Flow-Injection Analysis and Voltammetric Detection of NADH with a Poly-Toluidine Blue Modified Electrode

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Poly-Toluidine Blue film was prepared by electrooxidative polymerization at a glassy carbon electrode in a phosphate buffer solution. The resulting chemically modified electrode (CME) exhibited excellent electrocatalysis toward the oxidation of reduced nicotinamide coenzyme (NADH) with over a 450 mV decrease of the overpotential compared with that at a bare glassy carbon electrode. Two electrochemical determinations of NADH, cyclic voltammetry and flow injection analysis, were established based on the electrocatalytical performance of the resulting modified electrode. Under an identical determinate condition, the voltammetric detection for NADH gave a detection limit of 3.3 µmol L⁻¹ with a linear concentration range of 9.1 µmol L⁻¹ to 1.8 mmol L⁻¹. As a detector in a flow-injection system, the CME gave a detection limit of 0.1 µmol L⁻¹ for NADH with a linear concentration range of 1.0 µmol L⁻¹ to 3.2 mmol L⁻¹. Obviously, flow-injection analysis is superior to voltammetric detection for its lower detection limit and wider detectable linear range.

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Introduction

The analysis of reduced nicotinamide coenzymes remains important in enzyme assays, since over 250 dehydrogenase enzymes use the oxidized form of this ubiquitous coenzyme as cofactors to participate in organism metabolism. A variety of analytical methods for NADH analysis have been proposed in recent years, such as spectrophotometry,¹ fluorometry,¹ chemiluminescence,² bioluminescence and amperometry.² Among these, amperometry³,⁴ has received considerable attention, since it has a great advantage over the conventional photometry in easy handling, automated procedure, small sampling volume and enhancement in the sensor characteristics of selectivity, accuracy and precision.⁵–¹⁰ Amperometry is based on the electrocatalytical oxidation of NADH at a chemically modified electrode. Since NADH is irreversibly oxidized with a great overpotential at an unmodified solid electrode, such as carbon and platinum, it is impossible in the direct determination of NADH at a bare electrode. Numerous efforts contributed to accelerating the electron-transfer between NADH and the electrode surface using the immobilization of various mediators on the electrode surface.¹¹–¹⁶ Phenothiazine and phenoxazine dyes as mediators modified on the electrode surface were investigated recently.¹⁷–⁴ The dyes exhibit great electrocatalytical activity toward NADH oxidation, and their catalytic reactions undergo a two-electron-proton mechanism.²⁵ Adsorption is a common immobilization of these dyes on a solid electrode surface.²⁶–²⁸ However, the utility of the adsorption by the inter-molecule force is often afflicted with mediator dec ompistion.²⁹,³⁰ Phenothiazine and phenoxazine dyes with an amine group can be electropolymerized onto the electrode surface,³,²⁶,³¹–³⁶ and the dyes immobilized by electropolymerization are more stable and more active in the electrocatalytical oxidation of NADH than those by adsorption, since more dyes can be coated on the electrode by electropolymerization, which produce a three-dimensional distribution of the mediators. Voltammetric detection for NADH using a poly-Toluidine Blue modified electrode was reported.³¹–³³ However, flow-injection amperometry for NADH has not yet been presented with this modified electrode. Compared with voltammetric detection, flow-injection analysis has advantages over voltammetric detection for NADH determination on the litter background current. Hence, it can gain a higher sensitivity and a lower detection limit. Therefore, it is worth investigating flow injection amperometry for NADH. In this work, we focused on preparing a stable poly-Toluidine Blue film modified electrode and its use in the flow-injection determination of NADH.

Experimental

Materials and apparatus

NADH was obtained from Sigma, and the exact concentration of the coenzyme solution was determined by spectrophotometrically, described by Chi and Dong.⁴ Toluidine Blue (TB) was obtained from the Second Reagent Factory of ShangHai, and recrystallized in methane before use. Other reagents used were analytically pure. NADH and L-ascorbic acid solution were freshly prepared. Phosphate buffer solutions were prepared with proper amounts of H₃PO₄, NaH₂PO₄, Na₂HPO₄ and Na₃PO₄. All solutions were prepared with doubly distilled, deionized water.

Electrochemical experiments were conducted on a CHI660 electrochemical workstation (CH Instruments, USA). A three-electrode system was used with a saturated calomel reference
Preparation of CMEs

The glassy carbon electrodes were polished with 1.0, 0.3 and 0.05 µm alumina slurry, respectively, rinsed with distilled water and ultrasonicated in alcohol. They were then rinsed with distilled water again before activation of the electrode.

The electrode after pretreatment was activated in a 0.1 mol L⁻¹ phosphate buffer solution (pH 6.68) by anodization at a potential of 2.0 V vs. SCE for 26 s, and the surface area of the electrode was determined to be 0.08 cm² for a flow-injection electrode in 0.1 mol L⁻¹ phosphate buffer (pH 6.68), and its electrochemical polymerization potential was 0.8 V (Fig. 2). If the potential was swept toward the positive direction over 0.7 V, the oxidation current increased abruptly, revealing that Toluidine Blue was polymerized initially at this potential on a glassy carbon electrode. A pair of new redox peaks appeared at around −0.1 V after the second potential scanning on the cyclic voltammograms. Moreover, with successive scanning, the redox peaks current increased continuously; meanwhile, the anodic peak potential shifted in the positive direction and the cathodic peak potential in the negative direction.

Preparation of CMES

The glassy carbon electrodes were polished with 1.0, 0.3 and 0.05 µm alumina slurry, respectively, rinsed with distilled water and ultrasonicated in alcohol. They were then rinsed with distilled water again before activation of the electrode.

The electrode after pretreatment was activated in a 0.1 mol L⁻¹ phosphate buffer solution (pH 6.68) by anodization at a potential of 2.0 V vs. SCE for 26 s, and the surface area of the electrode was determined to be 0.08 cm² for a flow-injection analysis and 0.50 cm² for other experimental method suggested by Chi and Dong. Then, the activated electrode was immersed in a 0.1 mol L⁻¹ phosphate buffer solution (pH 6.68) containing 5 × 10⁻⁴ mol L⁻¹ TB and swept by cyclic voltammetry at a scan rate of 50 mV s⁻¹ in the potential range of −0.70 V to 0.80 V for 20 cycles. The amount of TB immobilized onto the electrode surface was calculated from the peak area of a linear scan.

Results and Discussion

Electropolymerization of TB and its electrochemical property

Toluidine Blue was easy to be polymerized on a glassy carbon electrode in 0.1 mol L⁻¹ phosphate buffer (pH 6.68), and its electrochemical polymerization potential was 0.8 V (Fig. 2). If the potential scan was confined to the range of −0.7 to 0.6 V, a pair of reversible redox peaks was observed on the glassy carbon electrode, and the potential of a cathodic peak was −0.378 V and an anodic peak −0.326 V. However, providing that the potential was swept toward the positive direction over 0.7 V, the oxidation current increased abruptly, revealing that Toluidine Blue was polymerized initially at this potential on a glassy carbon electrode. A pair of new redox peaks appeared at around −0.1 V after the second potential scanning on the cyclic voltammograms. Moreover, with successive scanning, the redox peaks current increased continuously; meanwhile, the anodic peak potential shifted in the positive direction and the cathodic peak potential in the negative direction.

Two pairs of well-refined redox peaks (Fig. 4(a)) were observed when a glassy carbon electrode modified with the polymerized Toluidine Blue film was immersed in 0.1 mol L⁻¹ phosphate buffer solution (pH 6.68) and swept circularly at a scan rate of 20 mV s⁻¹ in the range of −0.7 to 0.45 V, among which, for couple I, the anodic peak potential was −0.167 V and the cathodic peak potential −0.259 V; for couple II, the anodic peak potential was 0.028 V and the cathodic peak potential −0.076 V. The formal potential, E°, which was taken as the mid-point of the anodic and cathodic peak potential, was −0.213 V for couple I and −0.024 V for couple II, respectively. As suggested by Chen et al., couple II corresponds to the polymer-type redox peak and couple I to the monomer-type redox peaks.

Electrocatalytic oxidation of NADH

The typical irreversible oxidation of NADH took place at a potential of 0.56 V vs. SCE with a high overvoltage of approximately 1.1 V for a NADH/NAD⁺ couple on a bare glassy carbon electrode in a 0.1 mol L⁻¹ phosphate buffer solution (pH 6.68) containing 1 mmol L⁻¹ NADH (Fig. 3). The electrochemical activity of NADH was considerably promoted by a decrease of over 450 mV in the overpotential under an identical condition when the poly-Toluidine Blue film was coated on the electrode (Fig. 4), demonstrating that the poly-Toluidine Blue film has excellent catalytic activity toward NADH oxidation. Its catalytic peak occurs at approximately 0.15 V, near to peak II of the redox potential for poly-Toluidine Blue film in a 0.1 mol L⁻¹ phosphate buffer solution (pH 6.68). Therefore, polymer-type TB is the main catalyst for NADH oxidation. The catalytic peak was obtuse, suggesting that NADH may permeate into the poly-Toluidine Blue film, and be oxidized on the electrode surface.
Cyclic voltammetric detection of NADH

Since the electrochemical activity of poly-Toluidine Blue is dependent on the solution pH, its catalytic oxidation of NADH is affected by the solution pH. As shown in Fig. 5, the catalytic current increased slowly from pH 3.0 and reached a maximum value at pH 6.68, then decreased abruptly with an increase in the pH above 6.68. A similar result was reported by Dong et al. for NADH oxidation at a Methylene Blue modified electrode. The effect of the solution pH was mainly due to the proton involved in the catalytic process.

The peak current of NADH oxidation linearly increased with increasing the NADH concentration in a 0.1 mol L\(^{-1}\) phosphate buffer solution (pH 6.68) on the cyclic voltammograms of NADH oxidation at the resulting modified electrode. The detectable linear range for NADH determination is 9.1 \(\mu\)mol L\(^{-1}\) -1.5 mmol L\(^{-1}\), and the detection limit is 3.3 \(\mu\)mol L\(^{-1}\). Under the above-mentioned condition, the voltammetric response of 1 mmol L\(^{-1}\) NADH remained stable for 44 h, and seven detections for 1 mmol L\(^{-1}\) NADH gave a 3.2% relative standard deviation.

Poly-Toluidine Blue film can electrochemically catalyze the oxidation of NADH; it also exhibits a strong electrocatalytical activity for L-ascorbic acid oxidation. Figure 7 shows the voltammetric behaviors of Poly-Toluidine Blue at a glassy carbon electrode in both the presence and absence of L-ascorbic acid. NADH and L-ascorbic acid share the same oxidative potential (0.1 V) at the modified electrode. Therefore, they interfere with each other in their determination when they coexist in the same solution. Similar results were also reported by Tanaka et al. in studying the electrocatalytical oxidation of NADH at a poly-thionine modified electrode. Other interferents investigated, such as dopamine, uric acid and 5-hydroxytryptamine, did not influence the flow-injection determination of NADH.

Flow-injection determination of NADH

The amount of catalyst (poly-Toluidine Blue) attached to the electrode surface can be controlled by the scan number during electropolymerization and calculated from the peak area in cyclic voltammograms. Figure 8 shows the catalytic current dependence on the catalyst coverage \((\Gamma)\). The catalytic current peaks at a catalyst coverage of 14 nmol cm\(^{-2}\) on the I-\(\Gamma\) curve. Therefore, the optimum value of the catalyst coverage for a flow-injection analysis of NADH on this glassy carbon
The catalytic current response was dominated by the applied potential. As shown in Fig. 9, on the poly-Toluidine Blue film modified electrode, the current response started at 0.0 V, and increased remarkably up to 0.2 V; from then on, the current response tended to be stable. A small current response was obtained at a bare electrode compared with that of the modified electrode, and the response current augmentation began from 0.35 V. The results from hydrodynamic voltammetry (Fig. 9) were in agreement with those from cyclic voltammetry (Fig. 4).

Figure 10 shows typical catalytic current responses to various concentrations of NADH at an applied potential of 0.20 V in a 0.1 mol L⁻¹ phosphate buffer solution (pH 6.68). Obviously, the catalytic current at the CME in the flow-injection analyzer increased with increasing the NADH concentration. Figure 11 shows the linear dependence of the catalytic current response to the NADH concentration in the range of 5 µmol L⁻¹ to 3.2 mmol L⁻¹. The detection limit was 0.1 µmol L⁻¹, obtained from the curve bottom.

\[
I_c (\mu A) = -0.003 + 0.07C_{\text{NADH}} (\mu mol L^{-1}), \ r = 0.9960
\]

As was demonstrated by the mutual interference between NADH and L-ascorbic acid when they coexisted in the same solution, the flow-injection analysis (Fig. 12) also confirmed the same result, which suggested that L-ascorbic acid interfered with the determination of NADH in practical analysis, and should be separated or diminished from a sample in the analysis procedure for NADH.

The stability and reproducibility of poly-Toluidine Blue in flow-injection analysis were examined by successive injections of 1 mmol L⁻¹ NADH (Fig. 13). The current response to several initial injections decreased conspicuously, and remained independent of time prolongation after the 30th injection remained stable for 24 h. The relative standard deviation was approximately 1.8%, obtained from 5 repetitive injections at random after the current response stabilized.

**Conclusion**

A stable poly-Toluidine Blue film was electrochemically polymerized onto a glassy carbon electrode. This CME exhibited excellent catalytic activity toward NADH oxidation; cyclic voltammetric detection can gain a linear concentration of NADH in the range of 9.1 µmol L⁻¹ to 1.5 mmol L⁻¹ with a detection limit of 3.3 µmol L⁻¹. When used as an amperometric...
advantage over cyclic voltammetric detection in promoting a
µ mol L –1 . Obviously, flow injection amperometry has an
applied potential, 0.20 V.

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Fig. 11 Plot of the catalytic current response vs. the concentration
of NADH, under the experimental conditions described above.

Fig. 12 Flow-injection response to: (a) 0.1 mmol L –1 L-ascorbic
acid and (b) 0.1 mmol L –1 NADH at PTB-GCE. Carrier phase, 0.1
mol L –1 phosphate buffer solution (pH 6.68); flow rate, 0.6 mL min –1 ;
applied potential, 0.20 V.

Fig. 13 Flow-injection response obtained for successive injection
of 1 mmol L –1 NADH over a period of 8 h. Carrier phase, 0.1 mol L –1
phosphate buffer solution (pH 6.68); flow rate, 0.6 mL min –1 ; applied
potential, 0.20 V.

detector for the determination of NADH in flow-injection
system, it can obtain a linear concentration of NADH in the
range of 5 µmol L –1 to 3.2 mmol L –1 with a detection limit of 0.1
µmol L –1 . Obviously, flow injection amperometry has an
advantage over cyclic voltammetric detection in promoting a
sensitive detection limit for NADH.

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