Electrochemical Polymerization of Azure Blue II and Its Electrocatalytic Activity toward NADH Oxidation

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Poly(azure blue II) (PABII) thin film modified electrode was successfully assembled on the surface of a glassy carbon electrode by means of electrochemical polymerization, which was carried out with cyclic voltammetric sweeping in the potential range of − 0.6 to + 1.3 V (vs. SCE) in Britton-Robinson buffer solution (pH = 9.8) containing 1.25×10^{-4} mol/L azure blue II. The effect of pH on the polymerization process of azure blue II and the electrochemical characteristics of the polymer-modified electrodes were studied in detail. The experimental results indicated that the electropolymerization of azure blue II could take place in basic or neutral media. The cyclic voltammograms of poly(azure blue II) thin film modified electrode showed the presence of two couples of redox peaks. The film modified electrode exhibited potent and persistent electrocatalysis for oxidation of dihydronicotiamide adenine dinucleotide (NADH) in phosphate buffer media with a diminution of the overpotential of about 410 mV and an increase in peak current. The presence of some divalent cations in an electrolyte can greatly enhance the electrocatalytic current for oxidation of NADH. The electrocatalytic current increased linearly with NADH concentration from 1.0×10^{-5} to 8.0×10^{-3} mol/L in the presence of 4.0×10^{-2} mol/L Mg^{2+} cation. The detection limit ($3s_{b1}/S$) was 5.0×10^{-6} mol/L, and the relative standard deviation of determination results was 4.2% for six successive determinations of 5.0×10^{-4} mol/L NADH in the presence of Mg^{2+} cation.

Keywords electrocatalytic oxidation, NADH, poly(azure blue II), electropolymerization

Introduction

Over 300 dehydrogenases are known to be dependent on the nicotiamide adenine dinucleotide coenzyme in its reduced (NADH) and oxidized (NAD^+) forms, and a monitoring of enzymatically generated NADH is the basis for the development of biosensors for the selected dehydrogenase. Hence, the detection of this coenzyme is important in both practical analysis and biochemical synthesis. The reported overpotential for oxidation of NADH at pH 7.0 is about 1.1 V at carbon and 1.3 V at platinum electrodes.[1,2] It is generally accepted that the direct oxidation of NADH at bare electrodes is totally irreversible and therefore requires a high overpotential. Moreover, the direct redox reactions at bare electrodes often suffer from pronounced pollution, which results in rather poor reproducibility. Therefore, many efforts have been made to reduce the overpotential and improve the kinetics of NADH oxidation.[3,4] One of the efficient ways to circumvent the high overpotential and to overcome the slow kinetics for the oxidation of NADH is to use an electrode modified with some mediators, which can undergo fairly fast redox reactions with NADH.[5,6] Generally, quinines,[7] redox dyes,[8,9] inorganic metal complexes,[10] catechols[11] and organic salts[12] are used as mediators. The redox dyes used for modified electrodes usually include phenazines,[13] phenothiazines[14-17] and phenoxazines.[18,19] The modification of electrode by dye is always achieved with one of the following methods:[20,21] (a) direct electropolymerization; (b) covalent attachment or adsorption; (c) incorporation in polymeric films containing the mediating functional group or macromolecular deposition; (d) other methods. Compared with methods (b), (c) and (d), electrochemical polymerization methods provide certain advantages, which are easy not only to prepare the stable chemically modified electrodes but also to yield modified electrodes with a three-dimensional distribution of mediators. Therefore, the catalytic activity and sensitivity can be improved.

Although there are many studies on the electrochemical oxidation of NADH using various modified electrodes, to our knowledge no report about the electrochemical oxidation of NADH with azure blue II electropolymerization modified glass carbon electrode has appeared. Azure blue II is one of the phenothiazine dyes (Scheme 1), which was used as a color-developing agent to determine some anions with spectrophotometric technology. The objective of this work is to optimize the electropolymerization conditions, to investigate the behaviors of the PABII modified electrode and to test the electrocatalytic activity of PABII toward NADH oxidation. The effects of solution pH and divalent cations on this electrocatalytic process have been studied.

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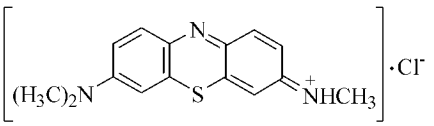
Scheme 1 Structure of azure blue II



Experimental

Chemicals

Azure blue II was obtained from Shanghai Chemical Reagent Company and NADH was purchased from Sigma Co. without further purification. Britton-Robinson buffer solutions with different pH were prepared by mixing the mixed acid (composed of 0.04 mol/L H_3PO_4, HAc and H_3BO_3) with 0.2 mol/L NaOH in proportion. The buffer solutions were used to control the acidity of the solutions. All other reagents were of analytical-reagent grade. Doubly distilled water was used throughout.

Apparatus

Electrochemical experiments were performed with a LK98 microcomputer-based electrochemical analyzer using a three-electrode electrochemical cell. The glassy carbon electrode with a diameter of 3 mm was used as the working electrode, which is modified as mentioned below. The reference electrode and counter electrode were a saturated calomel electrode (SCE) and a platinum wire electrode, respectively. All potentials in the text refer to SCE. High purity nitrogen was used for solution deaeration before all experiments.

Electrode preparation

Prior to each experiment, the glassy carbon electrode was first polished with 0.05 μm alumina slurry on a polishing cloth and cleaned by sonicating in doubly distilled water for 15 min. Subsequently, the working electrode was treated electrochemically by a continuous cyclic potential sweep from -0.5 to 1.5 V at 50 mV/s in phosphate buffer (pH = 7.0) solution until a constant background current was obtained.

PABII modified electrodes were prepared by cyclic voltammetry at a glassy carbon electrode in the potential range of -0.6 to 1.3 V at a sweep rate of 100 mV/s in different buffer solution containing 1.25×10^{-4} mol/L azure blue II. The film thickness was dependent on the number of sweeps. The modified electrodes were rinsed with water and stored in phosphate buffer (pH = 7.0) for further use. The experimental temperature kept constant (25 ℃).

Results and discussion

Electrochemical polymerization of azure blue II and the voltammetric characteristics of the polymer-modified electrode

In Britton-Robinson buffer solution (pH = 9.8) containing 1.25×10^{-4} mol/L azure blue II, the cyclic voltammogram (Fig. 1) between -0.5 and $+0.6$ V shows a couple of redox peaks at -0.08 and -0.12 V at a glassy carbon electrode for the first cycle. These peaks were formed by single electron electrode reaction of azure blue II. With an increase in the number of cycles, the peak current increased and after a few cycles it remained constant. Furthermore, with the increase of cycles, both the anodic peak potential (E_{pa}) and the cathodic peak potential (E_{pc}) shifted in a positive direction, and kept constant after a few cycles. These phenomena can be explained by that adsorption process of azure blue II occurred at the glassy carbon electrode. With an increase in the number of cycles, more and more azure blue II were adsorbed on the surface of the electrode, leading to an increase of the peak current. After a few cycles the adsorption gradually reached equilibrium, the peak current no long change. The same phenomena were found for some other similar dyes.[22] Cyclic voltammogram of the glassy carbon electrode that adsorbed azure blue II in 0.1 mol/L phosphate buffer solution (pH = 7.0) is shown in Fig. 2. In Fig. 2, only a couple of redox peak appears at $+0.16$ and $+0.13$ V, which is near to the final peak potential shown in Fig. 1. Thus, it was suggested that azure blue II could be adsorbed on the electrode surface strongly and in this potential range the electropolymerization film could not be formed.

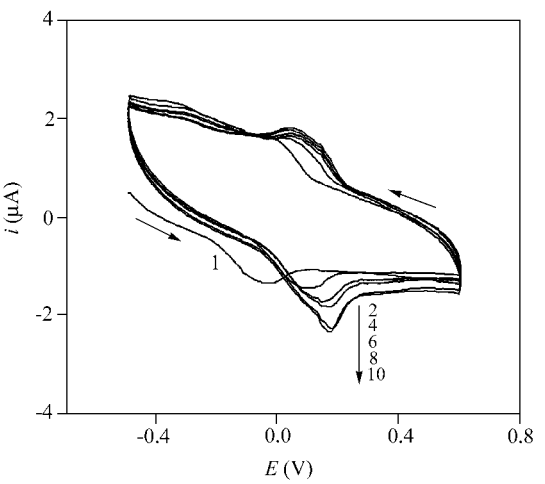


Fig. 1 Cyclic voltammograms of 1.25×10^{-4} mol/L azure blue II in pH 9.8 Britton-Robinson buffer solution between -0.5 and $+0.6$ V. Scan rate, 80 mV/s. 1, 2, 4, 6, 8 and 10 represent the number of scan cycle respectively.

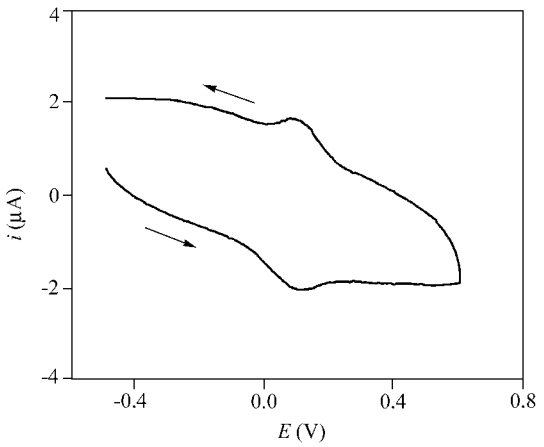
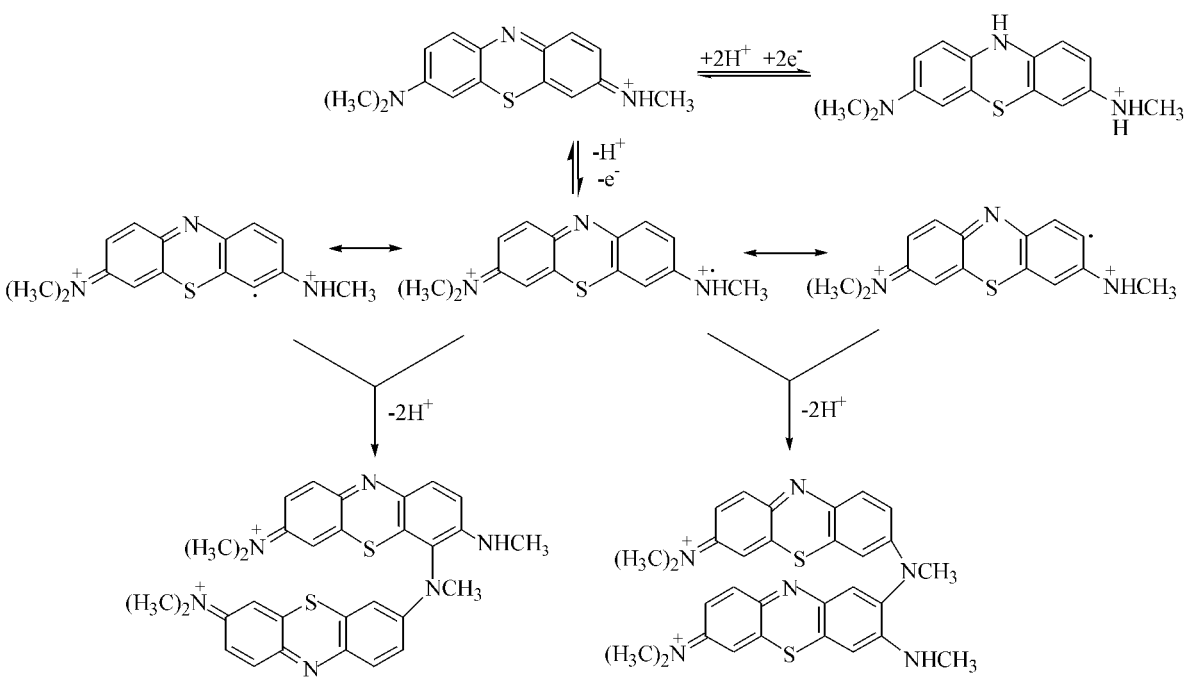


Fig. 2 Cyclic voltammetric response of the glassy carbon electrode adsorbing azure blue II in 0.1 mol/L phosphate buffer solution (pH 7.0) between −0.5 and +0.6 V; scan rate, 80 mV/s.

Experimental results indicated that electropolymerization of azure blue II was observed when the anodic sweep limit was extended to +1.3 V. Fig. 3 shows a continuous cyclic voltammogram of a glassy carbon electrode in Britton-Robinson buffer solution (pH = 9.8) containing 1.25×10^{-4} mol/L azure blue II. A couple of redox peak was observed with a formal potential of −0.16 V for the first0 cycle, which was near to the peak potential of the first cycle shown in Fig. 1. A shoulder peak appeared at about +1.0 V in the anodic process and a corresponding cathodic peak was not observed, indicating that the radical cation of azure blue II might be formed and has undergone following chemical reactions (Scheme 2). Upon continuous scanning, both the anodic peak potential and the cathodic peak potential gradually shifted in the positive direction and indicated a wide-range redox response. These peaks can not be related to a monomer redox couple (Fig. 4). Such a phenomenon could also be observed during the electropolymerization process of methylene green.[15] The wide-range redox response might indicate the redox nature of the polymer. With an increase in the number of scanning, the wide-range redox peak currents increased, but the shoulder peak current gradually decreased. When the electrode was subsequently removed from the electrochemical cell, rinsed with water, and immersed in phosphate buffer solution (pH = 7.0) containing no azure blue II, two couples of redox peaks are observed (Fig. 4). The results indicated that these peaks were not the redox peaks of the monomer but the electropolymerization of azure blue II. According to our experimental results and the reports about the electropolymerization of some other similar dyes,[17,23] the primary step for the azure blue II polymerization was the oxidation of the $-NHCH_3$ group of the azure blue II monomer. Then a radical cation was formed when the anodic potential was extended to +1.3 V. The unpaired electron can be delocalized in several forms and was sited with high probability either on $-NHCH_3$ group or at positions *ortho* to $-NHCH_3$ group. Radical dimerization can occur via carbon-nitrogen coupling routes to form a mixture of dimeric species. The electropolymerization process of azure blue II is proposed as in Scheme 2.

Scheme 2 Proposed azure blue II coupling scheme



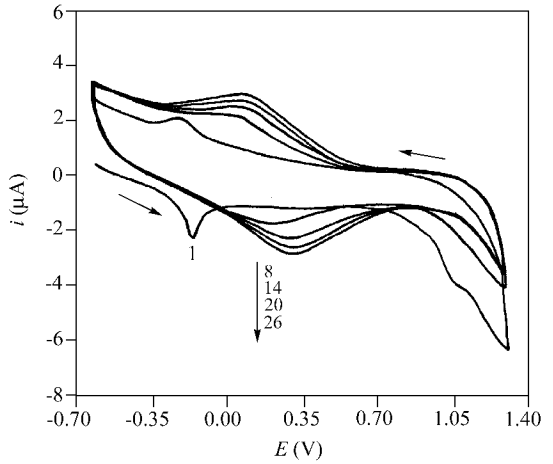


Fig. 3 Cyclic voltammograms of 1.25×10^{-4} mol/L azure blue II in pH 9.8 Britton-Robinson buffer solution between −0.6 and +1.3 V. Scan rate, 80 mV/s. 1, 8, 14, 20 and 26 represent the number of scan cycle.

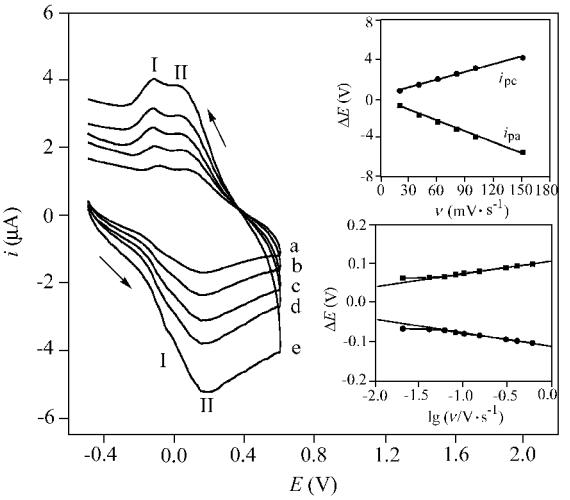


Fig. 4 Cyclic voltammetric response of the glassy carbon electrode modified with PABII in 0.1 mol/L phosphate buffer solution (pH = 7.0) at scan rates: (a) 40; (b) 60; (c) 80; (d) 100 and (e) 150 mV/s. The upper inset shows the dependence of peak current for couple II on the scan rate. Lower inset presents the dependence of the experimental variation of ΔE for couple II on the logarithm of scan rate.

The dimer of azure blue II would further form radical cations and produce oligomers of azure blue II. From the above scheme, it can be seen that there are two kinds of electroactive nitrogen atoms included in the PABII structure, one is the electroactive hexacyclic nitrogen atom contained in monomer units, the other is the electroactive nitrogen bridges between monomer units. Methylene blue is also one of the phenothiazine dyes and its basic structure is similar to that of azure blue II. Karyakin *et al*.[24] have studied the polymer of methylene blue modified glassy carbon electrode and found two redox couples in the cyclic voltammogram of poly(methylene blue). They pointed out that the redox couple at more negative potential corresponded to the monomer type redox activity, while the other redox couple at more positive potential corresponded to electrochemical reaction of polymer. According to the results obtained by Karyakin and his colleague, we considered that couple I in Fig. 4 could be ascribed to the redox reaction of the monomer units contained in PABII film, and couple II corresponded to the redox reaction of the nitrogen bridge.[16]

In our studies, it was found that the process of electropolymerization of azure blue II was also related to the pH value of the buffer solution containing the monomer. The cyclic voltammograms of electropolymerization for azure blue II in acidic solution showed that no shoulder peak current appeared in the anodic process and no wide-range redox peak currents increased with an increase in a number of potential cycles. The polymerization of azure blue II could not be obtained at the electrode surface because azure blue II lacks the primary amine groups and the *N*-substituted phenothiazines are difficult to be oxidized in acidic solution. But in neutral solution, the process of electropolymerization of azure blue II was almost the same as that in alkaline solution. Furthermore, the electrochemical behavior of PABII obtained in neutral solution was also similar to that of PABII formed in alkaline solution.

Cyclic voltammograms of the PABII modified glassy carbon electrode at various scan rates in phosphate buffer solution (pH = 7.0) are shown in Fig. 4. It can be seen that the peak currents are directly proportional to the scan rates in the range below 150 mV/s, indicating a surface-confined redox couple. The formal potential is −150 and +79 mV for couple I and couple II, respectively, at a scan rate of 80 mV/s. The formal potential is almost independent of the potential scan rate for sweep rates below 100 mV/s, suggesting facile charge transfer kinetics over this range of sweep rate and a transfer coefficient (α) of about 0.5. However, when the sweep rates exceed 100 mV/s, the peak to peak potential separations begin to increase, indicating the limitation arising from charge transfer kinetics.

Laviron derived general expressions for the linear potential sweep voltammetric response for the case of surface-confined electroactive species.[25] From this theory, the apparent charge transfer rate constant (k_s) for electron transfer between the electrode and surface deposited layer as well as the transfer coefficient (α) by measuring the variations of the peak potentials with scan rate can be determined. The peak to peak potential separation (ΔE_p) will be close to zero when the electron transfer rate is sufficiently fast compared with the scan rate and will increase when such a condition is not met. However, non-zero ΔE_p values can also arise as a result of other factors, including uncompensated resistance and others. We have found that for scan rates above 100 mV/s, the values of ΔE ($E_p - E^{0'}$) were proportional to the logarithm of the scan rate as indicated by Laviron.[25] Using such a plot, the calculated values of α and k_s for PABII modified at the glassy carbon electrode surface were 0.56 and 0.97 s^{-1} for couple II,

respectively.

pH *dependence studies of the* PABII *films*

Since the electrochemical behavior of many electropolymerization modified electrodes formed with dyes was affected by pH, the effect of pH on the redox response of PABII film has been investigated. The results indicated that the anodic and cathodic peak potentials shifted toward a negative direction with increasing pH of the solutions. The formal potential ($E^{0'}$) of the redox couple II, taken as the average of positive and negative peak potentials, was pH dependent with a slope of 27 mV per unit of pH (Fig. 5a), which corresponded to a one-proton process. In addition, pH values of the solutions had a great effect on the difference of peak potentials, ΔE_p (Fig. 5b). It is also suggested that the hydrogen ion participated in the electrode reaction.

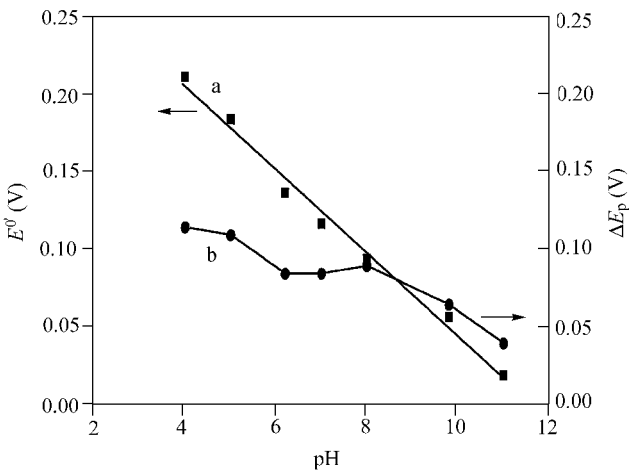


Fig. 5 Dependence of $E^{0'}$(a) and ΔE_p(b) of PABII for redox couple II on pH.

Stability of the modified electrode

Stability tests were carried out by repetitive scans in the potential range of -0.5 and $+0.6$ V in pH 7.0 phosphate buffer solution. The peak currents did not obviously decline after 100 cycles of repetitive scans. The PABII film electrode was also tested for prolonged use. It was placed in phosphate buffer solution (pH = 7.0) for 40 h and we found that the peak currents and peak potentials did not exhibit an obvious change before and after storage. Therefore, the PABII film can strongly adhere to the surface of the electrode and the modified electrode possessed a good stability.

Electrocatalytic oxidation of NADH *at the* PABII *modified electrode*

One of the objectives of this work was the development of a modified electrode capable of the electrocatalytic oxidation of NADH. This, in part, was responsible for the choice of azure blue II as a modifying agent since several redox dyes possessed high electrocatalytic oxidation activity for NADH.[15-17] In order to test the electrocatalytic activity of PABII film, the cyclic voltammograms were obtained in the absence and presence of 5.0×10^{-4} mol/L NADH, and the curves are shown in Fig. 6. In the absence of NADH (Fig. 6a), two couples of redox reaction for PABII film on the electrode can be observed. When 5.0×10^{-4} mol/L NADH was added, there was a dramatic enhancement of the anodic current and the cathodic current peak almost did not change, which is very characteristic of an electrocatalytic oxidation process. Simultaneously, the potential of anodic current peak shifts toward a positive direction after addition of NADH. The anodic peak potential for oxidation of NADH on the PABII modified electrode is about 0.28 V, while NADH is oxidized at about 0.69 V (Fig. 6e) at an unmodified glassy carbon electrode under identical conditions and exhibits a peak current lower than that observed with the modified electrode. Therefore, a decrease in overpotential of 410 mV and an enhancement of peak current are achieved with the modified electrode.

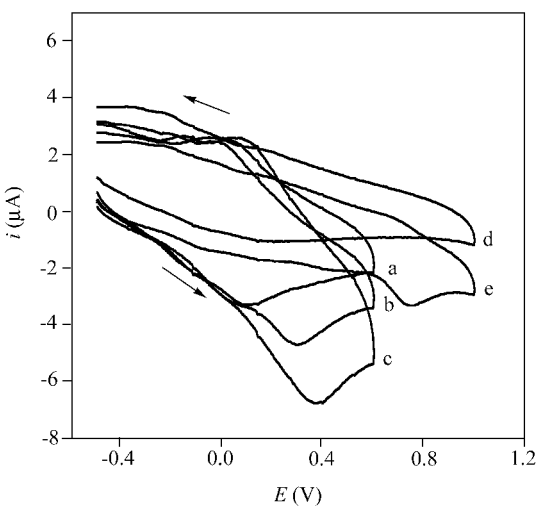


Fig. 6 Cyclic voltammograms of the PABII modified glassy carbon electrode in 0.1 mol/L phosphate buffer solution (pH = 7.0). (a) In the absence of NADH; (b) in the presence of 5.0×10^{-4} mol/L NADH; (c) in the presence of 5.0×10^{-4} mol/L NADH and 2.0×10^{-2} mol/L Mg^{2+}; (d) as (a) and (e) as (b) for the bare glassy carbon electrode.

Fig. 7 shows the cyclic voltammograms of the PABII modified electrode at various scan rates obtained in phosphate buffer solution (pH 7.0) containing 5.0×10^{-4} mol/L NADH. The anodic peak potential for the oxidation of NADH remains obviously constant for the scan rates up to 100 mV/s, suggesting a relatively high electron transfer rate constant for reaction between NADH and surface of PABII. However, a gradual shift of E_{pa} towards more positive potentials is observed for scan rates higher than 100 mV/s. The oxidation currents for NADH increase linearly

with the square root of the scan rate. These results show that the overall electrochemical oxidation of NADH at this electrode is controlled by the diffusion of NADH from solution to the redox sites of the modified electrode surface. The effect of pH on the anodic current was investigated from pH 4.0 to 11.0. The optimum pH range was 6.5 to 7.5.

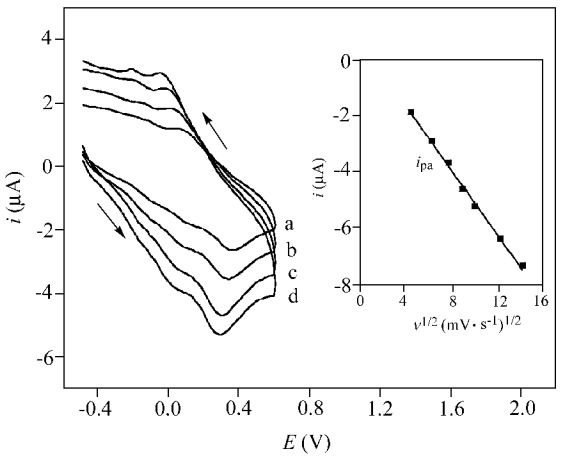


Fig. 7 Cyclic voltammograms of the PABII modified electrode in 0.1 mol/L phosphate buffer solution (pH = 7.0) containing 5.0×10^{-4} mol/L NADH at scan rates: (a) 40; (b) 60; (c) 80 and (d) 100 mV/s. The inset shows the variations of the electrocatalytic current with the square root of scan rate.

Effects of the cation on the electrocatalytic activity of the PABII *modified electrode toward* NADH *oxidation*

Katz *et al*.[26] recently reported that the presence of Ca^{2+} in the electrolyte gave rise to a considerable enhancement in the electrocatalytic oxidation of NADH at pyrroloquinoline quinone (PQQ) modified electrodes. These authors suggested that Ca^{2+} ion and PQQ can form the complex, Ca^{2+}-PQQ, and formation of the ternary complex between the Ca^{2+}-PQQ and NADH could be more efffficient in the electrocatalytic reaction than the formation of complex between immobilized PQQ and NADH. The Ca^{2+} ion might provide a favorable orientation of NADH molecules for its electrocatalytic process. Recently, a few of experimental results that some ions such as Ca^{2+} and Mg^{2+} can significantly enhance the anodic currents of NADH electrocatalytic oxidation at the glassy carbon electrode modified with 1,4-bis(3,4-dihydroxyphenyl)-2,3-dimethylbutane or 3,4-dihydroxybenzaldehyde have been reported.[27-29] In an attempt to increase the electrocatalytic efficiency of the oxidation of NADH at the PABII modified electrode and taking in account the above-mentioned results, we have carried out a series of experiments where a variety of cations were deliberately added to the electrolyte. The cyclic voltammograms of 5.0×10^{-4} mol/L NADH at the PABII modified electrode in phosphate buffer solution (pH = 7.0) in the absence and presence of 2.0×10^{-2} mol/L Mg^{2+} are shown in Fig. 6 (curves b and c).

In the presence of Mg^{2+} cation, the anodic electrocatalytic current was enhanced considerably in comparison with the absence of Mg^{2+} cation. The effects of other cations on the electrocatalytic oxidation of NADH have been investigated. In the presence of some divalent cations (*e.g.*, Mg^{2+}, Ca^{2+} and Co^{2+}), the anodic electrocatalytic current for NADH oxidation was enhanced considerably (approximately 1.6—1.8 times). Some other divalent cations (such as Ni^{2+} and Ba^{2+}) can also enhance the electrocatalytic current but with a smaller effect than Mg^{2+}, Ca^{2+} and Co^{2+}. However, the monovalent cations (Na^+, K^+ and NH_4^+) had no effects on the anodic currents for NADH oxidation. Effects of the cations on the electrocatalytic activity of the PABII modified electrode toward NADH oxidation depended on the concentration of cations. Using Mg^{2+} cation as an example, the relationship between the enhancement of electrocatalytic current and the concentration of cations was investigated. In order to compare the results obtained in the presence and absence of Mg^{2+} cation, the rate of i_{ion}/i_0 was obtained, where i_{ion} and i_0 represent the electrocatalytic currents of NADH oxidation in the presence and absence of Mg^{2+} cation, respectively. As shown in Fig. 8, in the case of Mg^{2+}, the maximum effect was obtained at concentration above 4.0×10^{-2} mol/L in phosphate buffer solution (pH = 7.0) containing 5.0×10^{-4} mol/L NADH. In our studies, we have also observed a decrease in electrocatalytic activity with cycling, although this phenomenon was not obvious and the loss became quite small after several cycles. But in the presence of some divalent cations, the decrease in electrode activity during potential cycling was much smaller than that in their absence. Therefore, those cations gave rise to an enhancement of not only the electrocatalytic current but also the stability. Our experimental results are similar to that reported by Katz *et al*.[26] and Moreno *et al*.[28,29] Therefore, we agree to the viewpoint that the reason of anodic current enhancement attributes to the formation of a ternary complex: PABII-NADH-ion.

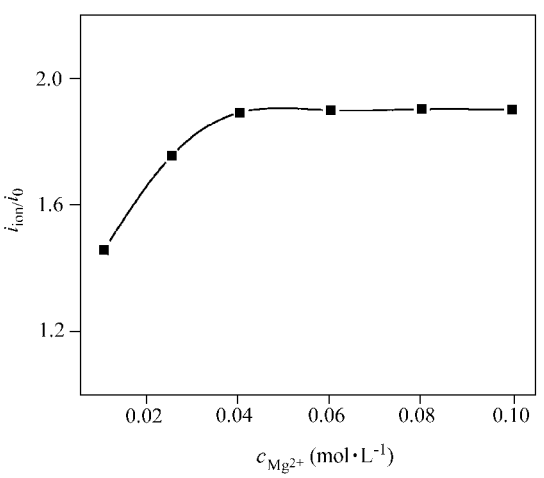


Fig. 8 Dependence of i_{ion}/i_0 on the concentration of Mg^{2+} cation.

Determination of NADH

The effect of increasing the NADH concentration on the voltammetric response of the PABII modified electrode was also investigated. The cyclic voltammograms were obtained in a series of concentration of NADH. Upon the addition of NADH, there was a dramatic enhancement in the anodic current. The relationship between the peak current and the concentration of NADH was different in the absence and presence of the divalent cations. The dependence of current response on the concentration of NADH was linear in the range of 1.0×10^{-4} to 5.0×10^{-3} mol/L NADH in the absence of the divalent cations. However, the electrocatalytic current increased linearly with NADH concentration from 1.0×10^{-5} to 8.0×10^{-3} mol/L in the presence of 4.0×10^{-2} mol/L Mg^{2+} cation. The detection limit was 5.0×10^{-6} mol/L, and the relative standard deviation of the results was 4.2% for six successive determinations at 5.0×10^{-4} mol/L NADH in the presence of Mg^{2+} cation.

Conclusions

Azure blue II was electrochemically polymerized at the glassy carbon electrode in basic or neutral media. The polymer-modified electrode exhibited a higher stability and a significant catalytic activity for the oxidation of NADH. Some divalent cations in electrolyte can enhance the electrocatalytic efficiency of the oxidation of NADH. Because of its linear catalytical response for NADH, it could be used for the determination of NADH. These properties may be very valuable in design of the biosensors based on dehydrogenases.

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