Revue Roumaine de Chimie, **2009**, *54*(9), 755–760

REAGENTLESS AMPEROMETRIC BIOSENSOR FOR NADH DETECTION

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Received March 25, 2009

A sensitive and selective amperometric biosensor, based on NADH-oxidase (NADOx**)** working in tandem with horseradish peroxidase (HRP), which is electrically connected to graphite electrode *via* Os-redox polymer (RP), was developed. Two designs were considered for the biosensor optimization: (i) the first one, [G/HRP-NADOx], exploited the direct electron transfer between HRP and the graphite electrode (G); (ii) the second one, [G/RP-HRP-NADOx], used RP as mediator. In both cases the enzyme matrix was cross-linked using poly(ethylenglycol) diglycidyl ether. The biosensors sensitivities, estimated as the slopes of linear ranges from the calibration curves, recorded in a single line flow injection setup, showed that G/RP-HRP-NADOx electrode (1.62 ± 0.05 mA M^{-1}) presents a higher efficiency than G/HRP-NADOx electrode (0.30 ± 0.03 mA M^{-1}).

INTRODUCTION

Nicotinamide adenine dinucleotide (NADH) is involved in a great variety of enzymatic reactions, being essential for the activity of about 300 dehydrogenases. That is why many attempts were made towards the achievement of sensitive and reproducible electrochemical detection of NADH. It is well-known that the direct oxidation of NADH at bare electrodes takes place at high potentials (> 1V), *via* radical cation intermediates, and the reaction products can easily adsorb onto the electrode surface, causing its rapid fouling.[1] To circumvent this problem, an important strategy consists in using chemically modified electrodes.[2] Different approaches were proposed, when either various mediators were adsorbed onto conventional electrode surface[2] or the electrode material was made from "organic metal", which exploits the electrocatalytic activity of conducting charge transfer complex.[3] However, all these approaches have as major drawback the poor stability of the modified electrodes.

A second main strategy for NADH oxidation is based on using enzymatic systems. Nicotinamide adenine dinucleotide oxidase (NADOx) is a NAD(P)H-flavin-dependent oxidoreductase, which in combination with many dehydrogenases has been successfully used in the biosensors technology for detection of: lactate,[4] malate,[5] acetaldehyde,[6] glutamate[7] or ethanol.[8] The detection schemes employed for these biosensors were based either on the electrochemical oxidation of H_2O_2 at an applied potential of about + 0.6 V *vs.* SCE,[4, 5, 8] or on the electrochemical reduction of H_2O_2[7, 9] at an applied potential between 0 and -0.1 V *vs.* SCE. However, both variants suffer because of electrochemical interferences (induced by high values of applied potentials) or chemical interferences (as a result of direct reaction between hydrogen peroxide and reducing agents existing in real samples).

NADOx, extracted from *Thermus thermophilus*, has been incorporated in mono-[6, 9] or bienzyme biosensors,[10] used for the amperometric detection of NADH. The first approach, using Prussian Blue as mediator for H_2O_2 detection, is characterized by a relatively poor stability. On the other hand, the proposed biosensor is not reagentless, due to the requirement of flavin mononucleotide cofactor in the surrounding solution. The second approach involves a free diffusing mediator (hydroxymethyl ferrocene), which represents a major drawback for the biosensor wide application.

In this work we report on the development of two new biosensor designs using NADOx working in tandem with horseradish peroxidase (HRP). One

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biosensor is based on a mediated detection scheme and the other exploits the direct electron transfer between HRP and the graphite electrode. In the mediated approach, HRP was electrically connected to graphite electrode *via* Os^{II}-redox polymer (RP). The amperometric response of both biosensors was evaluated in batch and in flow-injection systems. The investigated biosensors were both operated at low applied potential (-50 mV *vs.* Ag/AgCl, $KCl_{0.1M}$), where biases from interferences are minimal. Moreover, both proposed designs resulted in truly reagentless biosensors.

EXPERIMENTAL

Reagents and solutions

Recombinant NADOx (E.C. 1.6.99.3 from *Thermus thermophilus*) isolated and purified by GTP Technology, Toulouse, France as reported elsewhere[9] was delivered as an aqueous solution with an activity of 88.8 U mL^{-1} in a buffer containing 50 mM $NaPO_4$ and 250 mM NaCl (pH 7.4). HRP (E.C. 1.11.1.7) was purchased from Sigma Chem. Co. (St. Louis, MO, USA) as a lyophilized powder with an activity of 987 U mg^{-1} solid. NADH was obtained from Sigma-Aldrich (Poole, UK). The osmium redox polymer, (PVI_{10}dmeOs), was prepared by complexing poly(1-vinylimidazole) with [osmium (4,4'-dimethylbipyridine)$_2$Cl]$^{+/2+}$ as described by Ohara *et al.*[11] The two enzymes (NADOx and HRP) were cross-linked to the RP using poly(ethylenglycol) (400) diglycidyl ether (PEGDGE) from Polysciences (Warrington, PA, USA). The supporting electrolyte, used in all experiments, was 0.1 M phosphate buffer solution containing 0.1 M KCl (pH 7.2), prepared from Na_2HPO_4*$2H_2O$ and NaH_2PO_4*H_2O supplied from Merck (Darmstadt, Germany). Standard solutions of NADH were prepared daily by diluting the NADH stock solution (100 mM) with phosphate buffer. All reagents were of analytical grade and were used as received. If not otherwise specified, the solutions were prepared in deionized water (Milli-Q system; Millipore, Bedford, MA).

Biosensors preparation

Two types of biosensors, symbolized as G/HRP-NADOx and G/RP-HRP-NADOx, were prepared. Before modification, rods of spectroscopic graphite (Ringsdorff-Werke GmbH, Bonn-Bad, Germany, type RW001; 3.05 mm diameter) were polished on a wet fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI) and carefully rinsed with distilled water. 5 μL of a mixture containing 1.97 mg mL^{-1} NADOx and 6.67 mg mL^{-1} HRP were deposited on the electrode surface in order to prepare G/HRP-NADOx electrodes. The same procedure was followed to obtain G/RP-HRP-NADOx electrodes with the following mixture composition: 0.98 mg mL^{-1} NADOx, 3.33 mg mL^{-1} HRP, 0.83 mg mL^{-1} PVI_{10}–$Os(Me_2bpy)_2Cl$ and 0.42 mg mL^{-1} PEGDGE (freshly prepared aqueous solution). Each electrode was left to dry at room temperature. If not specified, all results are average values of three similarly prepared electrodes.

Electrochemical measurements

A single line flow-injection (FIA) system, consisting of a manual injection valve (Valco Instruments Co. Inc., Houston, TX, USA) with an injection loop of 100 μL, a peristaltic pump (Alitea AB, Stockholm, Sweden), a flow-through electrochemical cell,[12] a low current potentiostat (Zäta-Elektronik, Höör, Sweden) and a single channel chart recorder (Model BD 111, Kipp & Zonen, Delft, The Netherlands), was used to characterize the amperometric biosensors. The tubing connecting the peristaltic pump to the electrochemical cell was made of Teflon (0.5 mm i.d.).

The amperometric batch measurements were performed in a conventional three-electrode cell, under constant magnetic stirring by using a computer-controlled voltammetric analyzer BAS CV 50W (Bioanalytical Systems, West Lafayette, USA).

In both systems, the enzyme-modified graphite electrodes were used as working electrodes. The reference was a Ag/AgCl, $KCl_{0.1\ M}$ electrode and a Pt wire was used as counter electrode.

RESULTS AND DISCUSSION

Oxidase-based amperometric biosensors, characterized by minimal electrochemical interferences, usually exploit the electrical communication between a peroxidase, working in tandem with the oxidase, and the electrode. Taking into account that HRP is able to sustain direct or mediated electron transfer with carbonaceous electrode materials, two types of NADOx-based amperometric biosensors were developed. The working principle of these biosensors (Figure 1) is defined by the following reaction schemes:

(I) G/HRP-NADOx

$$NADH + O_2 \xrightarrow{NADOx} NAD^+ + H_2O_2 \quad (1)$$

$$\begin{cases} H_2O_2 + HRP_{red} \longrightarrow HRP_{ox} + H_2O & (2) \\ HRP_{ox} + 2e^- + 2H^+ \xrightarrow{electrode} HRP_{red} + H_2O & (3) \end{cases}$$

(II) G/RP-HRP-NADOx

$$NADH + O_2 \xrightarrow{NADOx} NAD^+ + H_2O_2 \quad (4)$$

$$\begin{cases} H_2O_2 + HRP_{red} \longrightarrow HRP_{ox} + H_2O & (5) \\ HRP_{ox} + 2Os(II) + 2H^+ \longrightarrow HRP_{red} + 2Os(III) + H_2O & (6) \\ Os(III) + e^- \xrightarrow{electrode} Os(II) & (7) \end{cases}$$

In both cases, the detection is performed at low applied potential,[13] well placed in the optimal domain for amperometric detection. It is worth mentioning that, due to the RP presence in the second design (Figure 1B), the mediated detection should provide higher biosensor sensitivity than that corresponding to direct electron transfer (Figure 1A).

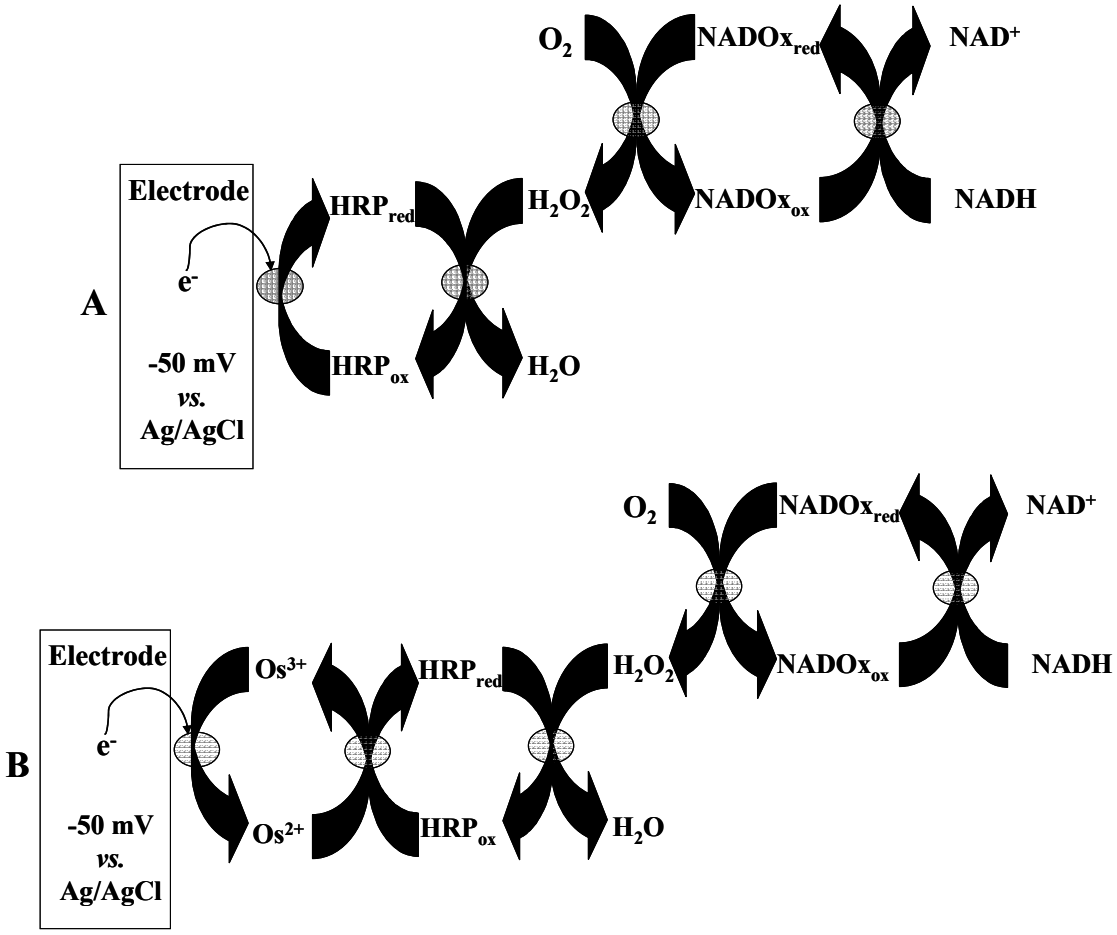

Fig. 1 – The biosensor working principle based on direct (**A**) and mediated (**B**) electron transfer.

As expected, the redox response of the surface confined RP is characterized by a well-defined pair of peaks (Figure 2), placed around 0.15 V *vs.* Ag/AgCl, $KCl_{0.1M}$. The peak current ratio ($I_{p,a}/I_{p,c} \sim 1.09$), the peak split (~100 mV), corroborate with the full widths at half maximum (higher than 90.6 mV[14]), proving that Os(III/II) redox couple exhibits a quasi-reversible behavior.

Comparing the electrochemical response observed at G/RP-HRP-NADOx in the presence and in the absence of NADH (Figure 2), a good electrocatalytic activity towards NADH oxidation was put in evidence. Thus, this effect is confirmed by the clear increase of the anodic peak current, simultaneously with the cathodic peak current decrease.

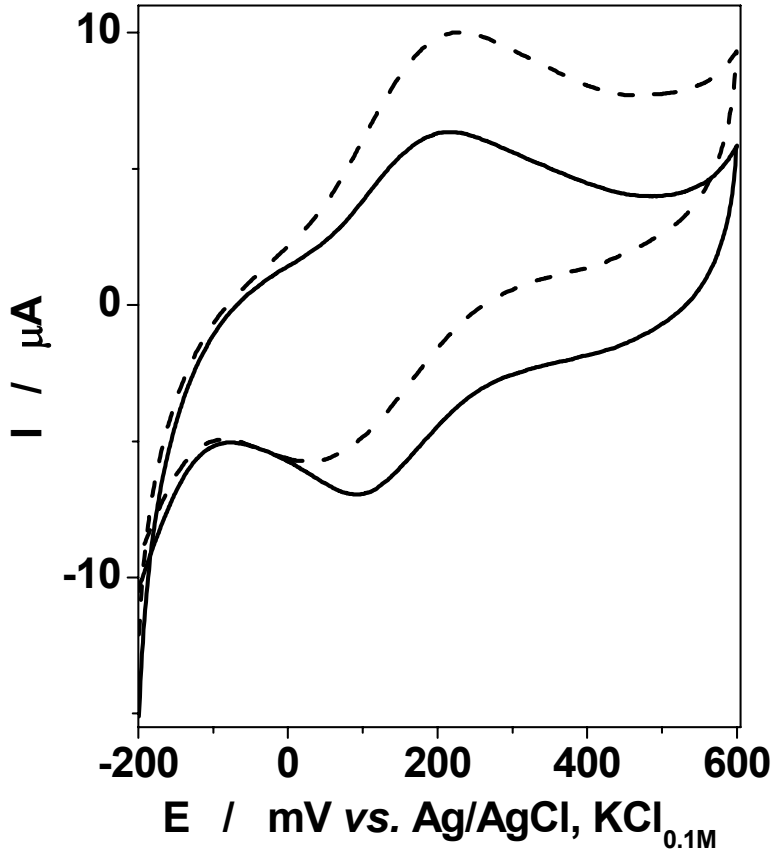

Fig. 2 – Cyclic voltammograms recorded at G/RP-HRP-NADOx electrode in absence (—) and in presence (------) of NADH. *Experimental conditions*: supporting electrolyte, 0.1 M phosphate buffer containing 0.1 M KCl (pH 7.2); starting potential, -200 mV *vs.* Ag/AgCl, $KCl_{0.1M}$; [NADH], 1 mM; scan rate, 5 mV s^{-1}.

The electrocatalytic efficiency, calculated as $EC = \frac{\left(I_{p,a}^{cor}\right)_{[NADH\neq 0]}}{\left(I_{p,a}^{cor}\right)_{[NADH=0]}}$, was found 1.4 (surface coverage of 120 nmol cm^{-2}; $I_{p,a}^{cor}$ stands for the base line corrected anodic peak current).

The amperometric responses to successive NADH additions, recorded at G-HRP-NADOx (Figure 3A) and G/RP-HRP-NADOx electrode (Figure 3B) in "batch" conditions, showed that at chosen applied potential of -50 mV *vs.* Ag/AgCl, $KCl_{0.1M}$, both detection schemes are working. However, the signal corresponding to the second one appears to be more stable.

Due to the relatively fast response, observed for both biosensors in "batch" measurements, further experiments were carried out in a single line flow-injection system (Figure 4). The obtained calibration curves point out that both modified electrodes obey Michaelis-Menten kinetics.

Additionally, Figure 4 clearly shows that G/RP-HRP-NADOx electrode is based on a more efficient detection scheme. This statement is quantitatively supported by the bioelectrochemical parameters listed in Table 1. Thus, irrespective of the method used for the evaluation of these parameters, the highest sensitivity was found for G/RP-HRP-NADOx biosensor. Moreover, the same biosensor exhibits a detection limit four times lower than that corresponding to the biosensor based on direct electron transfer between HRP and the electrode (Table 1).

It is interesting to notice that the sensitivity of G/RP-HRP-NADOx, 30.2 mA M^{-1} cm^{-2}, was ~ 50% higher than that reported for a similar electrode[7], GC/GA-BSA-RP-HRP-NADOx*, 20.5 mA M^{-1} cm^{-2} where BSA stands for bovine serum albumin, GA for glutaraldehyde and NADOx* for nicotinamide adenine dinucleotide oxidase, separated from *Bacillus licheniformis*.

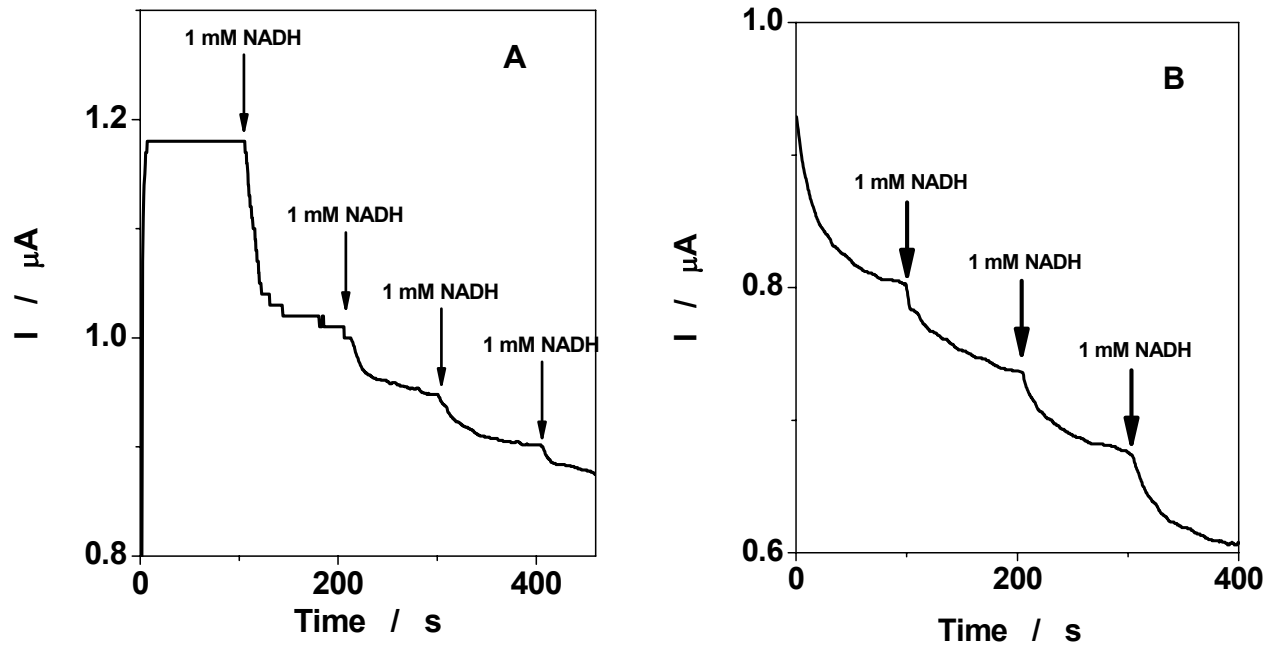

Fig. 3 – Amperometric responses recorded for NADH repetitive additions at G/HRP-NADOx (**A**) and G/RP-HRP-NADOx (**B**) electrodes. *Experimental conditions*: supporting electrolyte, 0.1 M phosphate buffer containing 0.1 M KCl (pH 7.2); applied potential, -50 mV *vs.* Ag/AgCl, $KCl_{0.1M}$.

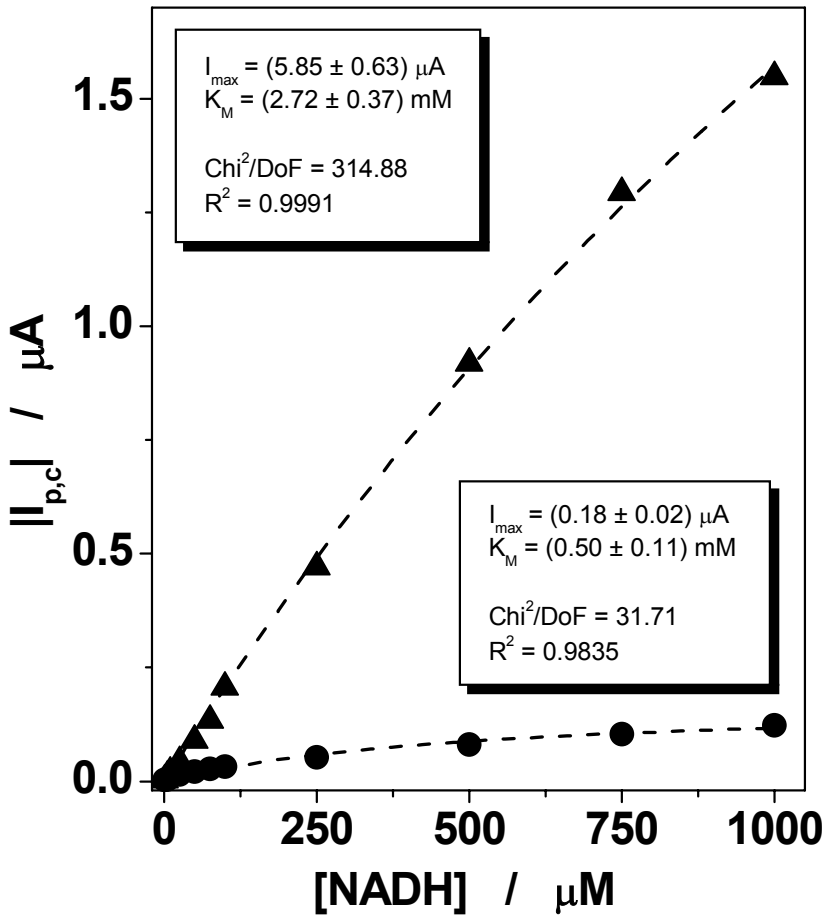

Fig. 4 – Calibration curves for NADH recorded at G/HRP-NADOx (●), and G/RP-HRP-NADOx (▲) electrodes. $|I_{p,c}|$ stands for the absolute value of the cathodic peak current. *Experimental conditions:* supporting electrolyte, 0.1 M phosphate buffer containing 0.1 M KCl (pH 7.2); applied potential, -50 mV *vs.* Ag/AgCl, $KCl_{0.1M}$; flow rate, 0.5 ml min^{-1}; dispersion coefficient, ~2.

Table 1

Bioelectroanalytical parameters for NADH detection at NADOx-modified graphite electrodes. Experimental conditions: see Figure 4

Electrode	Sensitivity (mA M^{-1})		Linear range (μM) R^2 / N	Detection limit* (μM)
	Slope of the linear range	Estimated as I_{max}/K_M ratio		
G/HRP-NADOx	0.30 ± 0.03	0.35 ± 0.06	10 – 100 0.9692 / 6	10.0
G/RP-HRP-NADOx	1.62 ± 0.05	2.14 ± 0.12	10 – 1000 0.9926 / 11	2.8

*estimated for signal / noise ratio equal to 3;
R - correlation coefficient for the linear regression;
N – number of experimental points.

CONCLUSIONS

Two types of NADOx-modified graphite electrodes, exemplifying a new NADH detection scheme, were reported. In both cases NADOx is working in tandem with HRP, but they differ because the last enzyme is electrically connected to the electrode using the direct or mediated (*via* Os-redox polymer) electron transfer.

The bioelectroanalytical parameters of the developed biosensors were compared for NADH amperometric detection. It was clearly demonstrated that the most sensitive biosensor design exploits the excellent electrical connection existing within the HRP-(Os-RP) system. The good electroanalytical performances recommend G/RP-HRP-NADOx electrode as a selective and sensitive sensor for NADH detection. This modified electrode represents a suitable amperometric transducer for constructing biosensors, based on NADH dependent dehydrogenases, useful for detecting various analytes of interest in biotechnological and biomedical applications.

Acknowledgments. The European Commission (NovTech project, contract no: HPRN-CT-2002-00186) and Roumanian National Council of Research in Universities (CNCSIS research grant TD 267/2007-2008) are acknowledged for financial support. GTP Technology France is acknowledged for providing NADOx.

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