Preparation of Poly(thionine)-Modified Electrode and Its Application to an Electrochemical Detector for the Flow-Injection Analysis of NADH

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Poly(thionine)-modified electrodes have been prepared using basal-plane pyrolytic graphite, glassy carbon and In-Sn oxide conducting glass as electrode substrates by an electrooxidative polymerization of thionine in both aqueous and acetonitrile media. It was found that the prepared films, the surface concentration of which can be controlled over the range of ca. 10⁻¹⁰ - 10⁻⁸ mol cm⁻² by appropriately choosing the electropolymerization conditions (e.g., solvent, concentration of thionine and electrolysis time), are electroactive (the formal potential is ca. 0.0 V vs. Ag/AgCl at pH 7.0) and chemically stable. The modified electrodes exhibited excellent electrocatalysis for NADH oxidation in neutral aqueous solutions, with an activation overpotential which is ca. 400 mV lower than that of a bare electrode. Further, the modified glassy carbon electrode was found to be promising as an amperometric detector for the flow-injection analysis of NADH, typically with a dynamic range of 5 µM - 1 mM.

Keywords Poly(thionine), electrocatalysis, NADH, FIA detector, modified electrode, electropolymerization

The analysis of reduced β-dihydronicotinamide adenine dinucleotide (NADH) is very important in enzyme assays to determine the concentration of enzyme substrates, since over 250 dehydrogenase enzymes use the oxidized form (NAD⁺) of this ubiquitous coenzyme to oxidize a substrate SH₂ to S⁻. A variety of analytical procedures for NADH analysis have been proposed, including spectrophotometry, fluorometry, chemiluminescence and amperometry. Among these, in the last decade, amperometric methods based on the electrocatalytic oxidation of NADH at modified electrodes, which chemically or physically immobilize various redox mediators for accelerating the highly irreversible oxidation of NADH on unmodified, conventional electrodes, such as platinum and carbon (the overpotential is typically of the order of 1 V), have received considerable attention because of the ease of handling, the use of a small sample volume, the ready adaptability in automated systems and the potential improvement of sensor characteristics, such as selectivity, accuracy and precision. In this connection polymer-modified electrodes with a three-dimensional distribution of mediators and of the so-called coated-wire type have also been studied based on the much larger catalytic response of polymer coatings than monolayer coatings due to the volume effect as well as the capability of miniaturization for in vivo sensor devices.

During the course of our continuing study concerning electrocatalysis at the polymer-modified electrodes, we recently found that the poly(thionine)-modified graphite electrode, which can be prepared by the electropolymerization of thionine, considerably electrocatalyzes the oxidation of NADH in neutral aqueous solutions and reported the preliminary results. In the present paper, we report on the preparation of the poly(thionine)-modified electrodes using various electrode substrates and their electrocatalysis for NADH oxidation and sensor characteristics when used as an amperometric detector for the flow-injection analysis of NADH. The electroactive (electrochemically reversible), chemically stable and water-insoluble properties of the thin films of poly(thionine) deposited on the glassy carbon electrodes enabled us to examine the characteristics of this modified electrode as an electrochemical thin-layer flow detector.

Experimental

Reagents

Reduced β-dihydronicotinamide adenine dinucleotide (NADH, Oriental Yeast Co. Ltd., Japan), L-ascorbic acid
(Kanto Chemical Co. Ltd.), dopamine (Kanto), NaClO₄ (Kanto) and uric acid (Wako Pure Chemical Industries, Ltd.) were used as received. Acetonitrile (Kanto) was purified by distillation over P₂O₅ and then over K₂CO₃. Thionine chloride of reagent grade was supplied by Tokyo Kasei Kogyo Co. Ltd. 0.2 M (1 M = 1 mol dm⁻³) phosphate buffer solution (pH 7.0), prepared by Na₂HPO₄·12H₂O and NaH₂PO₄·2H₂O (Wako), was used as the electrolyte and carrier in flow-injection experiments. Aqueous solutions were prepared from doubly distilled, deionized water or from that purified by passage through a Milli-Q purification train. A basal-plane pyrolytic graphite (BPG; Union Carbide Co.; area, 0.20 cm²), glassy carbon (GC; Bioanalytical Systems Inc. (BAS); area, 7.1×10⁻² cm²) disks and In-Sn oxide conducting glass (ITO; Matsuzaki Shinku Co. Ltd.; surface resistance, ca. 10 Ω/□) were used as the working electrode substrates. All of the other chemicals were of reagent grade and were used without further purification.

**Apparatus and procedures**

A standard three-electrode electrochemical cell was used for the electrochemical experiments. The electrode assembly comprised bare or poly(thionine)-modified BPG (GC or ITO) as the working electrode, an Ag/AgCl (KCl saturated) electrode as the reference electrode and a platinum electrode as the counter electrode. For cyclic voltammetry, a polarization unit (PS-07, Toho Technical Co. Ltd.) was employed with an X-Y recorder (WX-2400, Graphtec Co.). All electrochemical experiments were conducted under a nitrogen atmosphere at laboratory temperature (23±2° C).

The poly(thionine) film-modified electrodes were typically prepared by a potential-sweep electrolysis at 50 mV s⁻¹ in the potential range of -0.15 to 1.25 V (or -0.8 to 1.8 V) vs. Ag/AgCl in 0.1 M NaClO₄ aqueous solution (pH 1.0) containing 40 - 80 sM thionine (or 0.2 M NaClO₄ acetonitrile solution containing 1 mM thionine). The electrolysis time was typically 1 - 100 min. The electrodes thus prepared were soaked in a boiling water for a few minutes to dissolve any unreacted thionine monomer, adsorbed on the electrode surface and/or trapped in the polymer matrix. The surface concentration \( \Gamma \) (in units of mol cm⁻²) of the electroactive moieties within the poly(thionine) films could be controlled by appropriately choosing the electropolymerization conditions, such as solvent, concentration of thionine and electrolysis time; poly(thionine)-modified electrodes with \( \Gamma = \text{ca.} 1\times10⁻¹⁰ - 1\times10⁻⁸ \text{ mol cm}⁻² \) were typically used. The values of \( \Gamma \) were estimated by measuring the area of the cyclic voltammograms (for the oxidation-reduction reaction of the poly(thionine) film) obtained at slow potential scan rates (1 - 5 mV s⁻¹) in a 0.2 M phosphate buffer solution (pH 7.0). The film thickness of the poly(thionine) films prepared on electrode surfaces was measured with a stylus profiler (Dektak 3030, Veeco Instruments Inc., Sloan Technology Division, California) without stripping the films from the electrode surface.

The BAS-200 electrochemical analyzer along with a DM2M-1024 Solution Delivery Pump of the dual-plunger type and an injector (SV 1-507, Sanuki Kogyo Co. Ltd.) was used in the flow-injection experiments. Schematics of the flow-injection system and the measuring cell are shown in Fig. 1. The flow cell used for electrochemical detection was of the thin-layer design (LC-17A, BAS). The Ag/AgCl (3 M NaCl) reference electrode (RE-4, BAS), the potential of which was 0.015 V above that of the Ag/AgCl (KCl saturated) electrode, was positioned opposite to the working electrode, and the auxiliary electrode was positioned directly across the thin-layer channel (the gap: ca. 50 µm) from the working electrode. The working electrode was poly(thionine)-modified GC electrodes (MF-1000, BAS) and the auxiliary electrode was stainless steel. The instrument used for the flow-injection analysis was equipped with a GC electrode substrate as an electrochemical detector. The film-coated electrodes were equilibrated with a flowing supporting electrolyte (or mobile phase) solution, while the working potential was applied (typically at 0.1 V vs. Ag/AgCl). Amperometric measurements were made after the background current decayed to a steady-state value. The flow rate was varied over the range of 0.5 - 2.0 ml min⁻¹. The injected volume of sample solutions was 20 µl. The current–time output was recorded with a survocorder (SR 6342, Graphtec Co.).

**Results and Discussion**

**Preparation of poly(thionine)-modified electrodes**

Typical cyclic voltammograms of thionine at the BPG electrode in a 0.1 M NaClO₄ + HClO₄ aqueous solution
(pH 1) containing 40 µM thionine are shown in Fig. 2A. The irreversible anodic reaction, which commences at around 1.0 V, is considered to correspond to the oxidation of the NH2 groups of the thionine molecule (probably to the cation radicals31), as is usually accepted for most aromatic compounds with NH2 groups.32,33 The reversible response, which is observed over a wide range of potentials (ca. 0 to 0.5 V; its center is located around 0.25 V, assumed to be close to the formal potential $E^{\circ}$ of the poly(thionine) film at pH 1), corresponds to an oxidation-reduction reaction of the thionine ring. According to the redox reaction of thionine34, the following redox reaction may be speculated for the poly(thionine) film at pH 1:

$$\text{H}_2\text{N}-\text{S}═\text{NH}_2 \rightleftharpoons \text{H}_2\text{N}-\text{S}−\text{NH}_2$$

As can be seen from Fig. 2A, the current response increased continuously with successive potential scans and the electrode was covered with a purple, thin film with a fairly smooth surface. In this case, the surface concentration of the prepared film was estimated to be $2.4\times10^{-9}$ mol cm$^{-2}$ in a 0.2 M phosphate buffer solution (pH 7.0). The electropolymerization of thionine was also carried out in acetonitrile media, as reported recently.29 In this case, a well-defined reversible redox response, similar to that shown in Fig. 2A, was not observed during the electropolymerization (Fig. 2B), because the electrolyte solution contained no protons. However, when the film-coated electrode was transferred into aqueous solutions, it displayed the same redox response as that of the electrodes prepared from the aqueous media (Fig. 2C), indicating that the poly(thionine) films prepared from both media are essentially the same. The acetonitrile media were found to be more effective for preparing thicker films, because of the higher solubility of thionine.

A typical vertical sectional profile of the poly(thionine) film prepared on the ITO electrode is shown in Fig. 3. It is obvious that the film is continuous and that its thickness is almost uniform over the entire film.

**Electrocatalysis of poly(thionine)-modified electrodes for oxidation of NADH**

Figure 4 shows typical cyclic voltammograms that demonstrate the electrocatalytic activity of the poly-(thionine)-modified BPG electrode for NADH oxidation in a 0.2 M phosphate buffer solution (pH 7.0). The anodic peak potential for the oxidation of NADH at the bare BPG electrode is ca. 0.7 V (voltammogram c), being in agreement with the previous results.3-6,16,35 The $E^{\circ}$
The value of the NAD+/NADH redox couple has been reported to be −0.515 V vs. Ag/AgCl at pH 7. We can thus see that the overpotential for the NADH oxidation is considerably high, as well-known for conventional, unmodified electrodes. This is the primary reason for developing electrocatalytic materials that enable the oxidation of NADH at much lower overpotentials. The reversible redox response of the poly(thionine) film, itself (voltammogram a), can be observed at around 0.0 V. Voltammogram b observed in the presence of NADH shows a greatly enhanced oxidation current and a large negative shift in the anodic peak potential of about 400 mV, compared with that (voltammogram c) obtained at the bare BPG electrode. Essentially similar cyclic voltammetric behavior was also observed at the GC and ITO electrodes. These results demonstrate the electrocatalytic oxidation of NADH by the poly(thionine) film. The second anodic peak current observed at almost the same potential as that at the bare BPG electrode corresponds to the direct, unmediated oxidation of NADH that must penetrate the film to reach the electrode surface.

Sensor characteristics

Figure 5 shows a typical example of the flow-injection current–time profiles obtained for 99 repetitive injections of a 1 mM NADH solution over a period of ca. 5 h. Taking into account the fact that the peak current response corresponding to the oxidation of NADH as well as the interferent compounds becomes larger at more positive potentials (mentioned below), the optimum working potential was chosen at 0.1 V vs. Ag/AgCl in the flow-injection experiments. Note that the peak current obtained represents the difference in the currents obtained in the absence and presence of NADH. The peak response tended to gradually decrease during the first several tens of injections; after ca. 70–80 injections, however, it could be regarded as being almost constant, and was about 40% of the response for the 1st injection. Consequently, such a successive injection may be considered as being a “preconditioning” of the poly(thionine)-modified electrode to obtain a constant response. Further, we found a slower time-course of the current response, compared with such a “preconditioning”. After the preconditioned electrode was further immersed overnight in a 0.2 M phosphate buffer solution at room temperature, although the surface concentration actually remained unchanged, the current response decreased to about one-third of that obtained immediately after the preconditioning. In this case, however, the current response could be regarded as being virtually invariant during the course of additional flow-injection experiments for 1–2 h. These facts may suggest that poly(thionine) film is chemically stable and strongly adheres to the electrode surface; they also suggest that a slow morphological change of the film, which results in the observation of the slow time-course of the current response, occurs.

The characteristic flow injection response for 0.1, 0.5 and 1.0 mM NADH obtained at the electrode, which was kept overnight in a 0.2 M phosphate buffer solution after preconditioning, is shown in Fig. 6. It is apparent that a fairly reproducible response is obtained; that is, a very slight difference in the peak size at a given concentration is observed. The relative standard deviation of the 3 successive injections is less than 2%. In addition, it can be seen that the electrode exhibits a rapid increase in the current. The response times to reach the maximum signal are less than 10 s. The relatively fast response indicates a rapid replenishment of the solution, as desired.
The flow-rate dependence of the current response at a constant concentration of NADH was examined by recording the peak currents at different flow rates. A typical example of such experiments is shown in Fig. 7. The current response was found to decrease with increasing flow rate. For example, the peak current decreased from 87 nA at 0.5 ml min⁻¹ to 51 nA at 2.0 ml min⁻¹. This fact suggests that restricted diffusion of NADH to the sites for mediating its oxidation can be controlled by the time of exposure of the sample to the electrode, i.e., by varying the flow rate. A similar flow-rate dependence has also been reported for a chemically modified GC flow detector (for glucose) based on a bilayer-film coating of cobalt tetrakis(o-aminophenyl)porphyrin polymer film (inner film) and glucose oxidase enzyme film (outer film)²⁵, and for a poly(4-vinylpyridine)-coated GC flow detector for uric acid.³⁶ Such a flow-rate dependence is unique to the film-coated electrode system, and the related kinetics remain to be elucidated.

Figure 8 shows a typical calibration curve obtained at the poly(thionine)-modified GC electrode detector with $F=1.3 \times 10^8$ mol cm⁻². The present sensor possesses a dynamic range of 5 µM - 1 mM. The sensitivity, expressed as the initial slope of the current vs. NADH concentration, is 30 nA/mM. The current response of the sensor in the absence and presence of some interferent compounds (e.g., ascorbic acid, uric acid and dopamine) was also examined (Fig. 9), because these compounds are oxidizable in the range of a more negative potential than that (ca. 0.7 V) required to oxidize NADH at the bare graphite electrode (Fig. 10). The concentrations of ascorbic acid, uric acid and dopamine are 0.05, 0.32 and 0.02 mM, respectively, which are their individual standard values in human serum samples. Dopamine and uric acid exhibited no response, indicating that these interferences do not influence the sensor response under the conditions used. On the contrary, a large current response was observed for ascorbic acid. This is considered to be a result of the electrocatalyzed oxidation of ascorbic acid by the poly(thionine) film, as shown in...
Fig. 11.37 No electrocatalytic activity for the oxidation of uric acid and dopamine was observed. Thus, a "preseparation" of at least ascorbic acid and NADH is required for use of our poly(thionine)-modified electrode as an electrochemical detector for NADH. However, it is beyond the scope of this study to discuss this subject in further detail.

In conclusion, the poly(thionine)-modified electrode, which can be prepared by electropolymerization of thionine, possesses an excellent electrocatalytic activity for NADH oxidation in neutral aqueous media and it is promising as an amperometric detector for the flow-injection analysis of NADH.

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