ELSEVIER

Biophysical Chemistry 130 (2007) 122–131

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Immobilization of hemoglobin on electrodeposited cobalt-oxide nanoparticles: Direct voltammetry and electrocatalytic activity

Abdollah Salimi [a,b,*], Rahman Hallaj [a], Saied Soltanian [b]

[a] *Department of Chemistry, University of Kurdistan, P.O.Box 416, Sanandaj, Iran*
[b] *Nanotechnology Research Center, University of Kurdistan, P.O.Box 416, Sanandaj, Iran*

Received 21 June 2007; received in revised form 15 August 2007; accepted 17 August 2007
Available online 24 August 2007

Abstract

Cyclic voltammetry at potential range −1.1 to 0.5 V from aqueous buffer solution (pH 7) containing $CoCl_2$ produced a well defined cobalt oxide (CoOx) nanoparticles deposited on the surface of glassy carbon electrode. The morphology of the modified surface and cobalt oxide formation was examined with SEM and cyclic voltammetry techniques. Hemoglobin (Hb) was successfully immobilized in cobalt-oxide nanoparticles modified glassy carbon electrode. Immobilization of hemoglobin onto cobalt oxide nanoparticles have been investigated by cyclic voltammetry and UV–visible spectroscopy. The entrapped protein can take direct electron transfer in cobalt-oxide film. A pair of well defined, quasi-reversible cyclic voltammetric peaks at about −0.08 V vs. SCE (pH 7), characteristic of heme redox couple (Fe(III)/Fe(II)) of hemoglobin, and the response showed surface controlled electrode process. The dependence of formal potential ($E^{0'}$) on the solution pH (56 mV pH^{-1}) indicated that the direct electron transfer reaction of hemoglobin was a one-electron transfer coupled with a one proton transfer reaction process. The average surface coverage of Hb immobilized on the cobalt oxide nanoparticles was about 5.2536×10^{-11} mol cm^{-2}, indicating high loading ability of nanoparticles for hemoglobin entrapment. The heterogeneous electron transfer rate constant (k_s) was 1.43 s^{-1}, indicating great of facilitation of the electron transfer between Hb and electrodeposited cobalt oxide nanoparticles. Modified electrode exhibits a remarkable electrocatalytic activity for the reduction of hydrogen peroxide and oxygen. The Michaels–Menten constant K_m of 0.38 mM, indicating that the Hb immobilized onto cobalt oxide film retained its peroxidases activity. The biosensor exhibited a fast amperometric response <5 s, a linear response over a wide concentration range 5 μM to 700 μM and a low detection limit 0.5 μM. According to the direct electron transfer property and enhanced activity of Hb in cobalt oxide film, a third generation reagentless biosensor without using any electron transfer mediator or specific reagent can be constructed for determination of hydrogen peroxide in anaerobic solutions.

Keywords: Cobalt-oxide; Nanoparticles; Hemoglobin; Direct voltammetry; Electrodeposition; Biosensor

1. Introduction

Recently nanometer scale materials have attracted considerable attention due to their superior functional properties for a wide range of technological applications, including catalysis, optics, microelectronics, chemical sensors and biological biosensors. Metal and metal-oxide nanoparticles are capable of increasing the activities for many chemical reactions due to the high ratio of surface atoms with free valances to the cluster of total atoms. Furthermore, due to high surface area to volume ratio of nanoparticle derivatives materials, the size controllability, chemical stability and surface tenability provide an ideal platform for exploiting such nanostructures in sensing, biosensing and catalytic applications. In addition metal or metal oxide nanoparticles have been widely used in the field of electrochemistry where they have found applications in electroanalysis [1] and references therein], electrocatalysis [2,3] and biocatalysis, especially in sensors and biosensors fabrications [4].

In recent years, direct electrochemistry of proteins [5] and enzymes [6] is an interest subject because of its potential application in the study of the redox and electron transfer properties of biomolecules. Furthermore, reversible direct electron transfer between redox enzymes/proteins and electrode surface is important in mechanistic study of biological systems

* Corresponding author. Tel./fax: +98 871 6624001.
E-mail addresses: absalimi@uok.ac.ir, absalimi@yahoo.com (A. Salimi).

0301-4622/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2007.08.004

[7], biosensing enzyme electrodes, biofuel cells, bioelectronic systems [8] and constructing the third generation biosensors without using redox mediators [9,10]. It is difficult for enzymes and proteins to exchange electron with electrodes surface directly, because they usually have large and complex structure. In addition, the redox centers deeply immerse in the bodies and three dimensional structures hinder interaction with the electrode [11,12]. Therefore, the immobilization of proteins/enzymes on the electrode surfaces is an approach to achieving enhanced interfacial electron transfer [13]. Since the immobilization of enzymes on modified solid surfaces play a key role in the development of biosensors, bioreactors and bioelectronics devices, various types of films have been developed to immobilize different enzymes or proteins on electrode surfaces [14].

Hemoglobin (Hb), a heme protein that consists of a four polypeptide chain, each with one heme group which store and transport oxygen in the red blood cells, is an deal molecule for the study of electron transfer reactions of heme proteins and also for biosensing and electrocatalysis. Furthermore, Hb is known to have some intrinsic peroxidase activity because it has close similarity to peroxidase. Due to stability of Hb, the employment of hemoglobin for electrocatalytic reduction of hydrogen peroxide might be possible [15]. For the development of mediator-free Hb sensor, the important step is the realization of direct voltammetry of Hb. However, unlike some other small heme proteins, such as cytochromes, in most cases Hb is difficult to exhibit heterogeneous electron transfer process, which means that the electron transfer of hemoglobin is very slow. Therefore, no detectable current appears at conventional electrodes, even when rather large overvoltages are applied. However, the surfaces of the unmodified electrodes are incompatible materials that, in general, proteins undergo denaturation upon immobilization on bare or unmodified electrodes and consequently lose their bioactivities [16]. These inhibitions can be overcome by modifying electrodes with mediators and promoters [17,18] or incorporating enzymes and proteins into various films on electrode surfaces [19,20,21]. The incorporation, direct electrochemistry and electrocatalytic activity of hemoglobin in different polymer films, membranes or water soluble surfactants such as poly-3-hydroxyl butyrate membrane [22], poly amido amine dendrimer [23], triton X-100 [24], cetylpyridinium bromide [25], didodecyldimethyl ammonium bromide [26], SP sephadex membrane [27], chitosan biopolymer films [28], poly-vinyl sulfonate [29], poly cationinc poly (diallyldimethyl ammonium) [30] and silica sol–gel film [15] have been reported. Although direct electrochemistry was obtained for more modified electrodes, only few reversible electrochemical behavior of immobilized hemoglobin was observed and catalytic activity of the immobilized enzyme was low.

The combination of biological molecules and novel nanomaterials components is of great importance in the processes of developing new nanoscale devices for future biological, medical and electronic applications [31]. A large number of nanomaterials such as carbon nanotubes [32–34], clay nanoparticles [35] and nanometer-sized gold colloid particles [36] have been shown to be suitable for the incorporation of enzymes and proteins. Metal oxide particles and nanoparticles such as manganeous oxide [37], zirconium oxide [38], titanium oxide [39,40], tungsten oxide [41], iridium oxide [42], iron oxide [43] and zinc oxide [44] have been used successfully for immobilization and direct electrochemistry of enzymes and proteins and their applications for biosensor fabrication. Due to structure stability and small size of inorganic nanoparticles, they provided a favorable microenvironment for redox proteins and enzymes to transfer electrons with underlying electrodes and their application for electrochemical catalysis of various substances. In addition the small pores in metal oxide could act as substrate-transport channels to decrease the mass transfer resistance for efficient biocatalytic processes. Electrochemical co-deposition of enzymes and matrix is a convenient single step, which is fast and well controlled method [45]. To our best knowledge, no report has been reported using cobalt-oxide for immobilization proteins to fabricate biosensor. Following our previous works for immobilization, direct electrochemistry and electrocatalytic activity of enzymes and proteins [46–48], in the present report, first cobalt oxide nanoparticles electrochemically deposited onto a GC electrode and then their biocompatibility for immobilization of Hb is investigated. Finally, direct voltammetry and bioelectrocatyalytic activity of immobilized protein on cobalt oxide nanoparticles were investigated by various spectroscopic and electrochemical techniques. The ability of biosensor for electrocatalytic reduction of hydrogen peroxide and oxygen was also evaluated.

2. Experimental

2.1. Chemicals and apparatus

Human hemoglobin (Hb, MW 66000) was purchased from Sigma and used without further purification. 5 mg ml^{-1} hemoglobin solutions (pH 7) were stored at 4 °C. The phosphate buffer solutions, PBS (0.1 M) were prepared form H_3PO_4, KH_2PO_4 and K_2HPO_4 and adjusting the pH were regulated with HCl and KOH solutions. H_2O_2 (30%w/w) was from Merck, its diluted solution was prepared daily. $CoCl_2$, and other reagents used were of analytical reagent grade. All solutions were prepared with double distillated water. Pure N_2 was passed through the solution to avoid possible oxygen action during the experiments. Ultraviolet and visible (UV–vis) absorption spectra were recorded with a Carry 1A UV–vis spectrometer (Perkin-Elmer instruments) on an ITO glass electrode. The morphologies of the surface were observed in a Vega-Tesacn electron microscope. Electrochemical experiments were performed with a computer controlled μ-Autolab modular electrochemical system (Eco Chemie Ultecht, The Netherlands), driven with GPES software (Eco Chemie). A conventional three-electrode cell was used with a SCE(sat KCl) as reference electrode, a Pt wire as counter electrode and a glassy carbon disk as working electrode. Voltammetry on electrodes coated with Hb–cobalt oxide was done in buffers containing no hemoglobin. All experiments were carried out at ambient temperature of 20±1 °C.

2.2. Preparation of cobalt oxide modified glassy carbon and indium tin oxide electrodes

To prepare a cobalt oxide modified electrode a GC electrode was polished with emery paper followed by alumina (1.0 and 0.05 μm) and then thoroughly washed with twice-distilled water. To remove adsorbed particles the electrode was placed in a bath ultrasonic cleaner. Cobalt oxihydroxide film was electrodeposited on the surface of glassy carbon electrode from 1 mM cobalt-chloride pH 7 phosphate buffer solution, using repetitive potential cycling (30 cycles at 100 mV s^{-1}) between 0.5 and −1.1 V [49]. Modified electrode was eventually washed with double distilled water and stored at ambient temperature (20 °C) before being used in experiments. The same procedure was used for electrodeposition of cobalt oxide nanoparticles onto an indium thin oxide (ITO) glass electrode.

2.3. Immobilization of hemoglobin onto a GC and ITO electrodes modified with cobalt oxide nanoparticles

Cyclic voltammetry was used for immobilization of hemoglobin onto cobalt oxide nanoparticles. After the deposition of cobalt oxihydroxide nanoparticles onto GC electrode, the electrode was immersed in fresh phosphate solution containing 5 mg ml^{-1} hemoglobin and the potential was repetitively cycled (20 scans) from 1.2 to −0.5 V at scan rate 50 mV s^{-1} for immobilization of hemoglobin. The effective area of the electrode modified with cobalt oxide nanoparticles was determined as 0.15 cm^2 from cyclic voltammogram of 1 mM $K_3[Fe(CN)_6]$ in buffer solution pH 7. This procedure also was employed for immobilization of hemoglobin and cobalt oxide nanoparticles on an ITO electrode. Finally, the modified electrode was removed from hemoglobin solution, washed with double distilled water and stored at refrigerator temperature (4 °C) before being used in experiments.

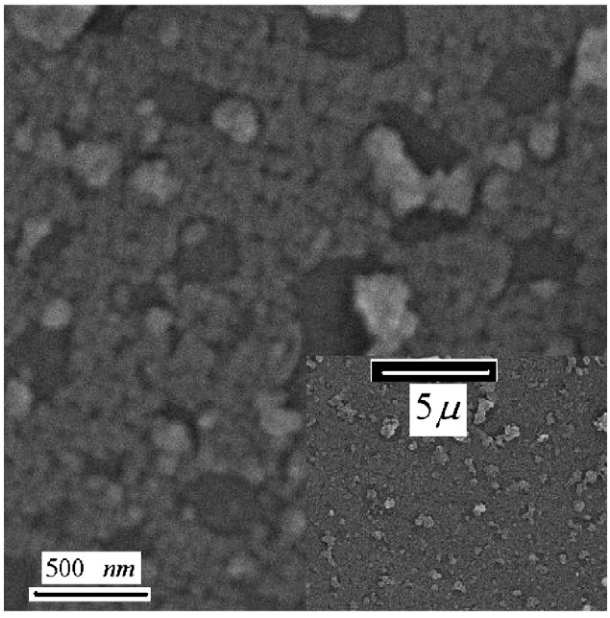

Fig. 1. SEM image of the electrodeposited cobalt oxide on glassy carbon, scale bare 500 nm. Inset is the SEM image with lower magnification for the same sample, scale bar is 5.0 micron.

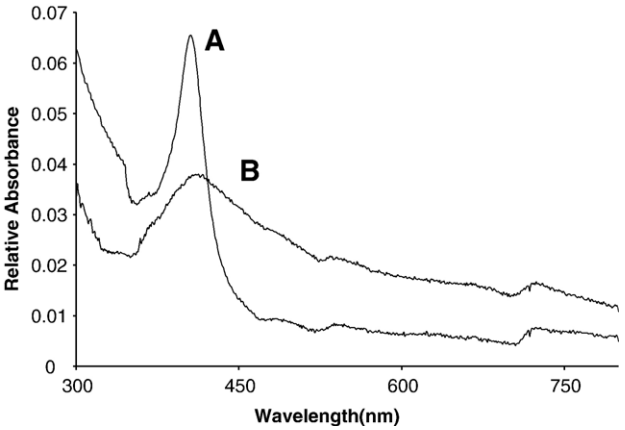

Fig. 2. UV–visible spectra of catalase in PBS (pH 7) phosphate buffer solution (curve A) and Hb-CoOx film on ITO electrode (curve B).

3. Results and discussions

3.1. Characterization of cobalt oxide nanoparticles

In order to investigate the formation and growth of the cobalt oxide particles, different parts of the electrode surface were observed by scanning electron microscope (SEM). Fig. 1 shows a typical image of an electrodeposited cobalt oxide film on glassy carbon electrodes. It can be seen that small particles with average size of less than 100 nm is almost uniformly distributed on the surface of electrode forming a thin layer. ED's analysis proves that these particles are cobalt oxide. In addition to well distributed small particles large agglomerated particles are also observed on the image. The inset of this figure shows SEM image of the same sample with lower magnification (the scale bar is about 5 μM). As we can see, a large agglomerated particle with the average size varies from under 200 nm to slightly less than 600 nm also observed on the image. The formation of cobalt oxide layer on the electrode surface was checked by recording cyclic voltammograms of the modified electrode in alkaline solution without cobalt ions (not shown).We observed a voltammogram same as others reported in the literature [49,50].

3.2. UV–visible spectroscopic analysis

It is well known that the shape and position of the sorbent absorption bands can provide information about possible denaturation of heme proteins [51], so UV–visible spectra was employed to investigate the interaction between hemoglobin and cobalt oxide nanoparticles. Fig. 2 shows the spectra of Hb in PBS (pH 7) and Hb immobilized onto cobalt-oxide film electrodeposited on ITO glass electrode. The hemoglobin cobalt oxide film (Curve B) showed similar soret absorption band at 407 nm to that of hemoglobin in pH 7 phosphate buffer solution (Curve A).The slight shift in soret band may be due to interaction between cobalt oxide nanoparticles and protein. Such interactions neither destroy the protein structure nor

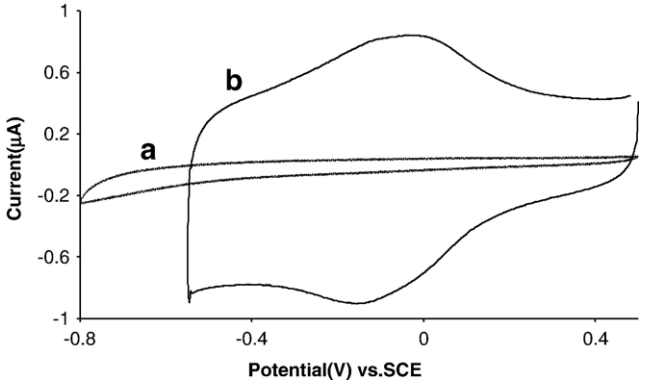

Fig. 3. CVs of glassy carbon electrode modified with cobalt oxide nanoparticles (a) and Glassy carbon electrode modified with cobalt oxide nanoparticles and Hb (b), electrolyte is PBS (pH 7), scan rate is 100 mV s^{-1}.

change the fundamental microenvironment of the biomolecules. This result indicates no observable denaturation of hemoglobin happened on cobalt oxide film.

3.3. Direct voltammetry of hemoglobin at cobalt oxide modified glassy carbon electrode

In order to investigate the electrochemical properties of Hb adsorbed onto cobalt oxide modified film, cyclic voltammogram of the modified glassy carbon electrode in 0.1 M phosphate buffer solution (pH 7) were recorded. As shown in Fig. 3 no redox peaks can be seen for GC electrode modified with cobalt oxide nanoparticles at potential range 0.5 to −0.8 V (voltammogram "a"). However, for GC electrode modified with CoOx–Hb a pair of reduction–oxidation peaks with formal potential −0.08 V is clearly observed (voltammogram "b"). The separation of anodic to cathodic peak potentials is (ΔE=60 mV) and ratio of anodic to cathodic peak currents is about one. This result indicates that hemoglobin undergoes a quasi-reversible redox process at the glassy carbon electrode modified with cobalt oxide nanoparticles. Thus, cobalt oxide film must have a great effect on the kinetics of electrode reaction and provide a suitable environment for the hemoglobin to transfer electron with underlying GC electrode. It is possible that cobalt oxide nanoparticles provided a three dimensional stage and some of the restricted orientations also favored the direct electron transfer between the protein molecules and the underlying electrode. Furthermore, the shapes of anodic to cathodic peaks were nearly symmetric, and the heights of reduction and oxidation peaks are the same. This behavior suggests that all of the electroactive HbFe(III) within the film are converted to HbFe(II) on the forward scan to negative potential and visa versa. The formal potential ($E^{0\prime}$), estimated as midpoint of cyclic voltammogram reduction and oxidation peak potentials

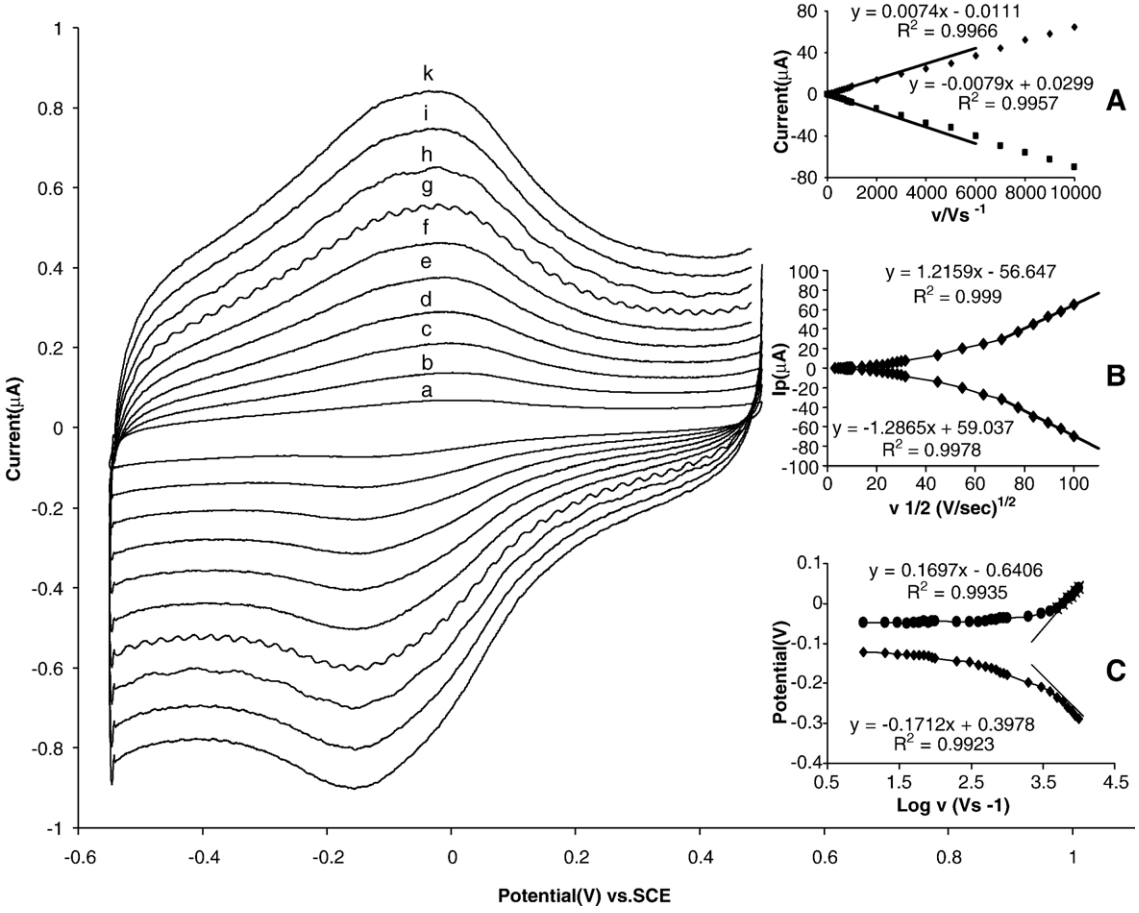

Fig. 4. CVs of Hb/ CoOx modified GC electrode at various scan rate in pH 7 PBS, (a) 10 (b) 20 (c) 30 (d) 40 (e) 50 (f) 60 (g) 70 (h) 80 (i) 90 and (k) 100 mV s^{-1}, Insets are plots of anodic and cathodic peak currents vs. scan rate (A) and square root of scan rate (B). (C) Plot of peak potential separations vs. log (scan rates).

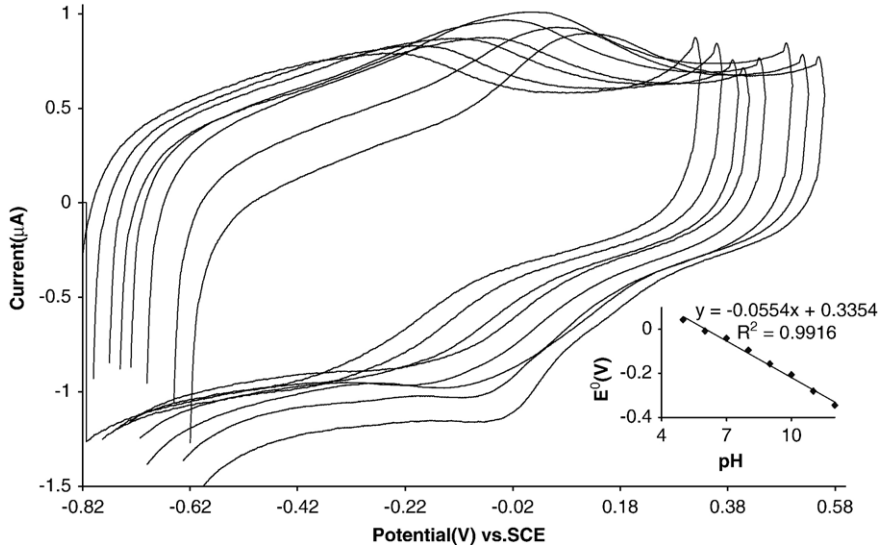

Fig. 5. Recorded cyclic voltammograms of Hb/CoOx modified GC electrode in different pH solutions, from right to left 5 to 12, scan rate 100 mV s^{-1}. Inset plot of formal potential vs. pH values.

(−0.08 V), about 100–200 mV is more positive than the hemoglobin formal potential at the surface of other modified electrodes presented in the recent works, such as; carbon paste silica sol–gel film [15] poly acryl amide hydrogel film [21], poly-3-hydroxybutyrate [22], polyamidoamine dendrimer [23], triton X-100 [24], cetylpyridinium bromide [25], didodecyldimethyl ammonium bromide [26], SP sephadex [27], nanometer-sized gold colloid particles [36], tungsten oxide [41], iron oxide multilayer film [43], chitosan and calcium carbonate nanoparticles [52] and compared with dissolved hemoglobin in an aqueous solution [53]. The high surface activation of the modified electrode decreases the formal potential of hemoglobin at cobalt oxide film in compared above mentioned films presented in the literature. To obtain the kinetic parameters of hemoglobin at cobalt oxide film the scan rate effect was investigated. Fig. 4 shows recorded cyclic voltammograms at

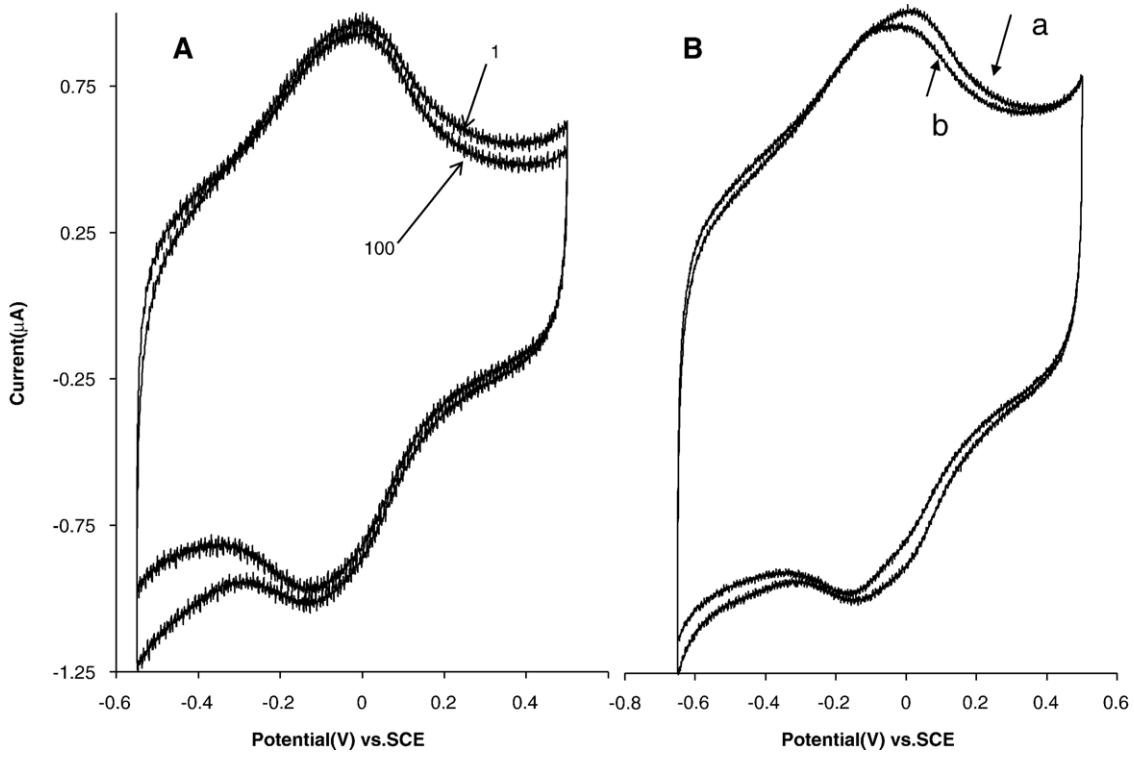

Fig. 6. (A) The 1st (a) and 100th (b) recorded cyclic voltammograms of GC electrode modified with Hb and cobalt oxide. (B) Recorded cyclic voltammograms of the fresh modified electrode (a) and after it kept at refrigerator for 3 days (b), electrolyte was PBS with pH 7 and scan rate was 100 mV s^{-1}.

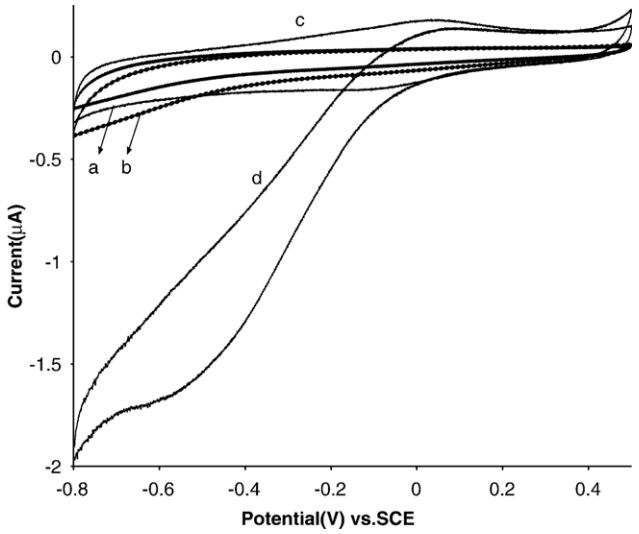

Fig. 7. Recorded cyclic voltammograms for (a) GC–CoOx film in buffer containing no H_2O_2 (b) as (a) in the presence 15 mM of H_2O_2, (c) and (d) as (a) and (b) for Hb/CoOx modified glassy carbon electrode, scan rate 20 mV s^{-1} and electrolyte was PBS (pH 7).

different scan rates. As shown in inset (A) of Fig. 4 the plots of peak currents vs. scan rates exhibits a linear relationship ($R^2 = 0.9957$), as expected for surface confined redox process. The peak to peak separation is about 60 mV at scan rates below 100 mV s^{-1}, suggesting facile charge transfer kinetics over this range of sweep rates. On the other hand it is found that at the scan rate of above 2.5 V s^{-1}, ΔE_p increases by increasing the scan rate. The values of peak-to peak potential separations are proportional to logarithm of the scan rate for scan rate higher than 2000 mV s^{-1} (inset C of Fig. 4). According to the Laviron theory the transfer coefficient (α) and electron transfer rate constant (k_s) can be estimated by measuring the variation of peak potential with scan rate [54]. The transfer coefficient (α) and heterogeneous electron transfer rate constant of hemoglobin are about 0.45 and 1.4 ± 1 s^{-1}, respectively. This value for electron transfer rate constant is comparable or higher than apparent heterogeneous electron transfer rate constant of hemoglobin at nanometer-sized gold colloid particles 0.49 s^{-1} [33], nanocrystaline titanium oxide film 0.137 s^{-1} [36], tungsten oxide 0.97 ± 06 s^{-1} [38] chitosan and calcium carbonate nanoparticles 1.8 s^{-1} [52] mesoporous silica modified glassy carbon electrode 92 ± 18 s^{-1} [55] and carbon nanotubes 0.062 s^{-1} [56]. This value shows that the electron transfer of hemoglobin on cobalt oxide nanoparticles is facile. It is assumed that the nanoparticles increase the surface area, active point for adsorbing hemoglobin and also make the film more porous for facilitating electron transfer. In addition, special nanostructure of cobalt oxide may act as molecular wire enhances the direct electron transfer of hemoglobin. The surface concentration of electroactive species Γ_c can be approximately

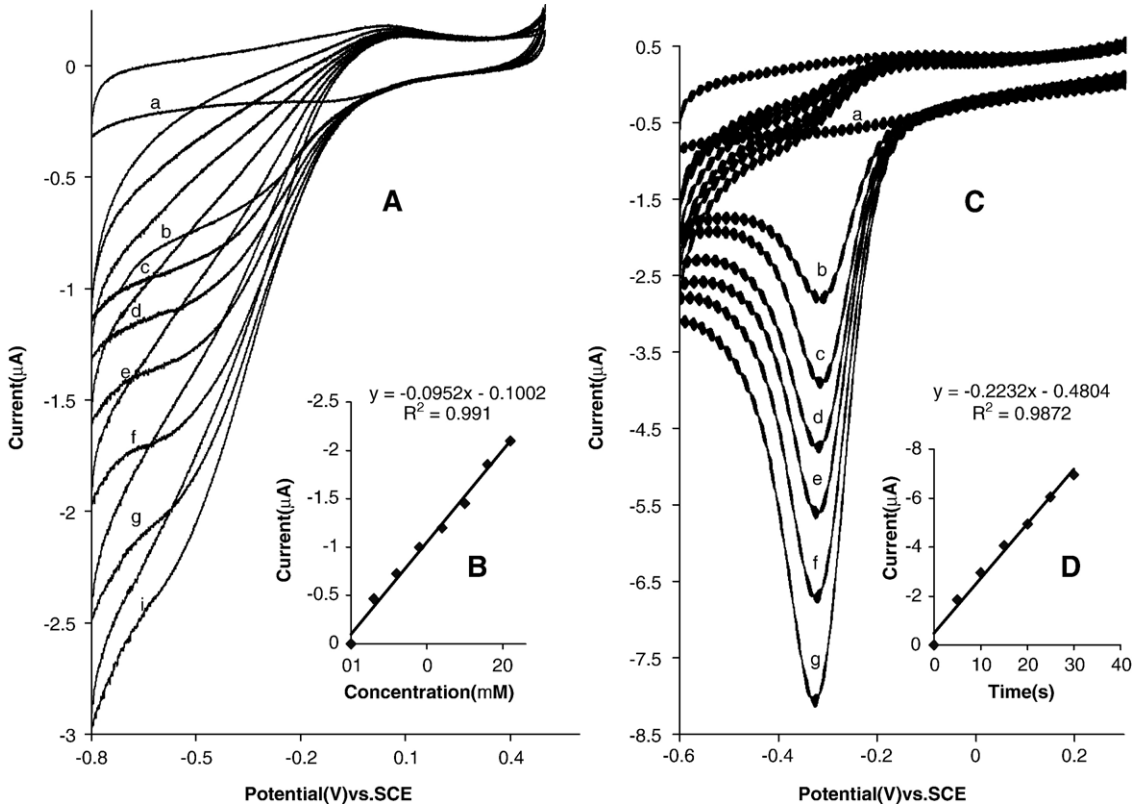

Fig. 8. (A) cyclic voltammetry response of Hb/CoOx modified GC electrode in the presence different concentration of H_2O_2 in PBS (pH 7) at scan rate 20 mV s^{-1}, (a) 0.0 (b) 3 (c) 6 (d) 9 (e) 12 (f) 15 (g) 18 and (i) 21 mM. (B) The catalytic response vs. hydrogen peroxide concentrations. (C) Recorded cyclic voltammograms of Hb/CoOx modified GC electrode for different bubbling time of oxygen (a) 0.0 (b) 6 (c) 12 (d) 18 (e) 24 (f) 30 and (g) 36 s. (D) Plot of peak current vs. bubbling times of oxygen.

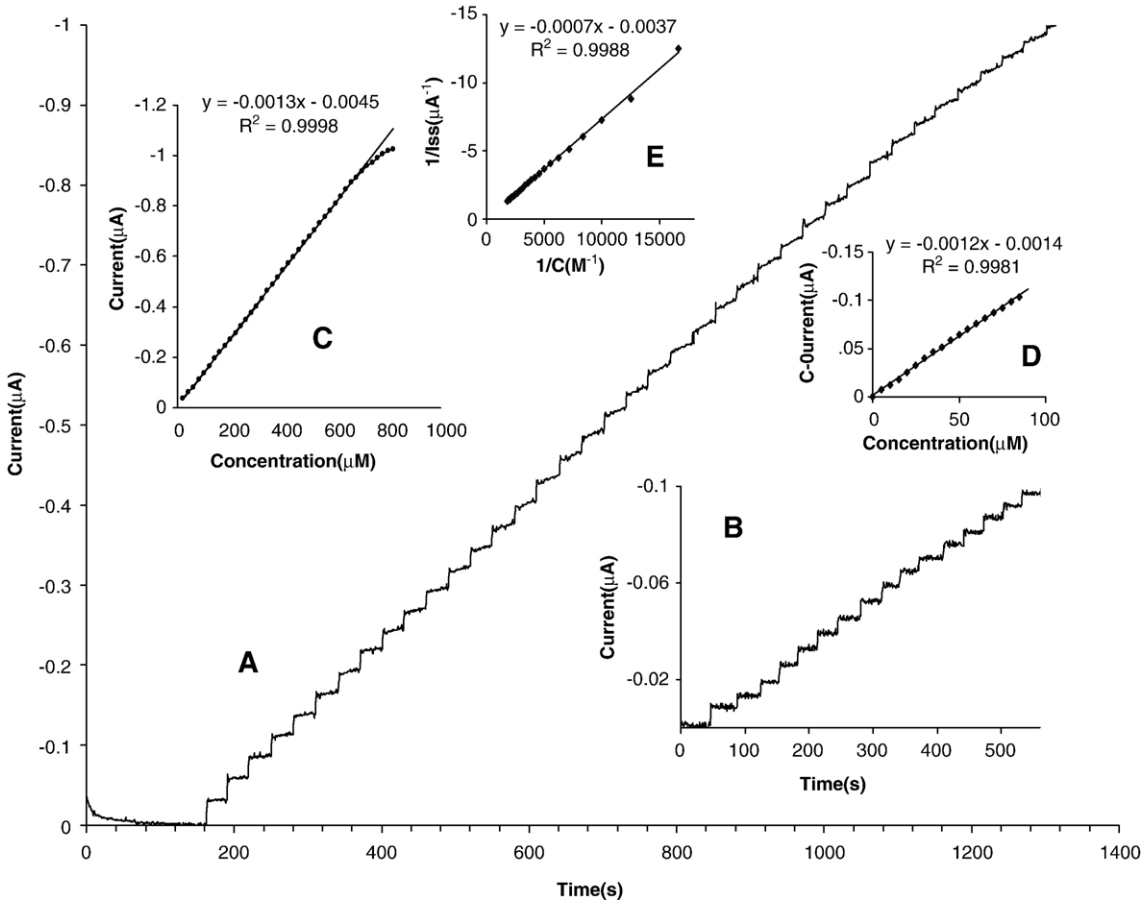

Fig. 9. Amperometric response of rotating Hb/CoOx modified GC electrode to H_2O_2, conditions −0.45 V constant potential, pH 7.0 and rotation speed is 2000 rpm, (A) successive addition of 20 μM and (B) 5 μM of hydrogen peroxide. (C and D) Plots of chronoamperometric current vs. H_2O_2 concentration. (E) Recorded plot for determination of K_M.

calculated from the slope of peak currents vs. scan rate. For a reversible surface reaction, the peak current has been given by the following equation [57]:

$$I_p = n^2F^2vA\Gamma_c/4RT \quad (1)$$

Where v is the sweep rate, A is the effective surface area (0.15 cm^2) of the modified electrode and the other symbols have their usual meaning. From the slope of cathodic peak currents vs. scan rate the calculated surface concentration of hemoglobin is 5.25×10^{-11} mol cm^{-2}, indicating a sub-monolayer of Hb immobilized onto cobalt oxide film.

3.4. pH effect, stability and reproducibility of Hb–cobalt oxide modified electrode

Cyclic voltammograms of modified electrode were recorded at different buffer solutions (pH 2–10) in the absence of oxygen (Fig. 5). A pair of well defined and stable redox peaks obtained for adsorbed Hb in different pH values. Both reduction and oxidation peak potentials of the Fe(III)/Fe(II) redox couple of hemoglobin at cobalt oxide nanoparticles modified GC electrode are negatively shifted by increasing pH values. The pH dependences of the peak potentials from pH 5 to 12 can be expressed as follows:

$$E^0 = -55.4\,\mathrm{pH} + 0.3354 \left(R^2 = 0.9916\right) \quad (2)$$

This slope is reasonably close to the theoretical value of −58.6 mV pH^{-1} at 25 °C for a reversible one proton coupled with one electron redox reaction process [58].

$$\mathrm{HbFe(III)} + H^+ + e^- \rightarrow \mathrm{HbFe(II)} \quad (3)$$

Furthermore, the same voltammogram can be obtained if the electrode is transferred from a solution with a different pH value back to its original buffer solution (pH 7). Long term stability is one of the most important properties for sensors, biosensors and bioreactors. The stability of Hb–cobalt oxide film electrodes was investigated by cyclic voltammetry. The working stability of the modified electrode was verified by monitoring the remaining amount of active Hb after successive sweeps of cyclic voltammograms, 100 cycles with scan rate of 100 mV s^{-1} in potential range 0.5 to −0.55 V (Fig. 6A). The peak height and peak potential of the immobilized enzyme remained nearly unchanged and amount of Hb remaining on the electrode surface were almost 97% of its initial value after 100 cycles. In addition no significant

decrease can be seen after replacing the electrolyte that has been used for 100 repetitive cycles with fresh buffer solution. The stability of the modified film was investigated by recording cyclic voltammogram of GC–Hb–CoOx electrode in buffer solution (pH 7), after storing it in refrigerator (4 °C) for 3 days (Fig. 6B). The enzyme electrode retained 96% of its initial current response after intermitted use over 3 days. High stability of modified electrode is related to the chemical stability of cobalt oxide film, the interaction between Hb and cobalt oxide and strong adsorption of Hb on cobalt oxide nanoparticles. Therefore, the Hb–cobalt oxide modified glassy carbon electrode, can be used as a biosensor due to its long term stability and excellent electron transfer rate constant.

In order to study the reproducibility of the biosensor and reliability of fabrication procedure, five glassy carbon electrodes modified with cobalt-oxide nanoparticles and Hb were prepared independently. Cyclic voltammograms of biosensors were recorded in buffer solution. The relative standard deviation (RSD) value of measured cathodic peak currents was 6%. Furthermore, the five electrodes showed acceptable reproducibility based on the current determination of 10 mM hydrogen peroxide (RSD 3%). For Hb–CoOx nanoparticles modified glassy carbon electrode, the relative standard deviation (RSD) determined by 6 successive assays of a 10 mM hydrogen peroxide sample was 2.5%. Good reproducibility and stability of the biosensor can be attributed to the natural features of cobalt oxide nanoparticles and the strong interaction between CoOx and Hb. Furthermore, high degree of biocompatibility of cobalt oxide provide good matrix to adsorb Hb and keep its native structure. A conclusion can be drawn from the above results that our immobilization procedure is efficient for retaining the electrocatalytic activity of Hb and preventing it from leaking out of the electrode.

3.5. Electrocatalytic reduction of hydrogen peroxide and oxygen at GC electrode modified with Hb and cobalt oxide

Hemoglobin such as other proteins and enzymes containing the heme group, have ability to reduce H_2O_2 and O_2 electrocatalytically based on the following equations:

$$HbFe(III) + H^+ + e^- \rightarrow HbHFe(II) \quad (4)$$

$$2HbHFe(II) + H_2O_2 \rightarrow HbFe(III) + 2H_2O \quad (5)$$

Electrocatalytic reduction of hydrogen peroxide and oxygen using Hb–CoOx nanoparticles modified GC electrode was investigated by recording cyclic voltammograms in the absence and presence hydrogen peroxide and oxygen. Fig. 7 shows cyclic voltammograms of cobalt oxide modified GC electrode and CoOx/Hb modified GC electrode in the absence and presence of H_2O_2. As shown when H_2O_2 was added to buffer solution reduction response was started at −0.05 V and an obvious catalytic reduction peak appears at the potential of −0.4 V (voltammogram "d"). For CoOx modified glassy carbon electrode, no redox response of H_2O_2 can be seen in the potential range from 0.5 to −0.8 V (voltammogram "b"). The reduction of overvoltage and increasing the peak current of hydrogen peroxide reduction confirm that Hb have high catalytic ability for H_2O_2 reduction. Therefore, Hb/CoOx nanoparticles are very suitable for fabrication third generation of mediatorless biosensors for determination of hydrogen peroxide. Cyclic voltammogram of the modified electrode in the presence different concentration of hydrogen peroxide was recorded (Fig. 8A). As shown in inset Fig. 8A peak currents linearly increased with increasing hydrogen peroxide concentration. The calibration plot is linear (correlation coefficient, 0.991) for a wide range of concentration (1–21 mM). The sensitivity and the detection limit (3σ) of the biosensor towards hydrogen peroxide were found to be 0.0952 μA/mM and 90 μM, respectively. When the biosensor immersed in buffer solution saturated with O_2, a large reduction peak could be observed while oxidation peak almost disappeared. In addition with increasing bubbling time of oxygen to buffer solution (increasing oxygen concentration) cathodic peak currents increased (Fig. 8B). The results indicate high electrocatalytic activity of Hb immobilized on cobalt oxide nanoparticles for oxygen reduction.

3.6. Amperometric detection of H_2O_2 at Hb/CoOx nanoparticles modified glassy carbon electrode

Fig. 9 shows the steady-state current response of hydrogen peroxide for the modified electrode with constant potential of −0.4 V. As shown, during the successive addition of 20 μM (chronoamperogram A) and 5 μM (chronoamperogram B) of H_2O_2, a well defined response is observed. The plot of response current vs. hydrogen peroxide concentration is linear over the wide concentration range of 5 μM to 0.7 mM. The calibration plot over the concentration range of 5–80 μM (16 points) has a slope of 1.2 nA/μM (sensitivity), correlation coefficient of 0.9981 and the detection limit of 0.6 μM at signal to noise ratio of 3. For hydrogen peroxide concentration higher than 600 μM, a response plateau was observed, showing the characteristics of the Michaelis–Menten kinetic mechanism. The Michaelis–Menten constant (K_M) gives an indication of the enzyme-substrate kinetics. The apparent (K_M) constant, can be obtained from the Lineweaver–Burk equation [59].

$$1/I_{ss} = 1/I_{max} + K_M/I_{max} \cdot 1/C \quad (6)$$

Here, I_{ss} is the steady-state current after the addition of substrate, C is the bulk concentration of substrate and I_{max} is the maximum current measured under saturated substrate solution. The Michaelis–Menten constant of the system (K_M) in this work is found to be 0.385 mM, implying that the Hb/CoOx modified glassy carbon electrode exhibits a higher affinity for hydrogen peroxide. The value of K_M for Hb in this work is smaller than that obtained at electrodes modified with immobilization of other enzymes and proteins such as; Hb sol–gel film carbon paste electrode,0.898 mM [15], Hb in poly-3-hydroxybutyrate membrane, 1.3 mM [22], Hb in triton X-100, 4.27 mM [24], Hb in a SP sephadex membrane, 1.9 mM [27], Hb immobilized onto ZrO_2, 1.77 mM [38], Hb immobilized onto mesoporous tungsten oxide, 1.84 mM [41], and Hb entrapped into nickel oxide film, 1.37 mM [46], Hb entrapped

in chitosan and $CaCO_3$ nanoparticles, 0.75[52], horseradish peroxidase(HRP)-based H_2O_2 sensor [59] and Hb immobilized in mesoporous silicas, 2.87 mM [60]. Therefore it clearly shows that the peroxidase activity of entrapped Hb in cobalt oxide nanoparticles is greatly enhanced, which is comparable to Hb immobilized onto different surfaces and modified layers. The smaller value of K_M validates that the immobilized Hb on cobalt oxide nanoparticles possesses higher enzymatic activity and the proposed electrode exhibits a higher affinity for hydrogen peroxide than other H_2O_2 biosensors.

4. Conclusion

Hemoglobin was successfully immobilized at GC electrode modified with electrodeposited cobalt oxide nanoparticles. The CoOx nanoparticles showed strong adsorption to Hb, leading to great enhancement of enzyme loading and improvement of its behavior due to their excellent biocompatibility. This method is advantageous when compared to other immobilization procedures because of the short immobilization time and no need for an electron transfer mediator or specific reagent. The biosensor exhibited good performance for electrocatalytic reduction of H_2O_2. Cobalt oxide nanoparticles in the biosensing interface not only could offer a friendly environment to immobilize Hb but also efficiently improved the electron transfer between Hb and electrode surface. Moreover, due to good biocompatibility, high adsorption ability and little harm to the biological activity of cobalt oxide nanomaterials, they have potential applications in the field of bioelectrochemistry, biosensors, bioelectronics and biofuel. The good performance of Hb/CoOx/GC electrode indicates these nanoparticles provided a suitable matrix for protein immobilization and well kept the bioactivities of protein. Finally the new matrix is strongly recommended for immobilization of many other enzymes or proteins for fabricating of third generation biosensors and bioelectronics devices.

Acknowledgments

The financial supports of Iranian Nanotechnology inventive, Management and Programming Organization of Kurdistan province and Research Office of University of Kurdistan are gratefully acknowledged.

References

[1] C.M. Welch, R.G. Compton, The use of nanoparticles in electroanalysis: a review, Anal. Bioanal. Chem. 384 (2006) 601–619.
[2] M. Chikae, K. Idegami, K. Kerman, N. Nagatani, M. Ishikawa, Y. Takamura, E. Tamiya, Electrochem. Commun. 8 (2006) 1375.
[3] A.N. Shipway, I. Willner, Nanoparticles as structural functional units in surface — confined architectures, Chem. Commun. (2001) 20352045.
[4] M. Yang, Y. Yang, Y. Liu, G. Shen, R. Yu, Platinum nanoparticles-doped sol–gel/carbon nanotubes composite electrochemical sensors and biosensors, Biosens. Bioelectron. 21 (2006) 1125–1131.
[5] F.A. Armstrong, Probing Metalloproteins by Voltammetry, Struct. Bonding, vol. 72, Springer, Berlin, 1990, pp. 137–230.
[6] A.J. Bard, M. Stratmann, Encyclopedia of Electrochemistry, Bioelectrochemistry, vol. 9, WILEY-VCH Verlag Gmbh, Weinheim, 2002, pp. 559–627, Chapter 17, Mediated Electron-transfer between Redox-enzymes and Electrode Supports.
[7] J.F. Rusling, Enzyme bioelectrochemistry in cast biomembrane-like films, Acc. Chem. Res. 31 (1998) 363–369.
[8] A. Heller, Electrical wiring of redox enzymes, Acc. Chem. Res. 23 (1999) 128–134.
[9] L. Gorton, A. Lindgren, T. Larsson, F.D. Munteanu, T. Ruzgas, I. Gazaryan, Direct electron transfer between heme-containing enzymes and electrodes as basis for third generation biosensors, Anal. Chim. Acta 400 (1999) 91–108.
[10] F.A. Armstrong, G.S. Wilson, Recent developments in faradic bioelectrochemistry, Electrochim. Acta 45 (2000) 2623–2645.
[11] L.G. Rodrigues, A.G. Wedd, A.M. Bond, Electrochemistry of xanthine oxidase at glassy carbon and mercury electrodes, J. Electroanal. Chem. 312 (1991) 131–140.
[12] Q. Li, G. Luo, J. Feng, Direct electron transfer for heme proteins assembled on noncrystalline TiO_2 film, Electroanalysis 13 (2001) 359–363.
[13] Z.H. Wen, B.X. Ye, X.Y. Zhou, Direct electron transfer reaction of glucose oxidase at bare silver electrodes and its application in analysis, Electroanalysis 9 (1997) 641–644.
[14] C. Cosnier, Biomolecule immobilization on electrode surfaces by entrapment or attachment to electrochemically polymerized films. A review, Biosens. Bioelectron. 14 (1999) 443–456.
[15] Q. Wang, G. Lu, B. Yang, Direct electrochemistry and electrocatalysis of hemoglobin immobilized on carbon paste electrode by silica sol–gel film, Biosens. Bioelectron. 19 (2004) 1269–1275.
[16] G. Wang, N.M. Thai, S.T. Yau, Preserved enzymatic activity of glucose oxidase immobilized on an unmodified electrode, Electrochem.Commun. 8 (2006) 987–992.
[17] W. Schuhmann, T.J. Ohara, H.L. Schimdt, A. Heller, Electron transfer between glucose oxidase and electrodes via redox mediators bound with flexible chains to the enzyme surface, J. Am. Chem. Soc. 113 (1991) 1394–1397.
[18] S. Yabuki, F. Mizutani, Y. Hirata, Hydrogen peroxide determination based on a glassy carbon electrode covered with a polyion complex membrane containing peroxidase and mediator, Sens. Actuators, B, Chem. 65 (2000) 49–51.
[19] Z. Zhang, S. Chouchane, R.S. Magiliozzo, J.F. Rusling, Direct voltammetry and catalysis with Mycobacterium tuberculosis catalase-peroxidase, peroxidases, and catalase in lipid films, Anal. Chem. 74 (2002) 163–170.
[20] X. Chen, H. Xie, J. Kong, J. Deng, Characterization for didodecyldimethylammonium bromide liquid crystal film entrapping catalase with enhanced direct electron transfer rate, Biosens. Bioelectron. 16 (2001) 115–120.
[21] H. Lu, Z. Li, N. Hu, Direct voltammetry and electrocatalytic properties of catalase incorporated in polyacrylamide hydrogel films, Biophys. Chemist. 104 (2003) 623–632.
[22] X. Ma, X. Liu, H. Xiao, G. Li, Direct electrochemistry and electrocatalysis of hemoglobin in poly-3-hydroxybutyrate membrane, Biosens. Bioelectron. 20 (2005) 1836–1842.
[23] L. Shen, N. Hu, Heme protein films with polyamidoamine dendrimer: direct electrochemistry and electrocatalysis, Biochim. Biophys. Acta 1608 (2004) 23–33.
[24] X. Liu, Y. Xu, X. Ma, G. Li, A third generation hydrogen peroxide biosensor fabricated with hemoglobin and triton X-100, Sens. Actuators, B, Chem. 106 (2005) 284–288.
[25] X. He, L. Zhu, Direct electrochemistry of hemoglobin in cetylpyridinuim bromide film: redox termodynamics and electrocatalysis to nitric oxide, Electrochem. Commun. 8 (2006) 615–620.
[26] S.M. Chen, C.C. Tseng, Comparison of the direct electrochemistry of myoglobin and hemoglobin films and their bioelectrocatalytic properties, J. Electroanal. Chem. 575 (2005) 147–160.
[27] C. Fan, H. Wang, S. Sun, D. Zhu, G. Wagner, G. Li, Electron transfer reactivity and enzymatic activity of hemoglobin in a SP sephadex membrane, Anal. Chem. 73 (2001) 2850–2854.
[28] H. Hung, N. Hu, Y.G. Zhou, Electrochemistry and electrocatalysis with heme proteins in chitosan biopolymer films, Anal. Biochem. 308 (2002) 141–151.
[29] L. Wang, N. Hu, Direct electrochemistry of hemoglobin in layer-by-layer films with poly(vinyl sulfonate) grown on pyrolytic graphite electrodes, Bioelectrochemistry 53 (2001) 205–212.

[30] P. He, N. Hu, G. Zhou, Assembly of electroactive layer-by-layer films of hemoglobin and polycationic poly (diallyldimethylammonium), Biomacromolcules 3 (2002) 139–146.
[31] I. Willner, Biomaterials for sensors, fuel cells, and circuits, Science 98 (2002) 2407–2408.
[32] G.C. Zhao, L. Zhang, X.W. Wei, Z.S. Yang, Myoglobin on multi-walled carbon nanotubes modified electrode:direct electrochemistry and electrocatalysis, Electrochem. Commun. 5 (2003) 825–829.
[33] A. Salimi, A. Noorbaksh, M. Ghadermarzi, Direct electrochemistry and electrocatalytic activity of catalase incorporated onto multiwall carbon nanotubes modified glassy carbon electrode, Anal. Biochem. 344 (2005) 16–24.
[34] J. Wang, M. Li, Z. Shi, N. Li, Z. Gu, Direct electrochemistry of cytochrome c at a glassy carbon electrode modified with single-wall carbon nanotubes, Anal. Chem. 74 (2002) 1993–1997.
[35] V.V. Shumyantseva, Y.D. Ivanov, N. Bistolas, F.W. Scheller, A.I. Archakov, U. Wollenberger, Direct electron transfer of cytochrome P450 2B4 at electrodes modified with non-ionic detergent and colloidal clay nanoparticles, Anal. Chem. 76 (2004) 6046–6052.
[36] H.Y. Gu, A.M. Yu, H.Y. Chen, Direct electron transfer and characterization of hemoglobin immobilized on a Au colloid-cysteamine-modified gold electrode, J. Electroanal. Chem. 516 (2001) 119–126.
[37] Y. Lvov, B. Munge, O. Giraldo, I. Ichinose, S. Suib, J.F. Rusling, Films of manganeous oxide nanoparticles with poly cation or myoglobin from alternate layer adsorption, Langmuir 16 (2000) 8850–8857.
[38] G. Zhao, J.J. Feng, J.J. Xu, H.Y. Chen, Direct electrochemistry and electrocatalysis of heme proteins immobilized on self assembled ZrO2 film, Electrochem. Commun. 7 (2005) 724–729.
[39] Q. Li, G. Luo, J. Feng, Q. Zhou, L. Zhang, Y. Zhu, Amperometric detection of glucose with glucose oxidase adsorbed on porous nanocrystalline TiO_2 film, Electroanalysis 13 (2001) 413–416.
[40] E. Topoglidis, C.J. Campbell, A.E.G. Cass, J.R. Durrant, Nitric oxide biosensors based on the immobilization of hemoglobin on mesoporous titania electrodes, Electroanalysis 18 (2006) 882–887.
[41] J.J. Feng, J.J. Xu, H.Y. Chen, Direct electron transfer and electrocatalysis of hemoglobineadsorbed onto electrodeposited mesoporous tungsten oxide, Electrochem. Commun. 8 (2005) 77–82.
[42] M.C. Rodriguez, G.A. Rivas, Glucose biosensor prepared by the dposition of iridium and glucose oxidase on glassy carbon transducer, Electroanalysis 11 (1999) 558–564.
[43] G. Zhao, J.J. Xu, H.Y. Chen, Fabrication, characterization of Fe_3O_4 multilayer film and its application in promoting direct electron transfer of hemoglobin, Electrochem. Commun. 8 (2006) 148–154.
[44] X. Zhu, I. Yuri, X. Gan, I. Suzuki, G. Li, Electrochemical study of the effect of nano-zinc oxide on microperoxidase and its application to more sensitive hydrogen peroxide biosensor preparation, Biosenso. Bioelectron. 22 (2007) 1600–1604.
[45] S. Cosnier, Biosensors based on immobilization of biomolecules by electrogenerated polymer films, Appl. Biochem. Biotechnol.. 89 (2000) 127–134.
[46] A. Salimi, E. Sharifi, A. NoorBakhsh, S. Soltanian, Direct voltammetry and electrocatalytic properties of hemoglobin immobilized on a glassy carbon electrode modified with nickel oxide nanoparticles, Electrochem. Commun. 8 (2006) 1499–1508.
[47] A. Salimi, E. Sharifi, A. NoorBakhsh, S. Soltanian, Direct electrochemistry and catalytic activity of catalase immobilized onto electrodeposited nano-scales islands of nickel oxide, Biophys. Chemist. 125 (2007) 540–548.
[48] A. Salimi, E. Sharifi, A. NoorBakhsh, S. Soltanian, Immobilization of glucose oxidase on electrodeposited nickel oxide nanoparticles: direct electron transfer and electrocatalytic activity, Biosens. Bioelectron. 22 (2007) 3246–3254.
[49] I.G. Casella, M. Gatta, Study of the electrochemical deposition and properties of cobalt oxide species in citrate alkaline solution, J. Electroanal. Chem. 534 (2002) 31–38.
[50] I.G. Casella, Electrodeposition of cobalt oxide films from carbonate solutions containing Co(II)-tartarate complexes, J. Electroanal. Chem. 520 (2002) 119–125.
[51] P. George, G.I.H. Hanania, Spectrophotometric study of ionizations in methemoglobin, Biochem. J. 55 (1953) 236–243.
[52] D. Shan, S. Wang, H. Xue, S. Cosnier, Direct electrochemistry and electrocatalysis of hemoglobin entrapped in composite matrix based on chitosan and CaCO3 nanoparticles, Electrochem. Commun. 9 (2007) 529–534.
[53] S. Song, S. Dong, Spectroelectrochemistry of the quasi-reversible reduction and oxidation of hemoglobin at a methylene blue adsorbed modified electrode, Bioelectrochem. Bioenerg. 19 (1988) 337–346.
[54] E. Laviron, General expression of the linear potential sweep voltammogram in the case of diffusionless electrochemical systems, J. Electroanal. Chem. 101 (1979) 19–28.
[55] Z. Dai, S. Liu, H. Ju, H. Chen, Direct electron transfer and enzymatic activity of hemoglobin in a hexagonal mesoporous silica matrix, Biosens. Bioelectron. 19 (2004) 861–867.
[56] Y.D. Zhao, Y.H. Bi, W.D. Zhang, Q.M. Luo, The interface behavior of hemoglobin at carbon nanotube and the detection for H2O2, Talanta 65 (2005) 489–494.
[57] J. Wang, Analytical Electrochemistry, VCH, New York, 1999.
[58] L. Meites, Polarographic Techniques, second ed., Wiley, New York.
[59] J. Li, S.N. Tan, H. Ge, Silica sol–gel immobilized amperometric biosensor for hydrogen peroxide, Anal. Chim. Acta 335 (1996) 137–145.
[60] Y. Xian, Y. Xian, L. Zhou, F. Wu, Y. Ling, L. Jin, Encapsulation of hemoglobin in ordered mesoporous silicas: influence factors for immobilization and bioelectrochemistry, Electrochem. Commun. 9 (2007) 142–148.