

# Direct Electron Transfer of Myoglobin on Zirconia Nanoparticles Modified Carbon Paste Electrode

<sup>1</sup>Sedigheh Banapour, <sup>2</sup>Manouchehr Mazdapour, <sup>3</sup>Zahra Dinpazhooh, <sup>4</sup>Fatemeh Salahi,  
<sup>5</sup>Mahdi Torkamani Noughabi, \*Masoud Negahdary, <sup>1</sup>Aram Asrari, <sup>6</sup>Faezeh Ghanami

<sup>1</sup>Department of Biology, Payame Noor University, Taft, Iran

<sup>2</sup>Department of Biology, Islamic Azad University, pharmaceutical science Branch, Tehran, Iran

<sup>3</sup>Department of chemical engineering, Islamic Azad University, Marvdasht Branch, Marvdasht, Iran

<sup>4</sup>Department of chemistry, Islamic Azad University, Firozabad Branch 74715, Iran

<sup>5</sup>Department of Basic Science, Gonabad University of Medical Sciences, Gonabad, Iran

<sup>6</sup>Department of Biology, Payame Noor University, Mashhad, Iran

\* **Corresponding Author:** Masoud Negahdary, Department of Biology, Payame Noor University, P.O. Box 19395-3697, Tehran, Iran. e-mail: Masoud.negahdary@hotmail.com

## Abstract

In this study, myoglobin (Mb) immobilized on carbon paste electrode that modified with zirconia nanoparticles to construct a novel biosensor for hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The prepared nanoparticles and electrodes were characterized by transmission electron microscope (TEM) and atomic force microscope (AFM). The resulting electrode displayed an excellent redox behavior for the myoglobin. The myoglobin showed a quasi-reversible electrochemical redox behavior with a formal potential of -0.428 V (versus Ag/AgCl) in 0.1 M PBS solution at pH 7.0 and temperature 25°C. The linear range of this biosensor for H<sub>2</sub>O<sub>2</sub> determination was from 20 to 450 μM. Moreover, the modified electrode demonstrated additional improved electrochemical properties, such as good reversibility (ipc/ipa ≈ 1) and high selectivity, good stability and repeatability.

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\* Corresponding Author: Masoud Negahdary, Department of Biology, Payame Noor University, P.O. Box 19395-3697 Tehran, Iran; e-mail: Masoud.negahdary@hotmail.com

**Keywords:** direct electron transfer, zirconia Nanoparticles, Myoglobin, carbon paste electrode

## INTRODUCTION

The direct electrochemistry of redox proteins is an important foundation for biosensors and bioreactors[1]. The results can also provide a model for the mechanistic study of the electron transfer between proteins in biological systems. The direct electrochemistry of redox proteins with bare electrodes is difficult to achieve due to the deep burying of the electroactive center and the unfavorable orientation of proteins[2]. So modified electrodes with nanoparticles lead to accelerate the direct electron transfer of redox proteins[3-4]. The interaction and direct electron transfer between redox proteins and electrode surface are of great importance for not only studying the electron transfer between biomolecules in biological system, but also investigating the novel biosensors [5]. Myoglobin (Mb) is an ideal model molecule for the study of electron transfer reactions of heme proteins, biosensing and electrocatalysis [6]. The determination of hydrogen peroxide is of considerable interest, because hydrogen peroxide is not only an important analyte in food, pharmaceutical, clinical, industrial and environmental analyses but also playing a key role as the product of the enzymatic reaction in coupled enzyme systems [7]. Several analytical techniques have been employed for this determination, such as titrimetric [8], spectrometry [9], chemiluminescence [10-12], but these techniques suffer from interferences, long analysis time and use of expensive reagents. Determination of hydrogen peroxide by biosensors is very easy and fast work. Recently, some oxide nanoparticles such as SiO<sub>2</sub> [13-14], TiO<sub>2</sub> [15–16], ZrO<sub>2</sub> [17-18], SnO<sub>2</sub> [19], and WO<sub>3</sub> [20] have also been used for immobilizing proteins and accelerating the electron transfer between the immobilized proteins and electrodes. Protein-nanoparticle systems have excellent prospects for interfacing biological recognition events with electronic signal transduction to design a new generation of biosensing devices with high sensitivity[21]. Direct electron transfer between redox proteins and electrodes is of practical and theoretical interest and can be improved by electrode or protein modification[22]. Zirconia nanoparticles are of great interest due to their improved optical and electronic properties with application as a piezoelectric, electro-optic and dielectric material[23]. Zirconia is also emerging as an important class of catalyst[24]. The synthesis of zirconia has been realized by physico-chemical methods such as sol–gel synthesis, aqueous precipitation, thermal decomposition and hydrothermal synthesis. Chemically modified electrodes have become very attractive due to high selectivity and sensitivity[25]. Chemically modified electrodes are devices containing a supporting electrode and a layer modified chemically[26]. The main purpose for this modification is to control the chemical and physical properties of the electrode/solution interface, thus, improving the reactivity and selectivity of the surface[26]. A heme group of myoglobin is bound in a hydrophobic cleft in the protein, and is key to the function of it: it is to the heme that oxygen binds[27]. The heme itself consists of an organic ring known as protoporphyrin that surrounds an iron atom. The iron is ligated to four nitrogens of the protoporphyrin, as well as to a histidine side-chain of myoglobin, which tethers the heme in the hydrophobic pocket. Maintenance of this heme iron in the reduced state (Fe<sup>2+</sup>) is imperative for oxygenation to occur[28]. Above reactions is very important in direct electron transfer.

## MATERIAL AND METHODS

### Reagents

Myoglobin purchased from Sigma. Zirconyl chloride octahydrate purchased from Sigma-Aldrich. Other Reagents purchased from Merck. The supporting electrolyte used for all experiments is 0.1 M pH 7 phosphate buffer solution (PBS), which is prepared by using 0.1 M  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$  solutions. All the reagents used were of analytical grade and all aqueous solutions were prepared using doubly distilled water[29].

### Apparatus

Surface morphological studies were carried out using Being Nano-instruments CSPM4000, atomic force microscope (AFM) and transmission electron microscope (TEM) measurement was conducted by using a DS-720 (Topcon Co. Ltd.), respectively. Cyclic voltammetry (CV) and square wave voltammetry were performed using an Autolab potentiostat PGSTAT 302 (Eco Chemie, Utrecht, The Netherlands) driven by the General purpose Electrochemical systems data processing software (GPES, software version 4.9, Eco Chemie). Electrochemical cell with three electrodes was used[30]; unmodified carbon paste electrode or carbon paste electrode modified with zirconia Nanoparticles was used as a working electrode, Ag/AgCl was used as a reference electrode while platinum wire was used as a counter electrode.

### Preparation of $\text{ZrO}_2$ nanoparticles

The  $\text{ZrO}_2$  nanoparticles were prepared according to the literature. Initially, 2.58 g  $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$  and 4.80 g urea were dissolved in 20.0 mL  $\text{CH}_3\text{OH}$  under stirring to form a colorless solution. The solution was transferred to a 20-mL Teflon-lined stainless steel autoclave, which was heated to 200 °C and maintained at that temperature for 20 h. The obtained white product was post-treated with sulphuric acid solution (0.167 mmol), and then calcined at 645 °C.

### Preparation of unmodified carbon paste electrode (CPE) and CPE modified by zirconia Nanoparticles

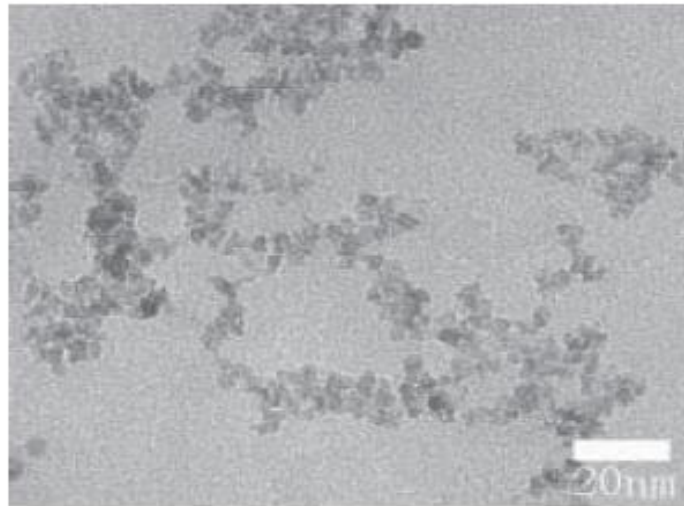
Unmodified carbon paste electrode was prepared by mixing 65% graphite powder and 35% paraffin wax. Paraffin wax was heated till melting and then, mixed very well with graphite powder to produce a homogeneous paste. The resulted paste was then packed into the end of an insulin syringe (i.d.: 2mm). External electrical contact was established by forcing a copper wire down the syringe. CPE modified with zirconia Nanoparticles was prepared by mixing 60% graphite powder and 30% paraffin wax with and 15% zirconia Nanoparticles. The surface of the electrode was polished with a piece of weighting paper and then rinsed with distilled water thoroughly.

## RESULTS AND DISCUSSION

### Microscopic characterization

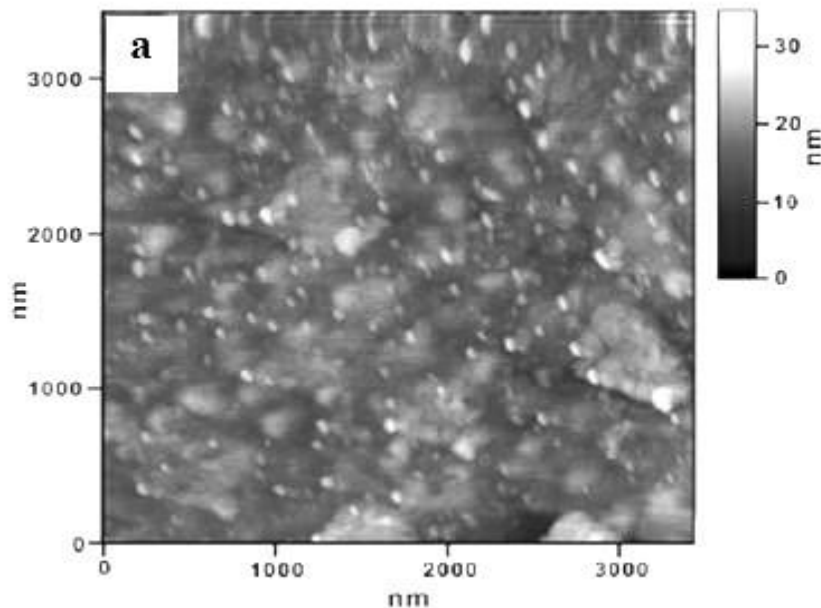
As it is well known, the properties of a broad range of materials and the performance of a large variety of devices depend strongly on their surface characteristics. For instance, the surface of a biomaterial/biomedical device meets the physiological environment, immediately after it is placed in the body or bloodstream and the initial contact regulates its subsequent performance

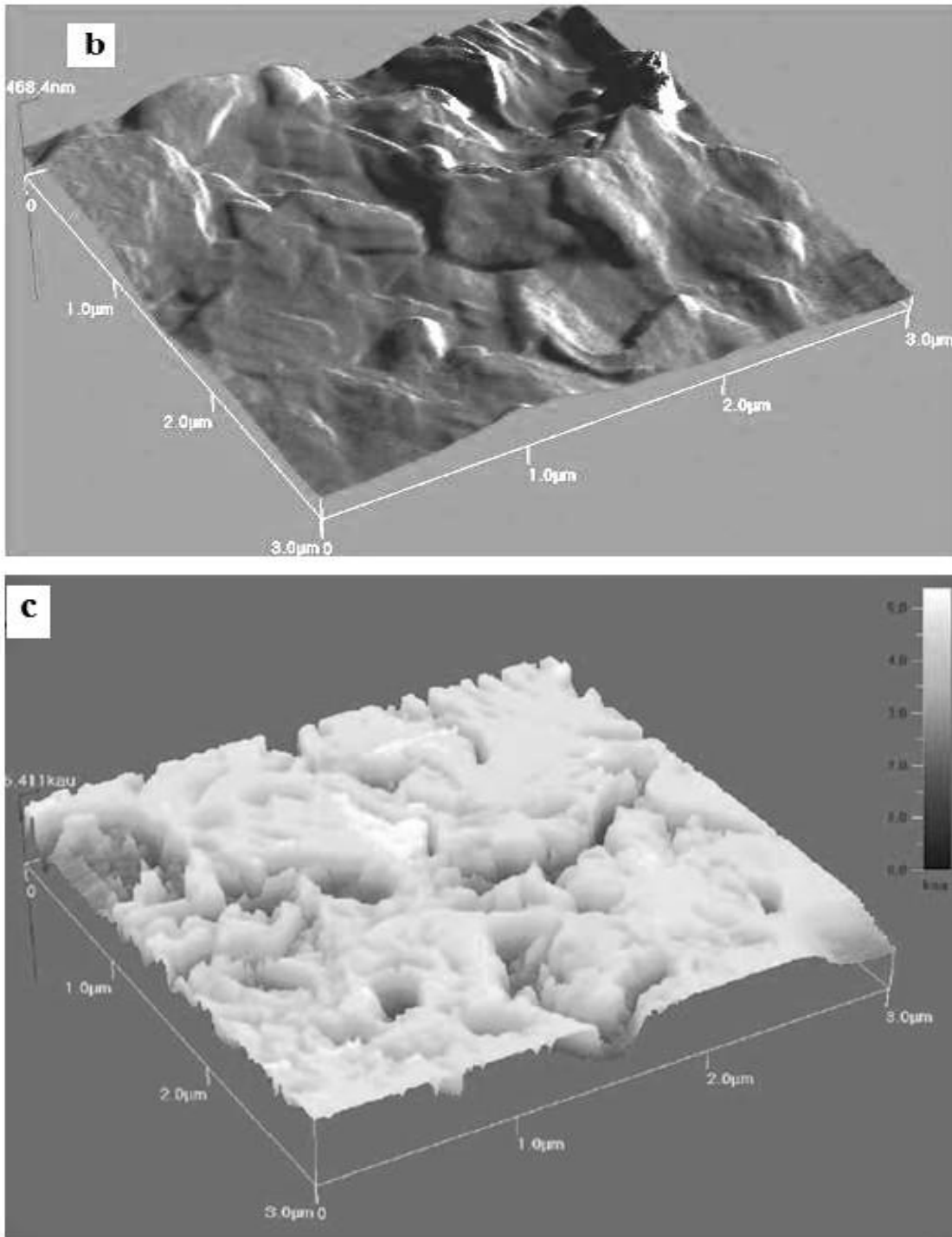
[31]. The average diameter of the synthesized  $\text{ZrO}_2$  nanoparticle is about 20 nm, and has a very narrow particle distribution. This statement is illustrated in figure 1. Fig. 1 shows a TEM picture of the  $\text{ZrO}_2$  nanoparticles.



**Fig. 1 TEM images of  $\text{ZrO}_2$  NPs, with diameter about 20 nm**

In the next study, initially synthesized zirconia nanoparticles were studied with atomic force microscope (AFM), that shown in figure 2(a). then bare Carbon paste electrode and Carbon paste electrode modified with zirconia nanoparticles were studied with atomic force microscope (AFM), that shown in figure 2(b) and figure 2(c) respectively.

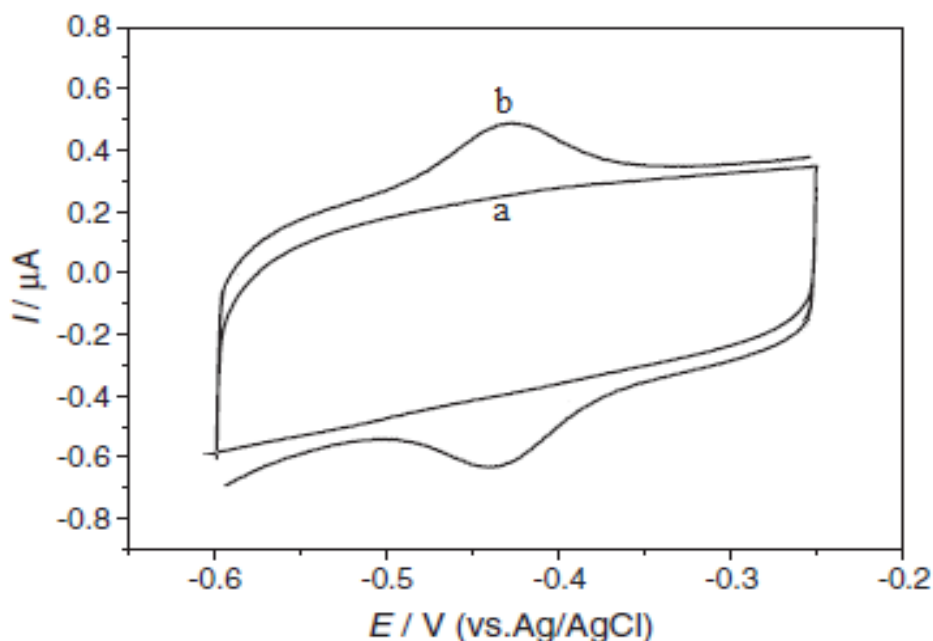




**Fig. 2** AFM images of (a)  $ZrO_2$  NPs, (b) 3D Wave Mode AFM image of a bare CPE,  $3 \mu m \times 3 \mu m$ , z-scale 468 nm, scan frequency 1 Hz, And (c) AFM Phase Mode scans of a carbon paste electrode modified with zirconia Nanoparticles.

### Direct electrochemistry of Mb/ZrO<sub>2</sub> nanoparticles /carbon paste electrode

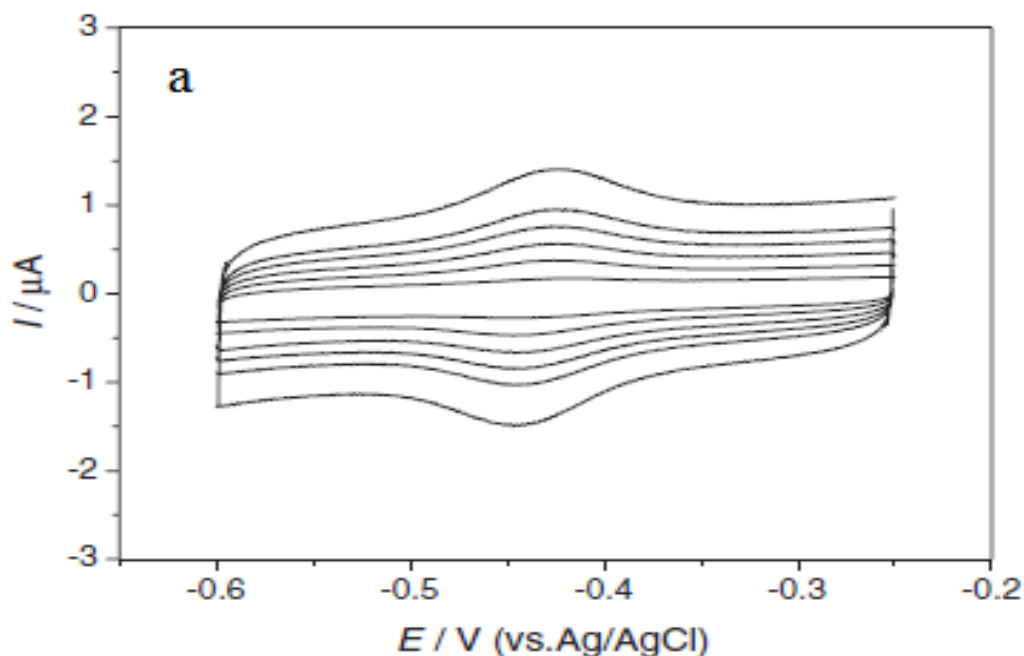
The integrity of the immobilized myoglobin construction and its ability to exchange electrons with the nanometer- scale ZrO<sub>2</sub> particles surfaces were assessed by voltammetry. A macroscopic electrode was required to attain a large enough myoglobin sample to yield detectable direct oxidation and reduction currents. The comparative CVs for the ZrO<sub>2</sub> NPs/CPE and Cyt c/ ZrO<sub>2</sub> NPs/ CPE electrodes in 0.1 M PBS (pH 7.0) were obtained. These voltammograms are demonstrated in Fig. 3 (a,b). From this Figure, it was noticed that there were no voltammetric response on ZrO<sub>2</sub> NPs/carbon paste electrode (Fig. 3a), which, Fig. 3b depicts a well-defined pair of oxidation–reduction (redox) peaks, observed on the Mb/ ZrO<sub>2</sub> NPs/ carbon paste electrode at 100 mv/s scan rate value. The Mb/ ZrO<sub>2</sub> NPs/ carbon paste electrode presented the reductive peak potential at -0.44 V and the corresponding oxidative peak potential at -0.415 V (at 100 mV s<sup>-1</sup>), illustrating the adsorbed myoglobin on the nanometer-scale zirconia particle surfaces. The difference of anodic and cathodic peak potential values was  $\Delta E = 0.025$  V. These redox peaks were attributed to the redox reaction of the myoglobin electroactive center. The formal potential ( $E^0$ ) for the myoglobin redox reaction on the Mb/ ZrO<sub>2</sub> NPs/ carbon paste electrode was -0.428 V with respect to the reference (Ag/ AgCl) electrode.

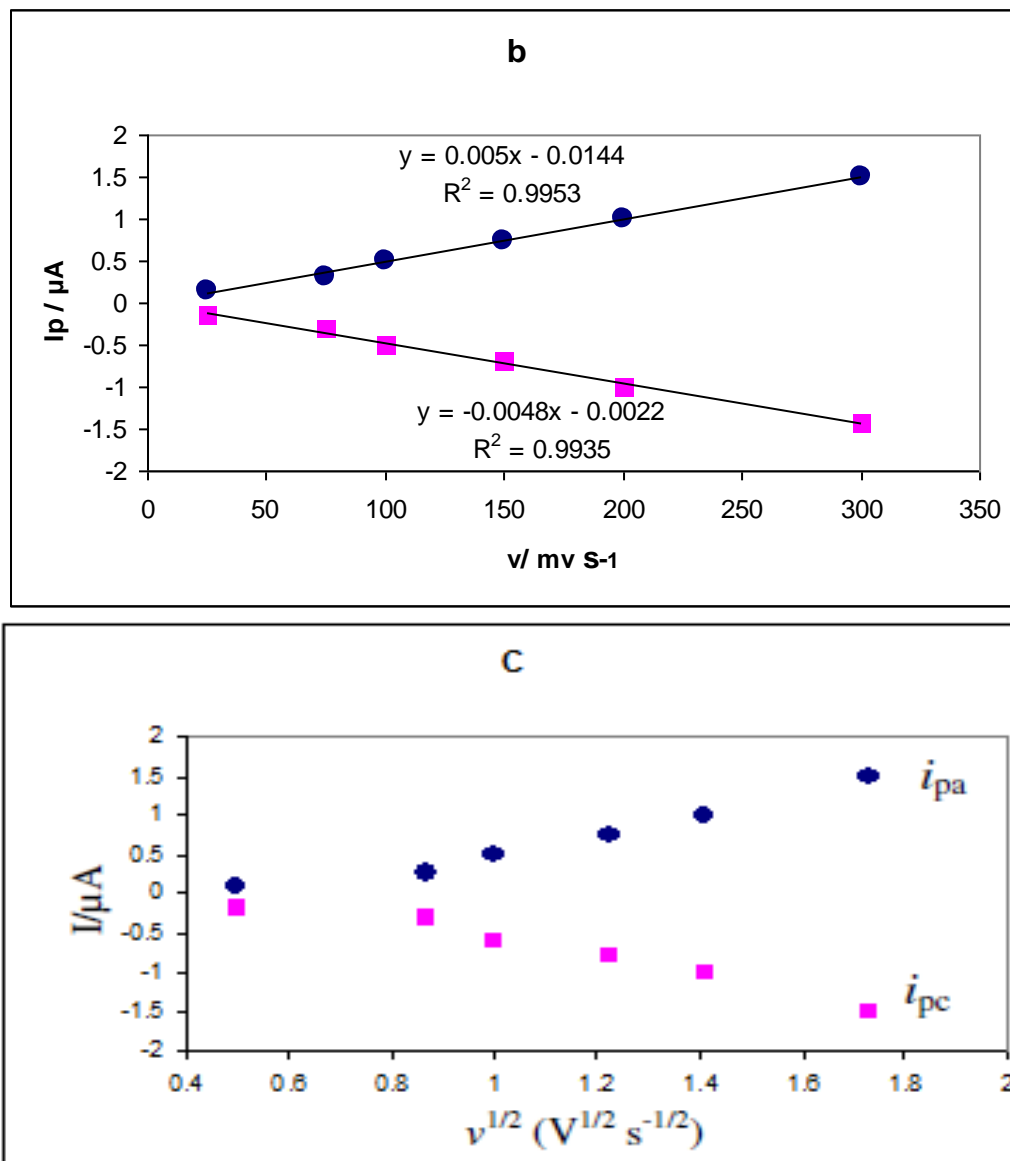


**Fig. 3** Cyclic voltammograms, using (a) the ZrO<sub>2</sub>NPs/CPE in 0.1 M phosphate buffer and (b) Mb/ ZrO<sub>2</sub> NPs/ CPE in 0.1 M phosphate buffer (scan rate: 100 mV/s).

The collected voltammograms in Fig. 4 a, substantiated this statement that the nanometer-scale zirconia particles could play a key role in the observation of the myoglobin CV response. On the grounds that the surface-to-volume ratio increases with the size decrease and because of the fact that the protein size is comparable with the nanometer-scale building blocks, these nanoparticles displayed a great effect on the electron exchange assistance between myoglobin and carbon paste electrode. To further investigate the myoglobin characteristics at the Mb/ ZrO<sub>2</sub>

NPs/ CPE electrode, the effect of scan rates on the myoglobin voltammetric behavior was studied in detail. The baseline subtraction procedure for the cyclic voltammograms was obtained in accordance with the method reported by Bard and Faulkner [30]. The scan rate ( $v$ ) and the square root scan rate ( $v^{1/2}$ ) dependence of the heights and potentials of the peaks are plotted in Fig. 4b and c. It can be seen that the redox peak currents increased linearly with the scan rate, the correlation coefficient was 0.9935 ( $i_{pc} = -0.0048v - 0.0022$ ) and 0.9953 ( $i_{pa} = +0.005v - 0.0144$ ), respectively. This phenomenon suggested that the redox process was an adsorption-controlled and the immobilized myoglobin was stable. It can be seen that the redox peak currents increased more linearly with the  $v$  in comparison to that of  $v^{1/2}$ .





**Fig. 4** (a) CVs of Mb/ ZrO<sub>2</sub> NPs/ CPE electrode in PBS at various scan rates, from inner to outer; 25, 75, 100, 150, 200 and 300 mV s<sup>-1</sup>, the relationship between the peak currents ( $i_{pa}$ ,  $i_{pc}$ ) vs., (b) the sweep rates and (c) the square root of sweep rates.

However, there is clearly a systematic deviation from linearity in this data, i.e. low scan rates are always on one side of the line and the high scan rate points are on the other. The anodic and cathodic peak potentials are linearly dependent on the logarithm of the scan rates ( $v$ ) when  $v > 1.0 \text{ V s}^{-1}$ , which was in agreement with the Laviron theory, with slopes of  $-2.3RT/\alpha nF$  and  $2.3RT/(1-\alpha)nF$  for the cathodic and the anodic peak, respectively [32]. So, the charge-transfer



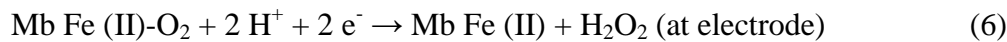
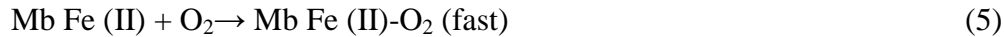
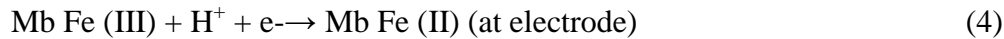
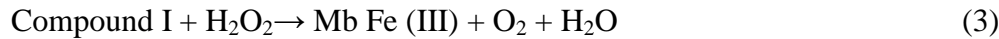
coefficient ( $\alpha$ ) was estimated as 0.5. Furthermore, the heterogeneous electron transfer rate constant ( $k_s$ ) was estimated according to the following equation [33-34]:

$$[\log k_s = \alpha \log(1-\alpha) + (1-\alpha) \log \alpha - \log \frac{RT}{nFv} - \frac{\alpha(1-\alpha)nF\Delta E_p}{2.3 RT}] \quad (1)$$

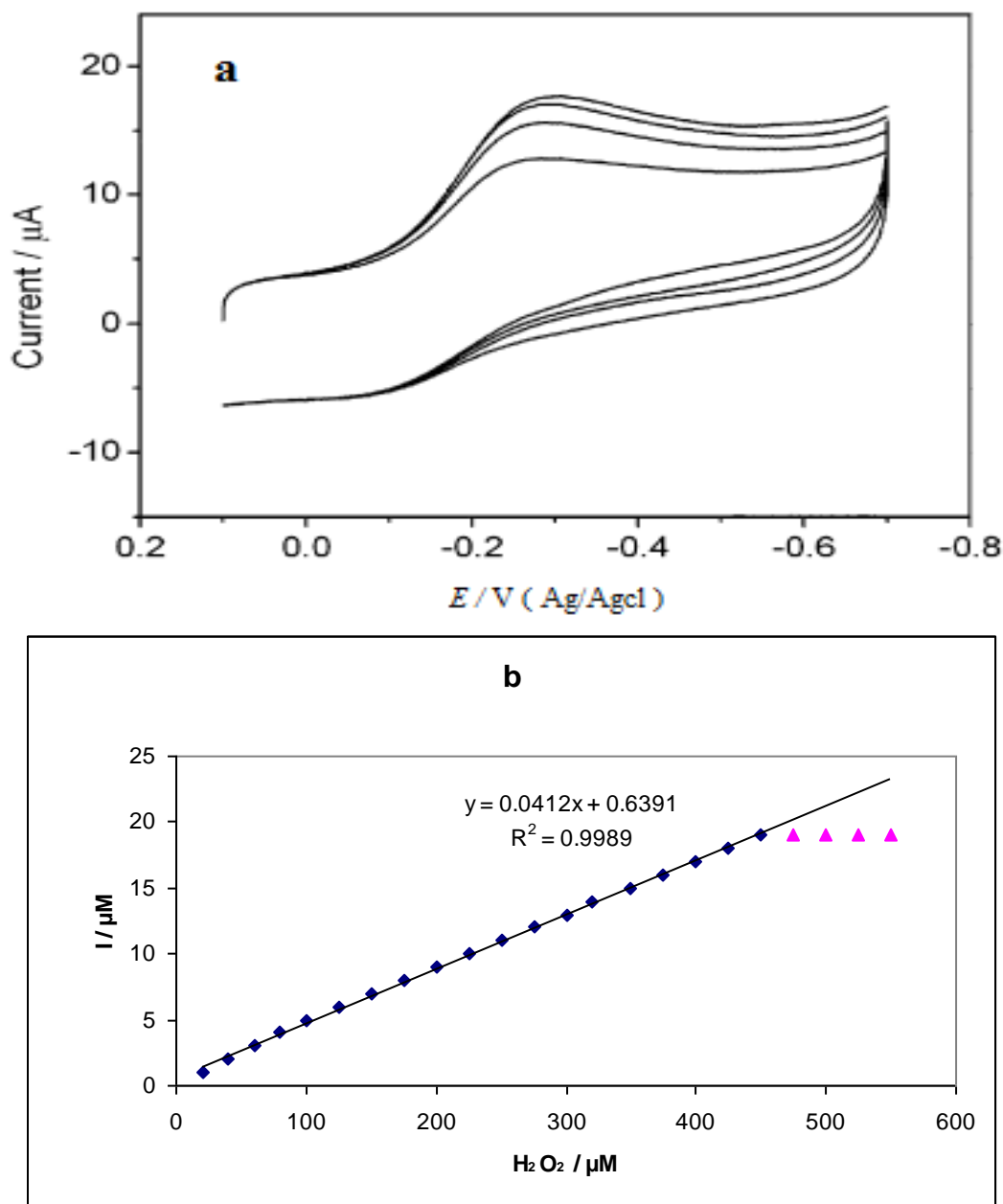
Here,  $n$  is the number of transferred electrons at the rate of determining reaction and  $R$ ,  $T$  and  $F$  symbols having their conventional meanings.  $\Delta E_p$  is the peak potential separation. The  $\Delta E_p$  was equal to 0.350, 0.4 and 0.45 V at 0.7, 1 and 2 V s<sup>-1</sup>, respectively, giving an average heterogeneous transfer rate constant ( $k_s$ ) value of 0.7 s<sup>-1</sup>.

### Electrocatalysis of Mb/ZrO<sub>2</sub>nanoparticles/CPE to reduction of H<sub>2</sub>O<sub>2</sub>

Upon addition of H<sub>2</sub>O<sub>2</sub> to 0.1M pH 7.0 PBS, the cyclic voltammogram of the Mb / ZrO<sub>2</sub> NPs/CPE electrode for the direct electron transfer of Mb changed dramatically with an increase of reduction peak current and a decrease of oxidation peak current (Fig. 5 a), while the change of cyclic voltammogram of bare or ZrO<sub>2</sub> NPs/ CPE was negligible (not shown), displaying an obvious electrocatalytic behavior of the Mb to the reduction of H<sub>2</sub>O<sub>2</sub>. The decreases of the oxidative peak current together with the increases of the reductive Mb / ZrO<sub>2</sub> NPs/CPE. The electro-catalytic process could be expressed as follows:



calibration curve (Figure 5 b) shows the linear dependence of the cathodic peak current on the H<sub>2</sub>O<sub>2</sub> concentration in the range of 20 to 450  $\mu\text{M}$ . In Figure 5 b, at higher concentration of H<sub>2</sub>O<sub>2</sub>, the cathodic peak current decreased and remains constant. Upon addition of an aliquot of H<sub>2</sub>O<sub>2</sub> to the buffer solution, the reduction current increased steeply to reach a stable value (Fig 5 b). This implies electrocatalytic property of electrode. Thus, this experiment has introduced a new biosensor for the sensitive determination of H<sub>2</sub>O<sub>2</sub> in solution. Figure .5. (a) Cyclic voltammograms obtained at an Mb/ZrO<sub>2</sub>NPs/CPE in 0.1M phosphate buffer solution (pH 7.0) for different concentrations of and (b) the relationship between cathodic peak current of Mb and different concentrations of H<sub>2</sub>O<sub>2</sub> (scan rate: 100 mVs<sup>-1</sup>).

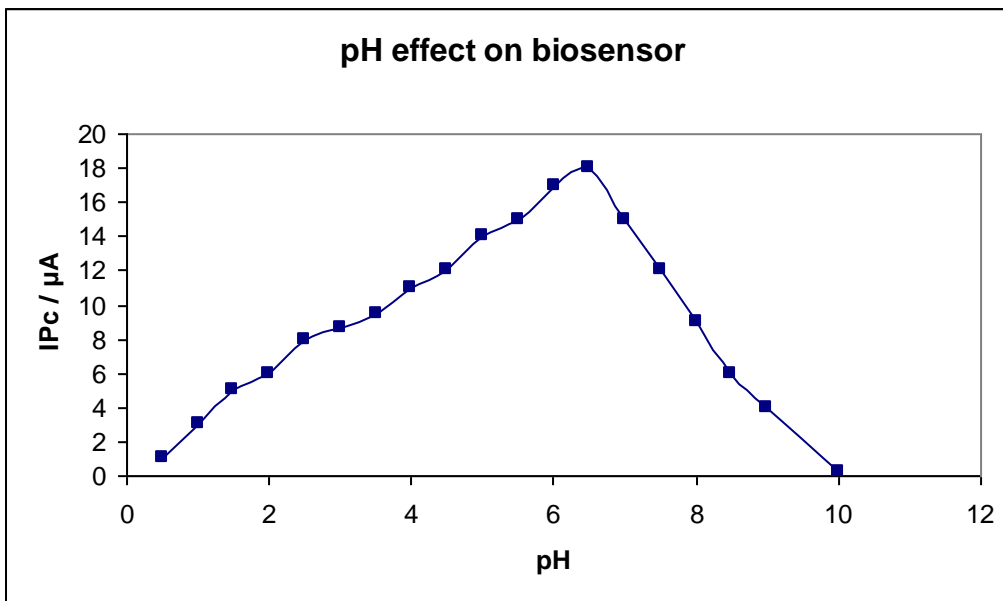


**Fig. 5 (A) Cyclic voltammograms obtained at an Mb/  $\text{ZrO}_2$  NPs/ CPE electrode in 0.1M phosphate buffer solution (pH 7.0) for different concentrations of and (B) the relationship between cathodic peak current of Mb and different concentrations of  $\text{H}_2\text{O}_2$  (scan rate:  $100 \text{ mVs}^{-1}$ ).**

#### **Effect of solution pH on the direct electron transfer of immobilized Mb**

In order to obtain an efficient biosensor for  $\text{H}_2\text{O}_2$ , the influence of pH and applied potential on the response of Mb/  $\text{ZrO}_2$  NPs/ CPE electrode were investigated. The change of chronoamperometric current with the pH under constant hydrogen peroxide concentration

(100.0 $\mu$ M) shown in Fig. 6. As can be seen, the maximum response appears at pH 6.6. So the buffer solution of pH 7.0 was selected for experiments.



**Fig. 6** Dependence of the current response of Mb/ ZrO<sub>2</sub> NPs/ CPE electrode to 50.0 $\mu$ M H<sub>2</sub>O<sub>2</sub> on the pH of buffer solutions.

#### Stability of the H<sub>2</sub>O<sub>2</sub> biosensor

The stability of Mb/ ZrO<sub>2</sub> NPs/ CPE electrode biosensor has been checked by carrying out experiments at the regular interval of a week and it has been found that Mb/ ZrO<sub>2</sub> NPs/ CPE electrode based optical biosensor retains its 93% activity after 15 days. The loss in the activity of biosensor is not due to the denaturation of myoglobin but it is due to the poor adhesion of zirconia Nanoparticles on the carbon paste electrode. For a result, interface materials have not high effect on operation of this biosensor.

## CONCLUSIONS

Myoglobin can be effectively immobilized in a ZrO<sub>2</sub> NPs/ CPE electrode. The Mb/ ZrO<sub>2</sub> NPs/ CPE electrode shows a fast direct electron transfer of Mb. Direct electron transfer of the Mb immobilized in ZrO<sub>2</sub> NPs/ CPE electrode was easily achieved. The immobilized Mb displays a high affinity and high response sensitivity to hydrogen peroxide. The sensor shows a good reproducibility and stability. This research provides an efficient strategy and a new promising platform for the study of electron transfer of proteins and the development of biosensors.

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**Received: February, 2012**