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Direct electrochemistry and electrocatalysis of myoglobin immobilized in hyaluronic acid and room temperature ionic liquids composite film

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ABSTRACT

A novel biocomposite film based on hyaluronic acid (HA) and hydrophilic room temperature ionic liquid 1-ethyl-3-methyl-imidazolium tetrafluoroborate ([EMIM][BF_4]) was explored. Here, HA was used as a binder to form [EMIM][BF_4]-HA composite film and help [EMIM][BF_4] to attaching on glass carbon electrode (GCE) surface, while doping [EMIM][BF_4] in HA can effectively reduce the electron transfer resistance of HA. The composite film can be readily used as an immobilization matrix to entrap myoglobin (Mb). A pair of well-defined and quasi-reversible redox peaks of Mb was obtained at the Mb-[EMIM][BF_4]-HA composite film modified GCE (Mb-[EMIM][BF_4]-HA/GCE) through direct electron transfer between Mb and the underlying electrode. The Mb-[EMIM][BF_4]-HA/GCE showed an excellent electrocatalytic activity toward the reduction of H_2O_2. Based on the [EMIM][BF_4]-HA biocomposite film, a third-generation reagentless biosensor could be constructed for the determination of H_2O_2.

1. Introduction

Recently, direct electrochemistry of redox proteins has aroused great interest, because it provides a foundation for fabricating biosensors without addition of mediators [1–3]. Furthermore, the direct electrochemistry of redox proteins can provide a good model for mechanistic studies of their electron transfer activity in the biological systems [4]. Unfortunately, it is difficult for the redox proteins to directly exchange electron with electrode surface because the denaturation and loss of electrochemical activities occurred when the proteins adsorbed directly on the electrode surface [5,6]. Therefore, finding new material with good biocompatibility for redox proteins immobilization on electrode surface is important to achieve their direct electrochemistry and keep their bioactivities.

Room temperature ionic liquids (RTILs) are compounds consisting entirely of ions that exist in liquid state around room temperature [7]. As attractive solvents, they possess unique properties such as negligible vapor pressure, low toxicity, wide potential window, high ionic conductivity and good solubility [8]. Several groups have reported increased thermal stability and activity of enzymes in aqueous mixture of ionic liquids as compared with conventional organic solvents or aqueous buffer solution [9–14]. Another interesting application is to incorporate RTILs into conventional matrices, such as cellulose [15], sol–gel-based silica [16], Nafion [17] and chitosan [18]. The combination of RTIL with conventional matrices can create unique materials that might open up new opportunities for the development of biosensors and biocatalysis.

Hyaluronic acid (HA) is a kind of linear polysaccharide consisting of linked disaccharide monomer units of glucuronic acid and *N*-acetylglucosamine. Because of its good biocompatibility and nontoxicity properties, HA hydrogel film had been widely used in biomedicine, tissue engineering, drug delivery and biosensors [19–21]. Ma et al. studied the direct electrochemistry of hemoglobin by casting hemoglobin-HA composite solution at pyrolytic graphite electrode [20]. Liu et al. adopted layer-by-layer method to assemble Mb and HA onto the basal-plane pyrolytic electrode and studied the electrochemistry properties of Mb [21]. To our knowledge, there was no report about the combination of RTIL with HA for study the directly electrochemistry of protein.

In the present work, a new biocomposite material based on HA and [EMIM][BF_4] was explored, which can be readily immobilized on the surface of GCE and form a stable film. Adding HA into RTILs can improve adhering ability of the composite film on electrode surface, meanwhile, doping RTILs in HA can improve conductivity of the composite film, so the combination of HA and RTILs shows more significant. A Mb modified electrode was constructed by simple entrapment Mb in the [EMIM][BF_4]-HA composite. Both biocompatibility of HA and intrinsic good conductivity of [EMIM][BF_4] enable the composite film to become an excellent platform for realizing direct electrochemistry and electrocatalysis of Mb along with good stability.

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2. Experimental

2.1. Apparatus and reagents

CHI660A electrochemical workstation (Shanghai Chenhua Co., China) controlled by a microcomputer with CHI660 software. A three-electrode system was used, where a saturated calomel electrode (SCE) served as the reference electrode, a platinum wire electrode served as the auxiliary electrode and a GCE or modified GCE served as the working electrode. All potentials reported were versus the SCE. DL-180 ultrasonic apparatus (Haitian Electronic Apparatus Company, Zhejiang, China) was applied in ultrasonic experiment. UV–Vis spectra experiments were carried out on a TU-1901 UV spectrophotometer (Purkinje General Instrument Co. Ltd., Beijing, China).

Mb and HA were purchased from Sigma and used as received. Mb (16 mg mL^{-1}) stock solution was prepared by 0.1 mol L^{-1} pH 7.0 phosphate buffer solution (PBS) and stored at a temperature of 4 °C. HA (2 mg mL^{-1}) solution was prepared by 0.1 mol L^{-1} pH 5.5 PBS. [EMIM][BF_4] was synthesized according to the literature [22]. H_2O_2 (30%) solution was purchased from Xi'an Chemical Reagent Company (Xi'an, China). PBS (0.1 mol L^{-1}) was prepared by mixing the stock solutions of Na_2HPO_4 and NaH_2PO_4.

2.2. Preparation of the Mb-[EMIM][BF_4]-HA modified electrode

Prior to use, the GCE (diameter 3 mm) was first polished with alumina slurry (followed by 1.0, 0.3 and 0.05 μm) and ultrasonically cleaned with ethanol and double distilled water then dried in nitrogen.

The amount of RTIL and HA can influence the quality of the modified electrode. Excessive amount of RTIL could cause instability of the electrode and the electrode would have poor conductivity if less RTIL is added. To obtain good cyclic voltammetric responses of Mb-[EMIM][BF_4]-HA/GCE, the concentrations and the mass ratios of HA, Mb and [EMIM][BF_4] in the mixture were optimized in control experiments. Typically, a homogenous solution containing 1.0 mg mL^{-1} HA, 12 mg mL^{-1} Mb and 6% (V/V) [EMIM][BF_4] was prepared by adding appropriate volume of HA solution, Mb solution and pure [EMIM][BF_4] into 5 mL 0.1 mol L^{-1} pH 7.0 PBS. Then 5 μL of the resulting solution was cast onto the surface of GCE by a microsyringe to prepare the Mb-[EMIM][BF_4]-HA/GCE. A beaker was covered over the electrode so that water can evaporate slowly in air and an uniform film electrode can be formed. The dried Mb-[EMIM][BF_4]-HA/GCE was stored at 4 °C in refrigerator when not in use. For comparison, HA/GCE, [EMIM][BF_4]-HA/GCE and Mb-HA/GCE was prepared with the same procedures as described above.

2.3. Procedure

The electrochemical measurements were carried out in 10 mL cells containing 0.10 mol L^{-1} pH 7.0 PBS. Prior to measurement, electrolytic solutions were purged with highly purified nitrogen for at least 30 min, then a nitrogen atmosphere was maintained over the electrochemical cell during the experiments. The cyclic voltammograms (CVs) were recorded from −0.9 to 0.2 V. All experiments were performed at room temperature.

3. Results and discussion

3.1. Characterization of the [EMIM][BF_4]-HA composite film

Potassium ferricyanide was selected as a probe to evaluate the performance of different electrodes. Fig. 1 compared the responses of GCE, HA/GCE and [EMIM][BF_4]-HA/GCE in 0.5 mmol L^{-1} $K_3Fe[CN]_6$ + 0.1 mol L^{-1} KCl solution at a scan rate of 0.10 V s^{-1}, respectively. A pair of well-defined CVs with a peak-to-peak separation (ΔE_p) of 0.086 V was observed at the bare GCE (Fig. 1a). After modified with HA, both anodic and cathodic peaks were decreased and ΔE_p was increased to 0.223 V (Fig. 1b), indicating the HA film acted as a blocking layer for electron transfer of ferricyanide. However, at the [EMIM][BF_4]-HA/GCE (Fig. 1c), the peak currents increased obviously and ΔE_p decreased to 0.153 V as compared with Fig. 1b, showing the addition of [EMIM][BF_4] could accelerate the electron transfer of ferricyanide [23]. Here, HA was used as a binder to form [EMIM][BF_4]-HA/GCE composite film and help [EMIM][BF_4] to attaching on the GCE surface, while doping [EMIM][BF_4] in HA can effectively reduce the electron transfer resistance of HA.

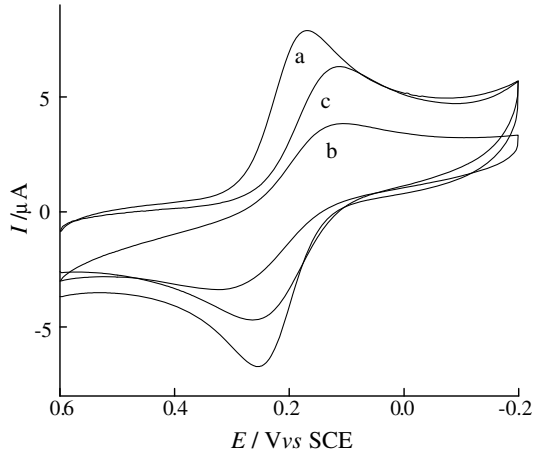

Fig. 1. CVs of (a) GCE, (b) HA/GCE, (c) [EMIM][BF_4]-HA/GCE in 0.5 mmol L^{-1} $K_3Fe[CN]_6$ + 0.1 mol L^{-1} KCl solution.

3.2. UV–Vis spectroscopy

UV–Vis spectroscopy is an effective means to probe into the characteristic structure of proteins. The Soret absorption band of heme may provide information about conformational change in the heme group region [10,24]. As can been seen from Fig. 2, Mb entrapped in [EMIM][BF_4]-HA composite film had a characteristic absorption band at 405.1 nm (Fig. 2b), same as that of native Mb

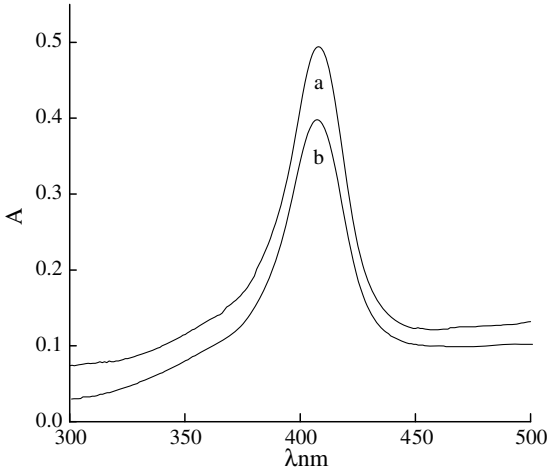

Fig. 2. UV–Vis absorption spectra of (a) Mb and (b) Mb-[EMIM][BF_4]-HA composite in 0.1 mol L^{-1} pH 7.0 PBS.

in pH 7.0 PBS (Fig. 2a). This suggested that Mb entrapped in the composite had a similar structure to the native Mb.

3.3. Direct electrochemistry of Mb-[EMIM][BF_4]-HA/GCE

Fig. 3A showed CVs of different Mb modified electrodes in 0.1 mol L^{-1} pH 7.0 PBS, respectively. A couple of ill-defined and not symmetric redox peaks corresponding to $MbFe^{III}/Fe^{II}$ was observed at the Mb-HA/GCE (curve a), which could be ascribed to the direct electron transfer between Mb and the underlying electrode. However, a pair of well-defined and nearly symmetric redox peaks of Mb was observed at the Mb-[EMIM][BF_4]-HA/GCE (curve b), with anodic peak potentials (E_{pa}) of −0.296 V, cathodic peak potentials (E_{pc}) of −0.394 V and ΔE_p of 0.098 V, respectively. The formal potential (E^0 estimated as $(E_{pa} + E_{pc})/2$) of Mb (Fe^{III}/Fe^{II}) was calculated to be −0.345 V, which was similar to literatures [25,26]. Compared with curve a, the current responses of Mb in curve b were remarkable augmented, demonstrating that the presence of [EMIM][BF_4] could greatly facilitate the electron transfer process between Mb and the underlying electrode. Thus, both biocompatibility of HA and inherent conductivity of [EMIM][BF_4] enable the composite to become an excellent platform for realizing the direct electrochemistry of Mb. The electrode reaction mechanism of Mb might be expressed by following equation [27]:

$$\mathrm{MbFe(III)} + e^- \rightarrow \mathrm{MbFe(II)} \quad (1)$$

The effect of scan rates on the response of the Mb-[EMIM][BF_4]-HA/GCE was shown in Fig. 3B. With scan rate increasing, both the reduction and oxidation peaks currents (I_p) were increasing linearly with the scan rates (Fig. 3B inset), suggesting a surface-controlled process. Furthermore, the E_{pa} of Mb shifted toward more positive direction and the E_{pc} shifted toward more negative direction with the increase of scan rate. Thus, the electron transfer rate constant (k_s) could be estimated from the ΔE_p value using the model of Laviron [28] for a surface-controlled electrode process. The k_s was calculated to be 4.21 s^{-1}. This value was higher than that of Mb on silk fibroin film modified graphite electrode (1.34 s^{-1}) [29] and clay-chitosan-gold nanoparticles modified glass carbon electrode (0.55 s^{-1}) [30], indicating the HA-[EMIM][BF_4] composite film had stronger promotion to the direct electron transfer of Mb.

Integration of reduction peaks at different scan rates gave nearly constant charge values. According to Faraday's law, the average surface concentration of electroactive Mb in Mb-[EMIM][BF_4]-HA/GCE was calculated to be 9.56×10^{-11} mol cm^{-2}. The result was larger than the theoretical monolayer coverage of 1.58×10^{-11} mol cm^{-2} for Mb [31], showing that several layers of Mb entrapped in the composite film participated in the electron transfer process.

The stability of the Mb-[EMIM][BF_4]-HA/GCE was investigated. The CVs of the Mb-[EMIM][BF_4]-HA/GCE changed only slightly after 40 continuous cycles, showing that Mb immobilized in the [EMIM][BF_4]-HA composite film was very stable. Conversely, the redox peaks of Mb almost disappeared after continuous potential cycling if no HA in [EMIM][BF_4] film, indicating HA could greatly improve [EMIM][BF_4] stably attached on the GCE surface.

3.4. Electrocatalysis of Mb-[EMIM][BF_4]-HA/GCE to the reduction of H_2O_2

The electrocatalytic activity of Mb-[EMIM][BF_4]-HA/GCE toward H_2O_2 was examined by cyclic voltammetry. Fig. 4 showed CVs of H_2O_2 at the Mb-[EMIM][BF_4]-HA/GCE. With increasing the concentration of H_2O_2, the reduction peak current increased obviously, while the oxidation peak current decreased to almost zero, showing a typical electrocatalytic reduction process of H_2O_2. This result illustrated that Mb in the composite film retained its bioactivity

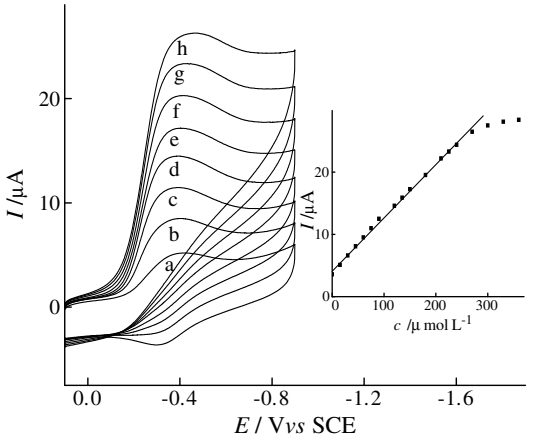

Fig. 4. CVs of Mb-[EMIM][BF_4]-HA/GCE in 0.1 mol L^{-1} pH 7.0 PBS with different concentration of H_2O_2. (a) 0; (b)30; (c) 60; (d) 90; (e) 120; (f) 150; (g)180; (h) 210 μmol L^{-1}. Inset: I_{pc} vs. *c*.

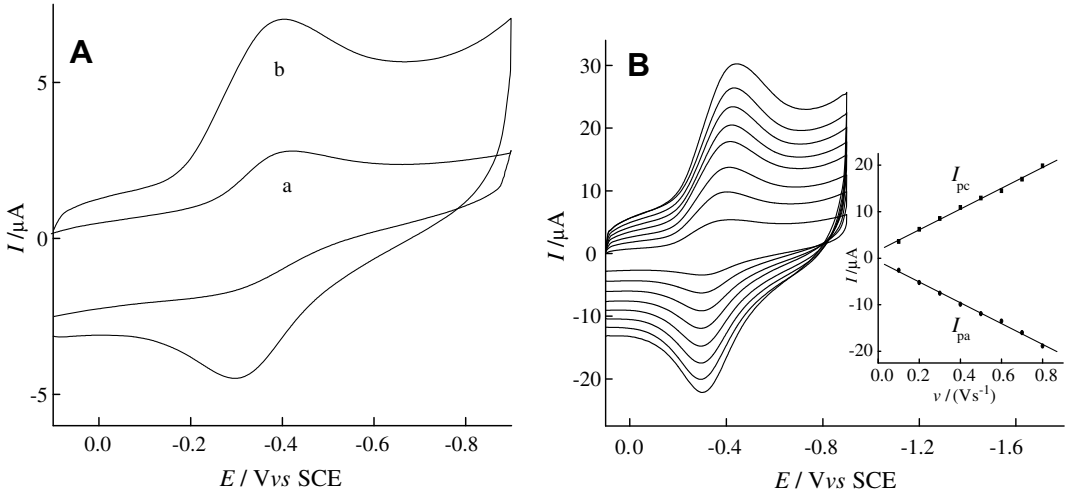

Fig. 3. (A) CVs of (a) Mb-HA/GCE and (b) Mb-[EMIM][BF_4]-HA/GCE in 0.1 mol L^{-1} PBS (pH 7.0). Scan rate: 0.10 V s^{-1}. (B) CVs of Mb-[EMIM][BF_4]-HA/GCE in 0.1 mol L^{-1} PBS (pH 7.0) at different scan rates (from inner to outer): 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 V s^{-1}. Inset: plot of I_p vs. *v*.

Table 1
Comparison of different protein-RTIL film modified electrodes for H_2O_2 determination

Electrode	Line range ($\mu mol\ L^{-1}$)	Detection limit ($\mu mol\ L^{-1}$)	References
HRP-chi-[BMIM][BF_4]/GCE	0.75–135	0.25	[18]
Cyt *c*/GNPs/RTIL/CNTs/GCE	50–1150	3.0	[33]
Chi-RTIL-HRP/Au	0.6–160	0.15	[34]
Cyt *c*/RTIL/BPGE	0–16	0.05	[35]
Mb-[EMIM][BF_4]-HA/GCE	2.0–270	0.6	This paper

HRP, horseradish peroxidase; Chi, chitosan; Cytochrome *c*, Cyt *c*; GNPs, gold nanoparticles; CNTs, carbon nanotubes; BPGE, basal plane graphite electrode.

and exhibited excellent electrocatalytic activity towards the reduction of H_2O_2. The mechanism of the electrocatalytic reduction of H_2O_2 could be expressed as follows [32]:

$$\text{ferric Mb} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (2)$$

$$\text{Compound I} + H^+ + e^- \rightarrow \text{Compound II} \quad (3)$$

$$\text{Compound II} + H^+ + e^- \rightarrow \text{ferric Mb} \quad (4)$$

A linear dependence between the catalytic peak current and the concentration of H_2O_2 was obtained in the range of 2.0–270 $\mu mol\ L^{-1}$ with a linear regression equation of I_{pc} (μA) = 0.087c ($\mu mol\ L^{-1}$) + 3.681 (r = 0.9982, n = 13) and a detection limit of 0.6 $\mu mol\ L^{-1}$ (S/N = 3) (Fig. 4 inset). The proposed Mb-[EMIM][BF_4]-HA/GCE for H_2O_2 determination was compared with other protein-RTIL film modified electrodes [18,33–35] and the results were listed in Table 1. It can be seen that this method can provide comparable linear range and detection limit.

When the concentration of H_2O_2 further increased, a platform emerged in the cathodic peak current, showing the characteristics of Michaelis–Menten kinetics. The apparent Michaelis–Mentan constant (K_m^{app}), which gives an indication of the enzyme substrate kinetics, can be obtained from the Lineweaver–Burk equation [36]:

$$1/I_{ss} = 1/I_{max} + K_m^{app}/I_{max}c \quad (5)$$

where I_{ss} is the steady-state current after the addition of substrate, I_{max} the maximum current measured under saturated substrate conditions and c is the bulk concentration of the substrate. K_m^{app} can be obtained by analysis of the slope and intercept of the plot of the reciprocals of the steady-state current versus H_2O_2 concentration. Based on the experimental data from Fig. 4 inset, the K_m^{app} of Mb-HA-[EMIM][BF_4]/GCE was calculated to be 0.29 $mmol\ L^{-1}$. The low value of K_m^{app} implied that the entrapped Mb possessed good affinity to H_2O_2.

4. Conclusion

A novel composite film based on biocompatible HA and conductivity [EMIM][BF_4] was prepared. Mb modified GCE was fabricated by entrapment Mb in the composite film. The results showed the [EMIM][BF_4]-HA composite film acted as an excellent platform for realizing direct electrochemistry and electrocatalysis of Mb. Based on the biocomposite film, a third-generation reagentless biosensor could be constructed for the determination of H_2O_2.

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References

[1] J.E. Frew, H.A.O. Hill, Eur. J. Biochem. 172 (1988) 261.
[2] F.A. Armstrong, H.A.O. Hill, N.J. Walton, Acc. Chem. Res. 3 (1994) 313.
[3] Y.T. Kong, M.N. Boopathi, Y.B. Shim, Biosens. Bioelectron. 19 (2003) 227.
[4] J.F. Rusling, Acc. Chem. Res. 31 (1998) 363.
[5] W. Zhang, G. Li, Anal. Sci. 20 (2004) 603.
[6] X. Lu, Z. Wen, J. Li, Biomaterials 27 (2006) 5740.
[7] Y. Liu, M. Wang, Z. Li, Y.H. Liu, P. He, J.H. Li, Langmuir 21 (2005) 618.
[8] H. Luo, S. Dai, P. Bonnesen, A. Buchanan, J. Holbrey, N. Bridges, R. Rogers, Anal. Chem. 76 (2004) 3078.
[9] S.F. Wang, T. Chen, Z.L. Zhang, D.W. Pang, Electrochem. Commun. 9 (2007) 1337.
[10] S.F. Wang, T. Chen, Z.L. Zhang, X.C. Shen, Z.X. Lu, D.W. Pang, K.Y. Wong, Langmuir 21 (2005) 9260.
[11] H.Y. Xiong, T. Chen, X.H. Zhang, S.F. Wang, Electrochem. Commun. 9 (2007) 1648.
[12] H.Y. Xiong, T. Chen, X.H. Zhang, S.F. Wang, Electrochem. Commun. 9 (2007) 2671.
[13] M.F. Machado, J.M. Saraiva, Biotechnol. Lett. 27 (2005) 1233.
[14] W.Y. Lou, M.H. Zong, H. Wu, Biocatal. Biotransform. 22 (2004) 71.
[15] M.B. Turner, S.K. Spear, J.D. Holbrey, R.D. Rogers, Biomacromolecules 5 (2004) 1379.
[16] Y. Liu, M. Wang, J. Li, P. He, H.T. Liu, J.H. Li, Chem. Commun. (2005) 1778.
[17] H.J. Chen, Y.L. Wang, Y. Liu, Y.Z. Wang, L. Qi, S.J. Dong, Electrochem. Commun. 9 (2007) 469.
[18] X.B. Lu, Q. Zhang, L. Zhang, J.H. Li, Electrochem. Commun. 8 (2006) 874.
[19] L. Lapcik Jr., L. Lapcik, S. De Smedt, J. Demeester, P. Chabrecek, Chem. Rev. 98 (1998) 2663.
[20] L. Ma, Y.N. Tian, Z.J. Rong, J. Biochem. Biophys. Methods 70 (2007) 657.
[21] H.Y. Liu, N.F. Hu, J. Phys. Chem. B 110 (2006) 14494.
[22] T. Nishida, Y. Tashiro, M. Yamamoto, J. Fluorine Chem. 120 (2003) 35.
[23] P. Yu, Y. Lin, L. Xiang, L. Su, J. Zhang, L. Mao, Langmuir 21 (2005) 9000.
[24] T.T. Herskovits, H. Jaillet, Science 163 (1969) 282.
[25] S.Q. Liu, H.X. Ju, Electroanalysis 15 (2003) 1488.
[26] Y.H. Wu, Q.C. Shen, S.S. Hu, Anal. Chim. Acta 558 (2006) 179.
[27] H.H. Liu, Z.Q. Tian, Z.X. Lua, Z.L. Zhang, M. Zhang, D.W. Pang, Biosens. Bioelectron. 20 (2004) 294.
[28] E. Laviron, J. Electroanal. Chem. 100 (1979) 263.
[29] Y.H. Wu, Q.C. Shen, S.S. Hua, Anal. Chim. Acta 558 (2006) 179.
[30] X.J. Zhao, Z.B. Mai, X.H. Kang, Z. Dai, X.Y. Zou, Electrochim. Acta 53 (2008) 4732.
[31] H.H. Liu, Z.Q. Tian, Z.X. Lua, Z.L. Zhang, M. Zhang, D.W. Pang, Biosens. Bioelectron. 20 (2004) 294.
[32] T. Tatsuma, H. MoriA. Fulishima, Anal. Chem. 72 (2000) 2919.
[33] C.L. Xiang, Y.J. Zou, L.X. Sun, F. Xu, Electrochem. Commun. 10 (2008) 38.
[34] F.N. Xi, L.J. Liu, Q. Wu, X.F. Lin, Biosens. Bioelectron. (2008), doi:10.1016/j.bios.
[35] S.F. Ding, W. Wei, G.C. Zhao, Electrochem. Commun. 9 (2007) 2202.
[36] R.A. Kamin, G.S. Wilson, Anal. Chem. 52 (1980) 1198.