

[Os(bpy)₂(PVI)₁₀Cl]Cl polymer-modified carbon fiber electrodes for the electrocatalytic oxidation of NADH

Huangxian Ju¹, Dónal Leech*

Département de Chimie, Université de Montréal, C.P.6128, Succursale Centre-ville, Montréal, Qué., Canada H3C 3J7

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Abstract

Carbon fiber microelectrodes modified with [Os(bpy)₂(PVI)₁₀Cl]⁺ films are described, where bpy is 2,2'-bipyridine and PVI is a poly(vinylimidazole) polymer. The electrochemical properties of the polymer-modified microelectrodes were studied using cyclic voltammetry, chronoamperometry and normal pulse voltammetry. A charge transport diffusion coefficient, D_{ct} , of $(1.6 \pm 0.2) \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ was estimated for these films using chronoamperometry at a modified microdisk carbon fiber electrode. A heterogeneous electron transfer rate constant of $(5.0 \pm 0.5) \times 10^{-4} \text{ cm s}^{-1}$ was evaluated by normal pulse voltammetry at the microcylinder electrode. The polymer-modified microelectrodes exhibit electrocatalytic activity for the oxidation of NADH. An apparent Michaelis–Menten constant of $0.23 \pm 0.02 \text{ mM}$ for the catalytic oxidation of NADH at the osmium-modified microcylinder electrode was evaluated and compared to the value obtained at rotating-disk polymer-modified macroelectrodes. The advantages of the modified microelectrodes for monitoring NADH levels are discussed.

Keywords: Microelectrode; Carbon fiber electrode; Osmium redox polymer; NADH; Modified electrode

1. Introduction

In recent years, there has been much interest in electron transport properties and electrocatalytic applications of redox polymer-modified electrodes. Much of this interest has focused on polymers containing stable redox couples of osmium bis(bipyridine) complexes [1–7]. A simple and convenient synthesis of osmium redox polymers is the substitution of one of the chloride ligands of an Os(bpy)₂Cl₂ complex with the pendant pyridine or imidazole nitrogen of a

preformed poly(vinylpyridine) (PVP) or poly(vinylimidazole) (PVI) polymer. The water-soluble [Os(bpy)₂(PVI)_nCl]Cl, where every *n*th imidazole is complexed to an osmium redox couple, can be conveniently adsorbed at electrode surfaces by dip-coating or drop and evaporation coating procedures [1]. Furthermore these polymers have been extensively used as electron transfer mediators between enzymes, such as glucose oxidase [2–5], lactate oxidase [6,7], peroxidases [8,9], tyrosinase [10] and laccase [11] and macroelectrode surfaces. Microelectrodes have many advantageous features when compared with macroelectrodes, such as small IR drops, larger current densities and application to in vivo detection. Malone et al. [12] have studied the electrocatalytic reduction

*Corresponding author.

¹Permanent address: Department of Chemistry, Nanjing University, Nanjing 210093, China.

of nitrite at an $[\text{Os}(\text{bpy})_2(\text{PVP})_{20}\text{Cl}]^+$ -modified micro-electrode. Heller's group modified a carbon fiber microdisk electrode [5] and carbon fiber arrays [7] with a quaternized $[\text{Os}(\text{bpy})_2(\text{PVP})_{10}\text{Cl}]^+$ polymer, cross-linked to glucose oxidase or lactate oxidase, to yield miniaturized probes for glucose and lactate, respectively.

In this work, we describe the preparation and characteristics of $[\text{Os}(\text{bpy})_2(\text{PVI})_{10}\text{Cl}]^+$ (Os-PVI₁₀)-modified carbon fiber microelectrodes. These modified electrodes show an electrocatalytic activity for the oxidation of NADH and could thus be useful in the construction of miniaturized probes for the detection of dehydrogenase enzymes and their substrates. The nicotinamide adenine dinucleotides (NAD(P)H) are key central electron carriers in biological redox reactions which relate to about 300 dehydrogenases [13]. Thus, selective determination of NADH and NADPH cofactors is very significant. A number of mediators, modified at electrode surfaces, have been developed for electrochemical determination of NADH [13–20]. Gorton and coworkers [13,14] studied NADH electrocatalysis by a series of phenoxazine dyes. Degrand and Miller [15] reported the electrocatalytic oxidation of NADH by 1,2-quinones. Electrodes modified with organic conducting salts [16], phenazines [17,18], poly(thionine) [19], quinone-derivatives [20] and inorganic mediators [21] have also been reported to electrocatalyze NADH oxidation. The use of the (Os-PVI₁₀)-modified carbon fiber microelectrodes in this report provides a simple and sensitive method for the detection of NADH. The Michaelis constant for the electrocatalytic NADH oxidation is determined from the steady-state response at the modified microcylinder electrodes without the need for electrode rotation. Verification of the Michaelis constant by rotating-disk voltammetry at an (Os-PVI₁₀)-modified macroelectrode illustrates the utility of this approach.

2. Experimental

2.1. Chemicals and materials

Poly-*N*-vinylimidazole was prepared by bulk free radical polymerization of vacuum-distilled *N*-vinylimidazole (Polysciences) using azo-isobutyronitrile (AIBN, Aldrich) as initiator. Synthesis and character-

ization of Os-PVI₁₀ was carried out according to literature methods [1,22]. β -Nicotinamide adenine dinucleotide, reduced form (NADH, 98%) was obtained from Sigma and used as received. All other chemicals were of analytical grade. All solutions were prepared with doubly deionized water purified with a Millipore Milli-Q water purification system. Carbon fibers (PAN type) of ca. 8 μm diameter were obtained from the Shanghai Synthetic Fiber Research Institute (China). Spurr low-viscosity embedding media (Polysciences) was used to seal the electrodes in glass rods.

2.2. Apparatus

Electrochemical measurements were performed with a BAS 100B/W electrochemical analyzer using a three-electrode system at room temperature ($24 \pm 1^\circ\text{C}$) placed in a faraday cage, with signal amplification by a PA-1 preamplifier (BAS, West Lafayette). Reference Ag/AgCl electrode (3.0 mol dm^{-3} NaCl, BAS), platinum wire counter electrode and carbon fiber microcylinder or microdisk electrodes were inserted into the cell through holes in the teflon cell cover. An ASFMRX rotator and glassy carbon macroelectrode (Pine Instrument Company, Grove City) were used for rotating-disk studies.

2.3. Procedures

The fabrication of carbon fiber microcylinder electrodes (CFMCE) was similar to that given previously [23]. The length of the cylinder exposed was approximately 3.5 mm, verified under a stereomicroscope. The electrode was rinsed with acetone and water, respectively, dried in air and then immersed in an aqueous solution of Os-PVI₁₀ (2 mg ml^{-1}) for approximately 10 s. The resulting polymer-modified microelectrode was dried in air for 10 min and then immersed into the electrochemical cell.

Carbon fiber microdisk electrodes (CFMDE) were prepared by subsequent polishing of the CFMCE with SiC paper and $0.05 \mu\text{m}$ alumina slurry (Beuhler) followed by ultrasonication in water. A radius of $4.0 \pm 0.4 \mu\text{m}$ for the microdisk electrodes was determined from steady state voltammograms of an acetonitrile solution containing 1.0 mmol dm^{-3} ferrocene with 0.1 mol dm^{-3} tetraethylammonium-perchlorate (TEAP) as supporting electrolyte, using

$2.4 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ as the diffusion co-efficient for ferrocene [24]. Polymer-modified microdisk electrode surfaces were prepared by drop-coating $2.0 \mu\text{l}$ of a 2 mg ml^{-1} Os–PVI₁₀ aqueous solution onto the disk and surrounding epoxy and glass.

Electrochemical measurements were performed in unstirred 0.1 mol dm^{-3} aqueous phosphate buffer, pH 7.4 (PB) unless otherwise stated. The timebase experiments (chronoamperometry) were carried out with a potential step from 0 to +0.5 V.

3. Results and discussion

3.1. Os–PVI₁₀-modified microelectrodes

The cyclic voltammograms (CVs) of an Os–PVI₁₀-modified CFMCE displayed well-defined oxidation and reduction peaks corresponding to the Os(II/III) redox couple, as shown in Fig. 1, yielding an average formal potential of +228 mV (vs. Ag/AgCl). Stable polymer films on the microcylinder electrode were obtained using a simple dip-coating method. The peak-to-peak separations for slow-scan CV (less than 40 mV s^{-1}) were typically less than 20 mV and the peak currents increased in direct proportionality to the scan rate as expected for a surface-immobilized redox couple. With increasing scan rate the peak-to-peak potential separation increased and the scan-rate

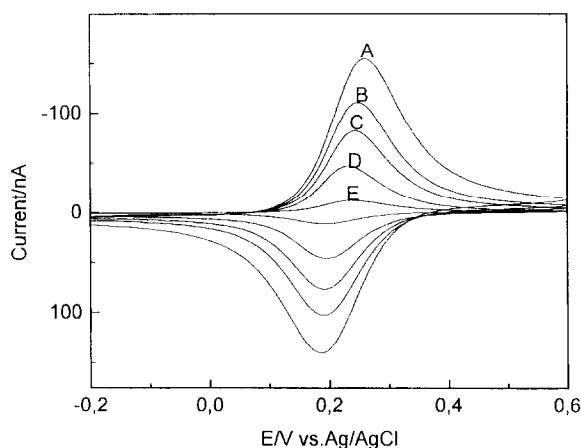


Fig. 1. Cyclic voltammograms of an Os–PVI₁₀-modified CFMCE in PB at scan rates of 100 (A), 60 (B), 40 (C), 20 (D) and 5 mV s^{-1} (E).

dependence of the peak currents became more complicated, indicative of a mixed linear and cylindrical semi-infinite diffusional charge transport within the redox film [25].

The CVs of Os–PVI₁₀-modified microdisk electrodes showed redox peaks similar to those obtained at the modified CFMCE. The peak current obtained at different scan rates for these electrodes was proportional to $v^{1/2}$ from 100 mV s^{-1} to 30 V s^{-1} as expected for a semi-infinite diffusion controlled process at these times. At scan rates less 100 mV s^{-1} , gradually more sigmoidal-shaped CV curves were observed as expected for a mixed thin-layer/microcylindrical response for a redox polymer-modified microdisk electrode [25].

An estimate of the osmium surface coverage can be obtained by integration of the charge passed upon electrolysis of the polymer-modified microcylinder electrode. Surface coverages of approximately $(3.8 \pm 0.6) \times 10^{-9} \text{ mol cm}^{-2}$ are routinely obtained using the dip-coating method. The concentration of electroactive osmium redox centers in the polymer was estimated by dividing the density of polymer by its formal molecular weight. The density of the dry film is approximately 1 g cm^3 [3] giving a concentration of $6.8 \times 10^{-4} \text{ mol cm}^{-3}$. The non-swollen layer thickness of our polymer films is thus estimated at 56 nm, which is about 0.7% of the diameter of the microelectrode.

Potential step chronoamperometry at the modified microdisk electrodes can be used to estimate the charge transport diffusion coefficient, D_{ct} , through the polymers. Although a steady-state response is not achieved, the current does not diminish significantly for times longer than 60 ms following a potential step through the Os(II/III) redox couple. A charge transport diffusion coefficient of $(1.6 \pm 0.2) \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ was estimated from the current at these times using the steady-state equation for microdisk electrodes [26]. This value is close to the results obtained by other researchers [1,27] with osmium-based redox polymer-modified macrodisk electrodes in various electrolytes.

Generally, the heterogeneous electron transfer rate constant for redox polymer-modified macroelectrodes is determined by normal pulse voltammetry (NPV) [28]. At a microcylinder electrode the current–potential relationship in NPV for a simple quasi-reversible

or irreversible electrode reaction can be expressed as [29]

$$E = E^* - \frac{RT}{(1-a)nF} \ln \left[z \left(\frac{1 + 0.571z^2(1 + e^{-\zeta})^2}{1 - z(1 + e^{-\zeta})} \right)^{1/2} \right], \quad (1)$$

$$E^* = E^0 + \frac{RT}{(1-1)nF} \ln \left[\frac{k^0 a}{Dh(\theta)} \right], \quad (2)$$

when $\theta' (=Dt/a^2)$ is less than 3×10^{-4} (i.e. when t , the sampling width, is less than 30 ms in this work), where

$$h(\theta) = 0.575\theta^{-1/2} \exp[5.20 \times \exp(-0.0188(7.5 - \log\theta)^{2.4})],$$

$\theta = D\tau/a^2$, τ = pulse width, $\zeta_- = (nF/RT)(E - E^0)$, $z = I/I_d$ and α is the anodic charge transfer coefficient. Typical normal pulse voltammograms for the oxidation and reduction of Os-PV1₁₀-modified microcylinder electrode are shown in Fig. 2. The sigmoidal shaped voltammograms are similar to those for solution-shaped redox species. With increasing sampling time, the currents decrease, and the half-wave potentials shift to more negative values for oxidation and more positive values for reduction processes. In the analysis of current–potential curves the logarithm of the second term on the right-hand side of Eq. (1) is plotted against the potential, E . For the same electrode process (sweep direction) these plots gave straight lines with similar slopes at different sampling times.

From the slopes and Eq. (1) charge transfer coefficients of 0.41 and 0.70 for oxidation and reduction can be estimated. The heterogeneous electron transfer rate constant, k^0 , obtained from the intercepts of these plots can thus be estimated as $(5.0 \pm 0.5) \times 10^{-4} \text{ cm s}^{-1}$. These values are similar to those obtained by Forster and Vos [1] in various electrolytes using NPV at an Os(PVI)₁₀-modified macrodisk electrode.

3.2. Electrocatalytic oxidation of NADH

Although the reversible potential for NADH/NAD⁺ couple is -0.56 V (vs. SCE) [30] the oxidation of NADH at an unmodified carbon fiber electrode is kinetically slow and requires high overpotentials. Cyclic voltammograms at both unmodified (A) and Os(PVI)₁₀-modified (B) microcylinder electrodes in pH 7.4 phosphate buffer, with and without addition of NADH, are shown in Fig. 3. At the modified electrode the anodic peak and plateau currents increase with increasing NADH concentration while the cathodic currents decrease, indicative of an electrocatalytic reaction. The change in anodic current at the modified electrode, measured at a potential of $+0.5 \text{ V}$, is approximately 35 times larger than the increase observed at the unmodified electrode upon addition of 4.0 mM NADH to the cell. The catalytic currents, i_k , were evaluated as the difference between the peak currents in the presence of NADH analyte, i_p , and the peak current observed in the absence of NADH, $i_{p,0}$. A

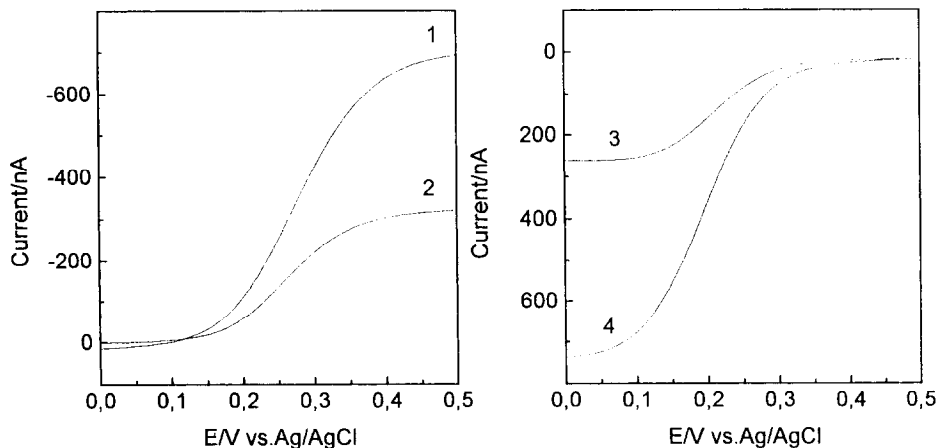


Fig. 2. Typical normal pulse voltammograms for the oxidation (1, 2) and reduction (3, 4) processes of an Os-PV1₁₀-modified CFMCE in PB. Sampling widths of (1, 4) 2 ms and (2, 3) 17 ms were used at a scan rate of 4 mV s^{-1} .

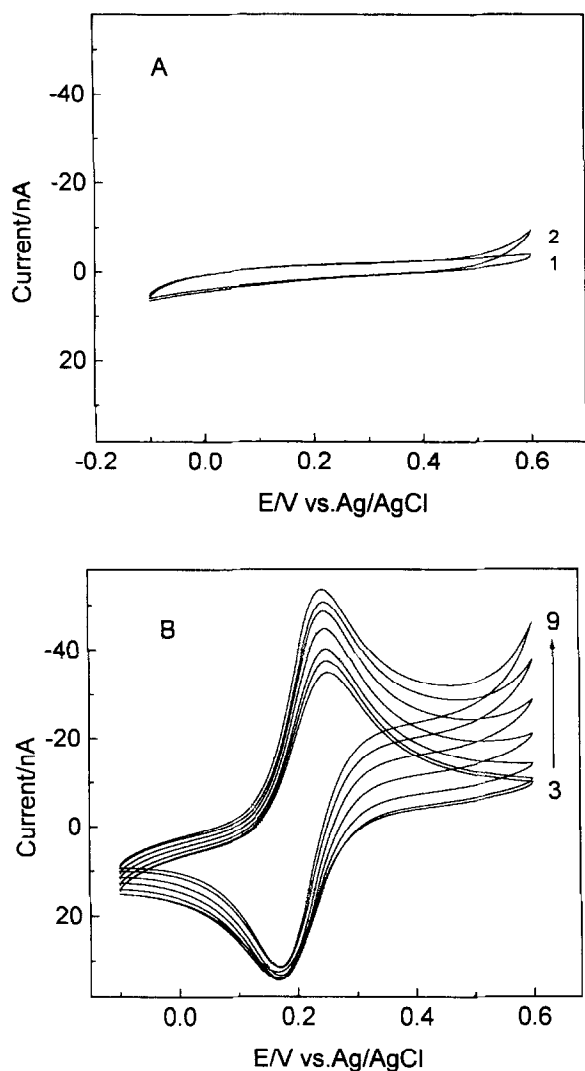


Fig. 3. Cyclic voltammograms of an (A) unmodified and (B) a modified microcylinder electrode in PB following addition of 0 (1 and 3) 0.01 (4), 0.1 (5), 0.4 (6), 1.0 (7), 2.0 (8) and 4.0 mM (2 and 9) of NADH to the cell. Scan rate is 20 mV s^{-1} .

Michaelis–Menten type response is observed for the catalytic current–NADH concentration curve (Fig. 4).

Operation of an amperometric sensor in a flow or steady-state system (batch analysis) at a constant applied potential can eliminate the background response of the catalytic redox couple and simplify data analysis. We have thus investigated the chronoamperometric response of the modified microcylinder electrodes in a static cell following addition of

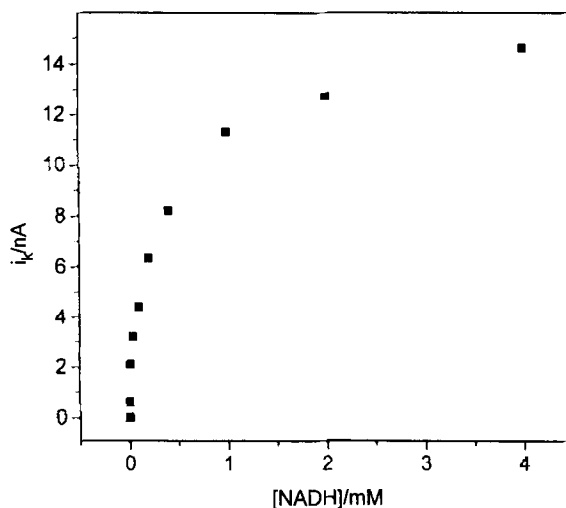
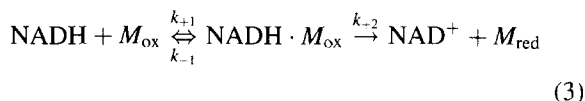


Fig. 4. Dependence of the catalytic current, i_k , on the concentration of NADH added to the cell, conditions as in Fig. 3.

NADH. At various concentrations of NADH all of the response curves reach steady values within a short time period after application of the pulse, without any convective stirring of the solution (Fig. 5). For example, the current reaches 90% of its steady-state value within 10 s for an NADH concentration as high as 4 mM. Furthermore, with the increase of NADH concentration the current increases and a Michaelis–Menten type response can be obtained (insert Fig. 5).

For thin polymeric films, where diffusional transport of both charge and substrate (NADH) can be neglected, the catalytic reaction for oxidation of NADH can be described by the formation and decomposition of an intermediate charge-transfer complex, similar to the kinetic mechanism of Michaelis–Menten (Eq. (3)) [13,14,31]:



with the apparent Michaelis–Menten constant, $K_M = (k_{-1} + k_{+2})/k_{+1}$ and the maximum current, $i_m = nFAk_{+2}$. In this case the steady-state catalytic current, i_k , can be expressed as [31]:

$$i_k = i_m \frac{[\text{NADH}]}{K'_M + [\text{NADH}]} \quad (4)$$

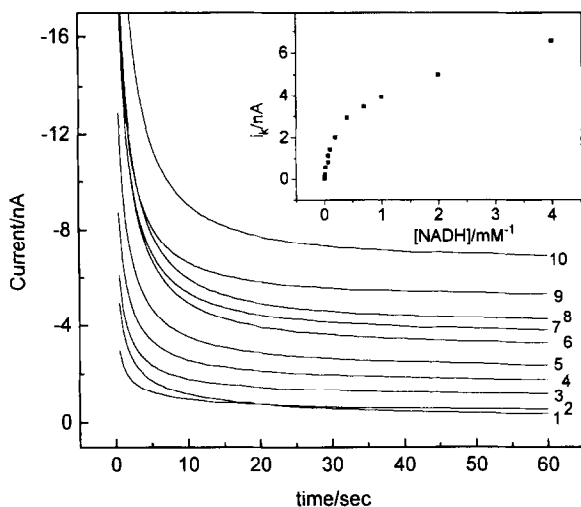


Fig. 5. Chronoamperometric curves obtained following a potential step from 0 to +0.5 V at an Os-PVI₁₀-modified CFMCE in PB containing 0 (1), 0.01 (2), 0.04 (3), 0.1 (4), 0.2 (5), 0.4 (6), 0.7 (7), 1.0 (8), 2.0 (9) and 4.0 mM (10) of NADH. The experiments were performed at the same modified electrode in new solutions.

Various approaches for data analysis have been presented by comparison to the determination of enzyme kinetic parameters [32]. The apparent Michaelis–Menten constant can be evaluated using Lineweaver–Burke (Eq. (5)), Eadie–Hofstee (Eq. (6)) or Hanes (Eq. (7)) transformations of the Michaelis–Menten equation (Eq. (4)) for reaction (3) above and

linear regression analysis of the corresponding plots (Fig. 6).

$$\frac{1}{i_k} = \frac{1}{i_m} + \frac{K'_M}{i_m} [\text{NADH}], \quad (5)$$

$$i_k = i_m - \frac{K'_M i_k}{[\text{NADH}]}, \quad (6)$$

$$\frac{[\text{NADH}]}{i_k} = \frac{K'_M}{i_m} + \frac{[\text{NADH}]}{i_m}. \quad (7)$$

From the slopes and intercepts of these plots an average K'_M of 0.23 ± 0.02 mM and i_m of 4.7 ± 0.2 nA can be evaluated. The apparent Michaelis–Menten constant evaluated is similar to that obtained for the catalytic oxidation of NADH at electrodes modified with adsorbed Meldola Blue (K'_M of 1.1 mM) [13], a Meldola Blue redox polymer (K'_M of 0.4–3.2 mM) [31] and electrodes modified with other 3,7-diaminophenoxazine derivatives such as the 3-anilino-7-dimethylamino-1,2-benzophenoxazonium ion (K'_M of 0.23 mM) [14]. In these reports the K'_M values were evaluated using rotating-disk voltammetry. In order to check the validity of our approach with microcylinder electrodes, we have evaluated K'_M for the catalytic oxidation of NADH at an Os-PVI₁₀ polymer-modified glassy rotating carbon macrodisk electrode. Conventional Koutecky–Levich plots at macroelectrodes modified with a thin polymer film (osmium surface coverage of 8×10^{-9} mol cm^{-2}) were linear, yielding K'_M values of 0.25 ± 0.05 mM. Thus the apparent

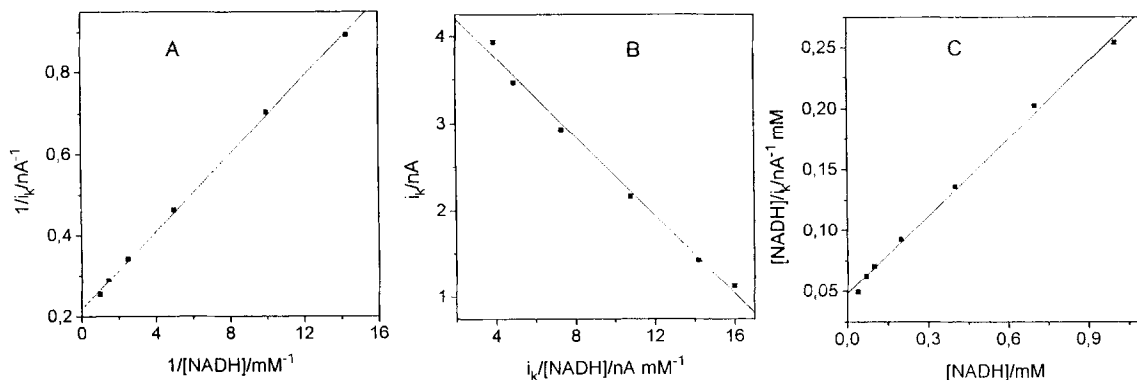


Fig. 6. Lineweaver–Burke (A), Eadie–Hofstee (B) and Hanes (C) analysis of the relationship between the catalytic current and NADH concentration for the determination of the apparent Michaelis–Menten constant at Os-PVI₁₀-modified CFMCEs. The experimental conditions are the same as in Fig. 5.



Fig. 7. Effect of pH on the chronoamperometric catalytic currents observed at an Os-PVI₁₀-modified CFMCE in PBS containing 0.7 mM of NADH. Other experimental conditions as in Fig. 5.

Michaelis–Menten constant evaluated using rotating-disk voltammetry at a modified macroelectrode are similar to the values obtained from analysis of the steady-state catalytic currents at stationary-modified microcylinder electrodes. Analysis using the microelectrodes can thus provide a simple method to study electrocatalytic parameters at mediator-modified electrodes.

The effect of the pH of the sample solution on the response of microcylinder-modified electrodes was examined in the pH range 2.2–12 (Fig. 7). The decomposition rate of NADH increases with increasingly acidic pH below pH 6. In alkaline solutions, the decrease in the catalytic currents could be due to film compaction resulting in restricted charge and substrate transport within the films. The catalytic response to NADH is thus optimum in the pH range 6–8.5.

4. Conclusions

We have demonstrated that microelectrodes modified with thin films of an osmium redox polymer can be useful for the detection of NADH levels. The modified microelectrodes exhibit similar electrochemical characteristics to their macroelectrode counter-

parts. A simple method for the evaluation of apparent Michaelis–Menten constants at these modified microelectrodes is presented and verified by comparison to results obtained at rotating macrodisk-modified electrodes. The steady-state catalytic currents obtained at the microelectrodes for NADH oxidation eliminate the need for rotating-disk experiments for the determination of the apparent Michaelis–Menten constants. The modified microelectrodes yield optimum responses at pH levels 6–8.5, which is useful if potential applications to clinical analysis of dehydrogenase enzymes and their substrates are to be exploited.

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