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Amperometric determination of lactate dehydrogenase based on a carbon fiber microcylinder electrode modified covalently with Toluidine Blue O by acylation

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Abstract

A method has been developed for the modification of a carbon fiber microcylinder electrode with acylation. The stability and surface coverage of the Toluidine Blue O-modified microelectrode were studied by cyclic voltammetry. The modified electrode showed significant activity for the electrocatalytic oxidation of NADH in pH 6.8–7.8 solution. The catalytic current increased linearly with increasing concentration of NADH from 4.0×10^{-5} to 1.5×10^{-3} M. A simple amperometric determination based on electrochemical detection of NADH produced from the enzymatic reaction of lactate with NAD^+ under the catalysic effect of lactate dehydrogenase (LDH) is reported. The experimental factors which had primary influence on the analytical performance were studied. The sensor had a linear response over a range of LDH concentrations from 5.0 U l^{-1} to 200 U l^{-1} at −0.2 V vs. SCE under optimum conditions. A satisfactory result was obtained for the determination of LDH in clinical blood samples.

Keywords: Acylation; Amperometric determination; Carbon fiber microcylinder electrode; Lactate dehydrogenase; Toluidine Blue O

1. Introduction

Many current works in the analytical field are devoted to chemically-modified electrodes and biosensors [1,2]. Some mediators have been fixed on the electrode surface by adsorption, electrochemical polymerization or polymer coating to efficiently facilitate electron transfer and to determine some biomolecules [1–4]. The irreversible adsorption of commercial dyes on a graphite electrode is a simple method for preparing modified electrodes [2,5]. In order to increase the stability of the modifying layer, Persson and co-workers [2,6,7] synthesized derivatives of Toluidine Blue O by introducing several aromatic rings to enhance π-electron overlapping with the carbonaceous surface. However, at a microelectrode modified by irreversible adsorption, no matter what the mediator is, even if it has a few aromatic rings, the stability of the modified microelectrode is very poor because of the high mass transport rate of

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the microelectrode [8,9], which makes the mediator, and its product, diffuse rapidly away from the surface during the process of electrochemical reaction. Thus, the catalytic efficiency declines greatly.

The application of carbon fiber microelectrodes (CFMEs) for electrochemical determinations has attracted great interest [8,9–13]. The preparation of modified CFMEs is usually done by using noble metal deposition [12], polymer coating [13,14] or covalent modification [15]. On the surface of a carbon electrode, the process of covalent modification is usually done by acylation [16–18]. Hajizadeh et al. [18] have used a crosslinking agent—triisocyanate—to fix thionin on a graphite electrode by acylation. The method has also been used to immobilize enzyme at a CFME by using carbodiimide [19,20]. However, to our knowledge, there has been no report until now of covalent modification by direct amidation with thionyl chloride at a CFME. In this work, Toluidine Blue O (TBO) was covalently bonded to the surface of a CFME for the first time. The stability of TBO-modified CFME is very good. The modified electrode can effectively catalyze the oxidation of NADH at a carbon fiber microelectrode.

The coenzyme NAD^+ and its reduced form NADH are used by over 250 dehydrogenases, and play a major role in many biological redox reactions. The direct electrochemical determination of NADH, particularly in a small system, is very important in clinical medicine. The electrochemical oxidation of NADH, whose normal formal potential is −0.56 V vs. SCE at pH 7.0 and 25°C, has been extensively studied at various modified electrodes [2,5,6,18,21–28]. Willner and Riklin [27] developed the amperometric sensors for NADH and malic acid by covalently linking pyrroloquinolinequione (PQQ) or PQQ and malic enzyme with 1-ethyl-3[3-(dimethylamino)propyl]-carbodiimide (EDC) and N-hydroxysulfosuceinimide sodium salt at a self-assembled cyteamine monolayer-modified Au electrode. Katz et al. [28] used cystamine and cysteamine to functionalize both Au and Pt electrodes and to covalently link PQQ with EDC; the modified electrodes can also catalyze the oxidation of NADH. However, little study of the direct electrochemical determination of NADH at a CFME has been reported. The electrocatalytic oxidation of NADH at a TBO- (and its derivatives) modified graphite electrode has been presented by Persson and co-workers previously [2,6]. However, if the TBO-modified carbon fiber microelectrode was prepared with their method, its stability was very poor [29]. Moreover, the determination of lactate dehydrogenase (LDH) with a TBO-modified CFME has never been studied. Here we report on the use of a TBO-modified microelectrode in biosensors for the monitoring of LDH based on the determination of NADH. The results will be very significant for the monitoring of enzyme in a small clinical system and the development of microbiosensors.

2. Experimental

2.1. Materials and reagents

TBO (B.S. grade) was obtained from the Chroma Chemical Reagent Company (UK), the reduced form of nicotinamide–adenine dinucleotide (NADH, >95%) and LDH (EC 1.1.1.27, Type XI, from rabbit muscle, 700 U mg^{-1}) were from Sigma (USA). These reagents were directly used as received without further purification. The reagents used for making up 0.2 M pH 2–12 phosphate buffer solutions, thionyl chloride ($SOCl_2$), and other reagents, obtained from chemical companies in The People's Republic of China, were of analytical-reagent grade. Pyridine was redistilled with P_2O_5 (8 g per 100 ml) and was stored over a 4 Å molecular sieve activated at 550°C. Water used in the experiment was doubly-quartz-distilled. Carbon fibers (PAN-type) with 6–7 μm diameter were obtained from the Shanghai Synthetic Fiber Research Institute. Epon 812 epoxy resin (New York, USA) was used to seal the electrodes.

2.2. Preparation of TBO-modified CFME

The method of fabrication of the CFME was similar to that given in a previous paper [9]. Briefly, a single carbon fiber was sealed in a glass capillary tube with epoxy resin. First, a CFME of

length 6–10 mm was washed thoroughly with acetone and distilled water in an ultrasonic bath, and pretreated electrochemically in 1.0 M H_2SO_4 solution with a triangular-wave potential sweep from −1.0 V to +2.0 V at a scan rate of 200 mV s^{-1} for 50 min. Next, the treated electrode was washed with doubly-distilled water and dried in air, and immersed in $SOCl_2$ for 30 min. Then the electrode was rinsed with tetrahydrofuran (THF) to remove $SOCl_2$ remaining on its surface, and dipped in anhydrous pyridine solution containing 1.0×10^{-3} M TBO for 15 min. During this process pyridine, as proton acceptor, deprotonated the oxidized TBO and gradually converted it into the imino form which reacted with the –COCl group on the electrode surface. Finally, the modified electrode was rinsed with phosphate buffer (pH 7.0) and stored in the same buffer solution.

2.3. Procedures

Electrochemical measurements were carried out with a Model BAS-100B Electrochemical Analyzer with a PA-1 Preamplifier (Bioanalytical Systems (BAS), West Lafayette, IN) to amplify current and filter out noise and an FPG-310 Color Plotter (Fujitsu Company, Japan) to record the voltammograms. A type 501 thermostat (Shanghai, People's Republic of China) was used to control the experimental temperature at 20 ± 0.1°C.

A three-electrode configuration with a saturated calomel electrode (SCE) as reference, Pt wire as counter and the above-modified electrode as working electrode was employed. After deaerating with pure N_2 for 10 min, the electrochemical measurements were carried out under a nitrogen atmosphere.

3. Results and discussion

3.1. Cyclic voltammogram of TBO-modified CFME

The cyclic voltammograms of the TBO-covalently-modified electrode in pH 7.0 buffer solution at a scan rate of 100 mV s^{-1} showed a couple of cathodic and anodic peaks (Fig. 1). Their peak potentials were at −276 mV and −228 mV ($E^{\circ\prime}$ = −252 mV, ΔE_p = 48 mV) respectively. The peak potentials shifted slightly in a positive direction in comparison with those of soluble TBO at a bare carbon fiber microelectrode [29] and the change in the normal formal potential was about 29 mV. The small change was similar to that resulting from acylation found in the literature [16,30], indicating that TBO on the electrode surface had been acylated.

At the beginning of the cyclic voltammetric sweep of the TBO-covalently-modified electrode, the cathodic and anodic peak currents decreased rapidly, then dropped gradually to a constant value and remained at this value for a very long time (see Fig. 1). Furthermore, no change in peak current appeared after the modified microelectrode had been dipped in pH 7.0 buffer solution for several weeks, indicating that the stability of the TBO-covalently-modified CFME was very good. However, the half life of TBO adsorbed on the CFME is only 55 min [29]. The better stability indicated that TBO was firmly fixed on the surface of the carbon fiber by a covalent bond. The decrease in peak current at the beginning of the sweep was due to the desorption of some adsorbed TBO. Because the differences in peak potentials of acylated TBO and adsorbed TBO,

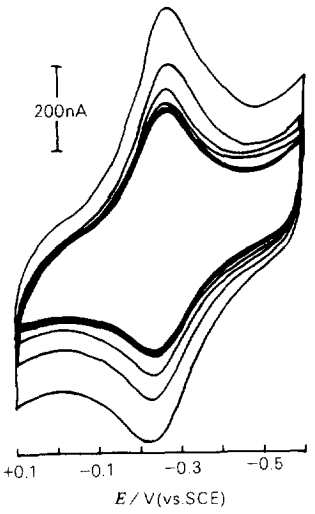

Fig. 1. Cyclic voltammograms of TBO-modified microelectrode in pH 7.0 phosphate buffer solution at 100 mV s^{-1}.

about 25 mV for the cathodic and 32 mV for the anodic peak, were small, only a single pair of peaks occurred, as shown in Fig. 1.

The reactions in the process of TBO covalent modification can be described as follows. First of all, a lot of carboxyl groups were formed on the surface of the carbon fiber during electrochemical pretreatment [15,20], they were then acylated by the reaction between –COOH and $SOCl_2$ to form –COCl groups under the usual conditions. Finally, these –COCl groups were amidated with TBO in the presence of excess organic base pyridine, as a proton acceptor, which removed the positive charge of TBO and converted it to the imino form, to form the amido link and to covalently bond TBO to the surface of the carbon fiber. Thus, this method of preparing a modified carbon fiber electrode can greatly enhance the stability.

3.2. Surface concentration of TBO at modified microelectrode

The surface concentration Γ of TBO can be calculated according to $\Gamma = Q/nFA = S/nFAv$, where Q is the charge consumed during complete reduction of TBO, S is the peak area of the cyclic voltammogram, and the other symbols have their usual meanings. At a microcylinder electrode with a geometric area A of 2.0×10^{-3} cm^{-2}, the surface concentration Γ_{exp} was 3.4×10^{-10} mol cm^{-2} ($n = 2$), which was determined from the steady cyclic voltammogram at 100 mV s^{-1} in pH 7.0 buffer solution.

Given that the horizontal section area of methylene blue, which is of similar structure to TBO, is 0.75 nm^2 [31], the theoretical mono-layer adsorbance Γ_{theory} of MB at an electrode is 2.2×10^{-10} mol cm^{-2}. Comparing $\Gamma_{exp,TBO}$ with $\Gamma_{theory,MB}$, TBO fixed on a carbon fiber surface was a monolayer due to the roughness of the pretreated carbon fiber surface, and almost the whole electrode surface was activated by electrochemical oxidation and acylation.

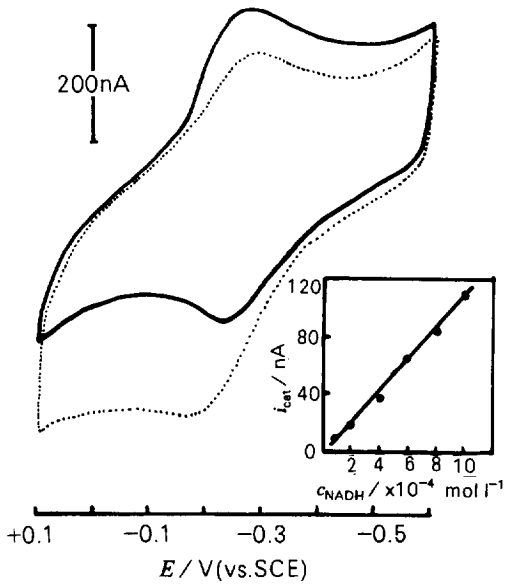

Fig. 2. Cyclic voltammograms of TBO-modified electrode in pH 7.0 buffer solution (——) and pH 7.0 buffer including 1.0×10^{-3} M NADH (· · ·); inset: relation between catalytic peak current and concentration of NADH at $v = 100$ mV s^{-1}.

3.3. Electrocatalytic oxidation of NADH at TBO-modified carbon fiber electrode

This work showed that the TBO-modified electrode was able to catalyze the oxidation of NADH in pH 7.0 phosphate buffer solution via an electron transfer reaction between acylated TBO and NADH at the heterogeneous boundary layer. The experimental results are shown in Fig. 2. When the buffer solution included 1.0×10^{-3} M NADH, the anodic peak current of the cyclic voltammogram increased, the cathodic peak current decreased, and the anodic peak potential shifted in a positive direction by about 60 mV, which is very characteristic of an electrocatalytic oxidation process. The shift resulted from the catalytic reaction between acylated TBO and NADH, which possibly changed the ratio of surface concentrations of oxidized and reduced forms of TBO and the electron transfer rate between the electrode and the TBO at the same potential. The catalytic mechanism can be expressed as follows:

$$TBO_{red} - 2e \rightleftarrows TBO_{ox}$$

$$NADH + TBO_{ox} \rightarrow TBO_{red} + NAD^+$$

The catalytic current i_{cat} ($=i_2 - i_1$, where i_1 and i_2 are the anodic peak currents of the modified electrode in buffer without and with NADH respectively) of NADH increased linearly with increasing concentration of NADH from 4.0×10^{-5} to 1.5×10^{-3} M (inset to Fig. 2) with a correlation coefficient of 0.992 and a relative standard deviation of 1.5% for six determinations with 1.0×10^{-3} M NADH.

3.4. Effects of experimental conditions on catalytic peak current

Fig. 3 shows the dependence of catalytic peak current on scan rate. At a high scan rate the catalytic peak current was proportional to $v^{1/2}$; however, at a lower scan rate the plots deviated from linearity. The catalytic current curve (broken line in Fig. 2) tended to a sigmoidal shape with the decrease of the scan rate. These are the characteristics of diffusion mass transport at a microelectrode. Considering that the peak potential was independent of v, the electrode process was controlled by the diffusion of NADH in solution [13,32]. Thus, the interfacial chemical reaction rate between bonded TBO and NADH was large, and the electrode process is similar to

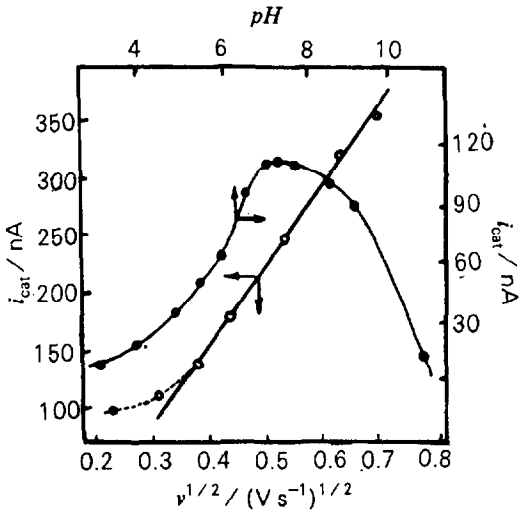

Fig. 3. Plots of catalytic peak current vs. $v^{1/2}$ at pH 7.0 (○) and vs. pH of solution at 100 mV s^{-1} (●).

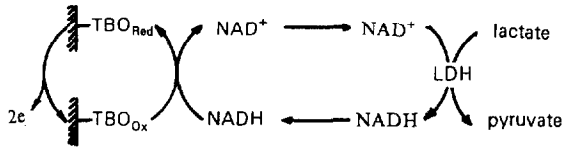

Fig. 4. Scheme of electrochemical response for LDH at a TBO-modified electrode.

the direct electrochemical oxidation of NADH at the electrode.

The catalytic peak current also depended greatly on the pH of the solution (Fig. 3). When the pH was low, the catalytic current was very small, and it increased with increase of pH. However, when the pH was >7.8, the current decreased with increase in pH. This phenomenon is similar to those described in Refs. [2] and [18], which resulted from both decomposition of NADH in acid solution and the rate of reaction between the TBO and NADH. With increasing pH the reaction rate and catalytic current decreased [2]. However, when the pH was <7, because the experiment was carried out after NADH was added to background for about 1 min in order to stir and deaerate, part of the NADH would decompose, which resulted in the decrease of the catalytic peak current. NADH is stable in neutral and basic pH solutions and the concentration of NADH did not change with time. Therefore, the catalytic peak current was a maximum between pH 6.8 and 7.8. A pH of 7.0 was used for the subsequent work [18].

3.5. Response of the modified microelectrode to LDH

When the pH 7.0 phosphate buffer solution included 1.0×10^{-3} M NAD^+, or 1.0×10^{-3} M NAD^+ and L-lactate, both the anodic and cathodic peak currents of the cyclic voltammogram of the TBO-modified microelectrode did not change. This result indicated that NAD^+ and L-lactate did not interfere with the electrode process of the TBO electrode. However, when the solution of 1×10^{-3} M NAD^+ and lactate included 100 U l^{-1} LDH, the cyclic voltammogram of the TBO-modified electrode showed the same change as in Fig. 2, indicating that LDH cata-

lyzed the oxidation of lactate with a simultaneous reduction of NAD^+ to produce NADH. The formed NADH diffused to the electrode surface and catalyzed the electrode reaction of TBO. With increasing LDH concentration, the catalytic peak current increased, which is the basis for the determination of LDH. The overall reaction process can be seen in Fig. 4.

3.6. Effect of experimental conditions on the amperometric response to LDH

In amperometric measurements, the potential dependence of LDH response (hydrodynamic voltammogram) in the range $-0.6-+0.1$ V indicated that the amperometric response of the modified electrode to LDH started at -0.35 V. With the positive shift of applied potential, the response rose sharply up to -0.2 V, and then exhibited almost a constant value. An applied potential of -0.2 V was chosen for subsequent work.

The effect of pH on the LDH response was very obvious. In the pH range 4–6.8, the amperometric response to LDH increased with increasing pH. The response remained constant at pH 6.8–8.5, and then decreased when pH was >8.5. A wider pH range of stable response to LDH than to NADH occurs because the reaction of lactate and NAD^+ under the catalysis of LDH is a process of releasing H^+, and therefore a high pH is advantageous to the positive reaction. However, too high a pH would result in both the decomposition of NAD^+ and the decrease of the reaction rate between NADH and mediator. Therefore, the amperometric determination was performed at pH 8.0.

NAD^+ concentration also affected the response of the modified electrode to LDH. At various LDH concentrations, all responses increased with increasing NAD^+ concentration, and remained constant when the NAD^+ concentration was larger than 1.0×10^{-3} M, which was selected as an optimum condition. In order to quicken the catalytic reaction of LDH, an excess lactate concentration of 5.0×10^{-3} M was selected.

3.7. Application of TBO-modified electrode in the measurement of LDH

Fig. 5 shows a typical trace of the steady state current–time response of the TBO-modified electrode at -0.2 V with successive injections of LDH (in 50 U l^{-1} steps). With the injection of LDH the response increased, and the time to reach constant response was very short (<10 s). The calibration plots of amperometric response vs. the concentration of LDH (inset to Fig. 5) showed a linear relationship. The linear response range was from 5.0–200 U l^{-1} with a correlation coefficient of 0.990. The relative standard deviation of results was 3.2% for five successive determinations at 100 U l^{-1}.

After a clinical human blood serum sample of 200 μl was injected into 1.8 ml pH 8.0 buffer solution including 1.1×10^{-3} M NAD^+ and 5.5×10^{-5} M lactate for 30 s, the amperometric response of the TBO-modified microelectrode was used to assay the LDH content without any interference. The average value of three determinations with an interval of 8 h was 23 U l^{-1} (SD = 0.5) in the diluted solution. Thus, the content of LDH in the human blood serum sample was 230 U l^{-1}. The result was close to the value of 225 U l^{-1} obtained by spectrophotometry (LDH–L method) at 340 nm wavelength. The advantages of the biosensor are that it has a

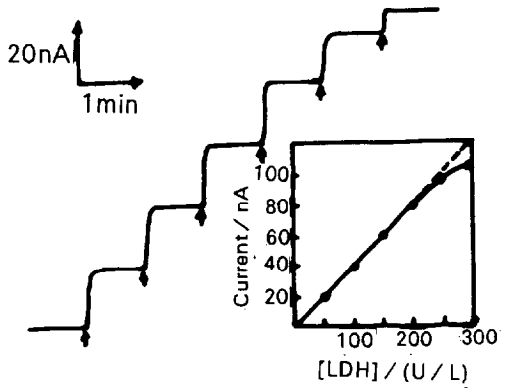

Fig. 5. Amperometric response to successive addition of 50 U l^{-1} LDH in pH 8.0 buffer including 1.0×10^{-3} M NADH and 5.0×10^{-3} M lactate with an interval of 1 min at -0.2 V. Inset: calibration curve of LDH at TBO-modified carbon fiber electrode.

shorter response time to LDH, it can detect LDH by a direct electrochemical method in a smaller system and it could potentially be used for in vivo and clinical analysis.

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References

[1] P.N. Bartlett and J.M. Cooper, J. Electroanal. Chem., 362 (1993) 1.
[2] B. Persson and L. Gorton, J. Electroanal. Chem., 292 (1990) 115.
[3] R. Santucci, M. Brunori, L. Campanella and G. Tranchida, Bioelectrochim. Bioenerg., 29 (1992) 177.
[4] M. Marchesiello and E.M. Genies, Electrochim. Acta, 37 (1992) 1987.
[5] L. Gorton, A. Torstensson, H. Jaegfeldt and G. Johansson, J. Electroanal. Chem., 161 (1984) 103.
[6] B. Persson, J. Electroanal. Chem., 287 (1990) 61.
[7] L.I. Boguslavsky, L. Geng, I. Kovalev, S.K. Sahni, Z. Xu, T.A. Skotheim, V. Laurinavichius, B. Persson and L. Gorton, Biosens. Bioelectron., 10 (1995) 693.
[8] R.M. Wightman and D.O. Wipf, in A.J. Bard (Ed.), Electroanalytical Chemistry, Vol. 15, M. Dekker, New York, 1989, pp. 267–353.
[9] H.X. Ju, H.Y. Chen and H. Gao, J. Electroanal. Chem., 361 (1993) 251.
[10] J. Wang and Q. Chen, Anal. Chem., 66 (1994) 1007.
[11] E.N. Navera, M. Suzuki, E. Tamiya, T. Takeuchi and I. Karube, Electroanalysis, 5 (1993) 17.
[12] J. Wang and L. Angnes, Anal. Chem., 64 (1992) 456.
[13] H.X. Ju, Y.G. Xun and H.Y. Chen, J. Electroanal. Chem., 380 (1995) 283.
[14] L.I. Netchiporouk, A.A. Shul'ga, N. Jaffrezic-Renault, C. Martelet, R. Olier and R. Cespuglio, Anal. Chim. Acta, 303 (1995) 275.
[15] P. Pantano and W.G. Kuhr, Anal. Chem., 63 (1991) 1413.
[16] J.C. Lennox and R.W. Murray, J. Electroanal. Chem., 78 (1977) 395.
[17] W.J. Albery and A.R. Hillman, Ann. Rep. Prog. Chem., Sect. C, 78 (1981) 277.
[18] K. Hajizadeh, H.T. Tang, H.B. Halsal and W.R. Heineman, Anal. Lett., 24 (1991) 1453.
[19] P. Pantano, T.H. Morton and W.G. Kuhr, J. Am. Chem. Soc., 113 (1991) 1832.
[20] E. Csöregi, L. Gorton and G. Marko-Varga, Anal. Chim. Acta, 273 (1993) 59.
[21] Z. Samec and P.J. Elving, J. Electroanal. Chem., 144 (1983) 217.
[22] C.J. McNeil, J.A. Spoors, D. Cocco, J.M. Cooper and J.V. Bannister, Anal. Chem., 61 (1989) 25.
[23] P.C. Pandey, Anal. Biochem., 221 (1994) 392.
[24] C. Cai, H. Ju and H. Chen, Anal. Chim. Acta, 310 (1995) 145.
[25] M. Vreeke, R. Maidan and A. Heller, Anal. Chem., 64 (1992) 3084.
[26] A.S.N. Murthy and Anita, Bioelectrochem. Bioenerg., 33 (1994) 71.
[27] I. Willner and A. Riklin, Anal. Chem., 66 (1994) 1535.
[28] E. Katz, T. Lötzbeyer, D.D. Schlereth, W. Schuhmann and H.-L. Schmidt, J. Electroanal. Chem., 373 (1994) 189.
[29] H.X. Ju and H.Y. Chen, Acta Chim. Sin., 52 (1994) 1118 (in Chinese).
[30] J.F. Evans, T. Kuwana, M.T. Henne and G.P. Royer, J. Electroanal. Chem., 80 (1977) 409.
[31] V. Svetličić, J. Clavilier, V. Žutić and J. Chevalet, J. Electroanal. Chem., 312 (1991) 205.
[32] K. Aoki, K. Tokuda and H. Matsuda, J. Electroanal. Chem., 199 (1986) 69.