Biosensors and Bioelectronics 33 (2012) 95–99

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

# Simultaneous detection of NADH and $H_2O_2$ using flow injection analysis based on a bifunctional poly(thionine)-modified electrode

Selvaraj Baskar, Jen-Lin Chang, Jyh-Myng Zen*

*Department of Chemistry, National Chung Hsing University, Taichung 402, Taiwan*

ARTICLE INFO

*Article history:*
Received 27 October 2011
Received in revised form
13 December 2011
Accepted 14 December 2011
Available online 8 January 2012

*Keywords:*
Poly(thionine)
Preanodized screen printed carbon electrode
NADH
Hydrogen peroxide

ABSTRACT

We report here a novel detection scheme for simultaneous detection of NADH and $H_2O_2$ based on a bifunctional poly(thionine)-modified electrode. Electropolymerization of thionine on a "*preanodized*" screen-printed carbon electrode effectively lowers the oxidation potential of NADH to 0.15 V (vs. Ag/AgCl). Since poly(thionine) is also a well known electrochemical mediator for $H_2O_2$ reduction, we further developed a poly(thionine)-modified ring disk electrode for simultaneous measurement of nicotinamide adenine dinucleotide (NADH) and hydrogen peroxide ($H_2O_2$) by flow injection analysis. By applying the optimized detection potentials of 0.2 V and −0.2 V at disk and ring electrodes, respectively, this system allows the simultaneous measurement of both analytes with good sensitivity (0.13 μA/mM for $H_2O_2$ and 0.34 μA/mM for NADH) and limit of detection (1.74 μM and 26.0 μM for NADH and $H_2O_2$). This opens the possibility of a whole series of biosensor applications.



## 1. Introduction

A great deal of research has been devoted to developing biosensors by coupling a selected oxidase or dehydrogenase enzyme with an effective amperometric detector of hydrogen peroxide ($H_2O_2$) or of nicotinamide adenine dinucleotide (NADH) (Gorton, 1986; Bartlett et al., 1991). Both NADH and $H_2O_2$ play an important role in biological processes. However, most of the enzyme based biosensors which have been developed use neither oxidase nor dehydrogenase and they detect subsequent product from the biological reaction to quantify the analyte concentration. Zhang et al. (1997) used both lactate oxidase and lactate dehydrogenase and improved sensitivity and detection limits by 8 and 5 times respectively, compared to those of single-enzyme systems. Wang et al. (2001) used glucose oxidase and glucose dehydrogenase, which produce $H_2O_2$ and NADH respectively, for the measurement of glucose and proposed that the integration of additional functional element like a low cost single use screen printed carbon electrode (SPCE) offers decentralized clinical testing of glucose.

There are also some studies of biological processes involving both NADH and $H_2O_2$. These include, oxidation of NADH by $H_2O_2$ (Hogg and Jago, 1970), oxidation of NADH by vanadium in the presence of $H_2O_2$ (Stankiewicz et al., 1991; Vijaya and Ramasarma, 1984), interference from NADH on detection of $H_2O_2$ with amplex red (Votyakova and Reynolds, 2004), oxidation of NADH in the presence of NADH-oxidase reducing $O_2$ to $H_2O_2$ (Jiang and Bommarius, 2004; Radoi et al., 2007), and measurement of serum lactate dehydrogenase activity (Tabata et al., 1994).

Other studies of biological processes which are affected by the presence of one by changes in the concentrations of either one of these molecules have investigated problems regarding modulation of mitochondrial function by $H_2O_2$ (Nulton-Persson and Szweda, 2001), determination of NADH and $NAD^+$ in a single cell under $H_2O_2$ stress (Xie et al., 2009), influence of NADH on redox state of hemoglobin (Olek et al., 2002). Clearly, the development of a dual sensor which catalyzes NADH oxidation and $H_2O_2$ reduction simultaneously can help to develop biosensors using both oxidase and dehydrogenase. Such biosensors can monitor and quantify levels of both NADH and $H_2O_2$ in biological studies and can help us to understand their mechanistic pathways.

Two problems associated with amperometric detection of NADH are the high overpotential required (Schmakel et al., 1975) and the electrode fouling due to the production of radical intermediates during NADH oxidation (Moiroux and Elving, 1979). Some researchers have used redox mediators to decrease the overvoltage required for NADH oxidation and also to avoid the electrode fouling problem (Hayes and Kuhr, 1999; Katakis and Domiguez, 1997). Poly(thionine) and carbon nanotube (CNT) have been used for both NADH oxidation and $H_2O_2$ reduction to decrease the overpotential and increase their sensitivity (Yang et al., 1998; Gao et al.,

* Corresponding author. Tel.: +886 4 22850864; fax: +886 4 22854007.
*E-mail address:* jmzen@dragon.nchu.edu.tw (J.-M. Zen).

0956-5663/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2011.12.025

2003). Since both CNT and thionine can oxidize NADH, they have been combined to detect NADH with lower potential and improved detection limits (Meng et al., 2008).

Herein, we report a novel system which can lower the overpotential of NADH oxidation without the help of any external material like CNT by polymerizing thionine on a "*preanodized*" screen printed carbon electrode (designated as SPCE*). Note that our previous study (Prasad et al., 2007) has demonstrated that a simple preanodization procedure can make the SPCE more electroactive toward NADH oxidation because of the generation of edge plane sites together with oxygen functionalities on the electrode surface during the preanodization process.

In the present paper, we report on the preparation of the poly(thionine)-modified SPCE* and its electrocatalysis for NADH oxidation and $H_2O_2$ reduction. X-ray photoelectron spectroscopy (XPS) was employed to characterize the modified electrodes. A flow injection analysis (FIA) system based on a poly(thionine)-modified ring disk electrode (SPRDE) was subsequently designed for simultaneous measurement of NADH and $H_2O_2$. By coupling the modified electrode with a wall-jet electrochemical cell, the NADH oxidation reaction at the disk electrode and the reduction of $H_2O_2$ at the ring electrode can be individually monitored. This system could, in fact, easily be coupled with any desired oxidase or dehydrogenase enzyme to detect many important biological analytes.

## 2. Experimental

### 2.1. Reagents and electrodes

All reagents were bought from Sigma and used as received and all aqueous solutions, were prepared with Millipore deionized water (18 MΩ cm$^{-1}$). Both NADH and $H_2O_2$ 10 mM stock solutions were prepared with mobile phase and stored under refrigeration. Carrier and sample solutions were prepared by suitable dilution of the stock solution before experiments. Both the SPCE (geometric area = 0.196 cm$^2$) and RDSPCE (disk = 3.14 mm$^2$ and ring = 7.07 mm$^2$, respectively) were purchased from Zensor R&D (Taichung, Taiwan). The measured average resistance was 85.64 ± 2.10 Ω cm$^{-1}$.

### 2.2. Apparatus

Electrochemical measurements were performed at a CHI 900 electrochemical workstation (Austin, TX, USA) with a three-electrode cell assembly. The dynamic amperometric experiments were carried out using an FIA system constructed with a Cole-Parmer microprocessor pump drive (BAS, PM-92E), a Rheodyne model 7125-sample injection valve (20 μL loop) with interconnecting Teflon tube, and a Zensor SF-100 electrochemical cell specifically designed for RDSPCE. Note that the flow injection system coupled with SPRDE was described in our previous reports (Hsu et al., 2006; Yang et al., 2009). X-ray photoelectron spectroscopy analysis (American Physical Electronics, Auger 670 PHI XI/ESCA PHI 1600) was performed at an Mg Kα radiation source (1252.6 eV) with a resolution of 0.1 eV (pressure inside the analyzer was maintained at 10$^{-9}$ Torr).

### 2.3. Analytical method

The fabrication of bare SPCE, SPCE/poly(thionine), SPCE*/poly(thionine) and RDSPCE*/poly(thionine) and the study of their electrochemical behavior was carried out by cyclic voltammetric method. The simultaneous detection of NADH and $H_2O_2$ at the RDSPCE*/poly(thionine) was accomplished by using an amperometric method integrated with an FIA system. All the detections of NADH and $H_2O_2$ were carried out without any sample treatment.

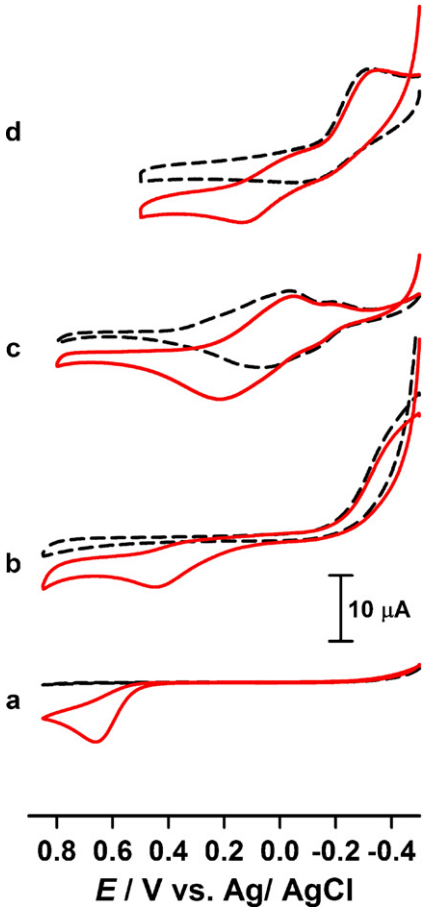


**Fig. 1.** Cyclic voltammetric responses of a SPCE (a), SCPE* (b), SPCE/poly(thionine) (c) and SPCE*/poly(thionine) (d) in 0.1 M, pH 7 PBS in the presence (solid line) and absence (dotted line) of 1 mM NADH at a scan rate of 10 mV s$^{-1}$.

The SPCE working electrode was equilibrated in 0.1 M, pH 7.4 PBS by cycling between 0.0 and 1.3 V at a scan rate of 100 mV s$^{-1}$ for 10 segments before being used in other electrochemical experiments. The SPCE*was prepared by applying 2.0 V for 300 s in 0.1 M, pH 7.4 PBS. The SPCE*/poly(thionine) was prepared by cycling between −0.5 and 1.1 V at 50 mV s$^{-1}$ in 100 μM thionine/0.1 M, pH 7 PBS on an SPCE* for 40 segments and then stabilized in the PBS solution. The same conditions as those used for SPCE*/poly(thionine) were used to prepare the RDSPCE*/poly(thionine). Curve fitting in the X-ray spectrum was performed by XPSPEAK41 system software (Version 4.1).

## 3. Results and discussion

### 3.1. Electrochemical behavior of NADH

Fig. 1 compares the cyclic voltammograms for the oxidation of NADH at various electrodes of a bare SPCE (a), SPCE* (b), SPCE/poly(thionine) (c), and SPCE*/poly(thionine) (d) all in pH 7 PBS. As can be seen, anodic oxidation of NADH takes place at a relatively high potential of ∼0.7 V (vs. Ag/AgCl) on a bare SPCE; whereas the SPCE* exhibits a significant shift in peak potential to ∼0.4 V. As reported in our previous study, this is due to the generation of edge plane sites together with oxygen functionalities on the electrode surface of the SPCE during the preanodization process (Prasad et al., 2007). As to the SPCE/poly(thionine), the NADH oxidation mediated by the redox potential of poly(thionine) occurs at ∼0.3 V with a further shift in oxidation potential compared to that

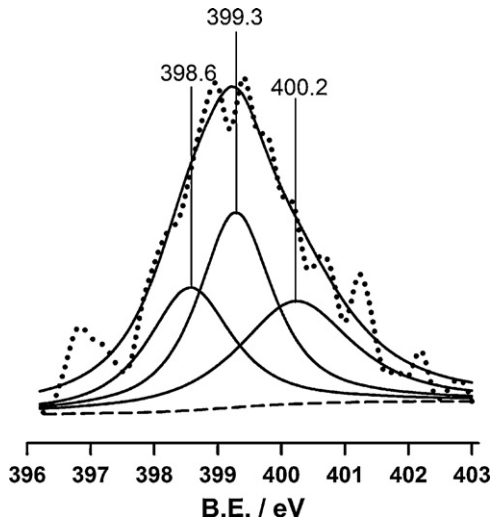


**Fig. 2.** Resolved N1s spectra of the as-prepared poly(thionine).

of the SPCE*. Note that this is consistent with the observation of a poly(thionine) modified electrode prepared by electropolymerization of thionine in neutral phosphate buffer in a study by Gao et al. (2003). The ability of poly(thionine) to decrease the overpotential of NADH oxidation is thus confirmed in this study. Since both SPCE* and poly(thionine) can help to oxidize NADH, we expect that they can combine to detect NADH with lower potential and an improved detection limit. Indeed, as shown in Fig. 1(d), the NADH oxidation shifts to an even lower potential of 0.15 V when poly(thionine) is prepared on the SPCE*. This again is believed to be related to the edge planes and carbonyl functional groups generated during preanodization which help in enhancing the electron transfer behavior, as will be discussed in a later section (Prasad et al., 2008; Banks and Compton, 2005). Overall, the application of SPCE* upon electropolymerization of electron transfer mediator poly(thionine) shows the best performance in decrease of the overvoltage required for NADH oxidation. Moreover, the SPCE*/poly(thionine) was also found to eliminate the electrode fouling problem.

### 3.2. Surface characterization

Since the SPCE*/poly(thionine) shows good electrocatalytic behavior toward the oxidation of NADH, the as-prepared electrode was characterized by XPS analysis. Surface characterization can provide direct evidence for the formation of poly(thionine) as well as for the oxygen functionalities on SPCE*. Our previous studies showed that the preanodization process can increase the edge plane sites on the electrode to cause facile electron transfer in the electrochemical reaction of the SPCE* (Prasad et al., 2007, 2008). Physical characterization of the electrode surface by Raman spectra shows an increase in *D* band (edge plane) at ~1360 $cm^{-1}$ on the SPCE* which proves that the generation of edge planes occurs during the preanodization process (Prasad et al., 2008). The generated oxygen functionalities on SPCE* was also confirmed using XPS analysis (Prasad et al., 2007).

As to the formation of poly(thionine), as shown in Fig. 2, the resolved N1s spectrum gave three different peaks, at 398.6, 399.3, and 400.2 eV. The peak at 398.6 eV corresponds to the heterocyclic nitrogen atom and the peak at 400.2 eV comes from the amino nitrogen. As to the presence of the new state of nitrogen, at 399.3 eV, according to Kessel and Schultze (1990) and Camalli et al. (1990), this kind of nitrogen atom might originate from the bonding between an amino nitrogen atom in one thionine molecule and a carbon atom in another one. Thus, this peak shows that the

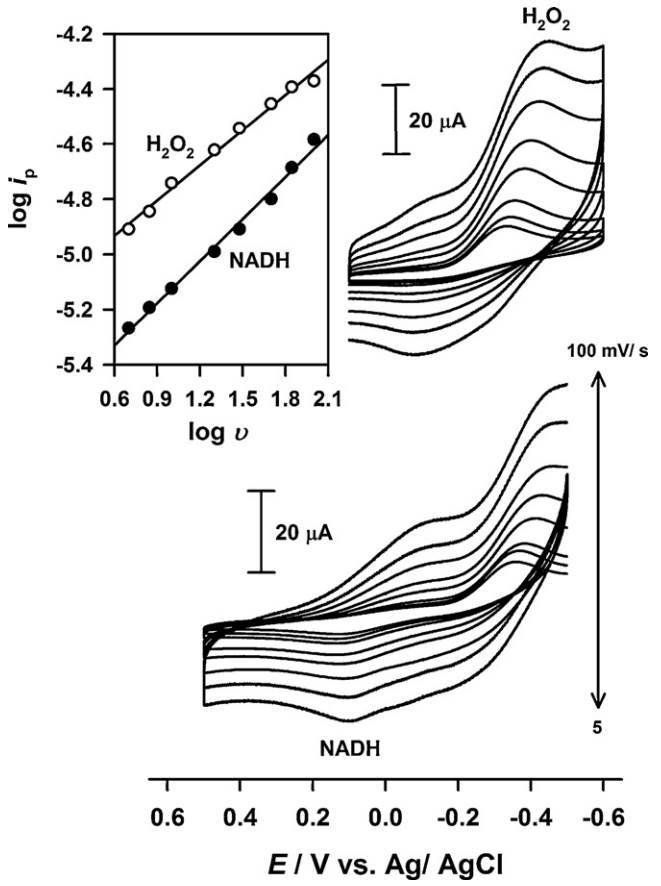


**Fig. 3.** Cyclic voltammetric responses of SPCE*/poly(thionine) in 1 mM NADH and 1 mM $H_2O_2$ at various scan rate and the plot of log($\upsilon$) vs. log($i$) for NADH and $H_2O_2$.

generation of polymer and the electropolymerization of thionine took place under our experimental conditions.

### 3.3. Analytical application

As stated earlier, we aimed at the development of a detection scheme for biosensor applications based on the above described bifunctional poly(thionine)-modified electrode. Fig. 3 exhibits the cyclic voltammograms of the SPCE*/poly(thionine) in the presence of 1 mM each of NADH and $H_2O_2$ at various scan rates. The inset shows the relationship between peak current and scan rate for NADH and $H_2O_2$. A slope of ~0.5 from the log($i_p$) vs. log($\upsilon$) plot suggests that both the oxidation of NADH and the reduction of $H_2O_2$ are diffusion-controlled at the SPCE*/poly(thionine). Since the use of a disposable sensor together with the application of FIA is certainly attractive in terms of practical application, a dynamic amperometric method coupled with the FIA system was developed to detect NADH and $H_2O_2$ simultaneously.

To evaluate the possibility for simultaneous detection of NADH and $H_2O_2$, the interference between NADH and $H_2O_2$ was studied using the SPCE*/poly(thionine). Fig. 4(A) provides a schematic representation of the proposed SPRDE system for simultaneous detection of NADH and $H_2O_2$ by FIA. The cyclic voltammograms obtained for the detection of NADH, $H_2O_2$ and a mixture of NADH and $H_2O_2$ show that virtually the same electrochemical behavior and peak current of NADH occurred in the absence/presence of 1 mM $H_2O_2$ (Fig. 4(B)). Similarly, no interference was observed from NADH in the detection of $H_2O_2$ (Fig. 4(C)). Since the signals of NADH and $H_2O_2$ at disk and ring electrodes can be individually monitored without interference from each other, the possibility of using

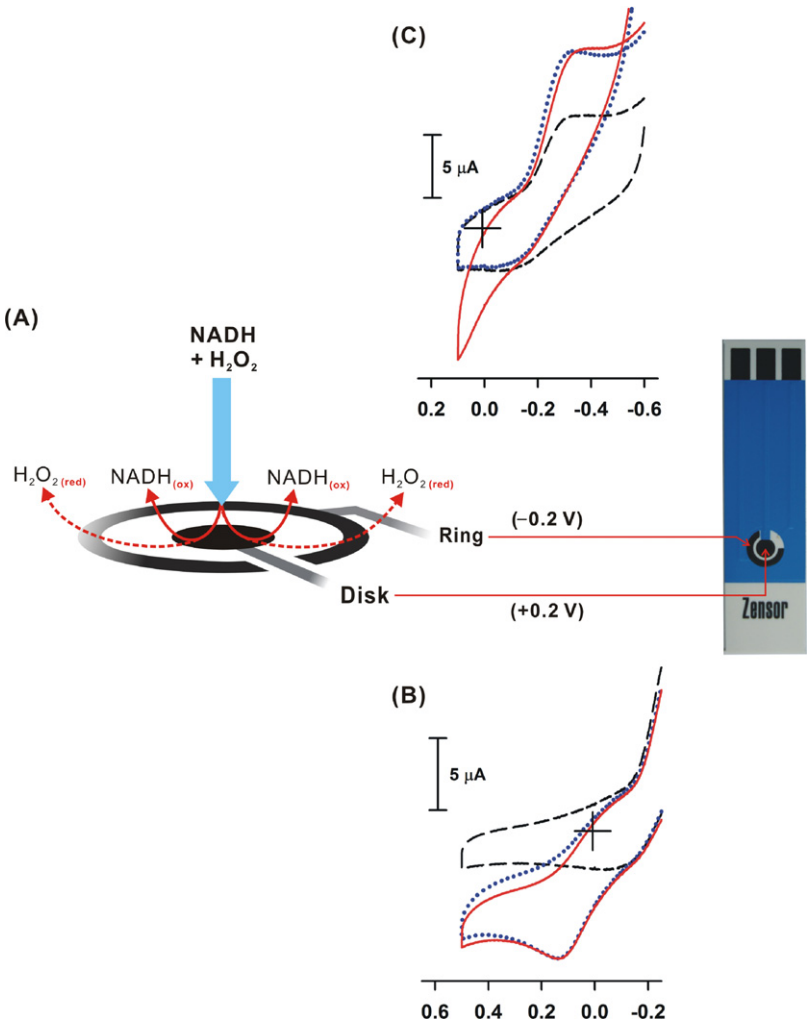


**Fig. 4.** (A) Schematic representation for simultaneous detection of NADH and $H_2O_2$ at the RDSPCE*/poly(thionine) by FIA. Cyclic voltammetric responses of NADH (B) and $H_2O_2$ (C) at the SPCE*/poly(thionine) in pH 7 PBS (dashed line), 1 mM NADH (solid line) and 1 mM NADH + $H_2O_2$ mixture (dotted line) a scan rate of 10 mV $s^{-1}$. Insert shows the photograph of a RDSPCE.

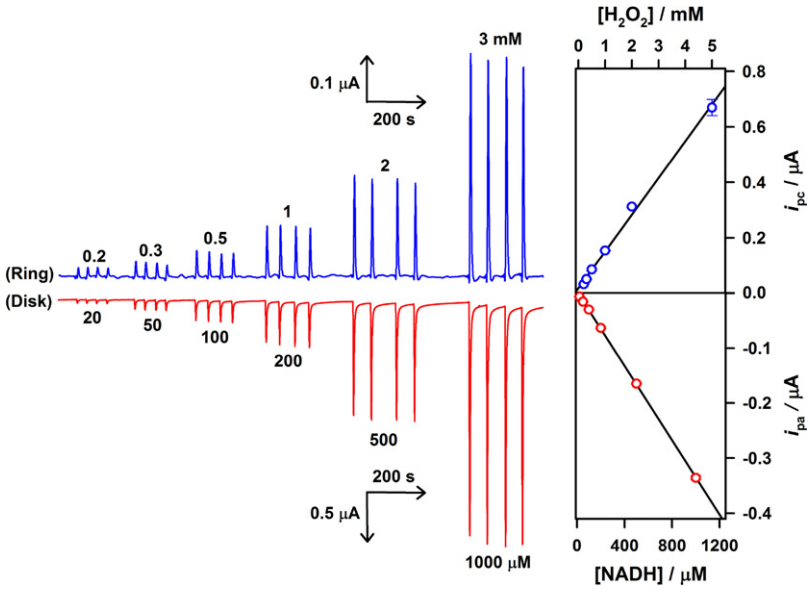


**Fig. 5.** Typical FIA responses and the obtained calibration curves for simultaneous detection of NADH and $H_2O_2$ at a flow rate of 0.7 mL $min^{-1}$ with applied potential of 0.2 V at disk electrode for NADH and −0.2 V at ring electrode for $H_2O_2$.

this bifunctional poly(thionine)-modified electrode for simultaneous detection of NADH and $H_2O_2$ in practical applications seems very promising.

In order to optimize the detection potentials of the ring and disk electrode for the best sensitivity in NADH and $H_2O_2$ determination, the detection potential were evaluated separately. Based on the hydrodynamic voltammograms of 100 μM NADH and 500 μM $H_2O_2$ in pH 7 PBS as carrier solution, the FIA responses for NADH show better results in the range of 0.15–0.3 V with the highest peak current at 0.2 V. A detection potential of 0.2 V at the disk electrode was therefore used in subsequent studies for NADH detection. In the case of $H_2O_2$, the signal was found to increase with the increase in potential negatively from −0.1 to −0.5 V. Since higher background signals were observed when the detection potential was more negative than −0.2 V, a detection potential of −0.2 V at the ring electrode was selected in subsequent studies on $H_2O_2$ detection. Note that the optimized detection potential of 0.2 V and −0.2 V at disk and ring electrode to determine NADH and $H_2O_2$, respectively, is quite consistent with the observation in cyclic voltammetry as shown in Fig. 3. The effect of flow rate was further examined based on peak current using a carrier solution of pH 7 PBS. The peak responses of both NADH and $H_2O_2$ increase with the increase of flow rate from 0.1 to 0.7 mL $min^{-1}$ and start to decrease beyond 0.7 mL $min^{-1}$. A flow rate of 0.7 mL $min^{-1}$ was thus fixed for the analytical applications. Overall, the RDSPCE*/poly(thionine) electrode was verified for use in the determination of NADH and $H_2O_2$ simultaneously by FIA.

Fig. 5 shows the typical FIA responses observed using the optimum FIA conditions (i.e., detection potential = 0.2 V (disk, NADH) and −0.2 V (ring, $H_2O_2$); flow rate = 0.7 mL $min^{-1}$) for detecting NADH and $H_2O_2$. As can be seen, the peak current of NADH (disk electrode) increased linearly in the concentration range of 20 μM to 1 mM with a slope value of 0.34 (μA/mM) with a detection limit of 1.74 μM. As to the current responses of $H_2O_2$ (ring electrode), a limit of detection of 26.0 μM and a linear range between 200 μM and 5 mM with a slope value of 0.13 (μA/mM) was observed. Furthermore, precision of the proposed system was further evaluated by repeating injections of 200 μM NADH and 500 μM $H_2O_2$. The relative standard deviations ($n$ = 15) for NADH and $H_2O_2$ were 2.9% and 3.0%, respectively. In other words, the SPCE*/poly(thionine) can very effectively eliminate the electrode fouling problem. Overall, the developed system for the detection of NADH and $H_2O_2$ was demonstrated with satisfactory results.

## 4. Conclusion

We have successfully developed a poly(thionine)-modified ring disk electrode for simultaneous detection of NADH and $H_2O_2$ using flow injection analysis. Polymerization of electron transfer mediator poly(thionine) on a disposable SPCE treated by a simple preanodization procedure can effectively lower the overpotential for the oxidation of NADH. This is believed to be related to the edge planes and carbonyl functional groups generated during preanodization to help in enhancing the electron transfer behavior. We have demonstrated that the novel ring-disk electrochemical detection scheme based on this bifunctional poly(thionine)-modified electrode is sensitive and precise for simultaneous detection of NADH and $H_2O_2$.

## Acknowledgment

The authors gratefully acknowledge financial support from the National Science Council of Taiwan.

## References

Banks, C.E., Compton, R.G., 2005. Analyst 130, 1232–1239.
Bartlett, P.N., Tebbutt, P., Whitaker, R.P., 1991. Prog. React. Kinet. 16, 55–156.
Camalli, M., Caruso, F., Mattogno, G., Rivarola, E., 1990. Inorg. Chim. Acta 170, 225–231.
Gao, Q., Cui, X., Yang, F., Ma, Y., Yang, X., 2003. Biosens. Bioelectron. 19, 277–282.
Gorton, L., 1986. J. Chem. Soc., Faraday Trans. I 82, 1245–1258.
Hayes, M.A., Kuhr, W.G., 1999. Anal. Chem. 71, 1720–1727.
Hogg, D.McC., Jago, G.R., 1970. Biochem. J. 117, 791–797.
Hsu, C.-T., Chung, H.-H., Lyuu, H.-J., Tsai, D.-M., Kumar, A.S., Zen, J.-M., 2006. Anal. Sci. 22, 35–38.
Jiang, R., Bommarius, A.S., 2004. Tetrahedron: Asymmetry 15, 2939–2944.
Katakis, I., Domiguez, E., 1997. Mikrochim. Acta 126, 11–32.
Kessel, R., Schultze, J.W., 1990. Surf. Interface Anal. 16, 401–406.
Meng, L., Wu, P., Chen, G., Cai, C., 2008. J. Electrochem. Soc. 155, F231–F236.
Moiroux, J., Elving, P.J., 1979. J. Electroanal. Chem. Interfacial Electrochem. 102, 93–108.
Nulton-Persson, A.C., Szweda, L.I., 2001. Biochem. J. 276, 23357–23361.
Olek, R.A., Antosiewicz, J., Caulini, G.C., Falcioni, G., 2002. Clin. Chim. Acta 324, 129–134.
Prasad, K.S., Muthuraman, G., Zen, J.-M., 2008. Electrochem. Commun. 10, 559–563.
Prasad, K.S., Chen, J.-C., Ay, C., Zen, J.-M., 2007. Sens. Actuators B 123, 715–719.
Radoi, A., Compagnone, D., Devic, E., Palleschi, G., 2007. Sens. Actuators B 121, 501–506.
Schmakel, C.O., Santhanam, K.S.V., Elving, P.J., 1975. J. Am. Chem. Soc. 97 (18), 5083–5092.
Stankiewicz, P.J., Stern, A., Davison, A.J., 1991. Arch. Biochem. Biophys. 287, 8–17.
Tabata, M., Koushima, F., Totani, M., 1994. Anal. Chim. Acta 298, 113–119.
Vijaya, S., Ramasarma, T., 1984. J. Inorg. Biochem. 20, 247–254.
Votyakova, T.V., Reynolds, I.J., 2004. Arch. Biochem. Biophys. 431, 138–144.
Wang, J., Chatrathi, M.P., Ibanez, A., 2001. Analyst 126, 1203–1206.
Xie, W., Xu, A., Yeung, E.S., 2009. Anal. Chem. 81, 1280–1284.
Yang, R., Ruan, C., Dai, W., Deng, J., Kong, J., 1998. Electrochim. Acta 44, 1585–1596.
Yang, T.-H., Liao, C.-Y., Chang, J.-L., Lien, C.-H., Zen, J.-M., 2009. Electroanalysis 21 (21), 2390–2394.
Zhang, W., Chang, H., Rechnitz, G.A., 1997. Anal. Chim. Acta 350, 59–65.