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ARTICLE *in* FOOD ANALYTICAL METHODS · APRIL 2014

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Simple and Rapid Amperometric Monitoring of Hydrogen Peroxide at Hemoglobin-Modified Pencil Lead Electrode as a Novel Biosensor: Application to the Analysis of Honey Sample

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Received: 17 June 2014 / Accepted: 31 August 2014

Abstract This paper describes the use of a pencil lead electrode (PLE) covered by hemoglobin (Hb) through a simple and rapid electroless method for the electroreduction of hydrogen peroxide. Some thermodynamic and kinetic parameters such as the number of electrons involved in the rate determining step, n_α, transfer coefficient, α, and the total electrons (n) involved in hydrogen peroxide reduction were determined. By attention to the findings, the possible mechanism for the H_2O_2 reduction at Hb/PLE was suggested. Also, the catalytic rate constant of the electrochemical process k and diffusion coefficient of hydrogen peroxide D were determined. The mean values obtained are 41.9 M^{-1} s^{-1} and 1.76×10^{-6} cm^2s^{-1}, respectively.

Finally, the ability of the electrode for the determination of hydrogen peroxide was investigated. In optimum conditions, the hydrodynamic amperometry was used for the determination of H_2O_2 at μM concentration level. It is found that the calibration graph is linear in the H_2O_2 concentration range 5×10^{-6}–245×10^{-6} mol L^{-1} with correlation coefficient of 0.999. The detection limit of the method was about 1×10^{-6} mol L^{-1}. This biosensor was successfully used for the determination of hydrogen peroxide in the tap water and honey samples using amperometric method.

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Published online: 11 September 2014

Keywords Hemoglobin · Pencil lead electrode · Hydrogen peroxide · Hb-modified electrode

Introduction

Hydrogen peroxide is a naturally occurring compound found in nature (oceans, lakes, rivers, rain, and snow) and in all life forms. The human body creates and uses hydrogen peroxide (free radicals) to destroy harmful bacteria, viruses, and fungi (Li et al. 2013; Wang et al. 2013). Also, H_2O_2 are considered as the biochemistry mediators of the cellular pathology and may be involved in the etiology of aging and progressive neurodegenerative diseases, such as Parkinson's disease (Song et al. 2011). For these reasons, rapid, accurate, reliable, and reagentless determination of hydrogen peroxide is very important in the fields of environmental protection, clinical control, neurochemistry, food, and industry (Xu et al. 2008). Numerous quantitative methods have been developed for the detection of H_2O_2. The most commonly used approaches include spectrometry (Matsubara et al. 1992), chemiluminescene (Kricka et al. 1996; Vasiliou et al. 2007), fluorimetry (Li and Dasgupta 2000), potentiometric titration (Gimeno et al. 2013), and amperometry (Toniolo et al. 2001; Pournaghi-Azar et al. 2010).

Electrochemical methods such as amperometric biosensors based on simple and economical immobilized redox proteins or enzyme-modified electrodes have been extensively employed for the determination of H_2O_2 because of their simplicity, high selectivity, and intrinsic sensitivity (Xiao et al. 1999; Lei et al. 2003; Song et al. 2006; Jia et al. 2002; Miao and Tan 2000; Yabuki et al. 2000; Morales et al. 1996; Lai et al. 2008;

Yagati et al. 2011; Wang et al. 2002; Kulys et al. 1993; Yang et al. 2008; Yu and Ju 2003). H_2O_2 can be detected using any enzymatic material as the electrocatalyst for electrochemical reduction (Yagati et al. 2011). Horseradish peroxidase (HRP) is the most commonly used enzyme for the construction of H_2O_2 biosensors (Lei et al. 2003; Song et al. 2006; Jia et al. 2002). But, it is still necessary to find other substitutes for HRP to increase properties or reduce the cost of a H_2O_2 biosensor (Wang et al. 2002).

The sensitive determination of hydrogen peroxide may be achieved by the use of hemoglobin (Hb)-modified electrodes, since Hb is known to show excellent selectivity for their substrates (Yang et al. 2008). Hemoglobin is a redox active protein consisting of four electroactive iron(II) heme groups. Electroactivity of hemoglobin is related to reversible conversion of Hb-Fe(II) to Hb-Fe(III). Hemoglobin can be used as a substitute of peroxidase to catalyse the reduction of H_2O_2 (Yagati et al. 2011; Wang et al. 2002; Kulys et al. 1993; Yang et al. 2008; Yu and Ju 2003; Xu et al. 2011; Sun et al. 2012; Xuan et al. 2012; Wang et al. 2012; Xu et al. 2010; Tan et al. 2009; Wang and Gu 2009; Liu et al. 2006; Liu et al. 2007; Salimi et al. 2006; Xu et al. 2012; Liu et al. 2012; Ren et al. 2013; Salimi et al. 2007; Zhou et al. 2002; Nadzhafova et al. 2004; Baghayeri et al. 2013; Zhang et al. 2012).

The development of low-cost and disposable electrodes for diverse electroanalytical applications is an active area of research. There have been a number of reports in the literature on the use of pencil graphite electrodes. These include the use of an inexpensive pencil graphite electrode for determination of dopamine and uric acid in blood (Alipour et al. 2013), measurement of DNA and RNA (Wang et al. 2000; Erdem et al. 2006), detection of cyclophosphamide using DNA (Palaska et al. 2007), detection of DNA hybridization (Hejazi et al. 2008; Pournaghi-Azar et al. 2008; Alipour et al. 2011), and as a solid-phase microextraction fiber (Djozan et al. 2007). Also, recent developments of pencil-drawn electrodes for building up chemiresistors, separation, and electrochemical devices have been reported (Dossi et al. 2013; Mirica et al. 2013; Mandal et al. 2012).

In our previous work, we have reported an electroless preparation path of the Hb film on the bare pencil lead electrode (PLE) surface, describing that the Hb film on the bare PLE surface is electroactive (Majidi et al. 2011). The major advantages of Hb/PLE are the simplicity, high stability of the Hb film on the PLE surface, and biocompatibility of this electrode. It was interesting from both fundamental and applied point of view to investigate the mechanism and kinetics of H_2O_2 reduction on Hb-modified electrodes. To our knowledge, until now, there is no detailed and perfect study on the electrochemical behavior and kinetic characteristic of H_2O_2 reduction at the Hb-modified electrodes and determination of its kinetics. The main purpose of the present work is to study the electrochemical behavior and kinetic characterization of H_2O_2 on the Hb/PLE using cyclic voltammetry and chronoamprometry.

Experimental

Chemicals and Reagents

Human Hb was obtained from Sigma and used as received. Methyltrimethoxysilane (MTMOS), methanol, hydrochloric acid, glycerol, and hydrogen peroxide (H_2O_2) were purchased from Merck. A total of 0.1 M phosphate buffer solution (PBS) pH 7 was prepared by mixing the stock solutions of Na_2HPO_4 and NaH_2PO_4. All solutions were made up with twice distilled water. All other chemicals were of analytical grade and used without further purification.

Electrode Preparation

The pencil graphite was obtained as pencil lead from Rotring Co. LTD, Germany (R 505210 N) of type H. All leads had a diameter of 2.0 mm. A pencil lead was used for preparation of the Hb/PLE as follows: The body of the pencil lead was tightly coated with a teflon band, and the surface was polished on a weighing paper to a smoothed finish before each use (Majidi et al. 2013). The polished surface of PLE was rinsed with doubly distilled water and then dried at room temperature.

Immobilization of Hb on the polished PLE was performed as follows: 2.5 μL of Hb solution (5 mg Hb dissolved in 200 μL phosphate buffer solution with pH 7 containing 5 μL glycerol) was spread onto the polished PLE surface and was allowed to dry at room temperature for 1 h. The silica sol solution was prepared by mixing 0.6 mL of MTMOS, 0.9 mL methanol, and 0.1 mL hydrochloric acid (0.1 M) and stirred for 5 min to homogeneous gel solution resulted. A total of 0.5 μL of this ormosil was spread onto the hemoglobin-modified PLE and was allowed to dry for 1 h and kept in a refrigerator at 4 °C overnight. Thin layer of silica sol film that formed at the Hb/PLE can avoid probably destructive and fouling effects of experimental medium such as pH variations and real sample matrix. This causes to increase the separation of Hb redox peaks while electrocatalytic behavior of Hb remains unchanged.

Finally, after washing the electrode surface using doubly distilled water, the Hb/PLE was obtained. The modified electrode was stored at 4 °C in a refrigerator while not in use.

Sample Preparation

This work was carried out on a local honey sample. The local honey sample obtained from Sabalan mountain in Iran. The samples were stored in the dark at room temperature prior to

analysis. The pretreatment could be described as follows: For determination of hydrogen peroxide, 2 g of honey was dissolved in 10 mL of purified water. One milliliter of the obtained solution was injected in the electrochemical cell containing 10 mL phosphate buffer solution (pH 7). The H_2O_2 content in the sample was determined by hydrodynamic amperometry according to the standard additions method.

Instrumentation

All voltammetric experiments were performed using a potentiostat/galvanostat AUTOLAB, model PGSTAT30. Amperometric experiments were performed using a Polarecord Metrohm, model 626. The utilized three electrode system was composed of a Hb/PLE as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum wire as the counter electrode. The pH measurements were performed using the pH meter (Metrohm 654). All potentials given in this article are referred to the SCE.

Electrolytic solutions were purged with highly purified nitrogen for at least 20 min prior to the series of experiments. A nitrogen environment was then kept over solutions in the cell to prevent the contact of the solution from oxygen.

FTIR spectra were obtained on a Tensor 27 FTIR spectrometer (Bruker).

Results and Discussion

FTIR Spectroscopic Characterization

Structural variations of Hb should first be considered after the protein is immobilized on PLE because Hb is sensitive to variation of the microenvironment around the heme site. FTIR spectroscopy is a sensitive technique to probe into the secondary structure of proteins. The profiles of the amide I and II infrared bands of Hb, especially, provide detailed information on the secondary structure of the polypeptide chain (Kauppinen et al. 1981). The amide I band (1,700–1,600 cm^{-1}) is attributed to the C=O stretching vibration of peptide linkage in the backbone of protein. The amide II band (1,620–1,500 cm^{-1}) is assigned to the combination of N–H bending and C–N stretching.

Figure 1 shows the spectra of pure Hb (curve a) and Hb immobilized on the PLE (curve b). As shown in Fig. 1, the spectra of amide I and II bands of Hb and that in the Hb film (1,655.3 and 1,544.7 cm^{-1}) are nearly the same. If Hb is denatured, the intensities of the amide I and II will significantly diminish or even disappear (Nassar et al. 1995; Song et al. 1992). Similarity of spectra (a) and (b) in Fig. 1 suggests that

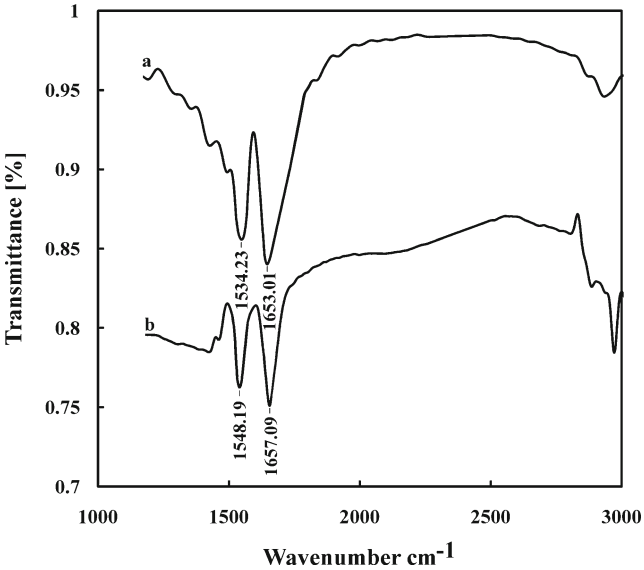

Fig. 1 FTIR spectra of the pure Hb (**a**) and Hb immobilized on PLE (**b**)

Hb retains the essential features of its native secondary structure on the PLE surface.

Preliminary Investigation

To get some information about the electrochemical behavior of H_2O_2 at bare PLE and Hb/PLE, a preliminary investigation using cyclic voltammetry seems to be useful. For this reason, CVs of bare PLE and Hb/PLE in the absence and presence of an aliquot of H_2O_2 were recorded. Figure 2 shows the bioelectrocatalytic response of the bare PLE (Fig. 2a) and Hb/PLE (Fig. 2b) in the absence and presence of 2 mM H_2O_2 in 0.1 M phosphate buffer solution of pH 7.0 at the scan rate of 20 mV s^{-1}. Curve (a) in Fig. 2a is related to bare PLE in PBS. Upon the addition of 2 mM H_2O_2 in the solution, any reduction peak relevant to H_2O_2 reduction did not appear on the bare PLE (curve b, Fig. 2a). As seen in curve (a) of Fig. 2b in the absence of H_2O_2, only the electrochemical behavior of Hb at Hb/PLE is apparent, as a pair of quasi-reversible anodic and cathodic wave appears in blank PBS. Upon addition of 2 mM H_2O_2 to the electrochemical cell, the cathodic peak increased and the anodic peak decreased (curve b, Fig. 2b), indicating a typical electrocatalytic reduction process of H_2O_2.

In order to monitor the variations of anodic and cathodic peaks of Hb with increasing H_2O_2 amounts, cyclic voltammograms related to increasing concentrations of H_2O_2 at Hb/PLE were obtained. Figure 3 shows the cyclic voltammograms of Hb/PLE in phosphate buffer solution (pH 7) containing different concentrations of H_2O_2 (0–25 mM) at the scan rate of 20 mV s^{-1}. Inset of Fig. 3 shows the plot of catalytic current versus H_2O_2 concentration. This figure shows that the reduction current increases linearly with the concentration of H_2O_2 in solution and at the high

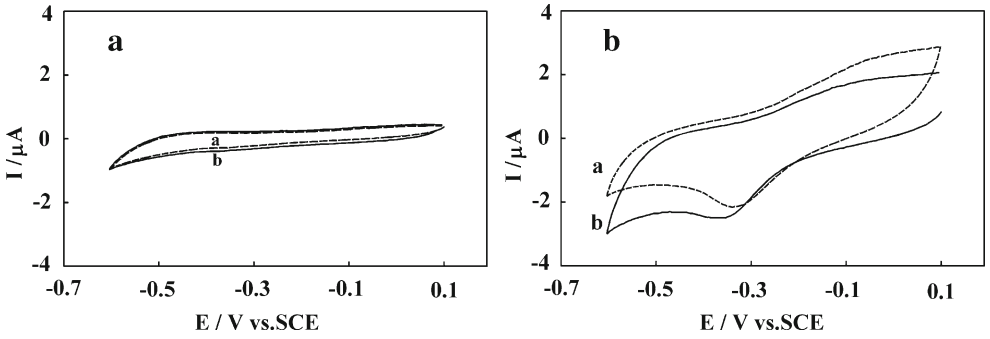

Fig. 2 **a** CVs of PLE in 0.1 M phosphate buffer solution (pH 7) without H_2O_2 *a* and containing 2 mM H_2O_2 *b* and **b** CVs of Hb/PLE in 0.1 M phosphate buffer solution (pH 7) without H_2O_2 *a* and containing 2 mM H_2O_2 *b* at the scan rate of 20 mV s^{-1}

concentrations of H_2O_2, oxidation peak of Hb disappeared. This behavior is typical of a mediated electrode process.

Effect of Scan Rate

Hemoglobin has certain intrinsic peroxidase activity due to its close similarity with peroxidase, so it can be employed to catalyze the reduction of hydrogen peroxide. Electrochemical catalytic reduction of hydrogen peroxide using Hb/PLE was investigated by cyclic voltammetry.

For the investigation of H_2O_2 electrochemical reduction, the effect of the scan rate on the H_2O_2 reduction peak was studied.

Figure 4a shows voltammograms of 5 mM hydrogen peroxide in the phosphate buffer solution at the scan rates from 20 to 200 mV s^{-1}. For the totally irreversible diffusion-controlled electrode processes, the following equation can be used for evaluation of the total number of electrons involved in the cathodic reduction of H_2O_2 (n) (Bard and Faulkner 2001):

$$I_p = 2.99 \times 10^5 n\,(\alpha n_\alpha)^{1/2} A C D^{1/2} v^{1/2} \quad (1)$$

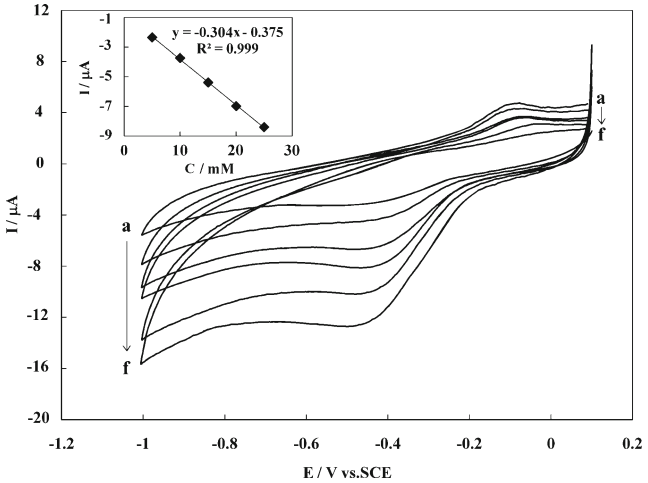

Fig. 3 Cyclic volammograms of H_2O_2 with increasing concentrations at Hb/PLE: **a** 0, **b** 5, **c** 10, **d** 15, **e** 20, **f** 25 mM H_2O_2 in 0.1 M phosphate buffer solution (pH 7). Scan rate: 20 mV s^{-1}. *Inset*: calibration graph

where n_α, α, D, and A are the number of electrons involving in the rate determining step, transfer coefficient, diffusion coefficient, and surface area of the electrode, respectively. C is the analyte concentration, and v is the potential scan rate.

The plot of the cyclic voltammogram peak current I_p versus $v^{1/2}$ should be linear, and the slope allows determining n (Fig. 4b). For this determination, the values of αn_α and D must already be determined.

In order to obtain information on the value of αn_α involved in the rate-determining step, a Tafel plot was drawn using background-corrected data from the rising part of the current–voltage curve at a scan rate of 20 mV s^{-1} (Fig. 4c). For 5 mM of H_2O_2, the slope of 7.96 (V per decade)$^{-1}$ was observed, which yields a value of about 0.5 for αn_α.

The value of αn_α can be obtained by another method according to the following equation valid for a totally irreversible diffusion-controlled process:

$$E_p = (b/2)\log v + \text{Const.} \quad (2)$$

where b indicates the Tafel slope.

Subjecting the cyclic voltammograms shown in Fig. 4a, a value of 63.8 mV per decade was obtained for b (Fig. 4d) and consequently a value 0.47 for αn_α.

By assuming $\alpha \sim 0.5$, the value of n_α from the two above different methods was obtained 1.

To get information on the final product of the H_2O_2-mediated reduction at the modified electrode, we have evaluated the total electrons (n) involved in the H_2O_2 reduction at a timescale of the cyclic voltammetric technique from Eq. 1.

Using the slope of I_p versus $v^{1/2}$ plot and considering αn_α equal to 0.5, $D = 1.76 \times 10^{-6}$ $cm^2 s^{-1}$ (calculated in the next section), $A = 0.03$ cm^2, $C = 5$ mM, it is found that the total number of electrons involved in the cathodic reduction of 1 H_2O_2 molecule is about 2.

With attention to our findings and previous studies (Olasehinde et al. 2008; Joseph et al. 2000), the possible mechanism for the mediated reduction of H_2O_2 at Hb/PLE can be expressed as follows:

$$\mathrm{Hb(Fe^{II})} + \mathrm{H_2O_2} \rightarrow \mathrm{Hb(Fe^{III})} + {}^{\bullet}\mathrm{O{\bullet}H} + \mathrm{H_2O} \quad \text{Slow} \quad (3)$$

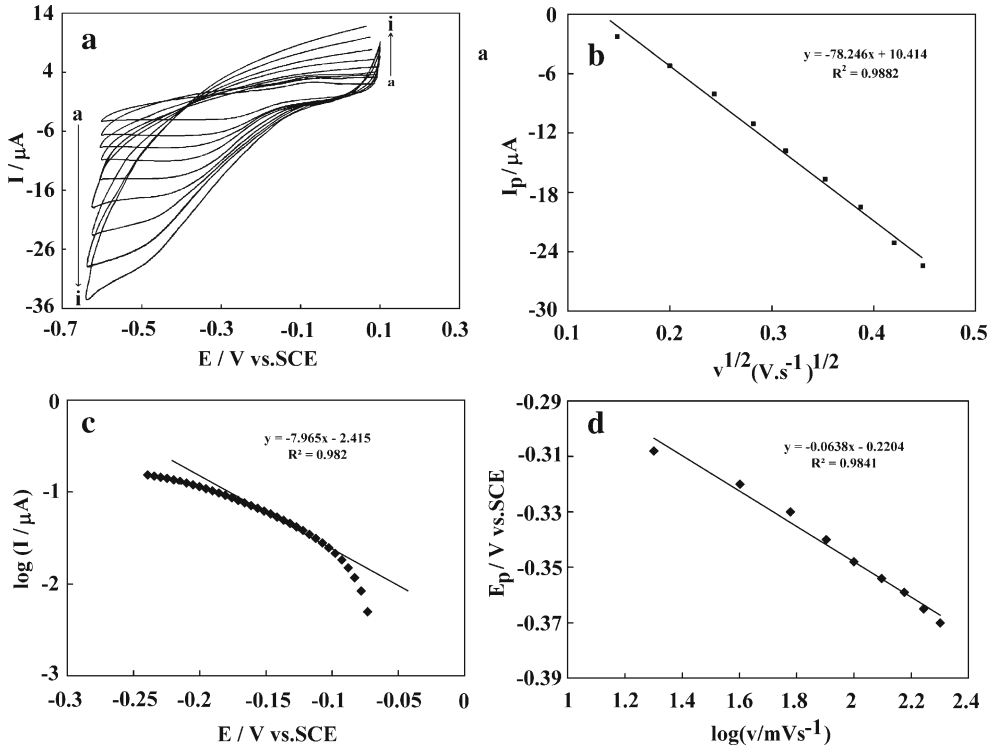

Fig. 4 **a** Cyclic voltammograms of Hb/PLE in phosphate buffer solution (pH 7) containing 5 mM H_2O_2 at the scan rates of 20, 40, 60, 80, 100, 125, 150, 175, 200 mV s^{-1}, **b** plot of variations of peak currents (I_p) versus $v^{1/2}$, **c** Tafel plot obtained from current–potential curves recorded at the Hb/PLE in the presence of 5 mM H_2O_2 at a scan rate of 20 mV s^{-1} at pH 7, **d** plot of variations of peak potentials (E_p) versus log v

$$Hb(Fe^{II}) + {}^{\bullet}O{\bullet}H \rightarrow Hb(Fe^{III}) + H_2O \quad \text{Fast} \tag{4}$$

$$Hb(Fe^{III}) + H^+ + e^- \rightarrow Hb(Fe^{II}) \tag{5}$$

The overall reactions of (3)–(5) would be

$$2Hb(Fe^{II}) + H_2O_2 \rightarrow 2Hb(Fe^{III}) + 2H_2O \tag{6}$$

$$2Hb(Fe^{III}) + 2H^+ + 2e^- \rightarrow 2Hb(Fe^{II}) \tag{7}$$

Chronoamperometry

Chronoamperometry as an effective electrochemical technique was used to evaluate the kinetics of H_2O_2-mediated reduction.

Calculation of Diffusion Coefficient (D)

Figure 5 represents the current–time profiles obtained by setting the working electrode potential at −0.55 V for Hb/PLE electrode in the absence (a) and in the presence of (b) 1, (c) 2, (d) 3, (e) 4, (f) 5 mM H_2O_2. As can be seen in this figure, choronoamperograms of analyte reach to steady state condition at about 3 s. So, we can conclude that the response time of electrode is 3 s. At long experimental times (t>1.5 s or $t^{-1/2}$< 0.8), where the electrocatalyzed reduction rate of H_2O_2 exceeds that of H_2O_2 diffusion, the current has a diffusional nature. In this region, the plot of I versus $t^{-1/2}$ gives a straight line (inset A, Fig. 5) and slope of such a line can be used for the estimation of the diffusion coefficient D of the substrate. From the slopes of these plots, the mean value of D was found to be 1.76×10^{-6} cm^2 s^{-1} for H_2O_2 which was comparable with previous reports (Pournaghi-Azar et al. 2010).

Inset B in Fig. 5 shows the plots of current sampled at a fixed time (elapsed times after application of the potential) as a function of H_2O_2 concentrations added to

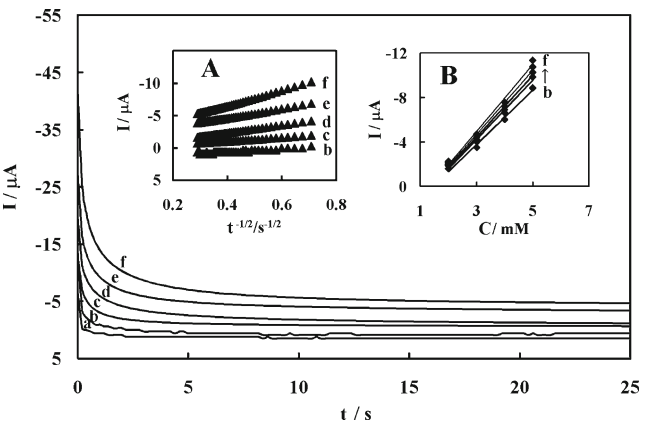

Fig. 5 Chronoamperograms obtained at the Hb/PLE in the absence (*a*) and in the presence (*b–f*) of 1, 2, 3, 4 and 5 mM H_2O_2, by setting the working electrode potential at −0.55 V. *Inset A*: plots (*b–f*) of I versus $t^{-1/2}$ for various concentrations of 1–5 mM; *inset B*: plots (*b–f*) of currents sampled at fixed times of 2.8, 2.2, 2, 1.8, and 1.6 s (elapsed times after application of the potential) as a function of H_2O_2 concentrations. Supporting electrolyte as in Fig. 2

the blank solution. Comparison of graphs b–f in inset B of Fig. 5 suggests that in all cases, there is a similar connection between currents measured at the fixed time and H_2O_2 concentration, but slope of the plot of current versus H_2O_2 concentration is slightly increased with decreasing elapsed time.

*Calculation of Catalytic Rate Constant (**k**)*

If the electron exchange processes at the electrode|Hb film interface is assumed to be fast, based on the experimental conditions, the rate-determining step must be one of the following processes (Bard and Faulkner 2001):

1. Diffusion of the H_2O_2 in the solution to the electrode surface
2. Diffusion of H_2O_2 through the Hb film
3. Diffusion of the electron in the film
4. Electron exchange between Fe(III) in the modifier thin layer and H_2O_2

When thin Hb films and high H_2O_2 concentrations are used, the contributions of H_2O_2 and electron diffusion within the film to rate limiting are negligibly small and mass transport process in the solution and the electron cross exchange between the Fe(III) in the film and H_2O_2 become dominant. Chronoamperometry as an effective electrochemical technique was used to evaluate the kinetics of H_2O_2-mediated reduction.

Figure 6 represents the current–time profiles obtained by setting the working electrode potential at −0.55 V for Hb/PLE in the absence (curve a) and in the presence of 5 mM H_2O_2 at pH 7 (curve b). As seen in Fig. 3, the behavior is typical of that expected for a mediated reduction.

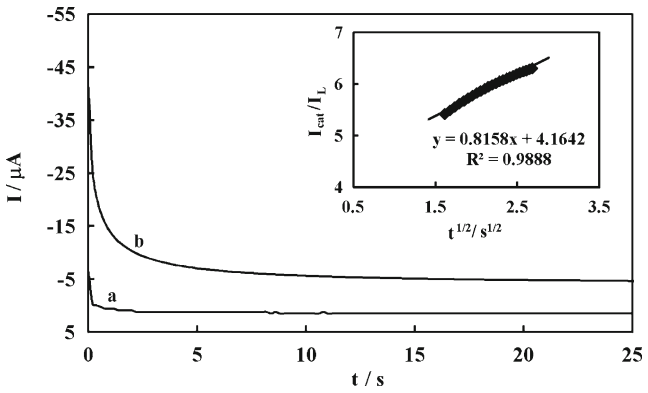

Fig. 6 Chronoamperograms obtained at the Hb/PLE in the absence (*curve a*) and in the presence of 5 mM H_2O_2 (*curve b*) at pH 7; potential step=−0.55 V. *Inset*: plot of variation of I_{cat}/I_L versus $t^{1/2}$. Supporting electrolyte as in Fig. 2

At intermediate times, where the reduction of Hb (Fe(III)) is almost complete, the catalytic current (I_{cat}) is dominated by the rate of the electron cross exchange between the Hb (Fe(II)) in the film and H_2O_2 (electrocatalytic rate), and the rate constant can be determined according to the method described in the literature (Galus 1994):

$$\frac{I_{cat}}{I_l} = \gamma^{1/2}\left[\pi^{1/2} erf\left(\gamma^{1/2}\right) + \exp(-\gamma)/\gamma^{1/2}\right] \quad (8)$$

where I_{cat} and I_l are the currents of the Hb/PLE in the presence and absence of substrate, respectively, and $\gamma = kct$ (c is the bulk concentration of H_2O_2) is the argument of the error function erf ($\gamma^{1/2}$). In the case that γ exceeds 2 (it is true in the present case), the error function is almost equal to 1, and Eq. 8 can be reduced to:

$$\frac{I_{cat}}{I_l} = \gamma^{1/2}\pi^{1/2} = \pi^{1/2}(kct)^{1/2} \quad (9)$$

where k and t are the catalytic rate constant ($M^{-1}\ s^{-1}$) and time elapsed (s), respectively. From the slope of the I_{cat}/I_l versus $t^{1/2}$ plot, we can calculate the value of k for a given concentration of H_2O_2. The inset of Fig. 6 shows such plot, constructed from the chronoamperograms for the Hb/PLE in the absence and presence of 5 mM H_2O_2, and the value for k was found to be 41.9 $M^{-1}\ s^{-1}$.

Hydrodynamic Amperometry

In order to evaluate the performance of the Hb/PLE as an amperometric device for determination of H_2O_2, the hydrodynamic amperometry was used at constant electrode potential. For choosing an optimum electrode potential, the hydrodynamic amperometry at various electrode constant potentials of −0.3, −0.35, −0.4, −0.45, −0.5, and −0.55 V was performed. The obtained results confirm that the highest amperometric response (i.e. slope of calibration graph) were obtained at −0.5 V (Fig. 7a). Similarly, the effect of Hb solution concentration (that is used for the modification of electrode) is also investigated and slope of calibration graphs constructed by PLE of different concentrations of Hb solution shown in Fig. 7b. As seen in Fig. 7b, the highest slope was obtained for 1 mg Hb/200 μL. The effect of pH on the electrode response was examined, and the variation of electrode response for 10 mM H_2O_2 versus pH was monitored by cyclic voltammetry. The results of this study showed that the highest response is related to pH 7 of the phosphate buffer solution as supporting electrolyte (Fig. 7c). In the optimized conditions, the electrode response was linear within the concentration range 5×10^{-6} to 245×10^{-6} mol L^{-1}.

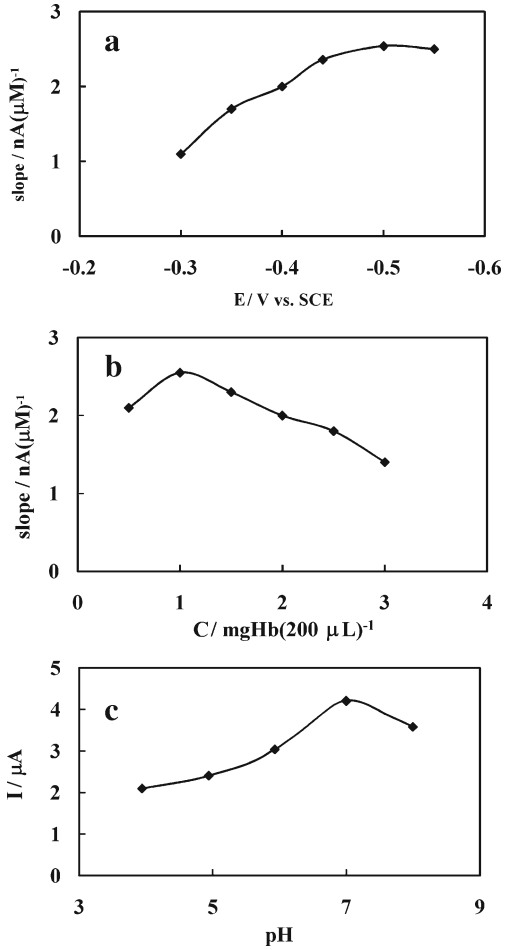

Fig. 7 Plot of hydrodynamic amperometric calibration curves slop of H_2O_2 versus (**a**) constant potential of electrode (**b**) concentration of Hb solution that used for the preparation of Hb/PLE and (**c**) variation of electrode response for 10 mM H_2O_2 versus pH monitored by cyclic voltammetry. Supporting electrolyte as in Fig. 2

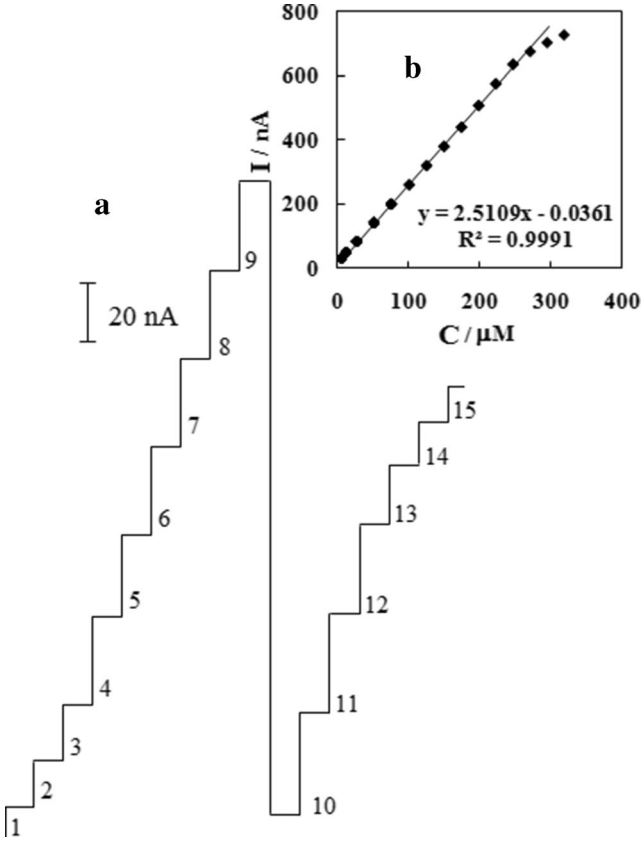

Fig. 8 **a** Hydrodynamic amperograms of Hb/PLE at constant electrode potential of −0.5 V in 0.1 M phosphate buffer solution of pH 7 for various concentrations of H_2O_2: *1*, 5 μM; *2*, 12 μM; *3*, 24.94 μM; *4*, 49.75 μM; *5*, 74.44 μM; *6*, 99.01 μM; *7*, 123.46 μM; *8*, 147.78 μM; *9*, 171.99 μM; *10*, 196.08 μM; *11*, 220.05 μM; *12*, 243.9 μM; *13*, 267.64 μM; *14*, 291.26 μM, and *15*, 314.77 μM and **b** calibration graph

The typical hydrodynamic amperograms and corresponding calibration graph are shown in Fig. 8. The amperograms of analyte reach to steady state condition at about 3 s. The analyte was added to electrochemical cell with interval times of 30 s.

The detection limit was 1 μM, which was calculated by means of $y_{LOD}\,(I)=y_B+3S_{y/x}$ equation and regression equation I (nA)=2.51c (μM)−0.036, where y_B is signal of the blank (here, intercept of calibration graph) and $S_{y/x}$ standard deviation of blank (here, standard deviation related to the calibration graph).

The Effect of Interferences in the Determination of H_2O_2

With the regard to H_2O_2 reduction potential, less interference can affect the detection of H_2O_2 except the dissolved oxygen. The interference from dissolved oxygen was studied. Figure 9 shows CVs of Hb/PLE in PBS (pH 7) before (curve a) and after deoxygenating of solution (curve b).

As can be seen in Fig. 9, the catalytic reduction peak current increased in the presence of oxygen, while the oxidation peak decreased, which demonstrates the catalytic reduction of O_2 by immobilized Hb at PLE. Thus, the presence of O_2 seriously interferes in the determination of H_2O_2. Therefore, deoxygenating of solution is necessary for recording accurate results. Thus, all solutions in this work were deoxygenated by bubbling highly pure nitrogen for at least 20 min and maintained under nitrogen atmosphere during the measurements.

On the other hand, the developed biosensor has enzymatic properties and acts as a peroxidase enzyme. Thus, the response of this biosensor for other substrates is negligible. However, the interference of some ions on H_2O_2 detection was investigated. The results showed that the peak current of 10 μM H_2O_2 was not affected by 0.1 M Na^+, K^+, HPO_4^{2-}, $H_2PO_4^-$, and Cl^-.

Stability of the Biosensor

The stability of Hb/PLE was investigated by recording repetitive cyclic voltammograms in 0.1 M PBS with pH 7 (Fig. 10).

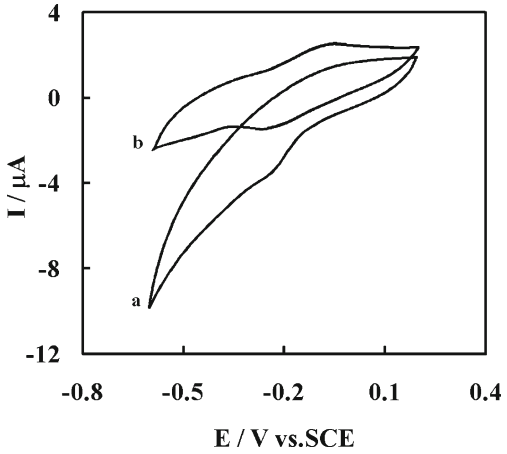

Fig. 9 CVs of Hb/PLE in PBS (pH 7.0) before (*curve a*) and after deoxygenating of solution (*curve b*)

As seen in Fig. 10, the peak height and peak potential of immobilized film remained approximately unchanged after 200 repetitive cycles in electrolyte solution.

This study show very good stability for Hb/PLE. In addition, no significant decrease can be seen after replacing the electrolyte having been used for 200 repetitive cycles with fresh electrolyte solution (results not shown).

Long-term stability is one of the most important properties of sensors, biosensors, and bioreactors. After storing of the Hb/PLE at 4 °C in refrigerator for about 5 days, the sensor retained about 98 % of its initial sensitivity for the reduction of H_2O_2, while a month later, 94 % efficiency was retained. On the other hand, the experiments on six electrodes that were made independently showed an acceptable reproducibility with the relative standard deviations of about 4.5 % for the current determinations of 1 mM H_2O_2. Therefore, the Hb/PLE can be used as a sensor due to its long-term stability. Reproducibility of the electrode response with the electrochemical techniques used in the present work (e.g. RSD of five replicate measurements of 1 mM of H_2O_2 was 1.9 %) confirmed that there was no any fouling phenomena, during the analyte reduction on the electrode and there were also no detachments of the catalyst (i.e. Hb).

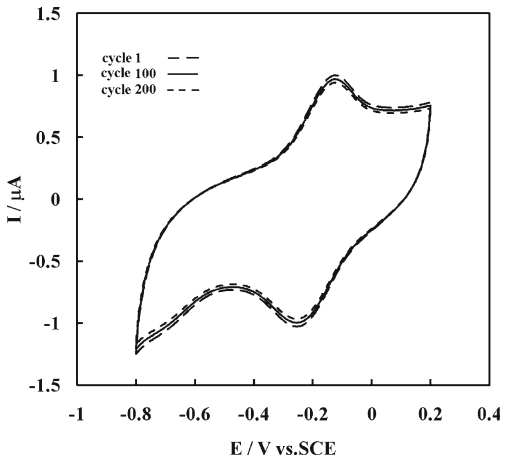

Fig. 10 CVs of Hb/PLE in 0.1 PBS with pH 7 (cycles 1, 100, and 200)

Determination of H_2O_2 in Real Sample

To demonstrate electrode suitability and potential application for sample analysis, the proposed method was applied to the analysis of H_2O_2 in tap water sample. With regard to the negligible amount of hydrogen peroxide in tap water, the sample was spiked with various amounts of H_2O_2 and a recovery test was performed (Table 1). The recovery was about 97.5 % suggesting that there was no interference from the matrix of tap water.

This method was also used for the determination of H_2O_2 in diluted honey sample. The sample solution was prepared as described at the Experimental section.

All honeys contain peroxide, which imbues them with antibacterial properties. It has been shown that the antibacterial activity of honey occurs due to hydrogen peroxide generation (Allen et al. 1991). Therefore, the determination of hydrogen peroxide is important in the characterization and selection of honey samples for its use as an antimicrobial agent. Hydrogen peroxide is generated by the enzyme glucose oxidase when honey is diluted, and maximum levels of hydrogen peroxide encountered in the diluted honeys are in the range of 1–2 mmol L^{-1} (Bang et al. 2003). Dilution is needed to decrease the acidity of the medium and for adjusting the pH for proper action of glucose oxidase. Weston stated that the level of hydrogen peroxide in honey is essentially determined by the amount of catalase, which originates from flower pollen, and glucose oxidase, which originates from the hypopharyngeal glands of bees (Weston 2000).

Franchini et al. showed that the amounts of hydrogen peroxide in 14 diluted honey samples with different

Table 1 Results of determination of hydrogen peroxide in the tap water and honey samples

Sample	Detected amount (μM)	Spiked (μM)	Average of founded (μM)	Recovery
Tap water	–	25	24.3	97.2 %
Tap water	–	50	48.8	97.6 %
Honey	44.8	25	69.2	97.6 %
Honey	44.8	50	93.5	97.4 %

Table 2 Comparison of developed sensor with other already reported ones for determination of H_2O_2

Electrode	Method	Linear range (μM)	LOD	LOQ	Ref.
Hemoglobin co-immobilized with silver–silver oxide nanoparticles on a bare silver electrode	Amperometry	2–50,000	2	–	Wang and Gu 2009
Fe_3O_4/CS-Hb-Fe_3O_4/CS-modified GCE	Amperometry	50–1,800	4	–	Tan et al. 2009
Hb/HZMS-SA-modified Au electrode	Amperometry	1.75–4,900	0.6	–	Xu et al. 2010
Hb-GNACs-modified GCE	Cyclic voltammetry	1–140	0.93	–	Xuan et al. 2012
Hb/PdNPs/GR–CS/GCE	Amperometry	2–1,100	0.66	–	Sun et al. 2012
–	Flow injection chemiluminescence	0.1–2	0.01	0.035	Vasiliou et al. 2007
Pt indicator electrode	Potentiometric titration	79–17,750	–	20	Gimeno et al. 2013
Hb/PLE	Amperometry	5–245	1	3.33	This study

geographical origin are in the range of 8–205 mg kg^{-1} (Franchini et al. 2008).

Amperogram obtained from the standard addition of H_2O_2 solution to electrochemical cell containing 0.1 M phosphate buffer solution (pH 7) and the diluted honey solution were used for the determination of H_2O_2. Taking into account the dilution of the honey sample, the H_2O_2 content in the original sample was calculated to be about 84.1 mg/kg. In order to confirm the accuracy of the method, sample solution was spiked with various amounts of H_2O_2 to check the recovery of the method.

The average recovery obtained from the standard addition of H_2O_2 solution to electrochemical cell containing honey solution and aliquot amount of H_2O_2 was found to be about 97 % (Table 1).

In Table 2, response characteristics of the proposed method are compared with those obtained by some other analytical and electroanalytical reported methods. In comparison with other methods reported for the determination of H_2O_2, the Hb/PLE can be considered as a sensor with low costs and easy preparation. In comparison with some other voltammetric methods, our method showed advantages in several aspects. For example, the preparation process of the Hb/PLE was simpler and is not time consuming. In comparison with other developed sensors, this sensor exhibits a low detection limit for H_2O_2 determination (see Table 2). On the other hand, we used Hb-modified electrode and amperometry method for the study of H_2O_2 reduction and determination of H_2O_2 in honey sample, for the first time. Moreover, since the Hb/PLE is biocompatible, a miniaturized Hb/PLE for in situ analysis can be developed.

Conclusion

The pencil lead electrode covered by a thin layer of Hb film exhibits the good electroactive characteristics for the hydrogen peroxide reduction and can catalyze the electroreduction of H_2O_2. The reaction pathway can be explained by cyclic voltammetry and chronoamperometry. Findings show that the rate-limiting step is one electron process and total number of electrons involved in the cathodic reduction of 1 H_2O_2 molecule is 2. The chronoamperometry can be used as an effective technique for determination of kinetics of the mediated reduction of H_2O_2. Finally, the capability of electrode for the determination of H_2O_2 was investigated by hydrodynamic amperometry. The hydrodynamic amperometry can be used as an effective technique for the determination of hydrogen peroxide at Hb/PLE, and this method successfully used for the determination of hydrogen peroxide in the tap water and honey samples.

Conflict of Interest Mir Reza Majidi declares that he has no conflict of interest. Mohammad Hossein Pournaghi-Azar declares that he has no conflict of interest. Afsaneh Saadatirad declares that she has no conflict of interest. Esmaeel Alipour declares that he has no conflict of interest.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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