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Full Paper

A Novel Amperometric Hydrogen Peroxide Biosensor Based on Catalase Immobilization on Poly(glycidyl methacrylate-co-vinylferrocene)

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Abstract- A novel amperometric hydrogen peroxide biosensor was fabricated for the determination of H_2O_2. Catalase (Cat) was immobilized on a glassy carbon electrode by poly(glycidyl methacrylate-co-vinylferrocene) (poly(GMA-co-VFc)). A polymeric electron transfer mediator, containing copolymers of glycidyl methacrylate (GMA) and vinylferrocene (VFc) with different molar ratios, have been prepared by free radical copolymerization. Amperometric response was measured as a function of concentration of H_2O_2, at fixed potential of +0.35 V vs. Ag/AgCl in a phosphate buffered saline (pH 7.5). The mediated hydrogen peroxide biosensor had a fast response of less than 7 s with linear range 0.5-14 mM. The sensitivity of the biosensor for H_2O_2 was 34 nA/mM.cm^2.

Keywords – Catalase, Hydrogen Peroxide Biosensor, Redox Polymer, Mediator, Ferrocene

1. INTRODUCTION

Hydrogen peroxide (H_2O_2) is a by-product of several highly selective oxidases, and also an essential mediator in food, biology, medicine, industry and environmental analysis [1,2].

The development of reliable, sensitive and accurate methods for hydrogen peroxide determination is becoming of practical importance. Many techniques have been employed for this determination, such as titrimetry [3], spectroscopy [4], chemiluminescene [5], but these techniques suffer from interferences, long analysis time and use of expensive reagents. Among these techniques, electrochemical technique based on enzyme biosensors has been extensively employed for the determination of H_2O_2 with simplicity, intrinsic selectivity and sensitivity [6-9]. As a result of the easy availability in high purity and low cost, catalase has been the most widely studied in the development of enzyme-based amperometric biosensors [10-12]. However, to improve the performance and long-term stability of the enzyme electrode, effective immobilization of Cat onto the transducer surface through suitable matrix is of great significance. Therefore, several valuable immobilization strategies and materials have been employed including entrapment [13], use of biological membranes [14,15], adsorption [16], covalent binding [17] and so on.

Hydrogen peroxide biosensors using catalase as a biological molecular recognition element are a simple and inexpensive methodology because molecular oxygen produced in the enzymatic reaction can easily be and amperometrically monitored by a Clark-type oxygen electrode [18-20]. Catalase is a heme-containing enzyme that is ubiquitous in aerobic organisms. It catalyzes the decomposition of hydrogen peroxide to water and oxygen:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The development of reagentless enzyme electrodes implies the covalent binding of enzymes and redox mediators on the sensor surface to prevent contamination of the sample by sensor components. Ferrocene derivatives are an excellent electron mediator which is widely used as mediators in the construction of mediated amperometric biosensor. Development of polymeric mediator for applications in sensor/biosensor is essential because polymers allow the incorporation of reagents to achieve reagent less devices. Direct attachment of the ferrocene-based mediators into polymeric films prohibits the leaching of the mediator. Some examples of redox copolymers where covalent attachment of ferrocene has been attempted are poly(vinylferrocene-co-hydroxyethyl methacrylate) [21], poly(N-acryloylpyrrolidine-co-vinylferrocene) [22], acrylamide copolymers [23] and multiwall carbon nanotubes [24].

In this article, we report on the construction, characterization, and application of a biosensor based on Cat covalently immobilized on poly(glycidyl methacrylate-co-vinylferrocene). A series of redox copolymers of poly(glycidyl methacrylate-co-vinylferrocene) were prepared by free radical copolymerization of vinylferrocene monomer with an epoxy group carrying comonomer (GMA). This work illustrates the versatility of an epoxy-based redox copolymer poly(glycidyl methacrylate-co-vinylferrocene) as an immobilization platform for the fabrication of enzyme electrode. The performance and

factors influencing the resulting biosensor were studied in detail. The resulted biosensor exhibited high sensitivity, good reproducibility, and long-term stability.

2. EXPRIMENTAL

2.1. Reagents

Catalase from bovine liver (EC 1.11.1.6) with a specific activity of 2800 units per gram of solid was obtained from Sigma. Glycdyl methacrylate (GMA) and 2,2'-azobis(isobutyronitrile) (AIBN) were purchased from Fluka and Across Chemical Co. and used without further purification. Reagent-grade vinylferrocene (VFc) was purchased from Aldrich Co. and used without purification. All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation and characterization of poly (GMA-co-VFc)

The redox polymer, having different compositions, was prepared according to our previous study (Fig. 1) [25]. A mixture of VFc and GMA at a known molar ratio was injected into a Pyrex tube, AIBN (1 mol % on the basis of total monomer concentration was same for all samples, 5 mol/dm^3). The mixture was degassed by using Argon gas and sealed under vacuum. After degassing, the tubes were placed in constant temperature baths controlled to 70 ^{o}C. After two days, the reaction mixture was added drop wise to rapidly stirring diethyl ether to precipitate the copolymer. Precipitated copolymer was washed with diethyl ether and reprecipitated in this manner two more times. Precipitated product was then dried under vacuum.

2.3. Fabrication of enzyme electrodes

To evaluate the electrochemical property, 10 μL of 1 wt % poly (GMA-co-VFc) in DMF was dropped onto the glassy carbon electrode. After drying at room temperature, the cyclic voltammetry (CV) of the copolymer was measured in 100 mM phosphate-buffered 0.8% saline (PBS, pH 7.5).

Cat was immobilized by covalent attachment on poly (GMA-co-VFc) coated glassy carbon electrode (GCE). 1 wt % solution of poly (GMA-co-VFc) in DMF was dropped onto the electrode and dried in air. Functional epoxy group carrying copolymer film electrode was immersed in 100 mM phosphate buffer (pH 7.5) for 2 h, and transferred to the same fresh medium containing Cat (2.0 mg/ml). Immobilization of Cat on the poly (GMA-co-VFc) film was carried out by continuously stirring the reaction medium at 24 ^{o}C for 24 h. After this period, electrode was removed from medium and washed with phosphate buffer (100 mM, pH 7.5).

2.4. Electrochemical measurements

Electrochemical measurements were performed using a CHI Model 842B electrochemical analyzer. A small Glassy Carbon working electrode (2 mm diameter), a Platinum wire

counter electrode (0.2 mm diameter), Ag/AgCl-Saturated KCl reference electrode and conventional three-electrode electrochemical cell were purchased from CH Instruments.

All amperometric measurements were carried out at room temperature. They were performed in stirred solutions by applying the desired potential and allowing the steady state current to be reached. Once prepared, the Cat electrode were immersed in 10 ml of a pH 7.5 100 mM PBS solution and the amperometric responses to the addition of known amount of hydrogen peroxide solution were recorded, respectively. The data are the averages of three measurements.

3. RESULTS AND DISCUSSION

3.1. Preparation of poly(GMA-co-VFc)

Poly (GMA-co-VFc) having different monomer ratios was prepared from glycidyl methacrylate and vinylferrocene monomers [25]. The present method was effective in that the reactive epoxy group was readily introduced into polymer support without any modification. In addition, the required functional group composition could also be fixed by changing the co monomer ratio in the polymer preparation mixture.

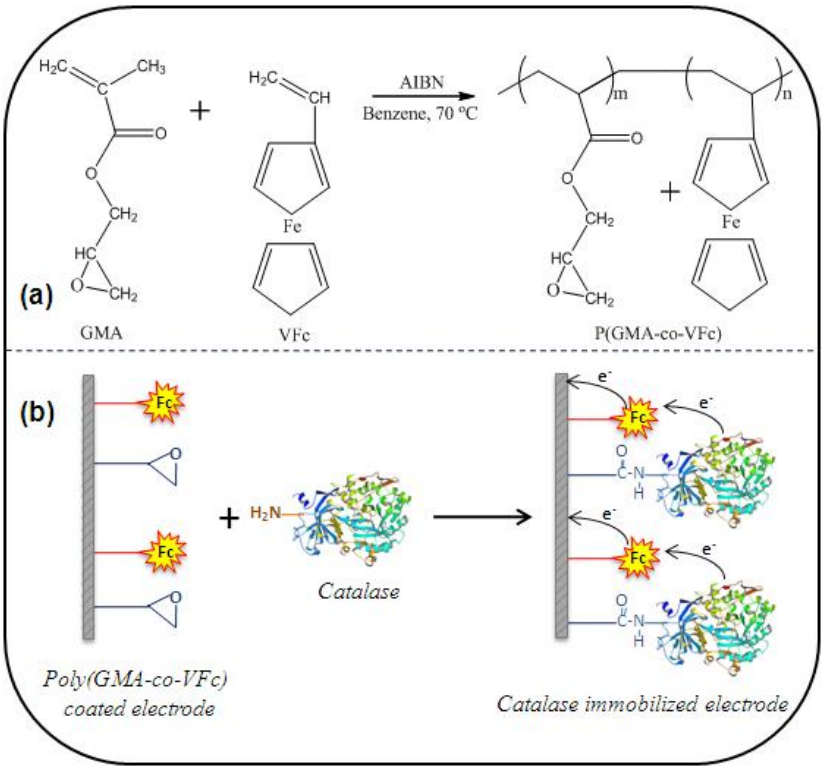

Fig. 1. (a) Preparation of poly (GMA-co-VFc) and (b) immobilization of enzyme via amine group onto Poly (GMA-co-VFc) film electrode

The epoxy group can bind the protein molecules via their amine, thiol, hydroxyl and carboxyl groups at the pH range where the enzyme is stable and does not lose activity. The C-N or O-C bonds formed between epoxy groups and biomolecules are stable, so that epoxy group containing supports are useful for enzyme immobilization. The chemical structure of poly (GMA-co-VFc) and the immobilization reaction via amine groups are presented in Fig. 1.

The Michaelis-Menten analysis results are summarized in Table 1. K_m^{app} and j_{max} values represent the linear response range and the dynamic range of the sensors, respectively. These results do not show the intrinsic property of enzyme, but show the properties each enzyme electrode.

Table 1. Apparent Michaelis-Menten constants and maximum current densities for enzyme electrodes

Abbreviation	K_m^{app} (mM)	j_{max} (nA.cm^2)
Poly(GMA-co-VFc)(0.2)	30.8	523
Poly(GMA-co-VFc)(0.4)	35.4	1355
Poly(GMA-co-VFc)(0.6)	24.5	909
Poly(GMA-co-VFc)(0.8)	71.2	724

Table 1 show that the catalytic current response depends on the composition of the copolymers. The catalytic response of the enzyme electrode takes a maximum with increasing the VFc composition up to 0.4 ratio.

3.2. Cyclic voltammograms of poly(GMA-co-VFc)

The cyclic voltammograms of poly(MTM-co-VFc) were obtained between 0.1 and 0.8 V in a PBS after the copolymer was casted onto a GCE. Fig. 2a show that the anodic and cathodic current of the copolymer film rise continuously with potential scans until a distinct redox couple of Fc occurred and reached steady state after 12-16 cycles. After equilibrium was established, the peak potentials Epa and Epc values remained constants. The steady state values for Epa and Epc of cyclic voltammograms shown in Fig. 2a are 0.33 and 0.54 V, respectively.

Typical cyclic voltammograms of the electrode loaded with a copolymer in PBS, when the scan rate was altered from 1 to 20 mV/s, is shown in Fig. 2b. A linear correlation between anodic peak current, Ipa, and square root of scan rate, $V^{1/2}$, was obtained. This result indicates

that charge propagation in the polymer occurs by a diffusion-like process such as electron hoping among neighboring redox sites and counter ion motion. Initially, the ferric ion in the VFc units exists in both the reduced form (Fe(II)) and oxidized form (Fe(III)). During the forward scan, Fe(II) is oxidized to Fe(III), and subsequently an oxidation current peak is observed. During the reverse scan, Fe(III) is reduced. The difference of the redox peaks was increased with increasing a scan rate. The voltammetric behavior also indicates that ferrocene has been immobilized on the surface of the glassy carbon electrode.

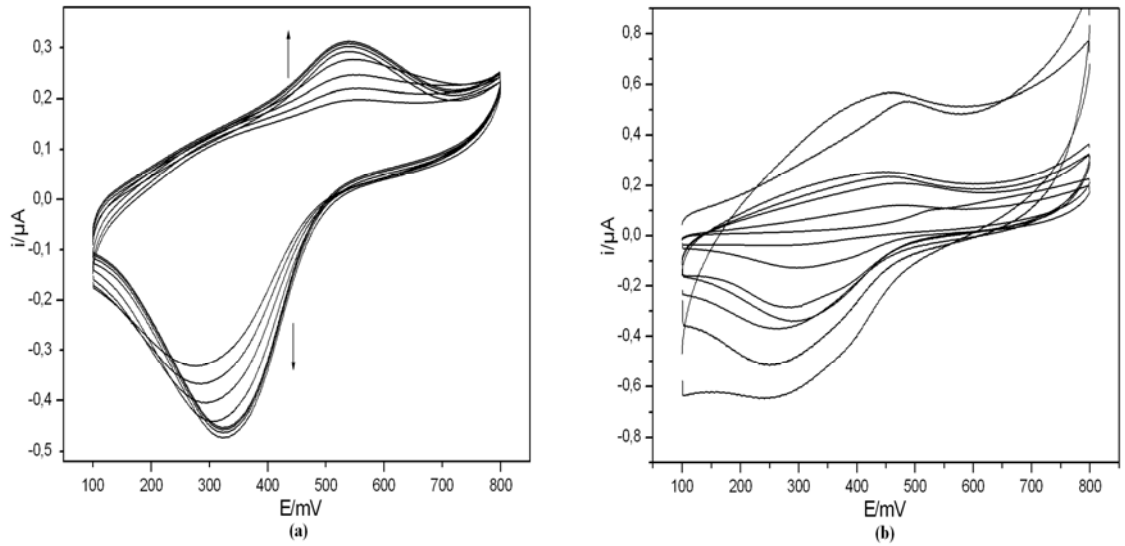

Fig. 2. Cyclic Voltammogram of poly(GMA-co-VFc) in 0.1 M PBS (pH 7.5) (a) at scan rate 5 mV/s (b) at scan rate of 1, 2.5, 5, 7.5, 10, 15 and 20 mV/s (from internal to external)

3.3. Catalytic current for enzyme electrode with different copolymer ratios

The steady-state catalytic current response of the enzyme electrodes containing GMA and VFc with different compositions as a function of H_2O_2 concentration at +0.35 V vs. Ag/AgCl are shown in Fig. 3. The catalytic current of enzyme electrode increases with increasing of the hydrogen peroxide concentrations. It is likely that this behavior follows the Michaelis-Menten kinetics, as seen many amperometric enzyme electrodes [26,27].

3.4. Steady-state amperometric response to hydrogen peroxide

Under optimized conditions the typical current-time response shows a good analytical performance (Fig. 4). The catalytic current change was monitored while aliquots of hydrogen peroxide were added in PBS solution. A well defined reduction current proportional to the hydrogen peroxide concentration has been observed. As shown in Fig. 4 during the successive addition of 0.5 mM H_2O_2, a well defined response is observed. The plot of response current vs. H_2O_2 concentration is linear over the concentration range 0.5 to 20 mM.

The calibration plot over the concentration range 0.5-14 mM has a slope of 34 nA/mM.cm^2 (sensitivity), a correlation coefficient of 0.9995 and the detection limit of 0.08 mM. An extremely attractive feature of the enzyme electrode, is its fast response time (i.e,<6 s).

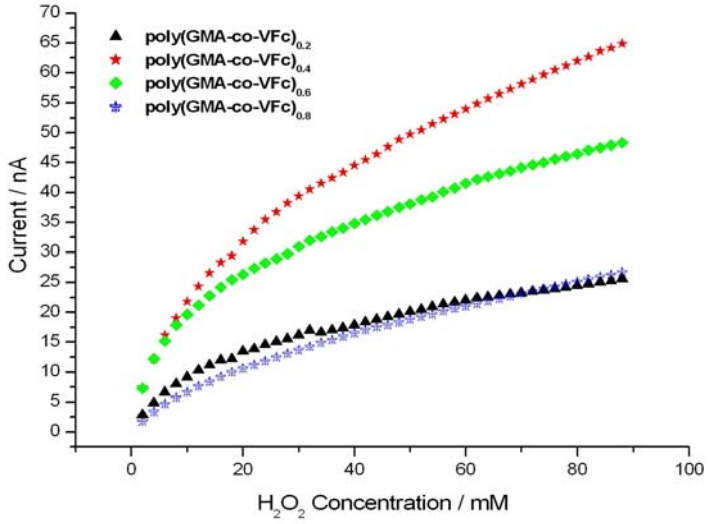

Fig. 3. Comparison of amperometric responses of the enzyme electrode at an applied potential +0.35 V fabricated with (a) GMA-co-VFc0.2, (b) GMA-co-VFc0.4 ,(c) GMA-co-VFc0.6 ,(d) GMA-co-VFc0.8 to successive addition of H_2O_2 at constant potential pH 7.5 10 mM PBS

When the concentration of H_2O_2 was higher than 16 mM, a response plateau was observed, showing the characteristics of the Michaelis-Menten kinetic mechanism. The apparent Michaelis-Menten constant (K_m^{app}), which gives an indication of the enzyme-substrate kinetics, can be calculated from the electrochemical version of the Lineweaver-Burk equation:

$$\frac{1}{I_{ss}} = \frac{I}{I_{max}} + \frac{K_m^{app}}{I_{max}^{c}}$$

Where I_{ss} is the steady-state current after the addition of substrate, c is the bulk concentration of the substrate and I_{max} is the maximum current measured under saturated substrate condition. The K_m^{app} was determined by analysis of the slope and intercept for the plot of the reciprocals of the cathodic current versus H_2O_2 concentration. The K_m^{app} value of the H_2O_2 biosensor was determined by steady-state amperometric response and found to be

40.7 mM for Cat immobilized on the electrode surface. The smaller K_m^{app} value means that the immobilized Cat possesses higher enzymatic activity, and the present electrodes exhibit higher affinity to hydrogen peroxide. The immobilization of Cat mentioned above appears to be beneficial to improve biosensor's performance.

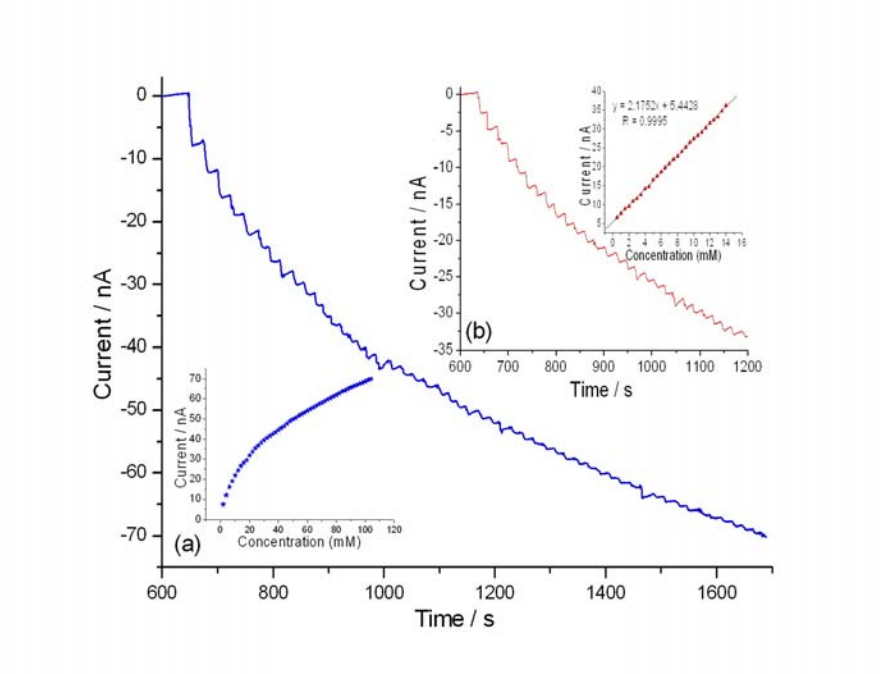

Fig. 4. Amperometric response of the enzyme electrode at at an applied potential +0.35 V pH 7.5 10 mM PBS, (a) successive addition of 2 mM and (b) 0.5 mM: inset plot of amperometric current vs. H_2O_2 concentration and linear calibration curve for determination of *K*m

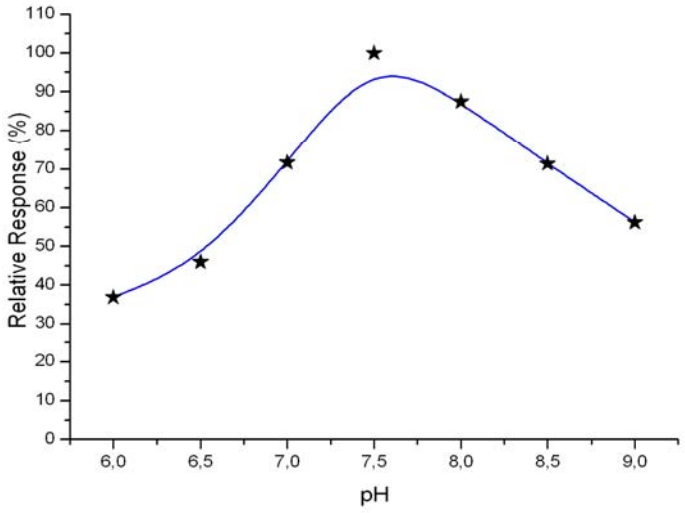

Fig. 5. Influence of pH values on the activity of the enzyme electrode in the presence of 10 mM H_2O_2 in PBS solution. Data collected with freshly prepared enzyme electrodes refer to the average of three experiments

3.5. The effect of pH

The effect of pH was studied between 6.0 and 9.0. As shown in Fig.5. the current response increased from pH 6.0 to 7.5, and decreased from pH 7.5 to 9.0, which was in agreement with that reported for soluble Cat [28]. For each point in Fig. 5, a new enzyme electrode was prepared in order to eliminate the errors that might arise from the reuse the enzyme-loaded copolymer.

3.6. The effect of temperature

The effect of temperature on the steady-state amperometric response was also investigated in the range of 25–50 °C, which was showed in Fig. 6. The response increased with the increase of temperature and reached a maximum at 40 °C, and then the response decreased. It might be caused by the denaturation of Cat or the instability of the films in the high temperature.

The dependence of amperometric current on temperature in an initial region can be expressed as an Arrhenius relationship

$i\ (T)=i_0\exp\{-E_a/RT\}$

where i_0 represents a collection of currents, R is the gas constant, T is the temperature in Kelvin degrees, and E_a is the activation energy. The activation energy for enzymatic reaction was calculated to be 2.87 kJ mol^{-1} from the slope of $I-1/T$ in the adoptive region of temperature.

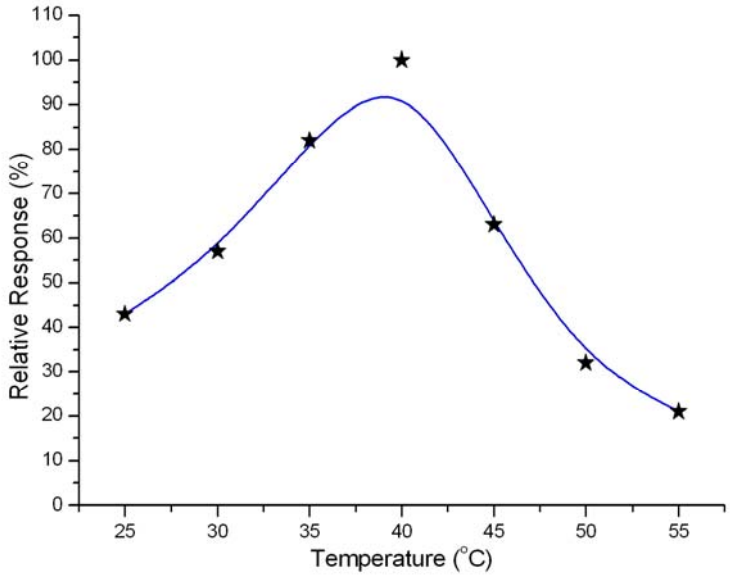

Fig. 6. The effect of temperature on the response of the Cat immobilized electrodes in the presence of 10 mM H_2O_2 in PBS solution. Data collected with freshly prepared enzyme electrodes refer to the average of three experiments

3.7. Reusability and storage stability

The operational stability Cat electrode was obtained by running measurements in the same day. Between each subsequent measurement electrodes stored at 4 ^{o}C in the buffer solution for 10 min. The first 20 measurements revealed the same response, an activity loss of 20 % was observed with the subsequent use (Fig.7a).

The response of the Cat electrode was measured of its response to 10 mM H_2O_2 for a period of 60 days. As shown in Fig. 7b the amperometric response of the enzyme electrode remained constant for 34 days, and then an activity loss of 45 % was observed. Covalent attachment of the catalase on the electrode may be responsible in protecting the enzyme from environmental effects and leaking.

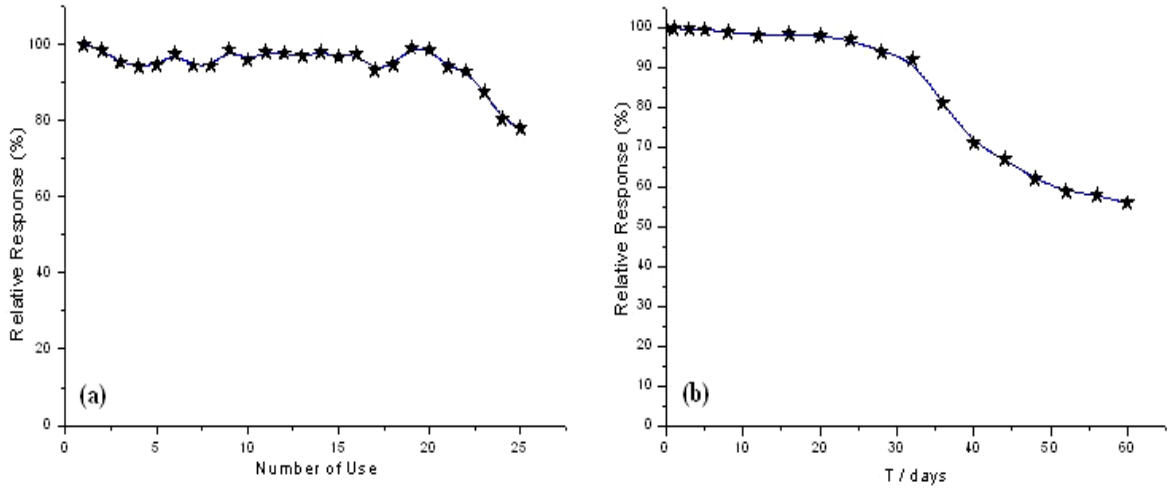

Fig. 7. (a) Reusability of the Cat immobilized electrode. Each data point represents the average of data collected by three electrodes (pH 7.5; ~25^{o}C). (b) Storage stabilites of Cat immobilized electrode. The amperometric responses of these enzyme electrodes are regularly checked during 50 days (pH 7.5; ~25^{o}C).

4. CONCLUSIONS

Poly (glycidyl methacrylate-co-vinylferrocene), as a polymeric mediator, has been shown to be an attractive material for the construction of hydrogen peroxide biosensor. Cat immobilized on the poly (GMA-co-VFc) film maintains its activity. The GMA-VFc copolymers show redox activity in the enzyme electrode, where the copolymer transfer electron between the active site of Cat and the electrode. We have developed a reliable, low cost and sensitive biosensor for H_2O_2. The biosensor had a variety of good characteristics including high sensitivity, good repeatability and reusability, rapid response and long term stability.

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