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Modified electrode approaches for nitric oxide sensing

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Abstract

Three different methods for the determination of nitric oxide (NO) in solution are described. These are based, respectively, on the use of a horseradish peroxidase (HRP) biosensor or on electrodes modified with films of redox-active transition metal complexes. In the case of the biosensor the enzyme was electrochemically immobilized onto a glassy carbon (GC) electrode. The activity of HRP is inhibited in presence of NO. Thus, the decrease in activity is correlated to the concentration of NO present in solution. The biosensor responds linearly over the range of 2.7×10^{-6}–1.1×10^{-5} M NO with a detection limit (5% inhibition) of 2.0×10^{-6} M. In the case of chemically modified electrodes, particular emphasis is placed on materials capable of catalyzing the oxidation of NO. In terms of electrocatalyst, the discussion will centre on electrodeposited films of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) and indium(III) hexacyanoferrate(III). The resulting sensors exhibited potent and persistent electroacatalytic activity towards the oxidation of NO with low detection limits (1 μM) and good linear relationship between the catalytic current and NO concentrations. In addition, interference due to the presence of nitrate and nitrite have been significantly reduced. According to these results, the described modified electrodes have been used as sensors for the determination of NO generated by decomposition of a typical NO-donor, such as *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). A critical comparison of the various methodologies employed is made.

Keywords: Nitric oxide; NO; Sensors; Modified electrodes

1. Introduction

In the last few years there has been an increasing interest in the development of methods for detecting and monitoring nitric oxide (NO) due to its importance in clinical and environmental analysis.

The half-life time of NO in physiological solutions is very short, 6 s [1,2]. As a result, the determination of NO becomes a challenging analytical problem and requires the design of specific sensors.

Different strategies based on direct or indirect methods have been reported for measurements of NO levels in a variety of biological systems [3]. Indirect methods relay on measurements of secondary species, such as breakdown products of

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NO metabolism (nitrite and nitrate). Other bioassays, based on the physiological effects of NO have been proposed, such as the stimulation of guanylate cyclase or inhibition of platelet aggregation [3]. The interaction of NO within biological systems has provided a key to the construction of optical biosensors [4].

Due to their inherent sensitivity, the electrochemical methods are specially well suited for the development of analytical approaches for NO. Thus, there have been some studies performed aiming at the development of electrochemically based sensors. These articles report on direct oxidation of NO at a platinum electrode at 0.8 V [5] and on its determination at modified carbon fiber electrode [6]. Wink et al. [7] have presented an overview of electrochemical techniques for NO determination. In this work, they describe the use of carbon fiber modified with films derived from various porphyrins, and they ascribe the lowering of the potential for NO oxidation to the modification of the carbon surface and not to the porphyrin film. On the other hand, Ciszewski et al. [8,9] have studied the role of nickel in conductive polymeric porphyrin films for electrocatalytic oxidation of NO. Electropolymerized nickel porphyrin films have also been used for the electrocatalytic oxidation of NO [10,11].

In recent times a number of chemical modifications of electrodes have been proposed for NO sensing. In general, the materials used for these modifications are metal complexes with different ligands such as macrocycles [12,13], which are attached to the electrode surface by direct adsorption [14], electropolymerization [15,16], electrodeposition [17] or self-assembling. In these types of sensors the oxidation or reduction current of NO at the electrode surface is the analytical signal used for the determination.

There continues to be a great deal of interest in the development of new materials capable of being incorporated into electrode surfaces in order to obtain better electrochemical NO sensors. In this way, electrodes modified with films derived from inorganic polymeric microstructures containing atoms of transition metals with different oxidation states (mixed-valence compounds [18]) have been extensively investigated. The use of carbon nanotubes [19] has also been studied.

Enzyme-based biosensors are highly selective devices, which rely on the specific binding of the target analyte (the substrate) to the active-site regions of the enzyme. Amperometric signals resulting from this biorecognition process have led to many useful enzyme electrodes. The response of these devices is often affected by the presence of inhibitors, which combine with the free enzyme in a manner that prevents substrate binding, having great influence on the velocity of the biocatalytic reaction. Indeed, such inhibitory effects have been exploited for indirect assays of the inhibitor. These approaches have been also used to develop methods to determine NO. Campanella et al. [20] reported the determination of superoxide and NO using their modulating action on three different enzymatic biosensor based on tyrosinase, superoxide dismutase and galactose oxidase.

In this paper, we describe and compare different electroanalytical strategies for the determination of NO based on either horseradish peroxidase (HRP) biosensors, making use of its inhibitory effect on HRP activity, or on the electrocatalytic oxidation of NO at modified electrodes.

2. Experimental

2.1. Materials

Peroxidase Type I and II (EC 1.11.1.7) from horseradish were obtained from Sigma as a powder containing 120 and 200 units per mg of solid, respectively and were stored as received at −20 °C. Glutaraldehyde (Grade I, 25% aqueous solution) was obtained from Sigma and stored below 0 °C. Nylon filter meshes of 150 μm pore size were purchased from Nytal. Hydrogen peroxide solution was purchased from Carlo Erba. The concentration of hydrogen peroxide solution was determined from its absorbance at 240 nm using an extinction coefficient of 39.41 $mmol^{-1}$ cm^{-1} [21]. The 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) was synthesized as previously described [22,23]. Reagent grade potas-

sium ferricyanide ($K_3[Fe(CN)_6]$, 99%) was obtained from Carlo Erba. Indium(III) nitrate pentahydrate ($In(NO_3)_3 \cdot 5H_2O$, 99.9%) and 1,4-benzoquinone were from Aldrich Chemical Company. High-purity NO gas was purchased from Air Liquide. Standard saturated NO solutions were prepared by bubbling NO gas through oxygen-free 0.1 M phosphate buffer solutions for 30 min. Aliquots of this saturated solutions were used to prepare solutions of known NO concentration, using a value of 1.9 mM for its concentration of saturation [24]. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was purchased from Sigma Chemical Co. and stored as received at −20 °C. 1.9 mM SNAP stock solution was prepared by dissolving 2.0 mg of SNAP in 4.8 ml of a deoxygenated solution of water containing EDTA at pH 9.0. The stock solution was freshly prepared for each experiment, since it is light-sensitive and slowly decomposes at room temperature. SNAP standards were prepared by dilution of the stock solution. Acetonitrile (AN; sds) was distilled in glass and dried over 4-A molecular sieves. Tetra-*n*-butylammonium perchlorate (TBAP; G.F. Smith) was recrystallized three times from ethyl acetate and dried under vacuum at 90 °C for 72 h. All other chemicals used in this work, such as sodium phosphate, potassium chloride, and sodium nitrite were reagent-grade quality and used as received without additional purification steps. Prepurified nitrogen gas was used to deaerate all solutions before use and flowed over the solutions during experiments to minimize the reaction of NO with oxygen. All measurements were carried out at room temperature. Water was purified with a Millipore Milli-Q system.

2.2. Instrumentation

Cyclic voltammetric and chronoamperometric studies were carried out with a BAS CV-27 potentiostat connected to a BAS X–Y recorder. The electrochemical experiments were carried out in a three-compartment electrochemical cell with standard taper joints so that all compartments could be hermetically sealed with PTFE adapters. This is important to ensure that there is no gas leakage to or from the electrochemical cell. A PTFE-covered GC disk electrode (geometric area, 0.07 cm^2) was used as working electrode. Prior to each experiment, GC electrodes were polished with 1 μm diamond paste (Buehler) and rinsed with water and acetone. A coiled platinum wire (5 cm) served as auxiliary electrode. All potentials are reported against a sodium-saturated calomel electrode (SSCE) without taking into account the liquid junction.

2.3. Enzyme immobilization and biosensor construction

HRP was immobilized onto a GC electrode surface by electrochemically induced cross-linking using glutaraldehyde (2.5% v/v) and serum albumin (BSA) (10% w/v) according to the procedure previously described [25].

2.4. Electrode modification

GC electrodes were modified with electrodeposited films of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) by holding the potential at 0.7 V for 5 min in a 0.1 mM solution of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) in AN (0.10 TBAP).

For GC electrodes modified with indium hexacyanoferrate (InHCF) films, they were prepared by electrochemical deposition through cycling of the potential from 0.0 to 1.0 V for 10 cycles in a solution containing 1.0 mM $In(NO_3)_3$ and 1.0 mM $K_3[Fe(CN)_6]$ in 0.5 M KCl, pH 2.0.

3. Results and discussion

Below we describe the three different approaches (described above; Section 2) for the determination of NO. We frame the discussion in terms of the aspects that are specific to each modality and then make comparisons of the analytical parameters including stability, sensitivity, detection limits, and interferences.

3.1. Determination of NO using a HRP based biosensor

HRP is a redox hemoglycoprotein whose active site in HRP-catalyzed reactions is the sixth coordination position of Fe(III) in the Fe(III) protoporphyrin IX prostetic group of the enzyme molecule [26]. In a single two-electrons process the HRP [Fe(III)] is oxidized by hydrogen peroxide to form water and an oxidized form of HRP, denoted HRP I. The reduction of HRP I back into the HRP [Fe(III)] occurs in two separate $1e^-$ step. The complete process can be represented by the following reaction sequence:

$$\text{HRP [Fe(III)]} + H_2O_2 + 2H^+ \rightarrow \text{HRP I} + 2H_2O \quad (1)$$

$$\text{HRP I} + 1e^- \rightarrow \text{HRP II} \quad (2)$$

$$\text{HRP II} + 1e^- \rightarrow \text{HRP [Fe(III)]} \quad (3)$$

The efficiency of the biocatalytic reaction clearly hinges on having all steps in the sequence be rapid. It is generally accepted that whereas steps 1 and 2 are indeed rapid, 3 on the other hand can be sluggish, this often requiring the use of an external redox mediator. This is the approach we have adopted in the design of a peroxidase based biosensor for H_2O_2 whose essential features are shown in the scheme of Fig. 1. The fundamental reaction is the two-electron oxidation of peroxidase in the presence of H_2O_2, the latter being reduced to water. The reduction of the enzyme back to its original form is carried out by electron transfer from the mediator (1,4-benzoquinone) in its reduced form. Thus, in the presence of hydrogen peroxide an external current will flow, the magnitude of which is a function of the peroxide concentration.

NO can react [27] directly with Fe(III) protoporphyrin, $[\text{Fe(III) (PP)}]^+$, yielding the porphyrin nitrosyl complex **B** according to the reaction:

$$\underset{\mathbf{A}}{[\text{Fe(III) (PP)}]^+} + \text{NO} \rightarrow \underset{\mathbf{B}}{[\text{Fe(II) (NO}^+\text{) (PP)}]^+}$$

The formation of the iron nitrosyl porphyrin complex is related to the partial inhibition of the reaction (1). Thus, a decrease in the activity of HRP in presence of NO must be observed.

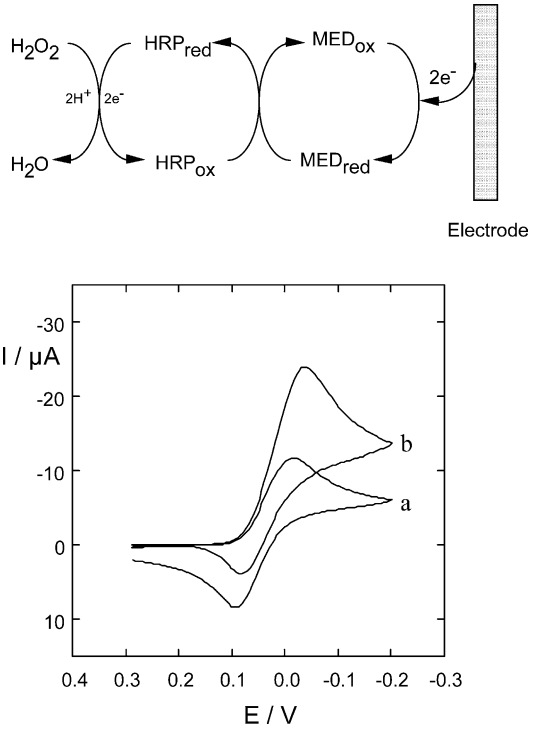

Fig. 1. Cyclic voltammograms (5 mV s^{-1}) obtained in 0.1 M phosphate buffer at pH 7.0 for HRP biosensor. Curve a: 1.0 mM 1,4-benzoquinone. Curve b: 1.0 mM 1,4-benzoquinone and 1.0 mM hydrogen peroxide. Inset: Schematic representation of biosensor response. HRP_{ox} and HRP_{red} are the redox forms of HRP and Med_{ox} and Med_{red} the redox forms of mediator.

The cyclic voltammetric response of the biosensor in the absence and presence of hydrogen peroxide as substrate using 1,4-benzoquinone as a soluble hydrogen donor mediator was used to assess the activity of the biosensor. Fig. 1a shows the cyclic voltammetric response of the biosensor in a pH 7.0 phosphate buffer solution containing 1.0 mM 1,4-benzoquinone but in the absence of hydrogen peroxide. The characteristic and well-behaved redox response ascribed to the reversible two-electron/two-proton process of the hydroquinone/quinone system is observed. Upon the addition of hydrogen peroxide to a concentration of 1.0 mM, an enhancement of the cathodic current (Fig. 1b) is clearly noted. Additional increases in the concentration of hydrogen peroxide resulted in concomitant increases in the cathodic peak current. In addition, no current was observed in the

anodic wave, which is consistent with a very strong electrocatalytic effect. Moreover, the biosensor response taken as the steady state current measured at −0.3 V was linear ($y = -0.003 + 1.37x$; $r = 0.9999$) to hydrogen peroxide concentration in the range from 0.1 to 3.0 mM.

For NO determination the biosensor response was assayed to 5.0 mM of H_2O_2 in the absence (control experiment) and presence of successive additions of aliquots of a saturated NO solution. The presence of NO results in a linear decay in the steady state current, that is, a linear decrease in the biosensor response as can be observed in Fig. 2. Each data point represents the mean of three replicate measurements, and the scatter of individual measurements from this value is indicated. The response in terms of % inhibition was linear over the entire range studied (2.7×10^{-6}–1.1×10^{-5} M), rapid (less than 30 s) and reproducible with a detection limit of 2.0×10^{-6} M, defined as the concentration of NO required to obtain 5% of inhibition, and a sensitivity of $2.68 \times 10^{6}\%I$ M^{-1}.

The biosensor response in the absence of NO after being stored in phosphate buffer under refrigeration overnight shows a decrease of 70% and inhibition of the response by NO was not observed. Thus, it can be used as a disposable biosensor only.

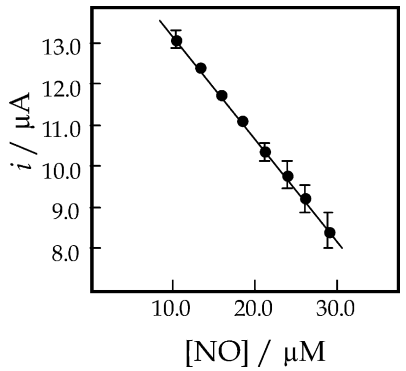

Fig. 2. Biosensor response, as the steady state reduction current (measured at −0.3 V) to NO concentration.

3.2. Determination of NO with chemically modified electrodes

3.2.1. Electrode modification

We have also studied different strategies for the analytical determination of NO based on the use of chemically modified electrodes exhibiting potent and persistent electrocatalytic activity towards the oxidation of NO. These modified electrodes have been used as amperometric sensor for the determination of NO in solution. The sensors were prepared by electrochemical deposition of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) (see Fig. 3) or indium(III) hexacyanoferrate(III) onto GC electrodes.

In the case of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II), the compound in acetonitrile solution adsorbed strongly on the electrode surface to form electroactive layers. Electrodeposition of films can be also achieved from acetonitrile solutions at constant potential of +0.7 V. If after the electrodeposition the electrode is removed from the solution, rinsed

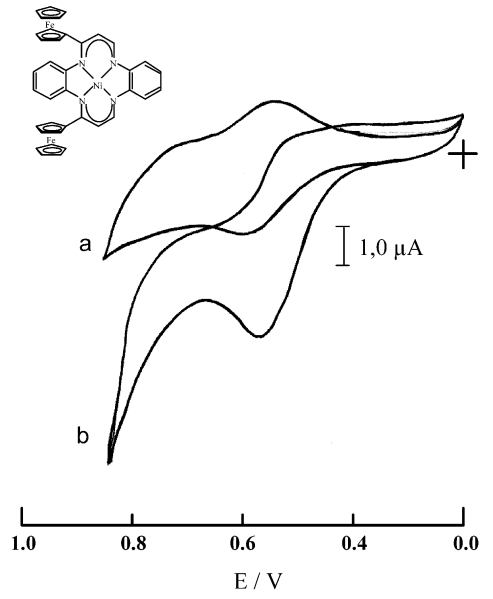

Fig. 3. (a) Cyclic voltammogram in aqueous 0.1 M phosphate buffer/0.1 M $NaClO_4$ for a GC electrode modified with an electrodeposited film of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II). (b) Upon addition of NO to a final concentration of 18.8 μM. Scan rate 10 mV s^{-1}.

with acetone, and placed in an acetonitrile (0.10 M TBAP) solution containing no dissolved compound, the cyclic voltammetric response exhibits two redox couples at +0.51 and +0.83 V, respectively. Potentials that are quite similar to those observed for the compound in solution. We ascribed the first to the simultaneous one-electron oxidation of the two non-interacting ferrocene units and the second is assigned to the metal-localized $Ni^{II/III}$ oxidation [17]. In aqueous solutions the films are also electroactive as can be seen in Fig. 3 a, which shows a cyclic voltammogram for a GC electrode modified with an electrodeposited film of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) in a phosphate buffer/$NaClO_4$ (0.1/0.1 M) at pH 7.0. A well-defined reversible voltammetric wave ascribed to the ferrocene/ferrocinium couple is observed. This reversible voltammetric wave has a formal potential of +0.55 V and the symmetrical shape anticipated for a surface-confined redox species. The films adhered strongly to the electrode surface and were stable to rinsing with acetone or water. Moreover, the films were quite stable to potential cycling, with less than 5% loss after 10 continuous cycles in phosphate buffer/$NaClO_4$ (0.1/0.1 M) at pH 7.0.

In a similar way, InHCF films can be electrochemically deposited on GC electrodes by continuous cycling from solutions containing 1.0 mM $In(NO_3)_3$ and 1.0 mM $K_3[Fe(CN)_6]$ in 0.5 M KCl at pH 2.0. Surface coverage values were determined from the integration of the cathodic wave yielding typically a charge of 90 μC which correspond to approximately 1.3×10^{-8} mol cm^{-2}. This clearly represents a multilayer coverage as we estimated a monolayer to represent about 10^{-9} mol cm^{-2}. The resulting modified electrodes in phosphate buffer/KCl at pH 6.5 exhibit the typical response for a reversible redox process confined on the electrode surface (Fig. 4a) with a formal potential quite similar to that observed during the electrodeposition step (+0.78 V) and a peak current ratio close to unity. In addition, the peak currents are directly proportional to the sweep rate for values up to 300 mV s^{-1}. At higher scan rates, the waves have a more diffusional shape, and there is a concomitant

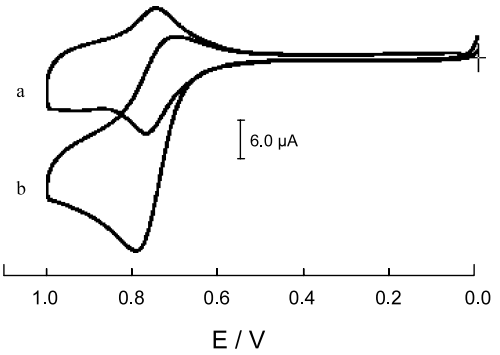

Fig. 4. (a) Cyclic voltammogram of an InHCF-modified GC electrode in the supporting electrolyte solution; and (b) upon addition of NO to a final concentration of 200 μM. Scan rate 10 mV s^{-1}.

increase in the ΔE_p values (data not shown). These deviations are probably due to charge limitations associated with the charge propagation in the film and they become more significant at higher coverages. In order to provide the necessary charge balance during the redox process in the film, the rapid transport of charge compensating cations must be assured. In this regard, Kulesza et al. [28] have reported that potassium ions are especially effective for electrodeposited films of InHCF.

The stability of these modified electrodes was assayed by continuous cycling in 50 mM phosphate/50 mM KCl, pH 6.5. After 15 min a loss of electroactivity of about 30% of the initial charge is estimated. However, the remaining electroactive material is quiet persistent.

3.2.2. Electrocatalytic oxidation of NO at modified electrodes

The electrocatalytic activity of electrodes modified as described above to the oxidation of NO in aqueous media was evaluated by comparing the voltammetric response of a modified electrode in a NO atmosphere with that obtained under atmosphere of nitrogen under otherwise identical conditions. In addition, the response for the modified electrode in the presence of NO was compared to that obtained for a bare GC electrode in a NO containing solution.

Fig. 3b depicts the cyclic voltammetric response for a GC electrode modified with an electrodepos-

ited film of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) in contact with pH 7.0 phosphate buffer solution (0.1 M) containing 0.1 M $NaClO_4$ and 18.8 μM NO. As can be observed, in the presence of NO the cyclic voltammogram exhibits an enhancement in the anodic wave with a decrease in the cathodic peak current which is consistent with a strong electrocatalytic effect.

Very similar results were obtained for electrodes modified with electrodeposited films of InHCF, as can be seen in Fig. 4, which shows the response of these electrodes in contact with a pH 6.5 phosphate buffer solution (50 mM) containing 50 mM KCl under a nitrogen atmosphere (a) and in a NO atmosphere (curve b). In the absence of NO (curve a) the well-behaved redox response previously described for the modified electrode, ascribed to the reversible redox couple of the indium(III) hexacyanoferrate(II)/indium(III) hexacyanoferrate(III) is observed. Upon the addition of NO to a concentration of 200 μM, the cyclic voltammogram exhibits a dramatic enhancement in the anodic wave with a decrease in the cathodic peak current. Both of these observations demonstrate a strong electrocatalytic effect in the oxidation of NO.

The appearance of the catalytic wave in both cases is ascribed to the oxidation of NO, since the peak current increases upon further addition. It is also clear that the peak potential for the oxidation of NO is shifted to less positive values, specially at the 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) modified electrodes relative to the bare GC where oxidation takes place at +0.95 V.

3.2.3. Analytical determination of NO

One of the objectives of these investigations was to develop amperometric sensors for NO determination. As mentioned earlier, in the presence of NO the electrochemical response of these modified electrodes exhibits a potent electrocatalytic effect. Moreover, the catalytic current (at 10 mV s^{-1}), measured as the difference of the peak currents in the presence and absence of NO, is proportional to NO concentration in solution. In the case of 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) modified electrodes the linearity between catalytic current and NO concentration is observed in all the range assayed (from 10.0 μM to 0.1 mM). However, for InHCF modified electrodes catalytic current increases linearly with NO concentration up to 300 μM and then levels off, which is probably due to kinetic limitations.

The corresponding calibration plots gave an excellent linear correlation ($r = 0.9998$) as can be seen, as an example, in Fig. 5. The sensitivity, obtained from the slope of the calibration plot, was 0.27 and 0.090 μA μM^{-1} for sensors based on 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) or InHCF, respectively. The detection limit of NO, calculated as three times the standard deviation of background current (3σ), was determined to be 1.0 and 5.0 μM for electrodes modified with 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) or InHCF, respectively. The relative standard deviation for five successive measurements of 10 μM of NO was 2.5%. On the basis of these results, the electrooxidation of NO onto 6,17-diferrocenyldibenzo[*b*,*i*]5,9,14,18-tetraaza[14]annulen]-nickel(II) or InHCF films can be used in the determination of this analyte with good sensitivity over a wide range of concentrations.

3.2.4. Nitrite and nitrate interferences

One of the main difficulties in the development of analytical applications of electrocatalysts for NO determination is the interference due to nitrite and nitrate, which are typically present in NO solutions and whose chemistry is very closely related to the chemistry of NO. Thus, its oxidation tends to be catalyzed by the same type of systems that catalyze NO oxidation. In order to reduce such interferences, electrodes are often coated with materials such as Nafion, which prevent anions from reaching the electrode surface via Donnan exclusion [15,29]. However, this fact is often accompanied by a drastic diminution in the analytical response and a loss of sensitivity probably due to kinetic limitations. Recently, Zhang et al. [30] reported that the selectivity of these sensors against NO increased covering the Nafion modified electrode with NO-selective gas permeable polymeric membranes.

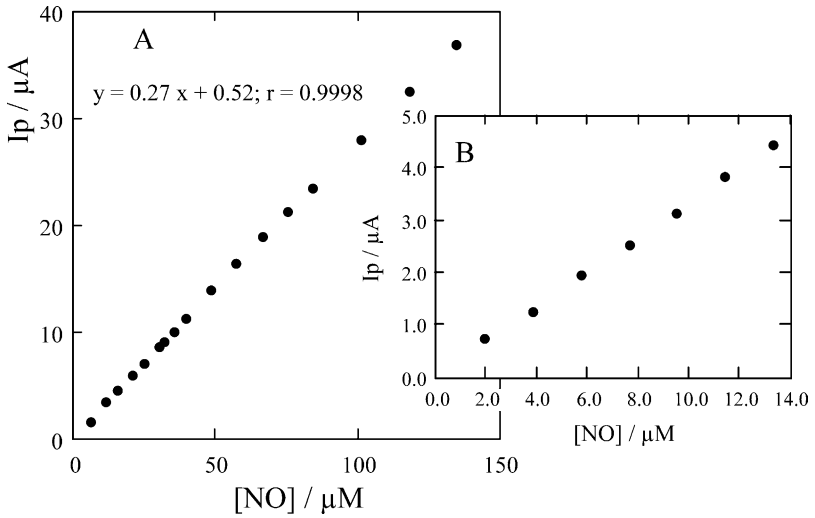

Fig. 5. Catalytic current (i_{cat}) as a function of NO concentration. Inset: linear range up to 14 μM.

The use of chemically modified electrodes that selectively electrocatalyze the oxidation of NO can provide an elegant way to prepare NO sensors with high performance characteristics. In order to assess this possibility and to determine if in the case of InHCF modified electrodes the interferent effects due to nitrite and nitrate could be minimized, we compared the electrochemical response of modified electrodes in solution containing 170 μM nitrite and 170 μM NO in a 50 mM phosphate solution (pH 6.5) containing 50 mM KCl. The electrochemical response obtained in the presence of nitrite was only 6% of that obtained for the NO alone in solution. In addition, when both NO and nitrite were present in the solution at a concentration of 170 μM each, the electrocatalytic wave is very similar to that observed in the absence of nitrite (depicted in Fig. 4b). These results suggest that InHCF-modified electrodes can be used in NO determination with very low interference of nitrite.

A similar set of experiments was performed to test the interference due to the presence of nitrate. In this case, the electrochemical response of the electrodeposited InHCF films and the electrocatalytic behavior of NO remain unaffected by the presence of nitrate ions, even at high concentration levels of this anion such as 0.5 M.

3.2.5. Determination of NO generated by a NO-donor

To test the potential utility of these modified electrodes as NO-sensors, we have studied the electrochemical response of InHCF-modified electrodes to NO generated by decomposition of *S*-nitroso-*N*-acetylpenicillamine (SNAP) in solution. SNAP (Fig. 6) is a stable analogue of endogenous *S*-nitroso compounds with therapeutic applications as vasodilator, smooth muscle relaxants, and lymphocyte activators. These therapeutic properties are related to the ability of SNAP to act as a NO-generator in vivo [31]. Under appropriate conditions, SNAP decomposes giving rise to the liberation of NO and the corresponding disulfide. The liberation of NO is a very slow process at physiological pH and temperature. However, it can be triggered by light and metal ion catalysis, especially by Cu^+ ions [32]. The release of NO from SNAP in our case was exclusively triggered by light in order to prevent possible electrochemical interferences associated with metal ions acting as catalysts.

Cyclic voltammograms (data not shown) of a bare GC electrode in phosphate solution containing 65 μM SNAP under nitrogen atmosphere exhibit an irreversible anodic wave with a peak potential at about +0.95 V. If the potential was cycled over the range of 0.0−+1.0 V, no addi-

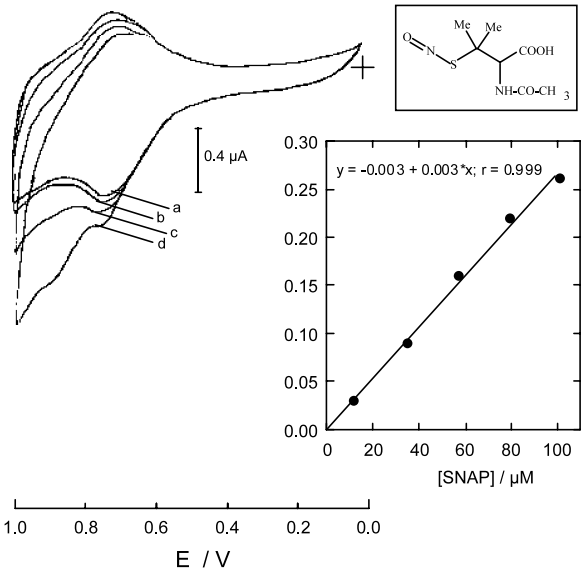

Fig. 6. Cyclic voltammogram of the InHCF-modified GC electrode in the supporting electrolyte solution (a); and containing 12 μM (b); 35 μM (c); 60 μM of SNAP (d). Scan rate 10 mV s^{-1}. Inset: Calibration curve for SNAP.

tional redox processes were observed by the continuous cycling. However, a continuous decrease in the current of the anodic wave ascribed to SNAP oxidation is observed, suggesting that the redox process is controlled by diffusion. On the other hand, the oxidation potential of SNAP is, at least, 100 mV more positive than the potential ascribed to the oxidation of the iron atoms in the InHCF films. Thus, the oxidation of NO generated during SNAP decomposition could be detected without significant interferences from the free SNAP molecules.

Fig. 6 shows cyclic voltammograms of the InHCF-modified electrode in the absence and presence of increasing amounts of SNAP. In the absence of SNAP (curve a) the electrochemical response is that previously described for the modified electrode. Upon addition of SNAP (scans b–d), the cyclic voltammograms exhibit a significant enhancement of the anodic peak current concomitant with a decrease in the corresponding cathodic peak current. This behavior can be ascribed to the electrocatalytic oxidation of NO generated by decomposition of SNAP in the solution. In addition, in presence of SNAP, a second anodic wave with a peak potential at + 0.95 V, ascribed to the irreversible oxidation of free SNAP molecules onto the modified electrode surface, can be observed. In addition, as can be seen in the inset of Fig. 6, the electrocatalytic peak current corresponding to the oxidation of generated NO increases linearly with the concentration of SNAP up to 100 μM and then levels off, probably due to kinetic complications in the decomposition reaction. In the linear range, statistical analysis gave a linear correlation of 0.999. The determination limit was estimated to be about 10.0 μM, and the relative standard deviation for five successive measurements in a solution containing 50.0 μM of SNAP was 3.0%.

4. Conclusions

We have presented three different approaches for the determination of NO in solution based on the inhibition of the enzymatic activity of HRP as well as on the use of electrodes modified with films of redox active transition metal complexes, respectively. In the first, we have employed surface immobilized HRP in conjunction with a redox mediator in solution (1,4-benzoquinone) to monitor NO via its inhibitory effect on HRP activity. A detection limit of the order of 2 μM was determined. Although this value is modest in its magnitude, the sensor exhibits a rapid response (< 30 s) and (as would be expected for an enzyme-based assay), it exhibits high selectivity and is furthermore relatively immune to interferences. Because it can be used as a disposable biosensor it is suitable for screening analysis rather than for continuous monitoring.

In the case of electrodes modified with redox active films of transition metal complexes, these devices are significantly more robust and stable and can be used in the determination of NO even in the presence of nitrite and nitrate. The limits of detection in this case were of the order of 1 and 5 μM for the devices based on the Ni and Fe complexes, respectively. These values are fully comparable to the one mentioned above for the HRP-based approach, but because of their robust-

ness, these are much more appealing for continuous monitoring applications.

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