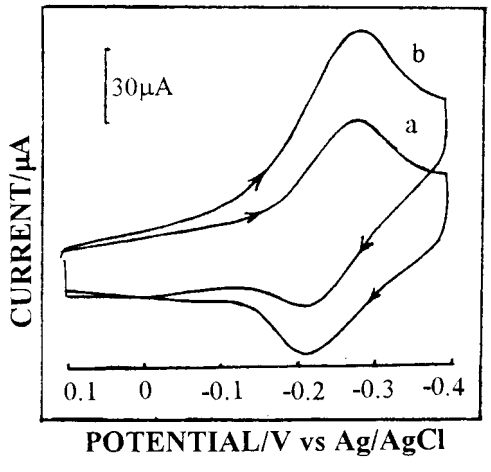



Fig. 5. Cyclic voltammograms of the $H_2O_2$ sensor in 0.1 M phosphate buffer solution (pH 6.5) in the absence (a) and presence (b) of 1 mM $H_2O_2$; scan rate 60 mV $s^{-1}$; the surface coverage of PMG is $8.6 \times 10^{-9}$ mol $cm^{-2}$.

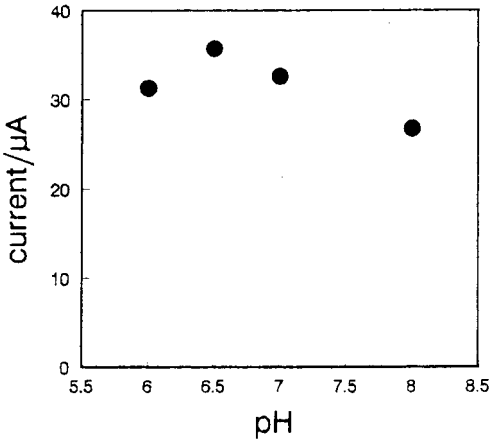



Fig. 6. Effect of pH on the PMG-mediated $H_2O_2$ sensor at 25 °C when $[H_2O_2] = 1$ mM in 0.1 M phosphate buffer solution.

formed during the reaction. Subsequently oxidized PMG is reduced to PMGH to produce the cathodic catalytic current at the sensor:

$$PMG^+ + H^+ + 2e^- \longrightarrow PMGH$$

The pH dependence of the electrocatalytic current for the $H_2O_2$ sensor has two causes. One cause is that pH affects the activity of HRP, the other is that pH affects the peak potentials of PMG. Therefore, both the oxidation and reduction reactions of the HRP catalysing cycle are affected by the pH of the working buffer. Concerning these two aspects, a pH range between 6.0 and 8.0 has to be selected. In this work, an optimum pH of 6.5 is chosen (Fig. 6) , which is not only critical for the PMG's peak potential proper for the catalytic reaction of HRP, but also guarantees the catalytic activity of HRP.

When the potential is set at −0.28 V, the reduction peak potential of PMG, and the current response of the $H_2O_2$ sensor at various $H_2O_2$ concentration can be determined and a calibration plot made. Figure 7 shows the current responses as a function of $H_2O_2$ concentration.

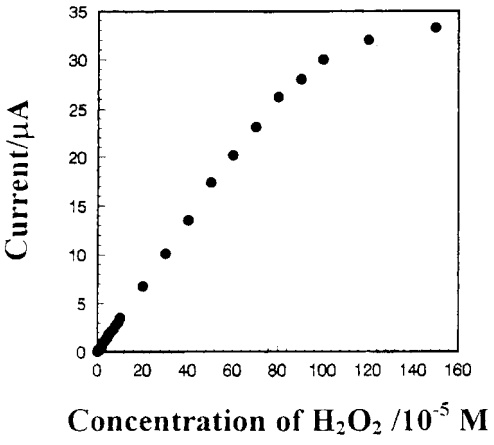



Fig. 7. Calibration plot for the PMG-mediated $H_2O_2$ sensor in 0.1 M phosphate buffer solution at 25 °C; the surface coverage of PMG is $8.6 \times 10^{-9}$ mol cm$^{-2}$.

## 4. Conclusion

We have achieved the immobilization of horseradish peroxidase in an electropolymerized methylene green redox film and constructed a novel $H_2O_2$ sensor. The catalytic reaction occurring on the $H_2O_2$ sensor in the presence of $H_2O_2$ demonstrates that the film of PMG cannot only immobilize the horseradish peroxidase but also effectively shuttle electrons between the enzyme's redox center and the base electrode. The electropolymerization of MG is carried out according to a two-step method from a neutral phosphate buffer solution, which only occurs on glassy carbon electrodes. The critical factor for the electropolymerization of MG lies in the operation of preanodization on a glassy carbon electrode, because a large amount of positive charges can be accumulated and used to create the cation radicals in order to achieve the formation of the polymer film of MG.

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