Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 67 (2005) 221–226
Talanta
www.elsevier.com/locate/talanta

A hydrogen peroxide biosensor based on Langmuir–Blodgett technique: Direct electron transfer of hemoglobin in octadecylamine layer

Fan Yin [a,b], Hoon-Kyu Shin [a], Young-Soo Kwon [a,*]

[a] *Department of Electrical Engineering, Dong-A University, Busan 604-714, South Korea*
[b] *Department of Chemistry, Changshu Institute of Technology, Changshu 215500, PR China*

Received 1 December 2004; received in revised form 21 February 2005; accepted 24 February 2005
Available online 23 March 2005

Abstract

The present paper describes the modification of hemoglobin (Hb)–octadecylamine (ODA) Langmuir–Blodgett (LB) film on a gold electrode surface to develop a novel electrochemical biosensor for the detection of hydrogen peroxide. Atomic force microscopy (AFM) image of Hb–ODA LB film indicated Hb molecules existed in ODA layer in a well-ordered and compact form. The immobilized Hb displayed a couple of stable and well-defined redox peaks with an electron transfer rate constant of $4.58 \pm 0.95\,s^{-1}$ and a formal potential of -185 mV (versus Ag/AgCl) in phosphate buffer (1.0 mM, pH 5.0) contain 0.1 M KCl at a scan rate of $200\,mV\,s^{-1}$, characteristic of Hb heme Fe(III)/Fe(II) redox couple. The formal potential of Hb heme Fe(III)/Fe(II) redox couple in ODA film shifted linearly between pH 5 and 8 with a slope of $-23.8\,mV\,pH^{-1}$, suggesting that proton took part in electrochemical reaction. The ODA could accelerate the electron transfer between Hb and the electrode. This modified electrode showed an electrochemical activity to the reduction of hydrogen peroxide (H_2O_2) without the aid of any electron mediator.

Keywords: Hemoglobin; Octadecylamine; Langmuir–Blodgett film; Electrocatalysis

1. Introduction

The fabrication of hydrogen peroxide (H_2O_2) biosensors based on redox protein immobilized on different electrodes has gained considerable interest because H_2O_2 can be extensively applied in different fields, such as food, clinic, pharmaceutical, industry and environment [1–11]. Owing to expensiveness and unstability of horseradish peroxidase (HRP) in solution, the more stable and cheap mimetic enzymes such as hemoglobin (Hb) [5–7], hemin [8], hematin [9], myoglobin [10] and cytochrome *c* [11] have been adopted to design H_2O_2 biosensor.

Hb is a molecule with four electroactive iron hemes, which can be used as an ideal model molecule for the study of electron transfer reactions of heme proteins and also for biosensing and electrocatalysis [12]. However, because of deeply embedded electroactive groups, adsorptive denaturation on electrodes, and unfavourable orientations at electrodes, Hb displays a rather slow electron transfer rate at conventional electrodes [13,14]. Direct electron transfer can be reinforced by immobilizing Hb onto electrode surface incorporated into stable films such as hydrogel [15], surfactant [16], cross-linking dextran [17], DNA [18], Nafion [19], biomembrane-like films [20,21], poly (ester sulfonic acid) [22], hexagonal mesoporous silica [23], colloid gold nanoparticles [24,25], and zirconium dioxide nanoparticles [26].

Langmuir–Blodgett (LB) method is a convenient tool for designing artificial system (LB films) with biological functions and has been applied to the manufacture of immunosensors [27], enzyme sensors [28,29], biomolecular microphotodiode [30,31], and biocatalysis membrane [32,33]. So far as we know, however, rare work has been reported yet on immobilizing Hb by LB technique to design a biosensor based on direct electron transfer of Hb. Generally, to form protein–lipid LB film, proteins are solved in subphase

* Corresponding author. Tel.: +82 51 2009609; fax: +82 51 2007743.
E-mail address: yskwon@dau.ac.kr (Y.-S. Kwon).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.02.021

firstly, and then amphiphilic reagents are spread on the surface of subphase. However, before deposition of protein–lipid LB films, macromolecular impurities, or proteins in subphase have a strong tendency to adsorb onto electrodes and create a passive layer that would block the electron transfer between the heme groups and the electrode surface [6].

In this paper, to avoid protein adsorption on electrode surface before deposition, Hb–ODA (Octadecylamine) film was formed by spreading Hb solution directly onto subphase covered with a layer of ODA. The direct electrochemical behaviour of protein at Hb–ODA LB film modified gold electrode was studied for the first time. The electrocatalytic reduction of H_2O_2 on electrode modified with Hb–ODA LB film was also investigated using cyclic voltammetry. Results obtained with electrode modified by Hb–ODA LB film are presented in this paper and discussed in detail.

2. Experimental

2.1. Materials and apparatus

Hemoglobin was purchased from Sigma. Octadecylamine was obtained from Aldrich (purity ≥ 97%) without further purification. All chemicals were of analytical-reagent grade or better. Ultra pure water with a resistance of 18.2 MΩ from an ultra pure water system (UP 900D, Hana Tech. Co., Korea) was used to prepare buffer, subphase, Hb solution, and other solutions used in this experiment.

Hb–ODA LB films were formed in a NIMA Langmuir–Blodgett Trough (Model 611 with a trough surface area of 20 cm × 30 cm and a trough volume of 450 cm^3). The surface pressure was measured by using a Wilhelmy balance equipped with a strip of chromatography paper suspending at the air–water interface. AFM measurements were performed with a Multimode^{TM} scanning probe microscope (Digital Instrument, USA) operating in contact model. Electrochemical experiments were carried out with a Potentiostat 263A (Perkin-Elmer, USA) and a three-electrode system. PQC gold electrode modified with Hb–ODA LB film was used as working electrode, with Ag/AgCl as reference electrode and a platinum wire as counter electrode.

2.2. Preparation of the modified electrodes

A 9 MHz AT-cut piezoelectric quartz crystal (PQC) (Seiko, Japan) with gold electrodes (5.0 mm in diameter) at both sides was firstly cleaned with a piranha solution (1:3 H_2O_2:H_2SO_4) for 5 min, and then rinsed with distilled water. After that, it was dried in flowing nitrogen.

A total of 1.0 M KCl and 0.001 M phosphate buffer (pH 7.0) solution was used as subphase to form Hb–ODA LB film. After 21.6 μg ODA (0.54 mg ml^{-1}, in chloroform) was spread onto subphase's interface for at least 10 min, 100 μl Hb solution (0.4 mg ml^{-1}, pH 7.0 phosphate buffer) was spread carefully onto the subphase's interface covered with a layer of ODA. Pressure–time (π–t) curve was recorded immediately to evaluate the equilibrium of ODA layer/Hb interaction. When the equilibrium between Hb and ODA monolayer was established, the pressure–area (π–A) isotherm was recorded in NIMA 611 at a barrier speed of 20 mm min^{-1}.

Only hydrophilic gold surface was used to transfer Hb–ODA LB film from air–water interface to electrode surface. The Hb–ODA LB film on substrate surface was obtained under surface pressure of 35 mN m^{-1} using vertical withdrawal method. The withdrawal speed was kept at 4 mm min^{-1}. Prior to use, the modified electrode was thoroughly rinsed with pure water. The modified electrode was stored in 0.01 M phosphate buffer (pH 7.0) at 4 °C when not in use.

2.3. Atomic force microscopy (AFM) measurement

Samples used in AFM measurement were prepared by depositing Hb–ODA LB film onto an Au(1 1 1)-coated silicon flakes. Before deposition, the surface of Au(1 1 1) was treated with piranha solution (1:3 H_2O_2:H_2SO_4) for 5 min, rinsed thoroughly with distilled water and dried in flowing nitrogen. After deposition, the sample was rinsed with distilled water and dried in flowing nitrogen. Then AFM images were obtained.

2.4. Electrochemical measurement

The electrochemical behavior of Au electrode covered with a layer of Hb–ODA LB film was studied in 100 ml phosphate buffer (0.01 M, pH 5.0) containing 0.1 M KCl after deaeration with pure Ar for 30 min. An Ar environment was then kept in the cell by blowing Ar continuously during measurements. Cyclic voltammetry (CV) was employed to record and monitor the signals. All experiments were performed at room temperature (21 ± 1 °C) controlled by air-condition.

3. Results and discussion

3.1. Preparation of Au electrode modified with Hb–ODA LB film

Fig. 1 shows the scheme of Hb–ODA LB film forming on air–water interface. After 21.6 μg ODA was spread onto the surface of subphase containing 1.0 M KCl and phosphate buffer (1.0 mM, pH 7.0) for at least 10 min, 100 μl Hb solution was carefully spread onto the subphase's interface covered with a layer of ODA. An increase of surface pressure was observed immediately (Fig. 2, curve a), which indicated that Hb molecules entered ODA layer. This behaviour was attributed to the modification of interfacial film by incorporating Hb molecules into the ODA membrane at air–water interface. The equilibrium time of Hb molecules was estimated as 2.5 h according to experimental results. When surface pressure reached quasi-equilibrium value,

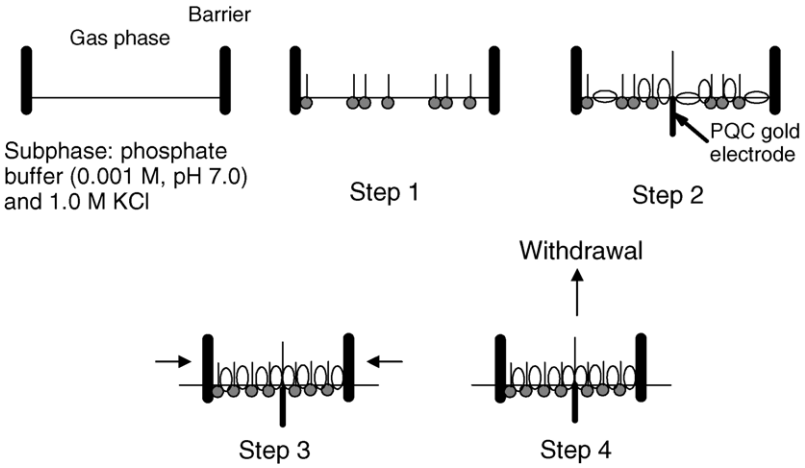

Fig. 1. Scheme of Hb–ODA LB film forming on air–water interface and deposition.

pressure–area (π–A) isotherm was recorded at a barrier speed of 20 mm min^{-1} and presented in Fig. 2 (curve b). Compared with π–A isotherm of pure ODA film (curve c), the surface film area increased under a given surface pressure, indicating that Hb molecules existed in ODA monolayer. Although it is difficult to estimate the exact surface pressure, the phase transition of Hb–ODA complex film from liquid-expanded (LE) state to liquid-condensed (LC) state was observed (Fig. 2, curve b), whereas no phase transition existed in pure ODA film (Fig. 2, curve c). The phase transition surface pressure of Hb–ODA complex layer was estimated at 30 mN m^{-1}. To obtain a compact and ordered Hb–ODA LB film on Au electrode surface, the surface pressure of 35 mN m^{-1} was chose to deposit the Hb–ODA LB film from air–water interface to PQC gold electrode surface by vertical withdrawal method under. The withdrawal speed was 2 mm min^{-1}.

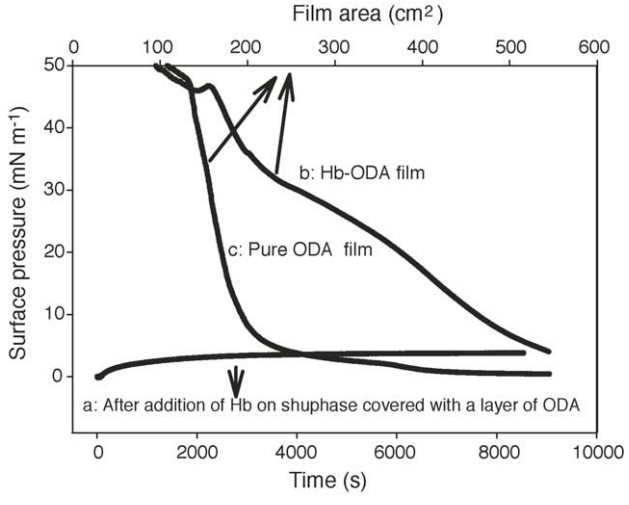

Fig. 2. Time-dependent variations of the surface pressure after spreading 100 μl Hb solution (0.4 mg ml^{-1}) on air–water interface covered with 21.6 μg ODA layer (curve a). Pressure–area (π–A) isotherms of the Hb–ODA layer (curve b) and pure ODA layer (curve c). Subphase, phosphate buffer (1.0 mM, pH 7.0) containing 1.0 M KCl; barriers speed, 20 mm min^{-1}.

3.2. AFM image of HB–ODA LB film

Atomic force microscopy was employed in this research to observe the structure of Hb–ODA LB film on Au(1 1 1) surface. The Au(1 1 1) surface covered with a layer of Hb–ODA LB film was prepared in the same way on PQC electrode. Fig. 3 shows typical contact mode AFM images of bare Au(1 1 1) and Au(1 1 1) covered with a layer of Hb–ODA LB film. Obviously, image of Au(1 1 1) surface with Hb–ODA LB film (Fig. 3B) was different from that of bare Au(1 1 1) surface (Fig. 3A). The compact and ordered structure of Hb–ODA LB film was observed, which was formed by aggregates of Hb molecules during compressing process. The image gave the evidence of Hb in Hb–ODA LB film on Au(1 1 1) surface.

3.3. Direct electrochemistry

Neither Au electrode covered with a monolayer of ODA LB film nor Hb molecules adsorbed directly on Au surface showed any electrochemical response in phosphate buffer (1.0 mM, pH 5.0) contain 0.1 M KCl (Fig. 4). However, when Au electrode was covered with a monolayer of Hb–ODA LB film, the cyclic voltammogram (scan rate 200 mV s^{-1}) displayed a pair of well-defined redox peaks (Fig. 4). It is obvious that the redox peaks of Au electrode modified by Hb–ODA LB film was attributed to the redox of the electroactive centres of Hb molecules in ODA layer. Thus the heme groups in Hb molecules still remained their structure and electrochemical activity. The anodic and cathodic peak potentials of Hb–ODA film on Au electrode were −168 and −202 mV (versus Ag/AgCl), respectively. The formal potential (E_0'), taken by the average value of the anodic and cathodic peak potentials, was −185 mV (versus Ag/AgCl). The comparison between the electrochemical behaviour of Hb in presence and absence of ODA suggested that ODA played an important role of facilitating the electron exchange between the Hb and Au electrode.

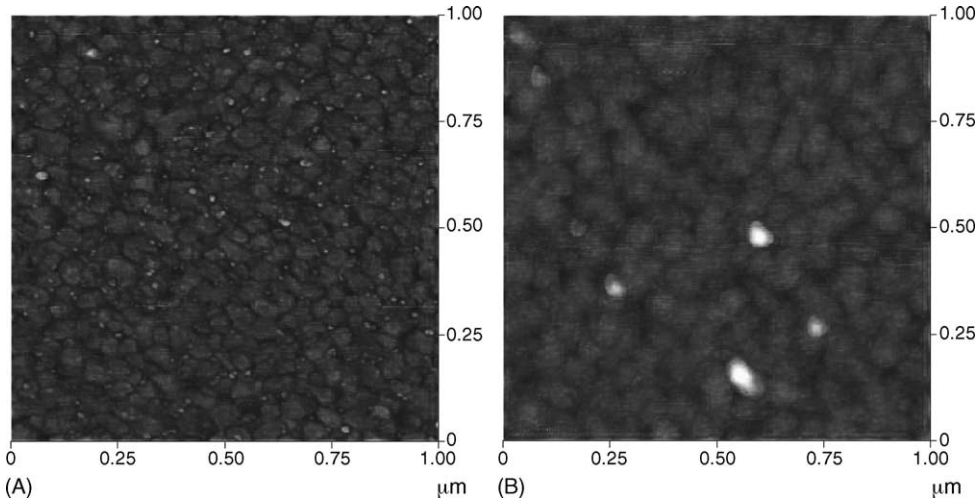

Fig. 3. Typical AFM images of Au(1 1 1) surface (A) and Au(1 1 1) surface covered with a layer of Hb–ODA monolayer deposited at surface pressure of 35 mN m^{-1} (B). The height scale is 20 nm.

The cyclic voltammograms of the Au electrode covered with Hb–ODA LB film showed a nearly equal height of reduction and oxidation peaks at the same scan rate (Fig. 5). With an increasing scan rate ranging from 50 to 900 mV s^{-1}, the anodic and cathodic peak potentials of the Hb showed a small shift and the redox peak currents increased linearly (Fig. 5, inset), indicating a surface-controlled electrode process. Supposing the charge transfer coefficient was between 0.3 and 0.7, the electron transfer rate constant, k_s, could be estimated with the formula $k_s = mnFv/RT$ when the peak-to-peak separation was less than 200 mV [34], where m is a parameter related to the peak-to-peak separation. According to the peak-to-peak separations of the cyclic voltammograms of the Au electrode covered with Hb–ODA LB film at 200, 300, 400, 500, 600, 700, 800 and 900 mV s^{-1}, the average k_s value was 4.58 ± 0.95 s^{-1}. This value is larger than that of Hb immobilized in the didodecyldimethylammonium bromide film [35], suggesting a reasonable fast electron transfer between the immobilized Hb and the Au electrode due to the presence of ODA layer.

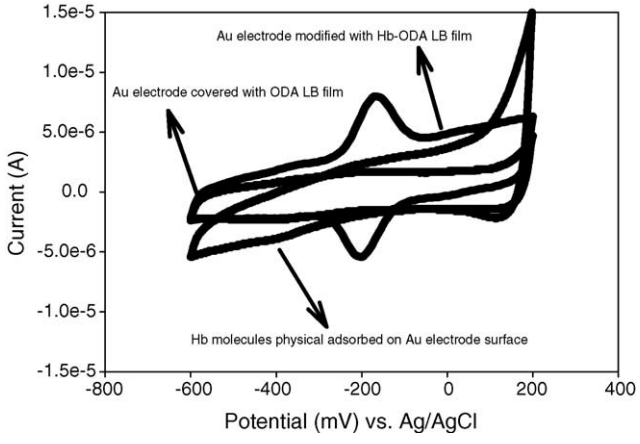

Fig. 4. Cyclic voltammograms of electrodes modified with different films.

3.4. Effect of solution pH on direct electron transfer of Hb

The direct electrochemistry of Hb immobilized on Au electrode by incorporated with ODA LB film showed a dependence on solution pH (Fig. 6). An increase of solution pH from 5.0 to 8.0 caused a linear negative shift in both cathodic and anodic peak potentials. The slope values of oxidation potential, reduction potential and formal potential were −9.5, −38.4 and −23.8 mV pH^{-1}, respectively. This behaviour was quite different from the reported dependence of these potentials on pH [6,35]. In these papers, the formal potentials were −55.7 mV pH^{-1} [6] and −56 mV pH^{-1} [35], respectively. It might be attributed to the hydrophobic environment of Hb incorporated with ODA LB film on Au electrode. It's difficult for hydroxonium ion to penetrate through this hydrophobic environment, entering Hb–ODA film and

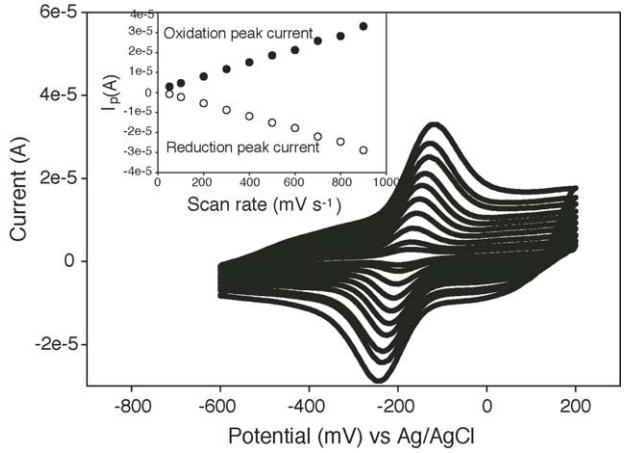

Fig. 5. Cyclic voltammograms of Hb–ODA LB film modified gold electrode in phosphate buffer (1.0 mM, pH 5.0) contain 0.1 M KCl at 50, 100, 200, 300, 400, 500, 600, 700, 800 and 900 mV s^{-1}. Inset: Influence of scan rate on oxidation and reduction peak current.

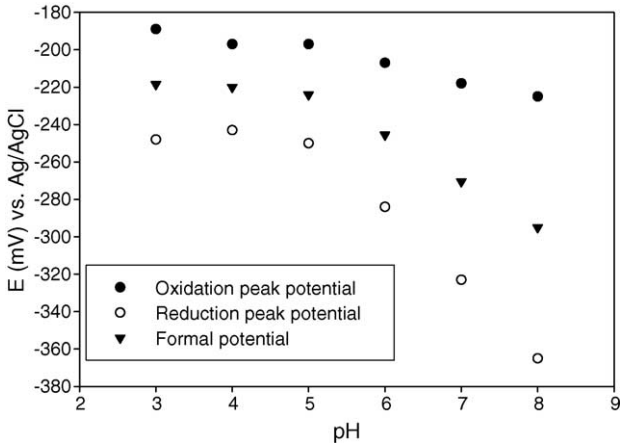

Fig. 6. Influence of pH on oxidation peak, reduction peak and formal potentials for Hb–ODA Lb film at 200 mV s^{-1}.

combining with Hb molecules during the redox process of Hb molecules, which would influence the dependence of the electrochemistry on solution pH to same extent. The pH independence region from 3.0 to 5.0 was observed in experiment. This phenomenon was also observed by Brunori and Taylor [36,37].

3.5. Electrocatalysis of Hb–ODA LB film to reduction of H_2O_2

With the addition of H_2O_2 to the electrochemical cell using a Hb–ODA LB film modified Au electrode as working electrode, an obvious increase of the cathodic peak was observed (Fig. 7), which was characteristic of an electrochemically catalytic reaction. On the other hand, no electrochemical signal corresponding to H_2O_2 on electrode modified with ODA LB film alone or with Hb adsorbed on it directly could be observed in the potential range of interest. Moreover, with the increase of H_2O_2 concentration, the cathodic peak current increased. According to experimental results, it was reasonable to think that the catalytic peak came from the interaction between Hb and H_2O_2. This result showed that Hb–ODA LB film can act as an effective catalyst to the reduction of H_2O_2. The catalytic current was linearly proportional to H_2O_2 concentration from 1×10^{-6} to 5×10^{-5} M with a correlation coefficient of 0.9982 ($n = 12$) (Fig. 7, inset). The detection limit was estimated to be 0.4×10^{-6} M, defined from a signal-to-noise ratio of 3.

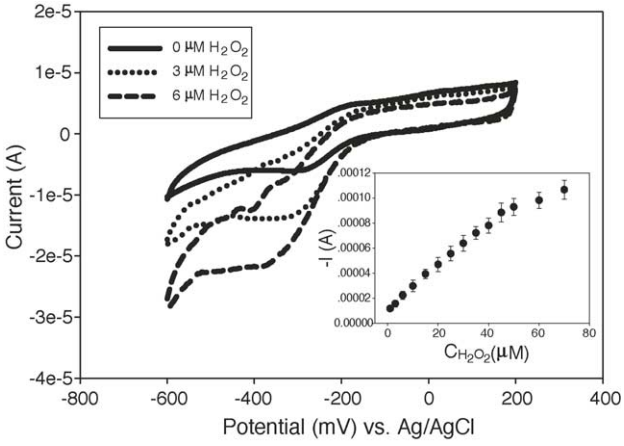

Fig. 7. Cyclic voltammograms at 200 mV s^{-1} in phosphate buffer (1.0 mM, pH 5.0) contain 0.1 M KCl: (a) no H_2O_2; (b) 3 μM H_2O_2; (c) 6 μM H_2O_2. Inset: Plot of the peak currents against the concentration of H_2O_2 in phosphate buffer (1.0 mM, pH 5.0) contain 0.1 M KCl. Scan rate: 200 mV s^{-1}.

The stability of the Hb–ODA LB film modified electrode was investigated by cyclic voltammetry measurements in the presence of 3 μM H_2O_2. The electrode was tested every day. When not in use, it was stored in a 0.01 M phosphate buffer (pH 7.0) at 4 °C. The sensor retained 85% of its original response after 7 days. This phenomenon may be attributed to the leakage of Hb from Hb–ODA LB film and the deactivation of Hb in Hb–ODA LB film.

The reproducibility of this sensor was investigated at a H_2O_2 concentration of 3 μM. The relative standard deviation (R.S.D.) was 4.8% ($n = 5$). For the reproducibility of four electrodes made from the same batch, the R.S.D. was 6.7% at 3 μM of H_2O_2.

4. Conclusion

In this paper, we reported a new method of making hydrogen peroxide biosensor by LB technique. This method provides simple and convenient experimental scheme to form Hb–ODA LB film on air–water interface with well-ordered and compact structure. Hemoglobin can be effectively immobilized on gold electrode surface by incorporated with ODA layer. Electrode modified with this Hb–ODA LB film proposes high electrochemical activity and shows a fast direct electron transfer of Hb. Redox peak currents increased linearly with the increase of scan rate, indicating a surface-controlled electrode process. The electron transfer rate constant was $4.58 \pm 0.95\ s^{-1}$. The slope values of oxidation potential, reduction potential and formal potential with pH were quite different from the reported dependence of them on pH. We thought that attributes to the difficulty of hydroxonium ion entering Hb–ODA film with hydrophobic environment and combining with Hb molecules during the redox process of Hb molecules. This modified electrode shows an electrochemical activity to the reduction of H_2O_2 without the aid of any electron mediator.

Acknowledgement

This paper is supported by the Dong-A University Research Fund in 2003.

References

[1] C.X. Lei, S.Q. Hu, N. Gao, G.L. Shen, R.Q. Yu, Bioelectrochemistry 65 (2004) 33.
[2] L. Wang, E.K. Wang, Electrochem. Commun. 6 (2004) 225.
[3] S.Q. Liu, H.X. Ju, Anal. Biochem. 307 (2002) 110.
[4] Y. Xiao, H.X. Ju, H.Y. Chen, Anal. Biochem. 278 (2000) 22.
[5] J.D. Zhang, M. Oyama, Electrochim. Acta 50 (2004) 85.
[6] Q.L. Wang, G.X. Lu, B.J. Yang, Sens. Actuators B 99 (2004) 50.
[7] S.Q. Liu, Z.H. Dai, H.Y. Chen, H.X. Ju, Biosens. Bioelectron. 19 (2004) 963.
[8] X. Wang, Y. Li, W. Chang, Anal. Chim. Acta 400 (1999) 135.
[9] J. Li, P.K. Dasgupta, Anal. Chem. 72 (2000) 5338.
[10] S.Q. Liu, H.X. Ju, Electroanalysis 15 (2003) 1488.
[11] H.X. Ju, S.Q. Liu, B.X. Ge, F. Lisdat, F.W. Scheller, Electroanalysis 14 (2002) 141.
[12] M. Fedurco, Coord. Chem. Rev. 209 (2000) 263.
[13] F.A. Armstrong, G.S. Wilson, Electrochim. Acta 45 (2000) 2623.
[14] J. Ye, R.P. Baldwin, Anal. Chem. 60 (1988) 2263.
[15] F.A. Armstrong, H.A.O. Hill, N.J. Walton, Acc. Chem. Res. 21 (1988) 407.
[16] J.F. Rusling, Acc. Chem. Res. 31 (1998) 363.
[17] H. Sun, N. Hu, H. Ma, Electroanalysis 12 (2000) 1064.
[18] M. Ciureanu, S. Goldstein, M.A. Mateescu, J. Electrochem. Soc. 145 (1998) 533.
[19] C. Fan, H. Wang, S. Sun, D. Zhu, G. Wagner, G. Li, Anal. Chem. 73 (2001) 2850.
[20] A.F. Nassar, J.F. Rusling, J. Am. Chem. Soc. 118 (1996) 3043.
[21] Q. Huang, Z. Liu, J.F. Rusling, Langmuir 12 (1996) 5472.
[22] J. Yang, N. Hu, Bioelectrochem. Bioenerg. 48 (1999) 117.
[23] Z. Dai, S. Liu, H. Ju, H. Chen, Biosens. Bioelectron. 19 (2004) 861.
[24] X.X. Xu, S.Q. Liu, B. Li, H.X. Ju, Anal. Lett. 36 (2003) 2427.
[25] S.Q. Liu, H.X. Ju, Analyst 128 (2003) 1420.
[26] S.Q. Liu, Z.H. Dai, H.Y. Chen, H.X. Ju, Biosens. Bioelectron. 19 (2004) 963.
[27] J. Yang, N. Hu, J.F. Rusling, J. Electroanal. Chem. 463 (1999) 53.
[28] V. Rosilio, M.M. Boissonnade, J. Zhang, L. Jiang, A. Baszkin, Langmuir 13 (1997) 4669.
[29] B.K. Oh, B.S. Chun, K.W. Park, W.C. Lee, W.H. Lee, J.W. Choi, Mater. Sci. Eng. C 24 (2004) 65.
[30] L. Caseli, M.E.D. Zaniquelli, R.P.M. Furriel, F.A. Leone, Colloids Surf. B 25 (2002) 119.
[31] C. Paternolli, P. Ghisellini, C. Nicolini, Mater. Sci. Eng. C 22 (2002) 155.
[32] S.Y. Oh, J.K. Park, C.B. Ko, J.W. Choi, Biosens. Bioelectron. 19 (2003) 103.
[33] J. Sánchez-González, J. Ruiz-García, M.J. Gálvez-Ruiz, J. Colloid Interface Sci. 267 (2003) 286.
[34] E. Laviron, J. Electroanal. Chem. 101 (1979) 19.
[35] H. Lu, Z. Li, N. Hu, Biophys. Chem. 104 (2003) 623.
[36] M. Brunori, J. Wyman, E. Antonini, A. Rossi-Fanelli, J. Biol. Chem. 240 (1965) 3317.
[37] J.F. Taylor, A.B. Hastings, J. Biol. Chem. 131 (1939) 649.