Fabrication of a novel Hydrogen peroxide Biosensor by immobilizing Microperoxidase on ANHDCT/GC electrode

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Abstract

Introduction: Enzymes play an important role in the metabolic processes of organisms as akind of typical biological macromolecule and special catalyst. Reversible redox transformation of enzymes at an electrode can be used to drive enzyme-catalyzed reactions

Aim : Here, we study the dirct electrochemical behaviour of MP at ANHDCT / glassy carbon Electrode. MP can display unique bioelectrocatalytical properties in the reduction of H_2O_2 by direct electron transfer from electron-donating electrod through the active site of the enzyme and to peroxide in solution.

Material and Method: MP/ ANHDCT electrode was fabricated by casting the mixture of MP and ANHDCT solutions onto glassy Carbon electrode (GCE). Direct electron transfer process of immobilized MP and its application as a biosensor for H_2O_2 , were investigated by using Cyclic Voltammetry.

Results: n This paper is considered to indicat that the MP | ANHDCT | GC electrode undertakes a direct electron transfer reaction and exhibits an excellent electrocatalytic response to the reduction of H_2O_2 in a Phosphate buffer solution (pH = 7) . The MP | ANHDCT | GC electrode can be used for determination of H_2O_2 concentration.

Conclusion : The MP / ANHDCT films may have a potential application in constructing the third-generation electrochemical biosensors based on mediator-free electrochemistry of the enzymes. Further work is in progress to extend to other redox enzyme-based biosensors.

Keywords : Microperoxidase (MP) , ANHDCT (6-Amino-5-Nitroso-4-Hydroxy-1,3-Diaza-5-Cyclohexene-2-Thioone) , Cyclic Voltammetry(CV) , Hydrogen peroxide (H_2O_2)

Introduction

Enzymes play an important role in the metabolic processes of organisms as a kind of typical biological macromolecule and special catalyst. Reversible redox transformation of enzymes at an electrode can be used to drive enzyme-catalyzed reactions.[1-3] The heterogeneous direct electron transfer reactions between redox proteins and electrode surface have been widely studied,and understanding of these reactions fundamentally can provide insight into physiological electron transfer process as well as impetus for the further Development of biosensors and bioelectrocatalytic systems. Electrical contact of redox enzymes with electrode surface is a key process in fabricating of enzyme electrodes for bioelectronic and

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biosensor applications. It is difficult for most enzymes to exchange electron with normal lectrode surface directly. The electron-transfer rates between large redox proteins and electrode surface are usually prohibitively slow because of the deep burying of the electroactive groups within the protein structure, the adsorptive denaturation of proteins onto electrodes, and the unfavourable orientations at the electrode.[4] Proteins with a heme group, such as Cythochrome C, hemoglobin (Hb) and peroxidases are enzymes defined as redox enzymes using an electron acceptor. Fabrication of highly selective, sensitive and stable biorecognition electrodes containing these biomolecules is an important research field for the construction of biosensors. Recently, various composite films such as polymers,[5-8] surfactants,[9-19] biomembranes [20-23] and nanoparticles [21-25] containing heme proteins were modified on electrode surfaces to obtain their direct electrochemistry. These films may provide a favourable microenvironment and thus enhance the electron transfer rates. Recently, independent and cyclic voltammograms (CV) of heme proteins in various films were reported in the absence of peroxides.[25,26-31] MP is a molecule with electroactive iron hemes, which can be used as an ideal model molecule for the study of electron transfer reactions of heme proteins and also for biosensing and electrocatalysis. Direct electron transfer can be reinforced by immobilizing MP onto electrode surface incorporated stable film such as ANHDCT. Here, we study the dirct electrochemical behaviour of MP at ANHDCT / glassy carbon Electrode. MP can display unique bioelectrocatalytical properties in the reduction of H_2O_2 by direct electron transfer from electron-donating electrod through the active site of the enzyme and to peroxide in solution.[32-34] The electron transfer process in detection of H_2O_2 at MP-modified electrodes can be described by following mechanism :

$$MP(Fe^{+3}) + H_2O_2 \xrightarrow{K_1} \text{Compound 1 } (Fe^{+4}{=}O\,,\, P^{\bullet +}) + H_2O$$

$$\text{Compound 1 } (Fe^{+4}{=}O\,,\, P^{\bullet +}) + 2e^- + 2H^+ \xrightarrow{K_s} MP(Fe^{+3}) + H_2O$$

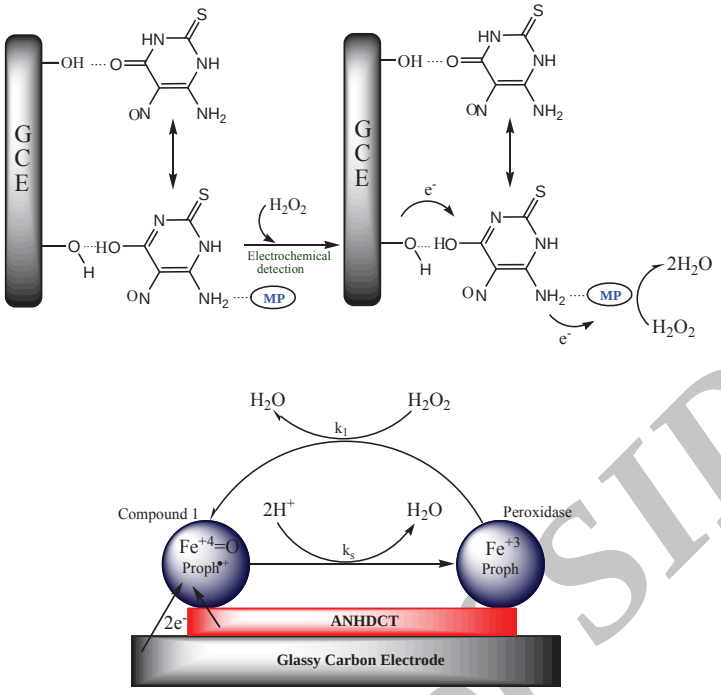
The electrode considered as an electron donor.[32] This process includes oxidation of ferriheme prosthetic group of MP (oxidation state +3) with H_2O_2 to compound 1 , representing oxidized MP with Oxyferryl iron (Fe^{+4}=O) and further direct reduction of compound 1 at the electrode surface to the initial MP state , Ferriperoxidase. Direct electron transfer between modified (ANHDCT /GC) electrode and active site of the enzyme occurs according to above mechanism , and with due attention to suitable orientation of ANHDCT on the surface of this biosensor , high sensitiveness toward H_2O_2 or organic hydroperoxides is possible. (Scheme 1)

Materials and methods

Experimental

Reagents

Microperoxidase (MP) was purchased from Sigma Chemicals Company. ANHDCT was prepared by Dr.A.R.Karimi and supplied to our group as a gift. Hydrogen Peroxide (30% W/W) was from Merck Chemicals Company. The dilute solutions of H_2O_2 was prepared daily. All other chemicals were of analytical grade. Phosphate buffer solution (PBS,0.1M) with various pH values were prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4 and adjusting the pH with 0.1M HCl and NaOH. Millipore filtered water was used for all aqueous solutions and rinsing.



Scheme .1- Schematic presentation of electron transfer from **ANHDCT** /GC electrode to MP

Preparation of MP | ANHDCT |GC electrode

Prior to coating, the glassy carbon electrode (GCE, geometric area 0.125 cm^2) was polished with alumina slurry (0.05 µm) on a polishing cloth and washed with nitric acid, etanol and deionized water. The MP| ANHDCT film was prepared on GC electrode by the following method :20 µl of 1.6×10^{-6} M MP and 20 µl of 10^{-5} M ANHDCT (in minimum amount of DMSO) solution were mixed and dropped on the pretreated GCE surface and allowed to dry for overnight. The modified electrode surface was rinsed with water.

Apparatuse and procedures

Cyclic voltammetry (CV) was carried out with a Autolab 4.9 electrochemical workstation (Ecochemie Instrument). All electrochemical experiments employed a three-electrode cell with a MP| ANHDCT |GC working electrode, a glassy carbon auxiliary electrode and an Ag/Agcl (sat. KCl solution) reference electrode. The buffers were purged with highly purified nitrogen prior to a series of experiments. A nitrogen environment was kept over solutions in the cell for exclusion of oxygen. All measurements were carried out at room temperature (25 ± 1°C). In order to determine the geometric surface area of GC electrode , with using cyclic voltammetry , we used $K_3Fe(CN)_6$ solution with following characteristics , and according to following equation , the geometric surface area of GC electrode was obtained :

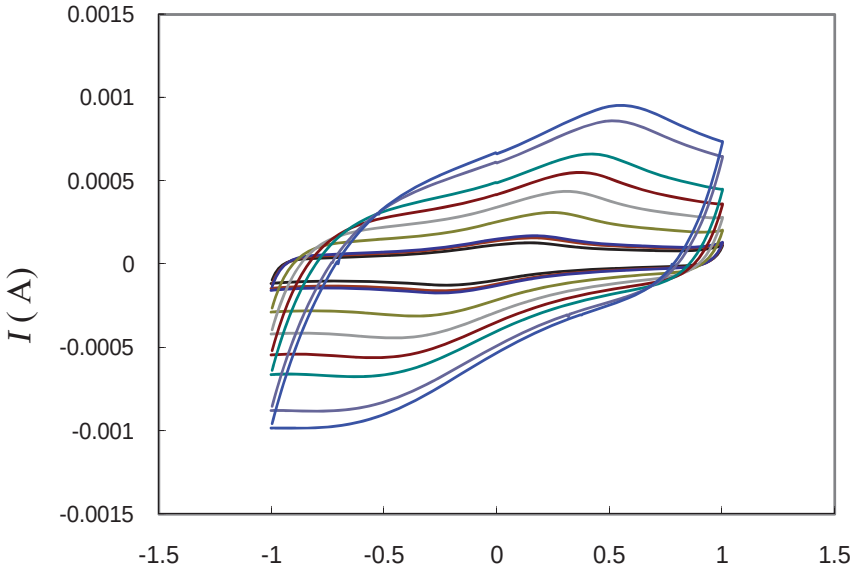
$$I_P = 2.69\times10^5 n^{\frac{2}{3}} A D^{\frac{1}{2}} \nu^{\frac{1}{2}} C_o$$

Where I_p is the peak current , A is the electrode geometric surface area , D is the diffusion coefficient (5.9×10^{-5}) , υ is the scan rate (0.1) and C_o is the concentration of $K_3Fe(CN)_6$ solution (10 mM). $\frac{cm}{s^2}$ $\frac{V}{s}$

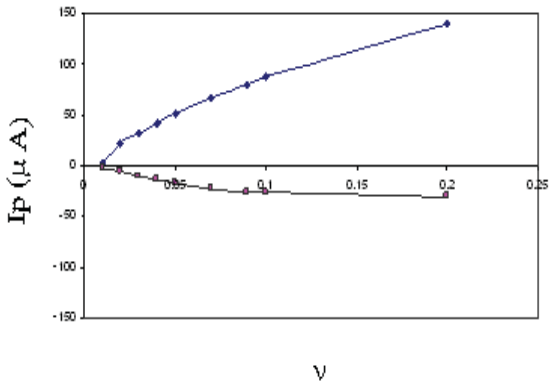
Results and discussion

Electrochemical characteristics of MP| ANHDCT film

The cyclic voltammograms (CVs) of a MP | ANHDCT film at scan rate from 0.01 to 0.8 V/s in 0.1 M Phosphate buffer solution (PH=7) are shown in Fig .1 .



	a	b	c	d	e	f
Scan rate v/s	0.08	0.07	0.06	0.5	0.04	0.03



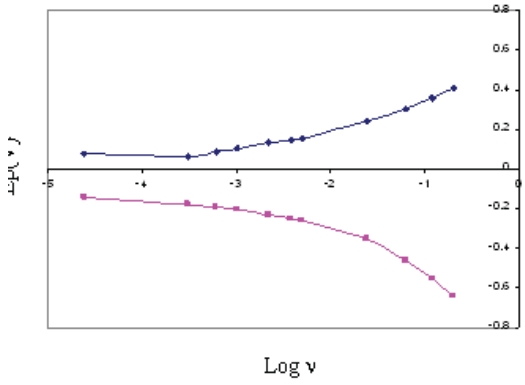


Fig.1- The Cyclic Voltammograms (CVs) of a MP | ANHDCT film in 0.1M Phosphate buferr solution (pH=7).
a- I_p increases linearly with scane rate . b- Line relationship between E_p and Log υ .

MP| ANHDCT film at GC electrode exhibited a well-defined redox waves with reduction and oxidation potentials of -0.142 and 0.078V respectively and formal potential of –0.032V(versus Ag/Agcl(sat.KCl)). Obviously, it was the electroactive center of MP that performed the redox reaction at the MP| ANHDCT modified GC electrode. The same GC electrode modified with MP alone gave much smaller and irreversible reduction in the same potential range. It presented strong evidence that MP and ANHDCT interact with each other and ANHDCT played the role of facilitating the electron exchange between the MP and GC electrode. The redox process was a typical quasi-reversible electrochemical process involving an active substance attached to the electrode. The reduction peak heights of the redox process were found to increase linearly with scan rate from 0.01 to 0.8 V/s (Fig.1a), which was characteristic of thin layers.[31]

The regression equation was deduced as

$$I_p\ (\mu A) = 0.0004\upsilon(V/s) + 3\times10^{-5}$$

According to Laviron's equation :

$$Ip = \frac{n^2F^2A\Gamma}{4RT}\upsilon$$

Γ is the surface coverage of the electrode reaction substance ($molcm^{-2}$) , A is the electrode area (cm^2) and n, I_p , F , R and T have their usual meanings. From the slope of the I_p versus υ plot, the average surface coverage (Γ) of MP immobilized on electrode surface was estimated to be 5.95×10^{-9} $molcm^{-2}$, indicating a multilayer of MP on electrode surface. According to following Laviron's equation :

$$E_P^{\ c} = E^{\circ\prime} - \frac{2.3RT}{\alpha nF} Log\frac{\alpha}{m} \qquad m = \frac{RT}{F}\frac{k}{n\nu}$$

$$E_P^{\ a} = E^{\circ\prime} + \frac{2.3RT}{(1-\alpha)nF} Log(\frac{1-\alpha}{m})$$

The anodic and cathodic peak potentials are linearly dependent on Ln υ : a plot of E_p versus Ln υ (Fig..1b) yields two straight lines with slopes of 0.0928 and -0.127 for the anodic peak and for the cathodic peak, respectively, so that the electron transfer coefficient (α) can be estimated as 0.56 and the electron transfer rate constant (k_s) can be estimated to be $1242s^{-1}$ according to the following Laviron's equation:

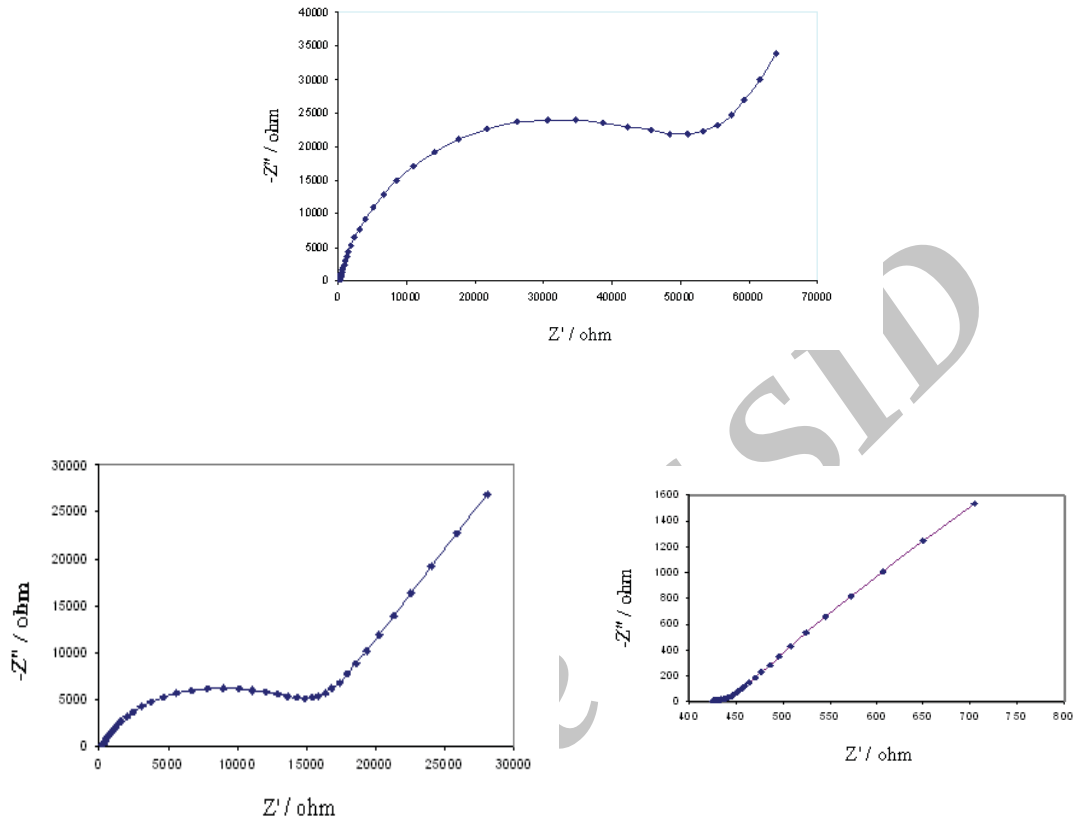
$$\Delta E_p = \frac{2.3RT}{(1-\alpha)nF}\left[\alpha Log(1-\alpha) + (1-\alpha)Log\,\alpha - Log\frac{RT}{nF} - LogK_s\ \right] + \frac{2.3RT}{(1-\alpha)\alpha nF} Log\,\upsilon$$

Characterization of ANHDCT |GCE with electrochemical impedance spectroscopy (EIS)

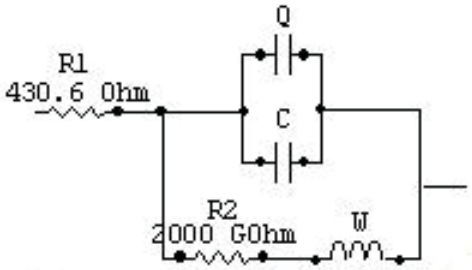
EIS was employed to investigate the impedance changes of the electrode surface in the modifying process. Fig.۲ shows the results of the EIS at ANHDCT /GCE , MP/GCE and MP/ ANHDCT /GCE in the phosphate buffer solution (pH=7).(Fig.2a,b,c)

Because of easy resonance of ANHDCT on the surface of electrode , it can play the role of electron-transfer mediator between surface of the electrode and MP. This compound enhances the electron-transfer rate and according to Impedance data for resitance toward electron-transfer , it can be seen that in the presence of ANHDCT, electron-transfer resistance is dramatically decreased. This subject illustrates that suitable orientation of

ANHDCT and its rigid structure provide easy electron-transfer between MP and electrode surface. Impedance data are shown in table 1.



(a) (b)



(c)

Fig 2- Impedance spectrums of MP | GCE , ANHDCT | GCE
b - The electrode potential was -0.142V ; the frequency range was 0.1Hz to 10000Hz and amplitude (rms) was 0.01V. Randle's equivalent circuit is shown in
C- (C=1PF , W=0.1×10^{13} Ohm and Q items are $Y_0=0.3977\times10^{-3}$, n=0.8817)

Table 1- Impedance data

Electrode	R_s / 10^2 Ohm	R_p / 10^4 Ohm	CPE / 10^{-6} F
MP | GC	2.510	76	0.262
S-Compond | GC	2.150	2.14	0.0432
5. MP | ANHDCT |GC	-2.164	0.0646	10.4

Effect of pH on CV

The changes in the anodic and cathodic peaks were examined as a function of the pH on the respons of the MP | ANHDCT | GC electrode in various pH values.

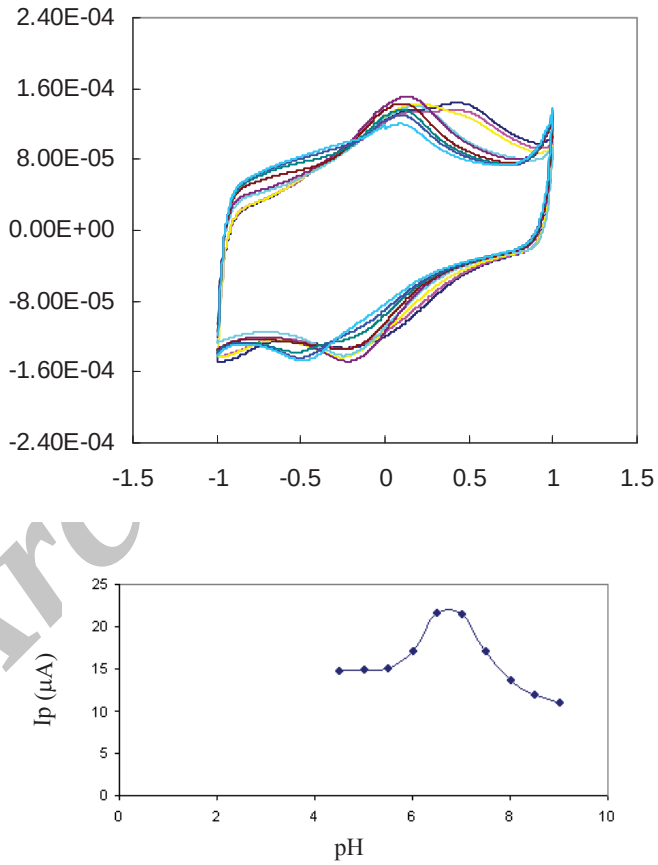


Fig.3-The cyclic voltammograms of MP | ANHDCT |GC electrode in pH range of 5-9. pH=7 is the optimum pH for the study of electrochemical behavior of MP| ANHDCT | GC electrode (Fig.3a).

In Fig.3. shows the cyclic voltammograms of modified electrode in the pH rang of 5- 9. the results showed that both the cathodic and the anodic peak potentials of MP

negatively shifted with an increase in pH. The pH dependence of the anodic peak potential are deduced as follows :

$$E_p^a = -0.0719pH + 0.5362$$

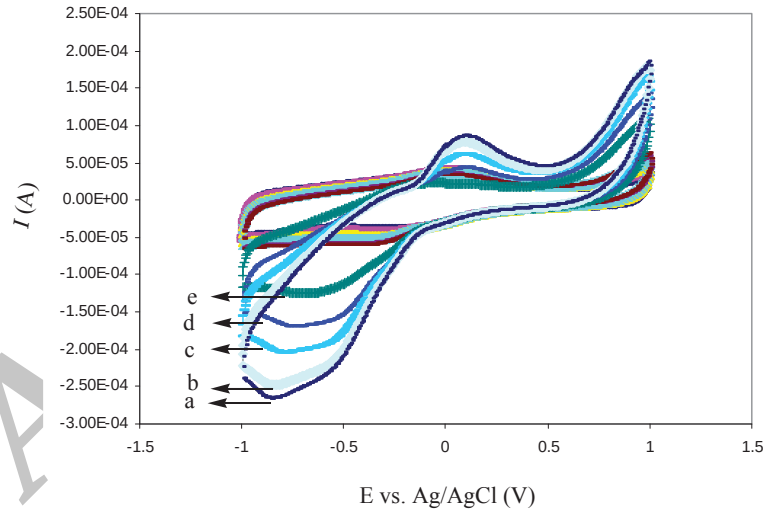
This equation is reasonably close to the following theoretical equation for a reversible process:

$$Ep = -0.059\ pH$$

The effect of pH on the anodic peak current of the electrochemical respons of MP | ANHDCT | GC electrode was examined (Fig.3a). As can be seen, maximum peak height was at around pH = 7 , Therefor, pH = 7 is the optimum pH for the study of electrochemical behaviour of MP | ANHDCT | GC electrode .

Respons of the MP | ANHDCT | GC electrode to Hydrogen peroxide

The response of MP | ANHDCT | GC electrode toward H_2O_2 was also studied. Fig.۴ shows the cyclic vlotammograms of the modified electrode after successive addition of 1 µM H_2O_2. Concentrations of H_2O_2 was determined with UV spectroscopy. The cyclic voltammograms were significantly changed .



	a	b	c	d	e
C_{H2O2}	40µM	30 µM	20 µM	10µM	9µM

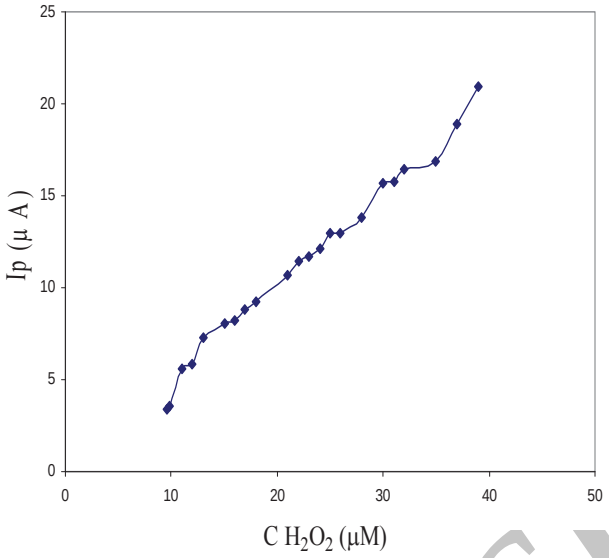


Fig.4- the cyclic vlotammograms of the modified electrode after successive addition of 1 μM H_2O_2 were significantly changed. Fig.4a, shows the linear relationship between the catalytic reduction peak current and the H_2O_2 concentration in the range of 9×10^{-6} to 4×10^{-5} M can be used for determination of H_2O_2 concentration.

the reduction peak current in the presence of hydrogen peroxide is much higher than that in its absence, and it was also observed the great increasing in the reduction peak current with increasing the concentration of hydrogen peroxide. The oxidation peak was disappeared. The disappearance of the oxidation peak shows that the oxidation rate of MP by H_2O_2 is very fast , indicating the peroxidase activity of the MP immobilized in the ANHDCT film. However , the direct electro-reduction of H_2O_2 is not observed at the ANHDCT modified electrode. These results indicat that the MP | ANHDCT | GC electrode undertakes a direct electron transfer reaction and exhibits an excellent electrocatalytic response to the reduction of H_2O_2 in a Phosphate buffer solution (pH = 7) . The MP | ANHDCT | GC electrode can be used for determination of H_2O_2 concentration. The calibration curve (Fig.4a) shows the linear relationship between the catalytic reduction peak current and the H_2O_2 concentration in the range of 9×10^{-6} to 4×10^{-5} M .

Conclusions

A novel , electrochemical H_2O_2 biosensor was fabricated by immobilizing MP on a self-assembled ANHDCT / GC electrode. The ANHDCT film provided a suitable orientation for enzyme molecules and a necessary pathway of electron transfer between MP and the electrode surface. In summary , ANHDCT can accelerate the electron transfer between the MP and the electrode. The ANHDCT can also keep the electrochemical activity of the MP to catalyze the reduction of H_2O_2 . The MP / ANHDCT films may have a potential application in constructing the third-generation electrochemical biosensors based on mediator-free electrochemistry of the enzymes. Further work is in progress to extend to other redox enzyme-based biosensors.

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