

## Electropolymerized poly(Toluidine Blue)-modified carbon felt for highly sensitive amperometric determination of NADH in flow injection analysis

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### Abstract

Poly(phenothiazine) films were prepared on a porous carbon felt (CF) electrode surface by an electrooxidative polymerization of three phenothiazine derivatives (i.e., Thionine (TN), Toluidine Blue (TB) and Methylene Blue (MB)) from 0.1 mol/L phosphate buffer solution (pH 7.0). Among the three phenothiazines, the poly(TB) film-modified CF exhibited an excellent electrocatalytic activity for the oxidation of nicotinamide adenine dinucleotide reduced form (NADH) at +0.2 V vs. Ag/AgCl. The poly(TB) film-modified CF was successfully used as working electrode unit of highly sensitive amperometric flow-through detector for NADH. The peak currents (peak heights) were almost unchanged, irrespective of a carrier flow rate ranging from 2.0 to 4.1 mL/min, resulting in the measurement of NADH (ca. 30 samples/hr) at 4.1 mL/min. The peak current responses of NADH showed linear relationship over the concentration range from 1 to 30  $\mu\text{mol/L}$  (sensitivity: 0.318  $\mu\text{A}/(\mu\text{mol/L})$ ; correlation coefficient: 0.997). The lower detection limit was found to be 0.3  $\mu\text{mol/L}$  ( $S/N = 3$ ).

**Key words:** poly(phenothiazine); Toluidine Blue; electropolymerization; carbon felt; NADH; flow injection analysis

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### Introduction

Reduced dihydronicotinamide adenine dinucleotide (NADH) is a cofactor of a number of dehydrogenases. Thus, the development of highly functional detection system for NADH is important for the construction of NADH-dependent dehydrogenases-based biosensors which are applicable for wide fields such as food industry, environmental monitoring and medical analysis (Curulli et al., 1997; Gorton et al., 1991; Jana and Raj, 2006; Karyakin et al., 1994; Lobo et al., 1997; Pariente et al., 1995; Silber et al., 1996; Zhang et al., 2007).

Among various detection methods, electrochemical method usually provides simple and relatively convenient procedure and highly sensitive results. Although NADH can be directly oxidized at electrode surface, relatively high applied potential is required (e.g., +0.6 V vs. Ag/AgCl at carbon electrode), leading to electrochemical interferences by co-oxidizable species in real samples. To avoid such problems and reduce the applied potential, the redox-active organic dyes have successfully been

employed as electron mediators for the electrocatalytic oxidation of NADH.

Adsorption of these dyes on the electrode surface is simple and convenient (Kimura and Niki, 1985; Yan et al., 2005), but the electrode stability is not necessarily sufficient, probably due to the leakage of the mediator from the electrode surface. To overcome this drawback, redox active organic dyes were immobilized covalently on the self assembled monolayers prepared on the electrode surface (Ohtani et al., 1997; Schlereth et al., 1994, 1995). However, the sensitivity of these monolayers modified electrodes is not necessarily sufficient (sub mmol/L order), due to the limitation of the surface coverage of the immobilized dyes.

Electropolymerization is powerful tool for the development of the modified electrodes. Electropolymerized materials usually have some unique properties which are not peculiar to the corresponding monomers. From this view point, the electropolymerization of phenothiazine derivatives provides a substantial improvement of the corresponding modified electrodes in relation to the electrocatalytic NADH oxidation. These electrochemically produced redox-active (electrochemically reversible) and

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chemically stable polymers with three dimensional distribution of mediators possess the following advantages as compared with adsorbed and covalently modified mediators; prolonged operational stability, and high sensitivity (Dilgin et al., 2007; Gao et al., 2003; Karyakin et al., 1993, 1994; Ohsaka et al., 1993; Shi et al., 2008; Tanaka et al., 1993; Zeng et al., 2006).

The usefulness of porous three-dimensional macro-electrodes as electrochemical flow-through detectors has been demonstrated. The low diffusion barrier of porous matrix facilitates mass transport, and high surface area permits higher coulometric efficiency, which enables highly sensitive determination even at higher flow rate. A reticulated vitreous carbon (RVC) is a primary choice of this purpose, and has been successfully introduced to an amperometric and coulometric detector units in flow-injection analysis (FIA) systems (Berrettoni et al., 2004; Strohl and Curran, 1979; Wang, 1981). On the other hand, a porous carbon felt (CF) is a microelectrode ensemble of micro carbon fiber with a random three-dimensional structure (Kato et al., 2000). As well as other porous electrodes, the CF has the extremely high surface area (estimated to be 0.3–30 m<sup>2</sup>/g) which allows large measurable current density and high electrolytic efficiency. Compared with the RVC, the CF has the following advantages: inexpensive, physically stable, easily-handling and easily manufactured to arbitrary shapes. Thus, CF would be alternative candidate as working electrode unit of electrochemical flow-through detector (Hasebe et al., 2007; Wang and Hasebe, 2009). The poly(phenothiazine)-modified electrodes have been prepared on various disk-electrodes: for example: gold film (Silber et al., 1996), basal-plane graphite (Ohsaka et al., 1993), glassy carbon (Tanaka et al., 1993; Karyakin et al., 1994), In-Sn oxide (Tanaka et al., 1993), carbon nanotube-nanowire composite (Zeng et al., 2006), nano-Au-modified glassy carbon (Shi et al., 2008) and screen printed carbon (Gao et al., 2003) electrodes. However, the preparation of poly(mediator) on porous electrode has not been reported so far, from our best knowledge.

In this study, we used three phenothiazines such as Thionine (TN), Toluidine Blue (TB) and Methylene Blue (MB), and prepared the poly(phenothiazine) films on the CF surface for the first time. The electrochemical properties and the electrocatalytic activity for the NADH oxidation were compared. Among them, the poly(TB)-modified CF, which exhibited superior performance, was chosen, and used as a working electrode unit of flow-through electrochemical detector for NADH. Various analytical performance characteristics were characterized.

## 1 Experimental

### 1.1 Reagents and materials

NADH was obtained from Oriental Yeast Co., Ltd., Japan. TN was obtained from Wako Pure Chemical Industry, Japan. TB and MB were obtained from Chroma-Gesellschaft Schmid GmbH Co., Ltd., Germany. These reagents were used as received. CF sheet (Nihon Carbon

Co., Ltd., Japan) was cut into appropriate size (i.e., 10 mm × 3 mm × 3 mm, with apparent volume 90 mm<sup>3</sup>, density 1.4 g/cm<sup>3</sup>, porosity more than 90%). Phosphate buffer solution (0.1 mol/L, pH 7.0) prepared by mixing KH<sub>2</sub>PO<sub>4</sub> (Wako) and K<sub>2</sub>HPO<sub>4</sub> (Wako) was used as electrolyte and carrier solutions. All aqueous solutions were prepared with doubly-distilled deionized water (Yokozawa Chemical Co., Ltd., Japan). All of other chemicals were of reagent grade and were used without further purifications.

### 1.2 Electrochemical methods

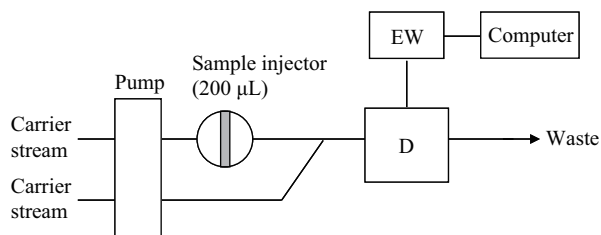
For the electrochemical experiments (cyclic voltammetry (CV), constant-potential amperometry), a single compartment three-electrode cell was used. CF with platinum lead wire (0.5 mm diameter, 99.99% from Tanaka Kikinzoku Kogyo Ltd., Japan) was used as working electrode together with Pt wire counter electrode (1 mm diameter) (Tanaka) and Ag/AgCl (3 mol/L NaCl) reference electrode (RE-1B, BAS, USA). Electrochemical measurements were carried out with ALS electrochemical workstation (ALS 611B, USA) connected to personal computer. For CV measurements, deoxygenized 0.1 mol/L phosphate buffer (pH 7.0) was used as an electrolyte solution. For constant-potential amperometry, air-saturated 0.1 mol/L phosphate buffer (pH 7.0) was used. All electrochemical measurements were carried out at room temperature.

### 1.3 Electropolymerization of phenothiazines

The poly(phenothiazine)-modified CF was prepared by a potential sweep electrolysis at 50 mV/sec in the potential range of −0.4 to +1.1 V (vs. Ag/AgCl) in 15 mL of 0.1 mol/L phosphate buffer (pH 7.0) containing 0.1 mmol/L phenothiazine derivatives. Prior to the potential scanning, the dissolved oxygen in electrolyte was removed by bubbling pure N<sub>2</sub> gas (99.99%, Hoshi Medical Co., Ltd., Japan) into the buffer solution at least 15 min. The resulting poly(phenothiazine)-modified CF was soaked in freshly prepared buffer and treated in an ultrasonic bath (40 kHz, AsOne, Japan) to remove unreacted monomer adsorbed on the surface or entrapped in the polymer. Unless otherwise stated, the cyclic scans 25 times were adopted for the preparation of poly(phenothiazine)-modified CF. During this potential sweep period, the solution was constantly stirred by magnetic stirring bar.

### 1.4 Flow injection analysis (FIA)

Figure 1 illustrates schematic diagram of FIA system based on the CF-based flow-through electrochemical detector. The electrochemical work station (ALS 6122A, USA) along with delivery pump of the double-plunger type (DMX-2000, Sanuki Kogyo Co., Ltd., Japan) and sample injector (SV 1-507, Sanuki Kogyo Co., Ltd., Japan) was used for flow-injection experiments. A home-made three-electrode flow-through cell is composed of poly(phenothiazine)-modified CF, platinum counter electrode, and Ag/AgCl (3 mol/L NaCl) reference electrode (RE-1B, BAS, USA). Counter electrode was opposed to the working electrode (the gap: ca. 2 mm), and reference



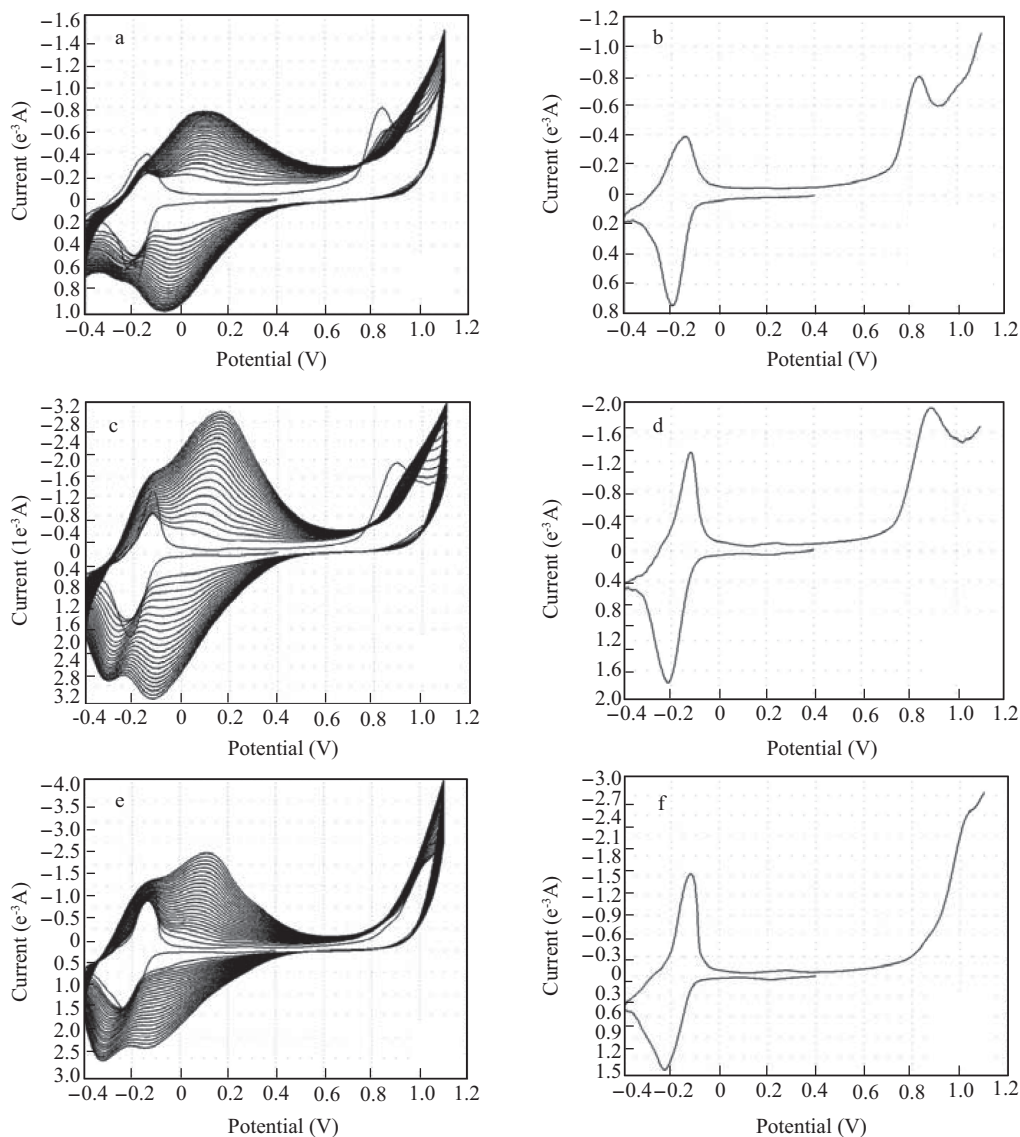
**Fig. 1** Schematic diagram of the CF-based flow-injection manifolds for the determination of NADH. D: CF-based electrochemical flow-through detector; EW: electrochemical workstation.

electrode was positioned at down stream of the working and counter electrodes. The working potential of +0.2 V (vs. Ag/AgCl) was applied during the FIA experiments. 0.1 mol/L phosphate buffer (pH 7.0) was used as a carrier solution (typically at 4.1 mL/min). After the background current had decayed to a steady-state value, NADH standard solutions (200 µL) were injected, and anodic peak current response (current-time output) was monitored.

## 2 Results and discussion

### 2.1 Electrochemical properties of poly(phenothiazine)-modified CFs

The phenothiazine derivatives can be polymerized by anodic oxidation from neutral aqueous media (Karyakin et al., 1999). The polymerization process involves the formation of cation-radical upon the electro-oxidation. Figure 2 shows cyclic voltammograms (CVs) recorded during the electro-oxidative polymerization of TN (Fig. 2a, b), TB (Fig. 2c, d) and MB (Fig. 2f, e). The new set of peaks (at the potential region from -0.2 to +0.2 V) increased during the repeated potential scans, which is shifted to the positive potential region compared with the redox activity of the monomers (Fig. 2b, d, and f). These sets of peaks are attributed to polymer-type redox activities, and indicates the growth of a redox active film on the CF surface. At high anodic potentials (more than 0.8 V), irreversible oxidation of phenothiazine monomer occurred



**Fig. 2** Cyclic voltammograms (CVs) of the electropolymerization of Thionine (a, b), Toluidine Blue (c, d), and Methylene Blue (e, f) onto the CF electrodes. Potential sweep rate was 50 mV/sec; starting potential was +0.4 V vs. Ag/AgCl and cycling was performed between -0.4 and +1.1 V vs. Ag/AgCl; electrolytes were 0.1 mol/L phosphate buffer (pH 7.0) containing 0.1 mol/L phenothiazine derivatives. (a), (c) and (e) were CVs recorded 25 cycles, and (b), (d) and (f) were 1st cycle.

and leads to appearance of oxidation peaks at 0.84 V for TN, 0.90 V for TB and 1.05 V for MB. These oxidation peaks are due to the formation of cation-radical species of primary and secondary amino groups of phenothiazine substitutes. These cation-radicals were obviously followed by chemical reactions finally resulting in polymerizations (Karyakin et al., 1993). Among these phenothiazines, TB showed the largest peak currents of polymer type redox activity after 25th cycle (Fig. 2c). In addition, preparation reproducibility of the poly(TB)-film was superior to poly(TN)- and poly(MB)-films. In the case of poly(TB)-film, covalent bonding formed via aromatic ring; namely amino group binds to aromatic ring "head-to-tail" bonding, which is similar to polyaniline structure (Karyakin et al., 1999).

Figure 3 shows the CVs of poly(phenothiazine)-modified CFs at various potential scan rate in 0.1 mol/L phosphate buffer (pH 7.0). In all cases, there are two redox couples. The negative couple correspond to a monomer-type redox activity, which is entrapped within the polymer film. Figure 3d is the dependency of cathodic peak current of polymer-type redox activities on the square root of scan rate ( $v^{1/2}$ ). In all cases, the anodic peak current increases linearly with the square root of scan rate between 0.01 and 0.1 mV/sec, which indicates that the reduction and the oxidation processes within the poly(phenothiazine) films are diffusion-controlled.

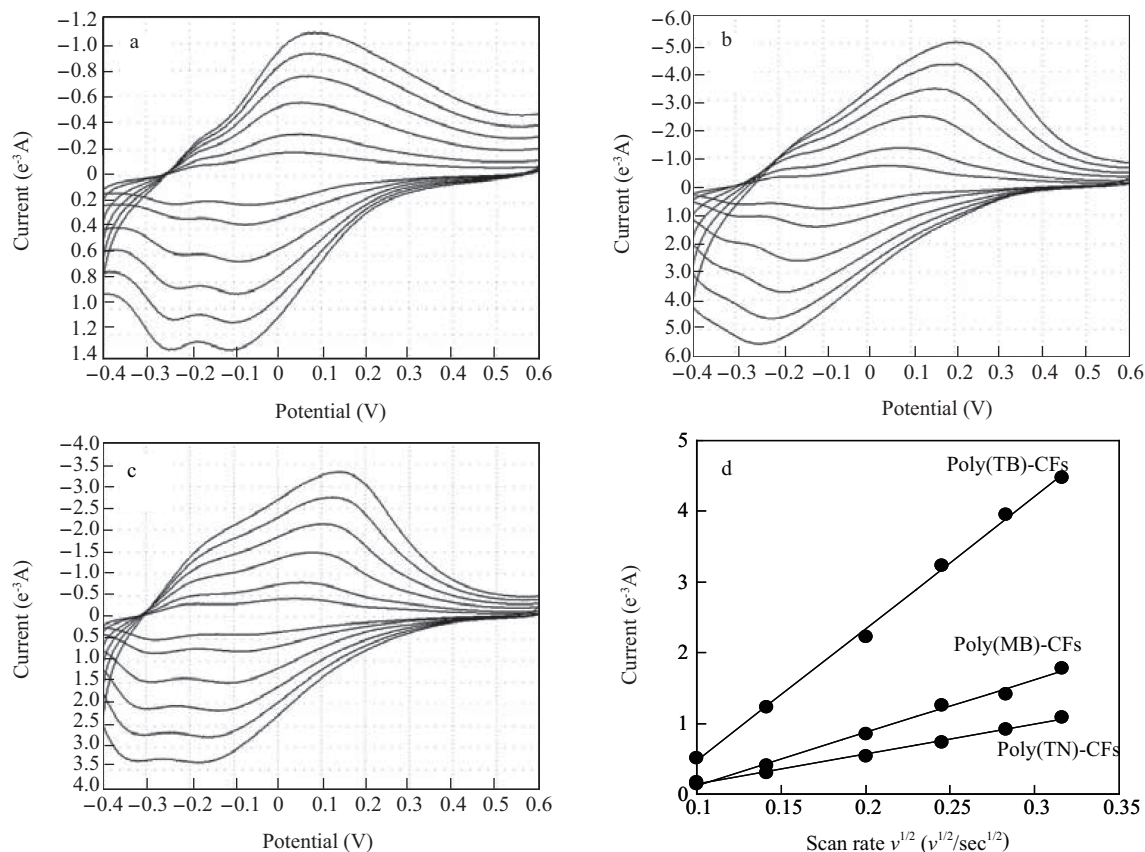
Figure 4 shows the CVs of poly(phenothiazine)-modified CFs demonstrating the electrocatalytic activity

for the oxidation of NADH in 0.1 mol/L phosphate buffer (pH 7.0). Before adding NADH, all poly(phenothiazine)-modified CFs exhibited two redox couples. After adding 1 mmol/L NADH, the oxidation current of polymer-type redox species increased, although the monomer-type redox wave almost no changed. These results indicate that the catalytic efficiency of polymer-type redox species is much higher than that of monomer-type redox species. The anodic peak potential obtained with NADH was ca. +0.3 V for poly(TN)-modified CF and ca. +0.2 V for poly(TB)- and poly(MB)-modified CFs. This voltamogram in the presence of NADH showed a large negative shift in the anodic peak potential of about 0.3–0.4 V, as compared with anodic peak potential (+0.6 V) that obtained at bare CF (data not shown).

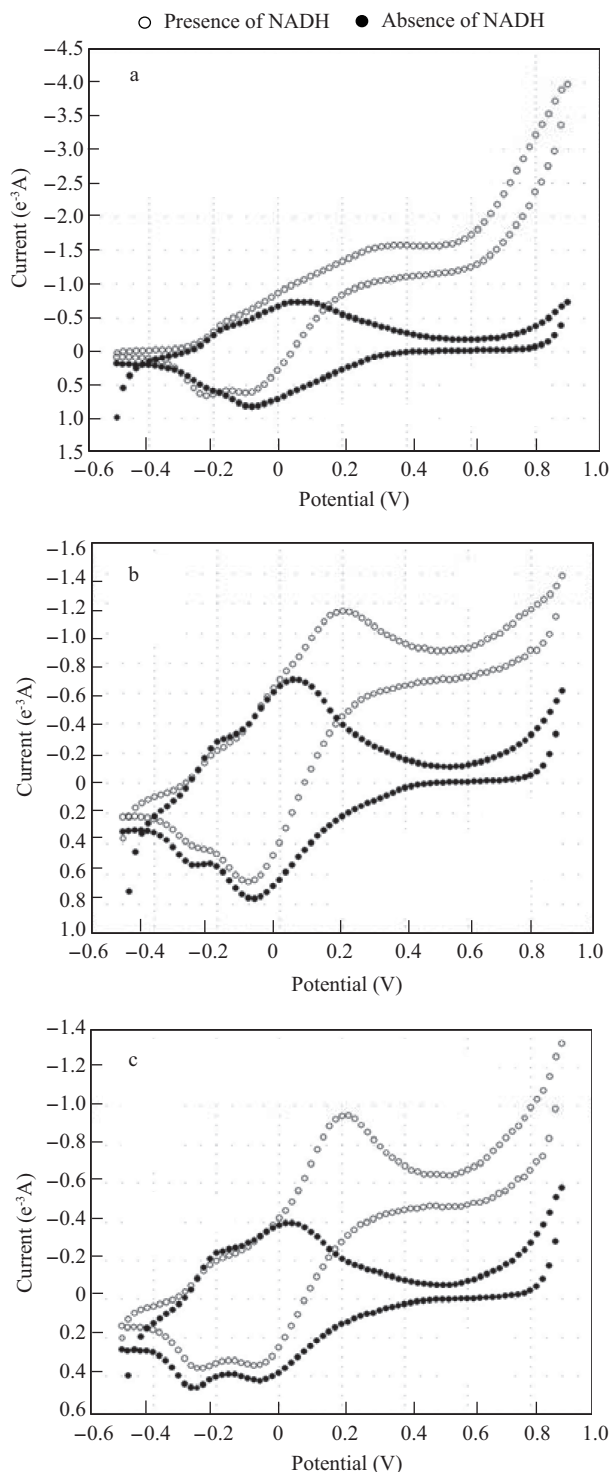
These behaviors are essentially similar to previous reports regarding on poly(TN)-film (Ohsaka et al., 1993; Tanaka et al., 1993; Gao et al., 2003), poly(TB)-film (Zeng et al., 2006) and poly(MB)-film (Karyakin et al., 1994; Silber et al., 1996). Among these three poly(phenothiazine)-modified CFs, the poly(TB)-modified CF exhibited the highest and the most reproducible catalytic currents, thus we selected poly(TB)-modified CF for subsequent flow-injection analysis experiments.

## 2.2 Flow injection analysis

Based on the result of CVs for the electrocatalytic oxidation of NADH (Fig. 4) by the poly(TB)-modified CF, we selected +0.2 V vs. Ag/AgCl as an applied potential

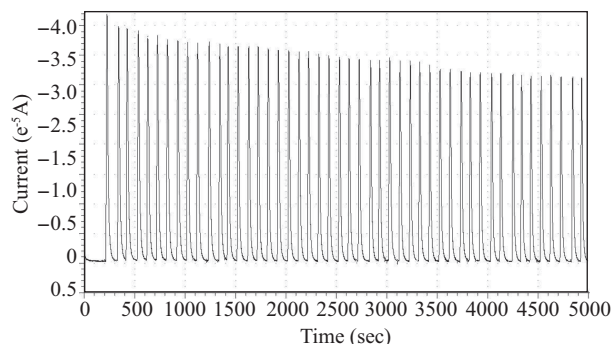


**Fig. 3** CVs of the poly(TN)- (a), poly(TB)- (b), and poly(MB)- (c) modified CFs at various scan rates. Potential scan rates; from inner to outer: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1 V/sec. Electrolyte was 0.1 mol/L phosphate buffer (pH 7.0). Starting potential was +0.6 V vs. Ag/AgCl. (d) relationship between cathodic peak currents and square root of scan rate obtained by poly(TB)-, poly(MB)- and poly(TN)-CFs.



**Fig. 4** CVs of poly(TN)- (a), poly(TB)- (b) and poly(MB)- (c) modified CFs in deoxygenized 0.1 mol/L phosphate buffer solution (pH 7.0) in the absence of and the presence of 1 mmol/L of NADH. Potential scan rate was 10 mV/sec. Starting potential was  $-0.5$  V vs. Ag/AgCl.

in the flow-injection experiments. Figure 5 shows typical flow-injection peaks obtained for 48 repetitive injections of 0.1 mmol/L NADH over a period of ca. 5000 sec. The peak current responses tended to gradually decrease during the first several tens of injections. This gradual decay would be attributed to the electrode fouling by oxidized intermediate products, or structural change in the poly(TB)-film. However, after 30–40 injections, it could be regarded as being almost constant, and the signal intensity

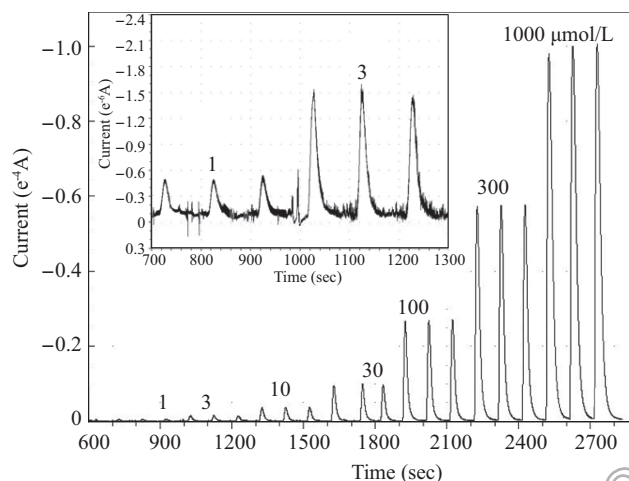


**Fig. 5** Flow-injection peaks obtained for repetitive 48 injections of 0.1 mmol/L NADH obtained by poly(TB)-modified CF-based flow-through amperometric detector. Carrier flow rate was 4.1 mL/min; 0.1 mol/L phosphate buffer (pH 7.0) was used as a carrier; applied potential was  $+0.2$  V vs. Ag/AgCl.

of these peaks was about 74% of the response of the 1st injection. The relative standard deviation of the 39 to 48th peaks was 0.75% ( $n = 10$ ). Therefore, the subsequent experiments were carried out after this “preconditioning” of the poly(TB) film-modified CF, to obtain the constant response.

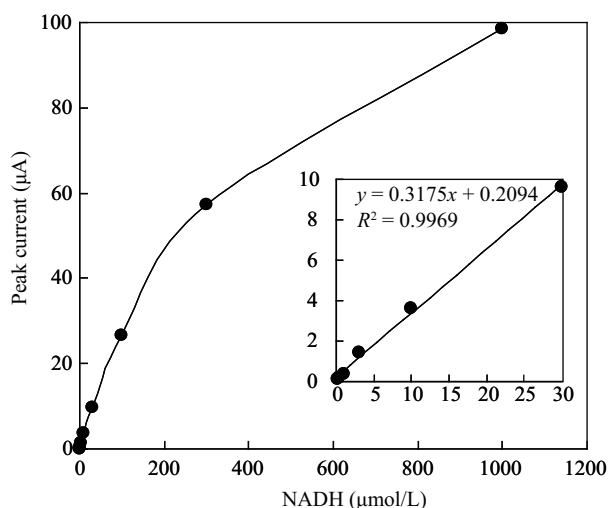
The effect of carrier flow rates on the peak current response for 0.1 mmol/L NADH was examined. Over the range from 2.1 to 4.1 mL/min, peak heights were almost unchanged, indicating that the overall electrocatalytic oxidation of NADH by the poly(TB) film-modified CF is a rate-limiting factors for the response. At a flow rate of 4.1 mL/min, the consecutive determination (ca. 30 sample/hr) was possible.

Figure 6 shows the typical flow-injection response of the poly(TB)-film modified CF for NADH solution of increasing concentration from 0.1 to 1000  $\mu\text{mol/L}$ . Well-defined and reproducible peak responses were observed for NADH solutions more than 1  $\mu\text{mol/L}$ . Figure 7 shows the calibration curves of NADH. The peak currents linearly increased up to 30  $\mu\text{mol/L}$  (slope, 0.318  $\mu\text{A}/(\mu\text{mol/L})$ ; correlation coefficient, 0.997), and the lower detection limit was found to be 0.3  $\mu\text{mol/L}$  ( $S/N = 3$ ). Based on the electrochemical Lineweaver-Burk plot, apparent Michaelis constant  $K_m^{\text{app}}$  was calculated to be 262  $\mu\text{mol/L}$ .



**Fig. 6** Typical flow-injection peaks to various concentration of NADH obtained by poly(TB)-modified CF-based flow-through amperometric detector. Other experimental conditions were the same as Fig. 5.





**Fig. 7** Calibration curve of NADH obtained by poly(TB)-modified CF-based flow-through amperometric detector. Inset graph is enlargement of lower concentration region. Each plot is an average of three measurements.

The sensitivity of this system based on the poly(TB) film-modified CF was superior to previous reports using disk electrodes (Silber et al., 1996; Ohsaka et al., 1993; Tanaka et al., 1993; Karyakin et al., 1994; Gao et al., 2003; Zeng et al., 2006), probably due to the features of CF (i.e., highly effective surface area, high porosity, and high electrolytic efficiency).

### 3 Conclusions

The poly(phenothiazine) film-modified CFs were prepared on the porous CF surface by electro-oxidative polymerization from 0.1 mol/L phosphate buffer (pH 7.0). Among TN, TB and MB, the poly(TB) film-modified CF exhibited higher and more stable electrocatalytic activities for the oxidation of NADH. The poly(TB)-film modified CF was successfully used as a working electrode unit of highly sensitive amperometric flow-through detector for NADH, which allows the determination of micro-molar level of NADH. This CF-based NADH-sensing system would be applicable to dehydrogenase-based various biosensors by combining the dehydrogenase-immobilized reactor or further modification of dehydrogenase onto the poly(TB) film.

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