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An amperometric nitrate reductase–phenosafranin electrode: kinetic aspects and analytical applications

Nancy F. Ferreyra, Velia M. Solís*

INFIQC, Departamento de Físico Química, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Pabellón Argentina, Ciudad Universitaria, Córdoba 5000, Argentina

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Abstract

The enzyme-catalysed reduction of nitrate was studied utilising *Aspergillus niger* nitrate reductase (NR) and phenosafranin in solution as the enzyme regenerator, working at lower potentials than that of the more common methyl viologen mediator. Cyclic voltammograms when enzyme, phenosafranin and substrate were together put in evidence the enzyme-catalysed reduction of nitrate, although with a relatively slow kinetics. From slope values not dependent on mediator concentration, the apparent Michaelis–Menten constant was evaluated.

Analytical parameters for the enzyme-modified electrode in the presence of phenosafranin for the determination of nitrate content in water were assessed, including a recovery assay for nitrate added to a river water sample. The stability of the electrode was checked.

Keywords: Nitrate Reductase; Phenosafranin/Phenosafranine; Nitrate; Enzymatic electrodes

1. Introduction

Although nitrate is not considered a particularly toxic substance, its presence in food, fertilisers and aquatic systems is of concern since this anion is a haemoglobin oxidant [1] as well as the starting point in the synthesis of nitrite and N-nitroso compounds, of recognised carcinogenic activity [2]. There are several analytical methods for nitrate quantification, but many of them require long sample pretreatment, besides interference problems from a variety of compounds [3]. In this sense, enzymatic electrodes can be applied to real samples with reasonable levels of sensitivity [4].

Nitrate reductases (NR), widely distributed in nature, are related to two main metabolic routes, the assimilatory uptake and utilisation of NO_3^- for biosynthetic purposes and the utilisation of this anion as oxidant in the respiration chain, under anaerobic conditions [5]. NR catalyse the reduction of nitrate to nitrite, with a pyridine nucleotide as the natural enzyme regenerator. The overall catalysed reaction is as follows.

$$NO_3^- + NAD(P)H + H^+ \rightarrow NO_2^- + H_2O + NAD(P)^+ \quad (1)$$

The general properties of these enzymes are fully reviewed in Refs. [6,7]. The enzymes are usually homodimers or homotetramers of subunits whose molecular weight is approximately 95 to 100 kDa or 50 kDa, respectively [8]. Each subunit contains FAD (the site for NAD(P)H oxidation), a b-type cytochrome and a molybdenum-pterin group (the site for nitrate reduction) in a 1:1:1 stoichiometry. Each cofactor domain constitutes an autonomous structural element and even isolated it retains its partial activity. Thus, the molybdenum domain is responsible for nitrate reduction, being also operative in the presence of synthetic electron donors [9,10].

Suitable redox mediators, which facilitate the electron transfer between the electrode and the enzyme, are critical for the proper performance of the bioelectrode. In this sense, organic redox dyes with sufficiently negative redox potentials have been proposed like phenothiazine and structurally

* Corresponding author. Tel.: +54-351-4334169/80; fax: +54-351-4334188.
E-mail address: vsolis@fisquim.fcq.unc.edu.ar (V.M. Solís).

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related oxygen or nitrogen heterocycles, triphenyl methane dyes, sulphonphtaleine dyes and viologens [9,11].

Since the work of Kiang et al. [12] using a dual enzymatic system and methyl viologen (MV) as mediator in an air gap electrode, several enzymatic electrodes including NR alone or combined with Nitrite Reductase have been employed. Different compounds have been used as enzyme mediators of NR, such as polythiophene-bipyridinium [13], MV derivatives like amphiphilic pyrrol viologen [14–16], durohydroquinone [17], methyl viologen (MV) [18,19], microperoxidase-11 [20–22], among others [9,10].

As a consequence of the redox potential of the molybdenum-pterin center in NR, mediators with redox potentials more positive than -200 mV vs. $Ag|AgCl|Cl^-$ cannot be applied. Methyl viologen and related compounds have the advantage of their relatively high solubility, although the redox potentials are very low. This fact poses serious difficulties with dissolved oxygen and possible interferents present in real samples. Furthermore, cross-reactions involving synthetic mediators and the enzymatic product may be present. In this sense, in a previous publication we reported a homogeneous chemical reaction between the methyl viologen radical cation and nitrite, and analysed the influence of this reaction on the chronoamperometric determination of nitrate at -0.800 V [23].

The purpose of our paper is to study the electro-catalysed reduction of nitrate on a nitrate-reductase modified electrode with phenosafranin (PS^+) as enzyme regenerator. The formal redox potential of this mediator, -0.437 V vs. SCE, is 0.238 V less negative than that of methyl viologen, -0.675 V [9,11]. Attention was paid to possible chemical reactions involving phenosafranin, as well as the incidence of the enzymatic and electrochemical steps on the electrode response. Also, the influences of the relative concentration of mediator and enzyme content for a given substrate concentration range are considered. The stability of the electrode was checked considering the incidence of the electrode lifetime and operation time.

2. Experimental

All solutions were prepared with ultra-pure water (18 MΩ cm^{-1}) from a Millipore MilliQ system. The background electrolyte solution was a phosphate buffer of 0.1 mol l^{-1} constant concentration, pH 7.5, prepared with H_3PO_4 (Carlo Erba) and Na_2HPO_4 (Merck). The mediator 3,7-diamino-5-phenylphenazinium chloride (phenosafranin) (Sigma) and $NaNO_3$ (J. T. Baker) were added to the background electrolyte in the desired concentrations. All chemicals were reagent grade and used as received. Solutions were prepared just before use, and carefully deoxygenated with high-purity nitrogen for 10 min prior to the measurements. A nitrogen atmosphere was maintained throughout the experiments, which were performed at room temperature.

The electrochemical measurements were done in 10-ml cells (Model VG-2 BAS) with a platinum wire as auxiliary electrode; the reference electrode was a $Ag|AgCl|Cl^-$(3 M), model RE-5B Mf 2052 BAS, all potentials being referred to this electrode. The working electrode was a glassy carbon (GCE) disk (Model Mf 2010 BAS), 5.7×10^{-2} cm^2 geometric area determined electrochemically. It was polished successively with wet alumina powder (Buehler) of particle size 1.0, 0.3, and 0.05 μm, copiously rinsed with ultra-pure water and sonicated for 1 min. Two cyclic linear potential scans between -0.45 and 1.35 V in 0.5 mol l^{-1} H_2SO_4 were done to check surface conditions. The enzymatic electrodes were prepared by dropping a small volume of enzymatic solution of known activity onto the electrode surface. NR, NAD[P]H (EC 1.7.1.2), from *Aspergillus niger* (Sigma) was used. The solvent was allowed to evaporate and the modified surface was covered with a cellulose dialysis membrane, fixed by an O-ring (Parker). This procedure allowed us to reach large enzyme concentrations, although the real value is not known. The membranes (cutoff 12 kDa, from Sigma) were treated prior to use according to manufacturer's specifications and stored in ultra-pure water. The enzymatically modified glassy carbon working electrode in the presence of the mediator is referred as GC/NR/PS bioelectrode.

Cyclic voltammetry measurements at different potential sweep rates v were performed with an Autolab (Eco-Chemie, Utrecht, Netherlands) equipped with a PGSTAT 30 potentiostat and GEPES 4.7 software package.

Chronoamperometric experiments using enzyme-modified electrodes with the mediator PS^+ added to the background electrolyte were performed with an OMNI 90 potentiostat and a Linseiss x-t recorder under convective conditions using magnetic stirring. A potential step was applied from the open circuit potential (around -0.040 V) to -0.750 V allowing the background current to stabilise prior to substrate addition. This value guarantees the maximum response, although lower potentials (closer to -0.475 V) can be applied. Enzymatic activities of NR solutions were checked with a Shimadzu UV 1601 Spectrophotometer. Assays were carried out under high purity nitrogen at room temperature in a cuvette containing the enzyme sample to be tested, 0.1 mol l^{-1} phosphate buffer pH 7.5, 2.5×10^{-3} mol l^{-1} $NaNO_3$ and $MV^{\cdot +}$. This radical was obtained by electrochemical reduction at -0.800 V of 2.25×10^{-3} mol l^{-1} MV^{2+} in an auxiliary three-electrode cell with a carbon mesh as working electrode, and then transferred under a nitrogen atmosphere to the cuvette. Re-oxidation of $MV^{\cdot +}$, coupled to the reduction of nitrate, was followed by recording the absorbance decrease at 601 nm. The reaction was as follows.

$$NO_3^- + 2MV^{\cdot +} + 2H^+ \xrightarrow{NR} NO_2^- + 2MV^{2+} + H_2O \qquad (2)$$

One unit of NR activity was the amount of enzyme catalysing the production of 1 μmol of nitrite per minute in phosphate buffer pH 7.5 at room temperature.

3. Results and discussion

As usual for amperometric bioelectrodes, we are interested in conditions for which the electrochemical regeneration of the mediator proceeds at constant potential under limiting current conditions. We performed cyclic voltammetry (CV) experiments in order to select that potential and to have straightforward information about the degree of reversibility and other general features of the electrochemical process.

Fig. 1 shows cyclic voltammograms recorded between 0.0 and -1.0 V in phosphate buffer pH $7.5 + 2.50 \times 10^{-4}$ mol l^{-1} PS^+ at potential sweep rates v in the range $(0.005 \le v \le 0.5)$ V s^{-1} with a bioelectrode containing the total amount of enzyme $U_E = 2.46 \times 10^{-2}$ U behind a dialysis membrane, in the absence of substrate. The electrochemical response observed was according to the electrochemical behaviour of PS^+ without any noticeable influence of the enzyme. A peak system at $E_{pc} = -0.500$ V and $E_{pa} = -0.470$ V was clearly defined, with $\Delta E_p = (0.035 \pm 0.005)$ V compatible with the transfer of two electrons, as expected. The formal potential, estimated as the average of the anodic and cathodic peak potentials $E^o = (E_{pa} + E_{pc})/2$, was (-0.475 ± 0.005) V. This value agrees with the one reported in Ref. [9] if the reference electrode used is taken into account. A linear relationship between the peak current I_P and $v^{1/2}$ was observed, pointing to the diffusional character of the process. The diffusion coefficients, evaluated from the anodic and cathodic slope assuming the reversible transfer of two electrons, were $D_O = (1.0 \pm 0.5) \times 10^{-5}$ cm^2 s^{-1} and $D_R = (8.9 \pm 0.5) \times 10^{-6}$ cm^2 s^{-1}, in good agreement with the literature [24]. We can conclude that under our experimental conditions, the mediator is reduced in a fast and reversible way. $E = -0.750$ V, a potential far beyond E_{pc}, was chosen as the potential to be applied during chronoamperometric measurements.

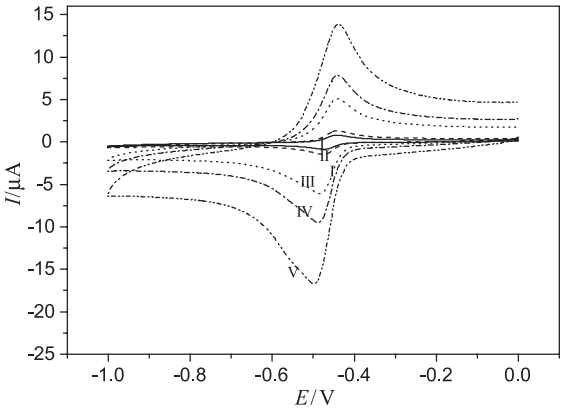

Fig. 1. I–E profiles recorded between 0.0 and -1.0 V in 2.50×10^{-4} mol l^{-1} PS^+ and 2.46×10^{-2} U of NR behind a dialysis membrane for different v/V s^{-1}: (I) 0.005; (II) 0.010; (III) 0.100; (IV) 0.200; (V) 0.500. Phosphate buffer 0.1 mol l^{-1}, pH 7.5.

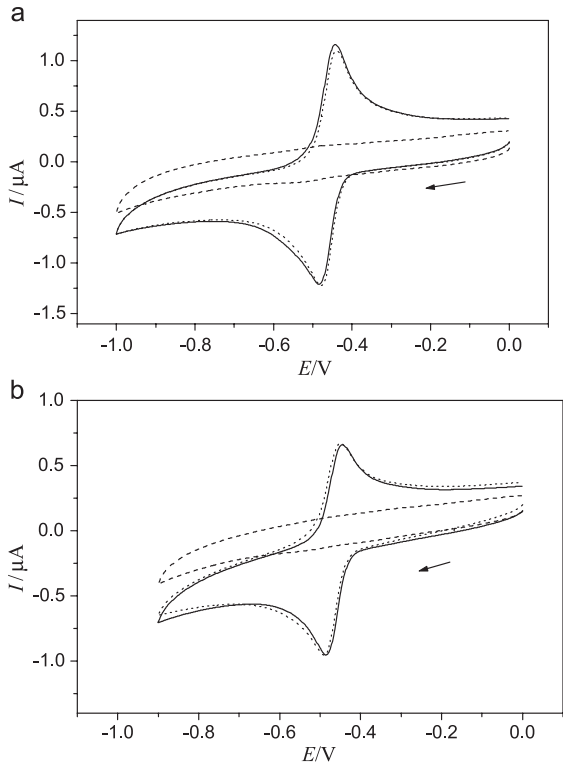

Fig. 2. Cyclic voltammograms on GCE at $v = 0.005$ V s^{-1} (a) (- - -) 0.1 mol l^{-1} phosphate buffer pH 7.5; (—) 2.66×10^{-4} mol l^{-1} PS^+; (. . .) 2.66×10^{-4} mol l^{-1} $PS^+ + NaNO_3$ 2.00×10^{-1} mol l^{-1}. (b) (- - -) 0.1 mol l^{-1} phosphate buffer pH 7.5, (—) 2.5×10^{-4} mol l^{-1} PS^+, (. . .) 2.5×10^{-4} mol l^{-1} $PS^+ + NaNO_2$ 5.0×10^{-2} mol l^{-1}. The arrows indicate the sense of the potential sweep.

3.1. Voltammetric behaviour of the GC/NR/PS bioelectrode

In order to discard possible interference reactions between the mediator and the substrate or product of the enzymatic reaction, current–potential profiles were recorded in the absence of NR in phosphate buffer pH 7.5 at 0.005 V s^{-1} with phenosafranin plus nitrate or nitrite at relatively high concentrations. Thus, Fig. 2a corresponds to voltammograms obtained with 2.66×10^{-4} mol l^{-1} PS^+ before and after the addition of 2.00×10^{-1} mol l^{-1} $NaNO_3$, whereas Fig. 2b shows the responses for 2.50×10^{-4} mol l^{-1} PS^+ in the absence and in the presence of 5.00×10^{-2} mol l^{-1} $NaNO_2$. The clear coincidence between voltammograms of PS^+ with and without nitrate or nitrite allowed us to discard any chemical interference between these substances and the mediator in the absence of the enzyme.

The electrocatalytic response of the bioelectrode with the enzyme in solution behind a dialysis membrane was also followed by cyclic voltammetry, analysing the dependence of the stationary current on the relative concentrations of mediator and substrate.

Fig. 3 shows CV profiles obtained with a bioelectrode containing 2.46×10^{-2} U of NR in 2.50×10^{-4} mol l^{-1} $PS^+ + 1.00 \times 10^{-2}$ mol l^{-1} NO_3^- in 0.1 mol l^{-1} buffer

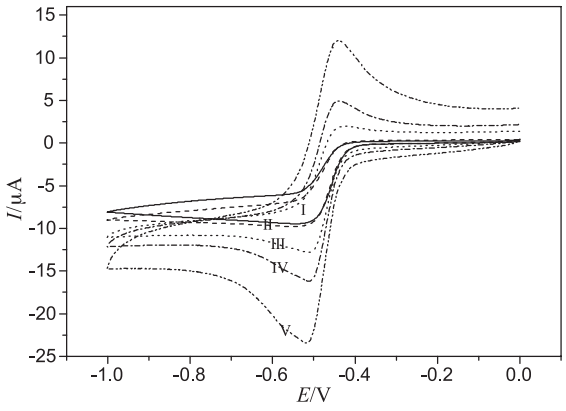

Fig. 3. Voltammetric experiments performed on GCE in 0.1 mol l^{-1} phosphate buffer pH 7.5 with 2.50×10^{-4} mol l^{-1} $PS^+ + 1.00 \times 10^{-2}$ mol l^{-1} $NaNO_3$ and 2.46×10^{-2} U of NR behind a dialysis membrane, for different v/V s^{-1}: (I) 0.005; (II) 0.010; (III) 0.100; (IV) 0.200; (V) 0.500.

solution pH 7.5, for v in the range $(0.005 \le v \le 0.500)$ V s^{-1}. The presence of the enzymatic reaction is detected by the increase of the reduction currents observed in the whole v range, when compared with the values obtained in the absence of the enzyme for the same v values; Fig. 1. The stationary regime was achieved only for v values lower than 0.010 V s^{-1} for which a clearly defined catalytic wave was observed. These results clearly point to the sluggish nature of the enzymatic reaction and, accordingly, v values higher than 0.005 V s^{-1} were avoided in the kinetic analysis. This fact has been reported before for other reductases, such as diaphorase [25].

We assume the classical reaction scheme proposed for the case in which mediator and substrate interact with the enzyme in an independent way (ping-pong mechanism), according to the following schematic reactions.

$$PS^+ + 2H^+ + 2e \rightleftarrows PSH_2^+ \quad (3)$$

$$NR_{(OX)} + PSH_2^+ \xrightarrow{k} NR_{(RED)} + PS^+ \quad (4)$$

$$NR_{(RED)} + NO_3^- \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [NR_{(RED)} - NO_3^-] \xrightarrow{k_{cat}} NR_{(OX)} + NO_2^- \quad (5)$$

where $NR_{(OX)}$ and $NR_{(RED)}$ stand for the oxidised and reduced forms of the enzyme.

If nitrate concentration $c_{NO_3^-}$ is high enough and U_E is kept constant, the incidence of the regeneration step, Eq. (4), on the voltammetric response can be analysed by varying c_{PS^+}.

Fig. 4a shows the voltammperometric profiles recorded from −0.200 to −0.750 V at 0.005 V s^{-1} in a 0.1 mol l^{-1} buffer solution pH 7.5 with a GC/NR/PS bioelectrode containing 4.1×10^{-2} U of NR for mediator concentration in the range $(5.0 \le c_{PS^+} \le 25) \times 10^{-6}$ mol l^{-1}. An enhancement of the cathodic currents proportional to the increasing mediator concentrations was observed. Currents measured at −0.650 V (steady-state) or peak currents in experiments similar to those of Fig. 4a but covering a wider range of mediator concentrations are plotted as a function of c_{PS^+} in Fig. 4b. A typical michaelian curve was obtained, with a linear response for $c_{PS^+} \le 2.5 \times 10^{-5}$ mol l^{-1}, as it is shown in the figure inset and a range of saturation effect at $c_{PS^+} > 1.75 \times 10^{-4}$ mol l^{-1}. Under this last condition, the regeneration step was no longer reaction rate-determining and a constant response independent of mediator concentration was observed; this interval of mediator concentrations is applicable for analytical determinations. For the mediator concentration range, at the relatively high $c_{NO_3^-}$ used in this case (see figure inset), the enzymatic regeneration step is contributing to the global rate of the process following a pseudo-first-order reaction scheme, Eq. (4) [25–27]. A complete kinetic analysis has been reported in Ref. [28]. According to this work, a large concentration of substrate in relation to mediator is not a sufficient condition for the pseudo-first-order approximation to be valid since, in addition, $kc^*_{med}/k_{cat} \ll 1$ has to be fulfilled. We have not

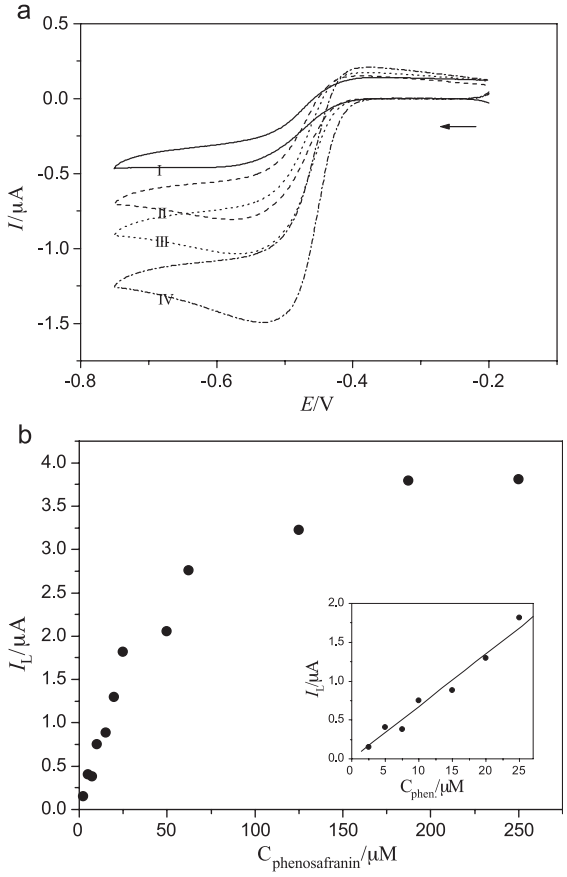

Fig. 4. (a) Cyclic voltammograms on GC/NR/PS at $v = 0.005$ V s^{-1}, in 5.00×10^{-2} mol l^{-1} $NaNO_3$ with 4.1×10^{-2} U of NR after the addition of different mediator concentrations, c_{PS^+}/mol l^{-1}: (I) 5.0×10^{-6}; (II) 1.0×10^{-5}; (III) 1.5×10^{-5}; (IV) 2.5×10^{-5}. (b) Limit currents I_L as a function of mediator concentration, c_{PS^+}, from cyclic voltammograms at $v = 0.005$ V s^{-1} in 5.00×10^{-2} mol l^{-1} $NaNO_3$ with 4.1×10^{-2} U of NR. Fig. inset: amplification of the linear response range. All solutions were prepared in 0.1 mol l^{-1} phosphate buffer pH 7.5.

measured k and k_{cat} for NR under our experimental conditions, but if we assume values not very different from those reported in Ref. [29] (400 M^{-1} s^{-1} and 100 s^{-1}, respectively) and $c_{PS^+} \leq 2.5 \times 10^{-5}$ mol l^{-1}, $kc_{PS^+}/k_{cat} \ll 1$ holds. Accordingly, the steady-state limiting current can be expressed as:

$$I_{lim} = nFAC^*_{med}(D_{med}kc^*_{enz})^{1/2}$$

where c^*_{med} and c^*_{enz} are the mediator and the enzyme concentrations, respectively, D_{med} is the mediator diffusion coefficient, k is the homogeneous bimolecular rate constant for the mediator–enzyme reaction and the other symbols have their usual meaning. From the slope of this plot, the value of $kc^*_{enz} = 4.37$ s^{-1} was estimated. As indicated in the experimental section, we do not have access to the enzyme concentration value.

Situations in which diffusion or the chemical regeneration step has incidence in the global reaction rate can be achieved if variable amounts of substrate are added to a solution containing a constant c_{PS^+}/U_E ratio. Fig. 5A shows I–E profiles at 0.002 V s^{-1} obtained for $c_{PS^+} = 2.5 \times 10^{-5}$ mol l^{-1} and $U_E = 4.3 \times 10^{-2}$ U after the addition of increasing amounts of nitrate, for which an enhancement of I_{max} was observed. In this case, c_{PS^+} corresponds to the linear range in Fig. 4B, and accordingly, a response kinetically controlled by mediator concentration has to be expected. This is the case for curves (II), (III) and (IV). In curve (I), the influence of the diffusion rate of the substrate is evident.

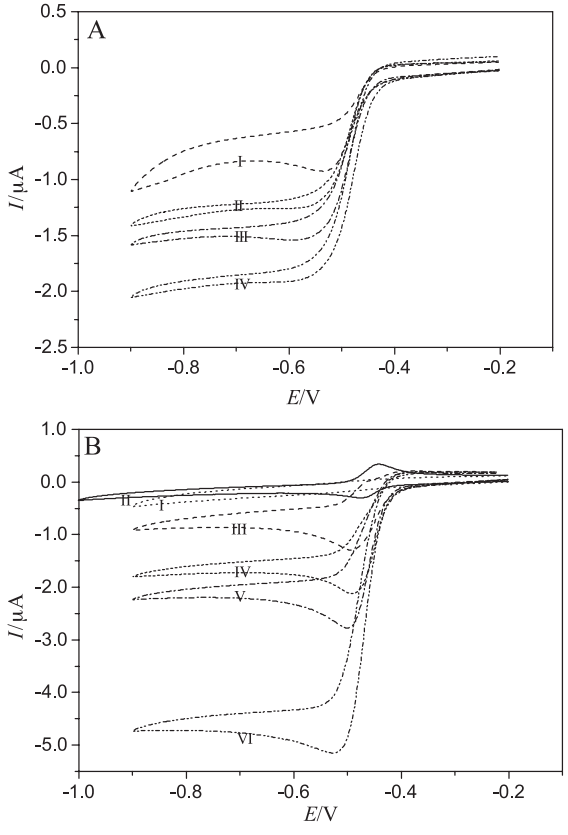

Fig. 5. Cyclic voltammograms on GC/NR/PS bioelectrode at $v = 0.002$ V s^{-1}, with enzymatic activity 4.3×10^{-2} U, phosphate buffer 0.1 mol l^{-1} pH 7.5 with different mediator and substrate concentrations. (A) $c_{PS^+} = 2.50 \times 10^{-5}$ mol l^{-1}; $c_{NO_3^-}$/mol l^{-1}: (I) 5.0×10^{-4}; (II) 2.00×10^{-3}; (III) 5.00×10^{-3}; (IV) 5.00×10^{-2}. (B): (I) phosphate buffer 0.1 mol l^{-1} pH 7.5; (II) phosphate buffer 0.1 mol l^{-1} pH $7.5 + 2.50 \times 10^{-4}$ mol l^{-1} PS^+; following curves, after the addition of increasing $c_{NO_3^-}$/mol l^{-1}: (III) 5.0×10^{-4}; (VI) 2.00×10^{-3}; (V) 5.00×10^{-3}; (VI) 5.00×10^{-2}.

Fig. 5B shows I–E profiles obtained with a bioelectrode containing the same amount of enzyme ($U_E = 4.3 \times 10^{-2}$ U) and substrate concentrations as in Fig. 5A but for a higher mediator concentration, $c_{PS^+} = 2.50 \times 10^{-4}$ mol l^{-1}. The blank experiments for nitrate alone and for phenosafranin without enzyme were also included (curves I and II, respectively). Maximum cathodic currents for curves III to VI are higher than those of Fig. 5A. In this case, according to Fig. 4B, the enzymatic reaction rate was not dependent on the regeneration step. Another fact to be noticed is the negative shift in peak potential for increasing $c_{NO_3^-}$. Whereas E_{pc} for curves III and IV in Fig. 5B were almost coincident, differences up to 0.06 V were observed for the most concentrated solution (Fig. 5B, curve VI). This potential shift has been predicted in the general mechanism for the catalytic reactions with reversible charge transfer analysed by Nicholson and Shain [30]. Nevertheless, due to the nature of enzymatic reactions, this negative shift for increasing nitrate concentration could be indicative of the complex dependence of the catalytic response on both, substrate and mediator concentrations within the reaction layer [31]. Equations developed in their classical publication predict a potential shift for increasing values of the kinetic parameter k_fRT/nFv, k_f being the pseudo-first-order rate constant for the catalytic process. In Fig. 5B, for voltammograms performed at constant v, the increase in the kinetic parameter can be associated to the acceleration of the enzymatic step in response to increasing amounts of substrate for a given c_{PS^+}/U_E ratio, changing the relative incidence of Eq. (5) and the substrate diffusion step on the global rate of the enzymatic process.

3.2. Chronoamperometric behaviour of the GC/NR/PS bioelectrode

The electrochemical response of the bioelectrode was also analysed by chronoamperometric experiments.

Fig. 6 shows a chronoamperometric profile obtained after the addition of successive aliquots of nitrate to a buffer solution at $E_A = -0.750$ V, selected from the voltammetric response (Fig. 1). In absence of the enzyme, no appreciable changes in the background current were detected (curve I), as expected. In contrast, when the enzyme was present, well-defined stationary currents (I_s) were observed (curve II).

Changes in I_s with $c_{NO_3^-}$ measured from chronoamperometric plots like that of Fig. 6 are shown in Fig. 7a, for $c_{PS^+} = 2.50 \times 10^{-4}$ mol l^{-1} and 14.0 U ml^{-1} NR enzymatic activity. Calibration plots of this type as well as the

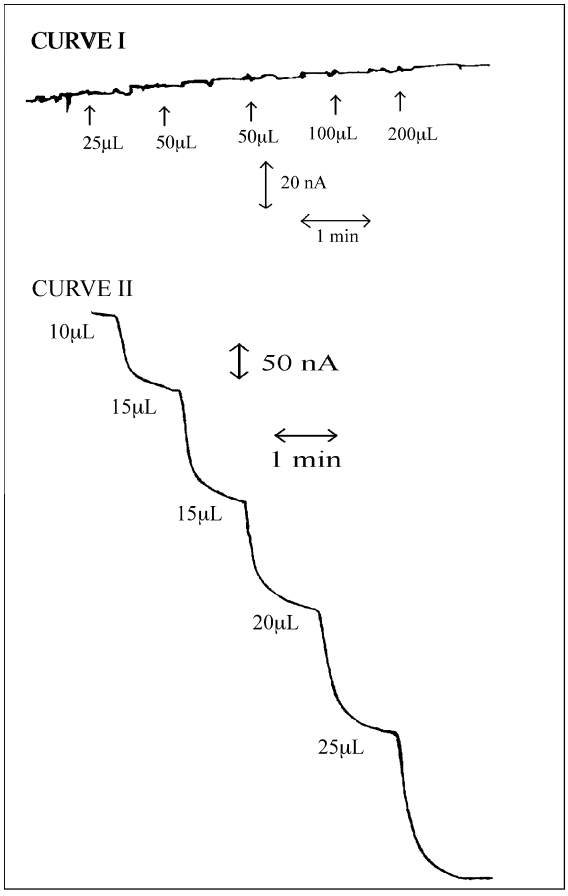

Fig. 6. Chronoamperometric response at −0.750 V on a GCE without (I) and with (II) NR for different aliquots of 2.5×10^{-2} mol l^{-1} $NaNO_3$ added to 10 ml of 0.1 mol l^{-1} phosphate buffer pH 7.5+ 2.5×10^{-4} mol l^{-1} PS^+. Convective conditions.

corresponding Eadie–Hofstee plots I_s vs. $I_s/c_{NO_3^-}$, Fig. 7b, were used for evaluating the kinetic behaviour as well as the analytical performance of the bioelectrode.

Under steady-state conditions for NR(ox) and for the enzyme–substrate complex, the approximated expression for the stationary current I_s, considering a "ping-pong" mechanism, is as follows [32,33].

$$I_s = \frac{I_m}{1 + \frac{K'_{NO_3^-}}{c_{NO_3^-}} + \frac{K'_{PS^+}}{c_{PS^+}}} \tag{6}$$

where I_m is the maximum current, directly proportional to the maximum enzymatic rate; $I_m = nFAk_{cat}c_{NR}$; $K'_{NO_3^-} = (k_{cat}+k_{-1})/k_1$ is the apparent Michaelis–Menten constant for the substrate and $K'_{PS^+} = k_{cat}/k$ is the kinetic constant for the enzyme–mediator interaction. Eq. (6) can be linearised in the following way.

$$I_s = \frac{I_m}{1 + \frac{K'_{PS^+}}{c_{PS^+}}} - \left(\frac{K'_{NO_3^-}}{1 + \frac{K'_{PS^+}}{c_{PS^+}}} \right) \frac{I_s}{c_{NO_3^-}} \tag{7}$$

Limiting expressions depending on the concentration range can be obtained. Thus, for $K'_{PS^+}/c_{PS^+} < (1 + K'_{NO_3^-}/c_{NO_3^-})$, Eq. (6) approaches

$$I_s = \frac{I_m}{1 + \frac{K'_{NO_3^-}}{c_{NO_3^-}}} \tag{8}$$

and Eq. (7) becomes the general Eadie–Hofstee expression for simple michaelian behaviour,

$$I_s = I_m - K'_{NO_3^-}\,(I_s/c_{NO_3^-}) \tag{9}$$

Comparison of Eqs. (7) and (9) draws attention to the fact that in order to obtain information about the apparent Michaelis–Menten constant for the substrate, the concentration ratio for mediator and substrate at a given total enzyme concentration has to be compatible with Eq. (9). In this case, slopes in Eadie–Hofstee plots have to be independent of c_{PS^+}. On the other hand, conditions under which c_{PS^+} are not high enough, Eq. (7) indicates slopes and ordinates dependent on c_{PS^+} and lower than those predicted by Eq. (9). The voltammetric analysis of the previous section is useful in order to find conditions under which

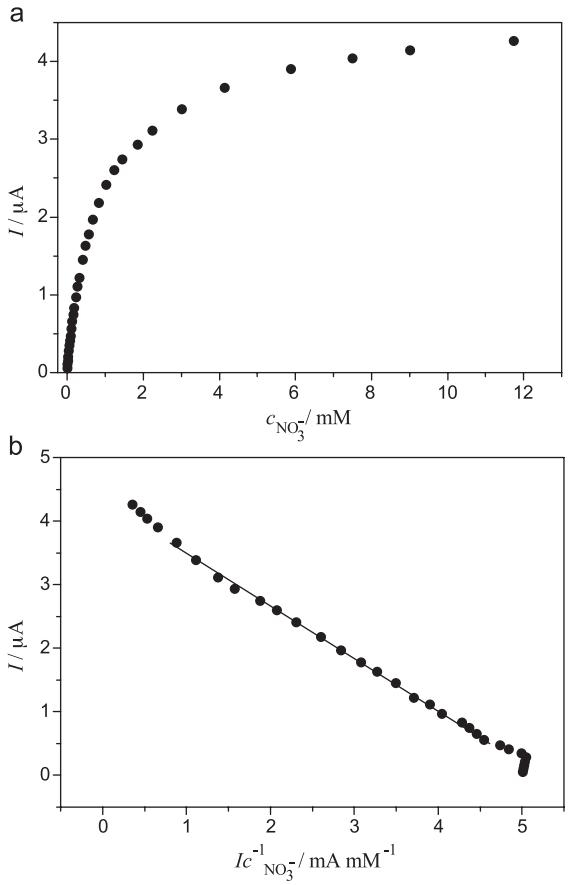

Fig. 7. (a) Nitrate calibration plots corresponding to c_{PS^+}/U_E/(mol l^{-1} U^{-1})3.6×10^{-3}. (b) Eadie–Hofstee plots corresponding to data of a.

Table 1
Apparent Michaelis–Menten constant for the NR in the catalysed reduction of nitrate for different enzyme mediator contents

Enzymatic activity ($\times 10^2$ U_E)	c_{PS^+} ($\times 10^4$ mol l^{-1})	K'_{NO_3} ($\times 10^4$ mol^{-1})
1.75	2.50	6.8
1.75	2.50	6.1
2.85	2.50	9.0
1.45	1.25	8.1
3.90	2.00	6.3
8.35	2.50	7.4
2.05	0.25	8.0
3.25	0.25	7.2
7.0	0.25	7.6

Values were arranged for decreasing c_{PS^+}/U_E from 1.43×10^{-2} to 3.57×10^{-4} mol l^{-1} U^{-1}.

the incidence of the enzyme regeneration step on the global enzymatic process is negligible, as it is the case for CV profiles in Fig. 5B.

Table 1 quotes the apparent K'_{NO_3} values evaluated from a set of Eadie–Hofstee plots like that of Fig. 7b whose kinetic parameters were $K_M=(8.09 \pm 0.06) \times 10^{-4}$ mol l^{-1}; $I_m=(427 \pm 2) \times 10^4$ μA. Within the experimental errors, all plots considered presented similar slopes, from which an average value of $K'_{NO_3}=(7.4 \pm 0.9) \times 10^{-4}$ mol l^{-1} ($n=9$) was evaluated. For the substrate concentrations involved, the constancy of the slope value is indicative of the validity of Eq. (9) under this mediator/enzyme concentration range.

Very few values for K'_{NO_3} with artificial mediators have been published. Our results, although for a different mediator, fall within the range reported in Ref. [34]: $K'_{NO_3}=(1-16) \times 10^{-4}$ mol l^{-1} but something higher than the range published in Ref. [7], $K_M=(0.4-2) \times 10^{-4}$ mol l^{-1}. For apparent K_M values obtained for enzymatic electrodes and redox mediators in solution, the following values have been reported: $K_M=(5.071 \pm 0.157) \times 10^{-4}$ mol l^{-1} with MV and values varying in the range $(4.152 \pm 1.400) \times 10^{-4}$ and $(1.081 \pm 0.119) \times 10^{-4}$ mol l^{-1} for other phenothiazines as regenerators [10]. To our knowledge, no data for K'_{NO_3} with phenosafranin have been published. It has to be stressed that our values are apparent and useful only for the particular case of the enzymatic electrode reported.

3.3. GC/NR/PS bioelectrode analytical parameters

Calibration plots of I_S as a function of c_{NO_3} similar to that shown in Fig. 7a were employed for the evaluation of the analytical parameters for nitrate quantification.

Experiments using electrodes with total enzyme content varying from 3.75×10^{-2} to 1.45×10^{-2} U, with c_{PS^+}/U_E from 5.3×10^{-3} to 8.6×10^{-3} mol l^{-1} U^{-1}, were performed. The response time was around 1 min. The detection limit, evaluated as the relationship between three times the noise signal and the sensitivity, ranged from 3.0×10^{-6} to 4.5×10^{-6} mol l^{-1} for the lower and higher enzyme content, respectively. The corresponding sensitivities ranged from $(6.0 \pm 0.1) \times 10^{-2}$ to (9.0 ± 0.4) A l mol^{-1} cm^{-2}. The response for a given enzyme content was proportional to the substrate concentration, but the sensitivity varied with the enzyme content in a nonlinear way. This fact suggests although low reproducibility in the immobilization procedure or other kinetic inhibitory effects.

In order to discard effects associated to a poor reproducibility of the enzyme-immobilization procedure, the response of two electrodes, prepared using 5 μl of 3.5 U/ml NR solution, respectively, was evaluated. Fig. 8 shows four calibration plots obtained with both bioelectrodes (solid and open symbols). Curve I shows the first calibration plot for each electrode. The almost coincident current values obtained are a clear indication for a reproducible preparation procedure.

We also analysed the decrease in the electrode activity as function of both, the using time or the lifetime. For this purpose, three chronoamperometric experiments were performed with each electrode: for the first one, they were performed successively in the same day, while the second electrode was employed to perform one chronoamperometric curve per day for 3 days, storing the electrode at 4 °C in phosphate buffer between experiments. The corresponding third calibration plot for each electrode is shown in Fig. 8, Curves II. The current decrease observed (65%) was the same for both electrodes, regardless the time elapsed in each case.

The low storage and operational stability of NR electrodes is a well-known fact. An exponential time decay of the enzyme activity, possibly caused by thermal or chemical inactivation of the enzyme, has been reported [35]. In our case, the same degree of deactivation was observed, as

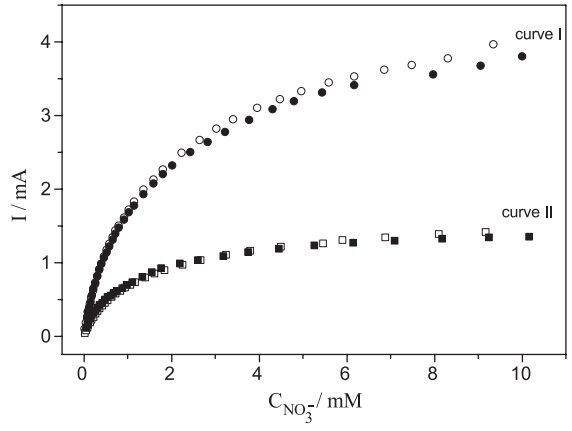

Fig. 8. Nitrate calibration plots for two electrodes (solid and open symbols) for $c_{PS^+}=2.50 \times 10^{-4}$ mol l^{-1} and 17.5×10^{-3} U NR enzymatic activity; curves I are the first calibration plots performed with each electrode. Curves II correspond to the third calibration plots for each electrode performed (□) successively in the same day and (■) one curve each day. The electrode was kept at 4 °C in phosphate buffer between experiments.

expected for a process related to the enzyme nature more than to the mediator involved.

Results in Fig. 8 indicate that the degree of inactivation was related on the number of enzymatic cycles more than on the lifetime of the immobilized enzyme. Besides chemical and thermal inactivation, changes in the coordination state of the molybdenum site with catalytic turnover reported in literature [36] may be one of the effects involved.

Chronoamperometric experiments were performed in order to evaluate the effect of nitrite inhibition under our experimental conditions. Again the competitive nature of the inhibition process was verified, in good agreement with the literature [7,36]. According to the reversible nature of the inhibition process, the low stability of the electrode could not be related to nitrite effects.

3.4. Analytical application of the GC/NR/PS bioelectrode

The amperometric GC/NR/PS bioelectrode was used for the quantification of nitrate in river water using the method of standard addition. The nitrate content of the sample was below the detection limit; therefore, a recovery assay was made in order to check the influence of the organic matrix and the presence of possible interferents in the analytical response of the GC/NR/PS bioelectrode. Fig. 9 shows the corresponding calibration plot, I_s vs. $c_{NO_3^-}$. The standard solution was 1.00×10^{-3} mol l^{-1} $NaNO_3$ prepared in buffer solution. Nitrate content present in the sample was $(4.36 \pm 0.05) \times 10^{-6}$ mol l^{-1} and the value experimentally determined was $(4.50 \pm 0.05) \times 10^{-6}$ mol l^{-1} with a regression coefficient of 0.995, indicating a recovery of 103.2%, which can be considered as very satisfactory. Concentration calculations were performed according to the procedure described in Ref. [37].

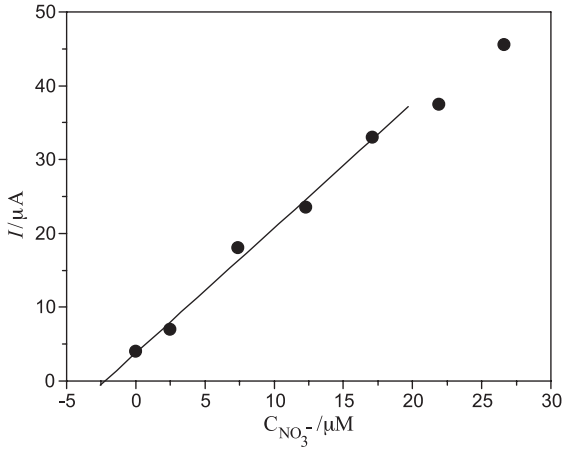

Fig. 9. Calibration plot for nitrate recovery assay performed at −0.750 V on GC/NR/PS bioelectrode with 4.7×10^{-2} U of NR for a solution containing 10.0 ml of water from Suquía River plus 5.0×10^{-5} mol l^{-1} PS, after the addition of 4.36×10^{-4} mol l^{-1} nitrate.

4. Conclusions

Phenosafranin proved to be a very suitable enzyme regenerator for *A. niger* NR. The mediator electrochemical process involved the reversible transfer of two electrons at a lower potential than that of methyl viologen, the more commonly used, which is advantageous in relation to the mitigation of interferences. Furthermore, no chemical reactions between the mediator and the enzymatic product were detected.

Cyclic voltammograms including enzyme, mediator and substrate put in evidence the enzyme-catalysed reduction of nitrate, although with a relatively slow kinetics. This type of experiments was useful in order to select the potential to be applied in the chronoamperometric determination of nitrate, as well as the adequate mediator concentration in relation to the enzyme content at the substrate concentration range of interest. Thus, for high substrate/mediator concentration ratios, the catalytic currents were dependent on mediator concentration. This has to be expected when the concentration of the oxidised form of the enzyme remains constant and the regeneration step follows a pseudo-first-order in the mediator. Kinetic information related to this reaction could be reached from these experiments if the enzyme concentration was known. On the other hand, CV with high mediator concentrations gave catalytic currents dependent on substrate concentration, conditions suitable for analytical purposes. Nevertheless, the relatively low solubility of phenosafranin and its tendency to get adsorbed on the dialysis membrane poses a real limit to the amount of enzyme to be incorporated, keeping a high c_{PS^+}/U_E ratio. Accordingly, the sensibility of the electrochemical response cannot be increased over a given limit. A more suitable immobilization procedure for the mediator, as the polymerisation of phenosafranin on the electrode surface, may allow overcoming this difficulty, as well as to work with mediator-free solutions [38]. In this sense, the polymerization of phenosafranin would be performed in conditions compatible with NR stability, in a similar way as reported for the polymerization of toluidine blue in a glucose reductase electrode [39].

The reproducibility of the electrode response was very good and gave suitable linear responses after four calibration plots, although with a marked decrease in sensibility. This diminution of the analytical response was proved to be dependent on the number of enzyme catalytic cycles more than on the lifetime of the electrode. The low operative stability of the enzyme remains an unsolved problem.

From the analytical parameters obtained, it can be concluded that the GC/NR/PS bioelectrode is very convenient for the determination of nitrate content in water. Also, the mediator concentration has to be adjusted considering its solubility, the enzyme content and the substrate concentration range to be analysed. The entrapment of the enzyme behind a dialysis membrane proved to be very suitable for

this relatively low enzymatic reaction rate, since high enzyme concentrations can be reached with very low amounts of enzyme. The good results obtained in a recovery assay of nitrate in river water indicated the absence of significant interferences in this matrix.

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