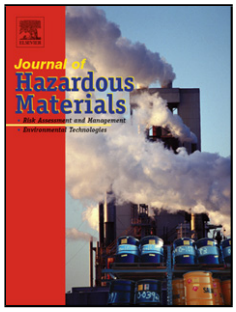

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Poly(neutral red) based hydrogen peroxide biosensor for chromium determination by inhibition measurements

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HIGHLIGHTS

- New amperometric peroxidase enzyme inhibition biosensors for Cr(III) and Cr(VI)
- Horseradish peroxidase (HRP) immobilized on poly(neutral red) carbon film electrode
- Improved analytical parameters compared to previous inhibition biosensors
- HRP inhibition mechanism was competitive for Cr(III) and uncompetitive for Cr(VI)
- Interference study demonstrated very good selectivity towards Cr(III) and Cr(VI)

Abstract

Amperometric hydrogen peroxide enzyme inhibition biosensors based on horseradish peroxidase (HRP) immobilized on electropolymerised neutral red (NR) or directly on the surface of carbon film electrodes (CFE) have been successfully applied to the determination of toxic Cr(III) and Cr(VI). Parameters influencing the performance of the biosensor including the enzyme immobilization method, the amount of hydrogen peroxide, applied potential and electrolyte pH were optimized. The inhibition of horseradish peroxidase by the chromium species was studied under the optimised conditions. Results from the quantitative analysis of chromium ions are discussed in terms of detection limit, linear range and sensitivity. The HRP kinetic interactions reveal mixed binding of Cr(III) with I_{50} = 3.8 μM and inhibition binding constant K_i = 11.3 μM at HRP/PNR/CFE electrodes and uncompetitive

binding of Cr(VI) with I_{50} = 3.9 μM and K_i' = 0.78 μM at HRP/CFE electrodes in the presence of H_2O_2 substrate. Interferences from other heavy metal ions were studied and the inhibition show very good selectivity towards Cr(III) and Cr(VI).

Keywords: amperometric biosensor; horseradish peroxidase; poly(neutral red); Cr(III); Cr(VI); enzyme inhibition.

1. Introduction

Chromium compounds are widespread in nature, including water, soil, plants and animals, as well as in atmospheric aerosols [1-3]. Chromium exists in different oxidation states of variable stability: 0, (II), (III), (IV), (V) and (VI) of which only elemental chromium does not occur naturally. The most common chemical species derive from Cr(III) and Cr(VI) which are non-degradable, and have mutagenic and carcinogenic properties [4-6], toxicity also depending on bioavailability [7]. Cr(VI) has strong oxidizing properties, occurs as soluble oxyanions and is highly pernicious for plants, animals and humans; its toxicity is considered to be 500–1000 times higher than that of Cr(III) [7-9]. Trivalent chromium is less toxic since it tends to form insoluble hydroxides; nevertheless, it can cause detrimental health effects after long-time exposure to high doses [6,7]. Environmental regulations define the upper limit values for total chromium and Cr(VI) concentrations in waters. For example, the U.S. Environmental Protection Agency stipulates that the maximum surface water contaminant level must not be above 50 μg/L for Cr(VI) and 100 μg/L for total chromium [4]. The development of a rapid and selective method for chromium species' determination is therefore necessary.

Analytical methods commonly used for chromium measurement in samples of environmental and biological origin include, as reported in [10,11]: spectrometry, inductively coupled plasma (ICP), chromatography coupled or not with atomic emission, and flame atomic absorption spectrometry (FAAS). However, even though these methods have high sensitivity and good reproducibility, they have drawbacks for routine analysis mainly due to the time needed and reagent consumption. Electrochemical sensors and biosensors for heavy metal ion determination have important advantages such as rapidity, effectiveness, simplicity, low

detection limit, and selectivity. Among electrochemical methods, enzyme inhibition biosensors have become very attractive for environmental monitoring [12].

It is well known that heavy metal ions inhibit the activity of enzymes. Enzyme inhibition-based biosensors appear to be very efficient for determining ions of these hazardous toxic elements, with high sensitivity and specificity. Enzyme-inhibition biosensors have been reported in the literature for the determination of heavy metal species such as: Co(II), Cd(II), Cu(II) and Ni(II) [13], Hg(II), Pb(II), Cd(II) [14] and Cr(III) [5]. The most used enzymes are urease [15,16], tyrosinase [5], acetylcholine esterase (AChE) [17] and horseradish peroxidase [18,19].

In the present work, an amperometric horseradish peroxidase based biosensor for inhibitive determination of toxic chromium ions is proposed for the first time. The aim of the investigation was to determine the influence of different chromium oxidation states (III and VI) on horseradish peroxidase activity. Optimization of the experimental conditions for maximising the biosensor response and the biosensor analytical characteristics is described, and comparison is made with the literature. The type of inhibition was determined and biosensor selectivity for chromium detection was evaluated.

2. Experimental

2.1 Reagents

All chemicals used in this work were of analytical grade and were used without further purification. Sodium acetate buffer solution (50 mM, pH 6), prepared from sodium acetate (Riedel-de-Haen) and acetic acid (Riedel-de-Haen) was used as supporting electrolyte for all the electrochemical measurements. Hydrogen peroxide 30% (w/w) was purchased from Riedel-de-Haën.

Neutral red (NR) from Aldrich was electropolymerised in potassium phosphate buffer pH 5.5 which was prepared using 0.025 M K_2HPO_4/ KH_2PO_4 from Panreac plus 0.1 M KNO_3 from Riedel-de-Haën.

Horseradish peroxidase (HRP, E.C. 1.11.1.7, 500 U/mg solid), glutaraldehyde (GA) (25% (v/v) in water) and bovine serum albumin (BSA) were obtained from Sigma. For the inhibition studies of Cr(III) and Cr(VI), the requisite amount of $Cr(NO_3)_36H_2O$ and $K_2Cr_2O_7$ (Merck) were dissolved in water.

All solutions were prepared using Millipore Milli-Q nanopure water (resistivity > 18 MΩ cm). Experiments were all carried out at room temperature (25±1ºC).

2.2 Electrochemical instrumentation and measurements

The amperometric and voltammetric experiments were performed with an Ivium CompactStat potentiostat (Ivium, The Netherlands), using a conventional three-electrode system. The working electrodes were modified carbon film electrodes (CFE). A platinum wire was used as counter electrode and all potentials were measured relative to an Ag/AgCl, saturated KCl reference electrode. Amperometric measurements were carried out in a stirred solution of 0.05 M sodium acetate buffer (pH 6.0) at -0.50 V.

2.3 Preparation of the modified carbon film electrode (CFE)

2.3.1 Electrode pre-treatment

Working electrodes with an exposed geometric area of ~0.20 cm^2 were made from carbon film electrical resistors of 2 Ω nominal resistance, length 6 mm and diameter 1.5 mm; the electrodes were prepared using the procedure described elsewhere [20,21].

Before electropolymerisation of NR, the bare electrodes were pre-treated by potential cycling from -1.0 to +1.0 V vs. SCE, at a scan rate of 100 mV s^{-1} in 0.1 M KNO_3 solution, for fifteen cycles, in order to decrease the background currents, increase the potential window, and ensure a reproducible electrode response [22].

2.3.2. Neutral red electropolymerisation

Neutral red (NR) is a phenazine dye which is soluble in water and ethanol [22]. A poly(neutral red) (PNR) modified carbon film electrode was prepared by electrochemical polymerisation from a fresh solution containing 1.0 mM of neutral red monomer, 0.025 M potassium phosphate buffer pH 5.5 plus 0.1 M KNO_3 by potential cycling 15 times between −1.0 and +1.0 V vs. Ag/AgCl at a potential sweep rate of 50 mV s^{-1}, as described in [22].

2.3.3. Enzyme immobilisation

Horseradish peroxidase (HRP) was immobilised onto the electrode surface by cross-linking with glutaraldehyde (GA) and bovine serum albumin (BSA) as previously used for other enzymes [23-25] in order to maintain the enzyme closer to its natural environment [26]. A mixture of 17 µL of 0.5 mg mL^{-1} HRP solution, 5 µL of 1 % BSA and 3 µL of 0.5 % GA was prepared; 7 µL of this mixture was pipetted onto the surface of the PNR-modified carbon film electrode and allowed to dry for 2 h at room temperature. The resulting cross-linked enzyme electrode was stored in phosphate buffer solution at 4ºC when not in use.

2.3.4. Biosensor response measurements

The HRP/PNR/CFE modified electrodes were immersed into a stirred acetate buffer solution (pH 6.0) and 1 mM of hydrogen peroxide (substrate) was added to record a steady-state current (I_0) before adding inhibitor. The concentration of added heavy metal ions (Cr(III) or

Cr(VI)) was increased stepwise, by adding defined volumes of an appropriately diluted solution to inhibit the enzyme activity, and the current decrease (I_1), which was proportional to the final concentration of inhibitor in solution was recorded. The percentage of inhibition (I (%)) due to the heavy metal ion inhibitor was evaluated according to the equation:

$$I\ (\%) = \frac{I_0 - I_1}{I_0} X\ 100$$

where I_0 and I_1 are the currents recorded before and after inhibition, respectively.

3. Results and Discussion

3.1 Cyclic voltammetry characterisation in the presence of H_2O_2

The developed biosensors were characterised by cyclic voltammetry in the absence and presence of hydrogen peroxide. Fig. 1 shows cyclic voltammograms of the enzyme electrode HRP/PNR/CFE and of the non-enzymatic electrode PNR/CFE measured without and with the addition of 20 mM H_2O_2 in 50 mM acetate buffer, pH 6.0. In both cases, a redox couple, corresponding to PNR oxidation/reduction is observed in the absence of H_2O_2. When peroxide is added, its reduction starts at -0.2 V and oxidation around +0.6 V; an enhancement of the PNR reduction peak current at -0.7 V is also observed. In the absence of enzyme, an increase of 33 % in the reduction response was observed for PNR/CFE at -0.7 V in the presence of 20 mM H_2O_2, but when HRP was immobilized on the PNR-modified carbon film electrode, HRP/PNR/CFE, there was a 77 % increase of the reduction current, indicating enzyme catalysis.

These observations illustrate that PNR, as well as HRP, plays a significant role, and this effect can be used to enhance the current generated. The same effect was seen in another hydrogen peroxide biosensor based on HRP and PNR, in which the enzyme was copolymerized together

with neutral red [27]. The observed increase in cathodic current might be explained by mechanisms where HRP catalyses the reduction of hydrogen peroxide to water.

The possible catalytic mechanism of HRP can be expressed by the following equations:

$$H_2O_2 + PNR_{red} \xrightarrow{HRP} PNR_{ox} + H_2O$$

$$PNR_{ox} + 2e^- \rightarrow PNR_{red}$$

3.2 Amperometric response to H_2O_2

Amperometric measurements were performed in stirred 50 mM acetate buffer solution (pH 6.0) at the working potential of -0.5 V vs Ag/AgCl using three different modified carbon film electrodes: HRP/CFE, PNR/CFE and HRP/PNR/CFE.

Amperometric responses of the HRP/CFE, PNR/CFE and HRP/PNR/CFE biosensors were investigated by sequentially increasing the concentration of H_2O_2. Fig. 2 inset shows the amperometric curve response of the biosensors upon successive addition of H_2O_2. When H_2O_2 was added, the HRP/PNR/CFE electrode responded rapidly to the increase in enzyme substrate concentration and achieved a steady-state current for each H_2O_2 concentration in 12 s.

Calibration curves for H_2O_2 at the three modified carbon film electrodes are shown in Fig. 2. Linear ranges were similar (up to 0.8 mM H_2O_2) for the two enzyme-based biosensors and longer for the mediator only biosensor (up to 1.8 mM H_2O_2). However, at higher hydrogen peroxide concentrations the response current approached saturation for all electrodes. The detection limits for HRP/CFE, PNR/CFE and PNR/HRP/CFE modified electrodes were calculated as 0.60, 0.50 and 0.03 μM respectively at a signal to noise ratio of 3 (Table 1). The highest sensitivity was obtained with the electrode modified with both PNR and HRP, which

was 600 times higher than when using only enzyme and a factor of 7 greater than PNR mediator only.

3.3 Optimisation of experimental conditions for inhibition

Experimental variables that can affect the performance of the inhibition biosensors using amperometry, namely the enzyme immobilization method, the constant applied potential, pH of the supporting electrolyte, and H_2O_2 concentration, were studied in order to optimise the inhibition response to chromium.

3.3.1 Influence of the immobilization method

The enzyme immobilization method is very important in developing a biosensor. Based on previous work where different immobilization methods were compared [23-25], horseradish peroxidase was chosen to be immobilized by cross-linking using glutaraldehyde and bovine serum albumin, due to a more sensitive response. The amount of immobilized enzyme was varied in order to evaluate which led to the best response towards hydrogen peroxide and metal ions. Different loadings were used: 0.1, 0.5, 1.0 and 2.0 mg mL^{-1}. The response toward peroxide increases with increase in enzyme concentration; however, the response to Cr(III) gradually decreases from 0.1 to 2.0 mg mL^{-1}. As a compromise between hydrogen peroxide and chromium ion response, an enzyme concentration of 0.5 mg mL^{-1} was chosen for use in future experiments, including those for Cr(VI) determination.

3.3.2 Influence of applied potential

Investigation of the influence of the potential on the percentage of inhibition was performed in the range of −0.2 V to −0.7 V vs Ag/AgCl. The effect of the applied potential on biosensor response is illustrated in Fig. 3. As can be seen, the response to H_2O_2 increases from −0.2 to −0.5 V, where the maximum was obtained, and then begins to decrease slightly (Fig.3a).

Therefore, a potential of −0.5 V vs Ag/AgCl was selected as the applied potential for amperometric measurements.

However, for inhibition measurements this potential might have to be shifted, owing to the catalytic mechanism, in which HRP catalyzes the reaction between H_2O_2 and the reduced form of PNR (see Section 3.1). Furthermore, the sensitivity of inhibition may depend on the amount of the reduced mediator (PNR) at the surface of the electrode. The degree of inhibition versus potential (Figure 3b) showed a similar behaviour to that of H_2O_2 (Fig. 3a), indicating that inhibition may be dependent on the amount of reduced PNR available in the vicinity of the enzyme on the surface of the electrode.

3.3.3 Influence of H_2O_2 concentration

The substrate concentration can influence the degree of inhibition [28]. Consequently, for an inhibition biosensor the amount of substrate has to be carefully adjusted [29]. The effect of substrate (H_2O_2) concentration on the inhibition of trivalent and hexavalent chromium by the enzyme electrode was thus examined. When the concentration of H_2O_2 was lower than 0.5 mM, the current response generated by the biosensor was as small as the inhibition by chromium and was not visible. However, in the case of competitive inhibition, at too high substrate concentration, all active sites of HRP enzyme will be occupied by the substrate and it will be insensitive to the inhibition by chromium ions. Hence, a concentration of 1.0 mM H_2O_2 was selected as the fixed concentration for further chromium ions amperometric measurements.

3.3.4 Effect of pH

The sensitivity of the enzyme biosensor depends significantly on the pH of the medium. Therefore, the effect of pH on the electrochemical behaviour of the HRP/PNR/CFE modified carbon film electrode over the range of pH 4.0–8.0 was investigated. The degree of inhibition

of the electrode for 1.0 μM of Cr(III) at different pH values of the electrolyte is shown in Fig. 4a. It is clearly observed that maximum inhibition is reached at about pH 6.0; for pH values below or above this, the degree of inhibition decreases.

The concentration of chromium ion corresponding to 10 % inhibition (I_{10}) was also determined at different pH. Fig. 4b displays the effect of pH on the variation of the I_{10} values due to Cr(III). The highest value was obtained at pH 8.0, followed by pH 4.0 and between pH 5.0 and 7.0, the values of I_{10} hardly change. However, the lowest value was at pH 6.0 which, consequently, was selected as the optimum pH for further experiments.

3.4. Determination of chromium by inhibition

Amperometric determination of Cr(III) and Cr(VI) was evaluated using all three modified electrodes: PNR/CFE, HRP/CFE, HRP/PNR/CFE; the possibility of direct interaction of chromium with peroxide was also verified in independent measurements with bare electrodes. No response for Cr(III) or Cr(VI) was obtained in the absence of enzyme, either at bare or at PNR modified electrodes, so that the response obtained can be attributed to enzyme inhibition. The inhibition results obtained at HRP/CFE and HRP/PNR/CFE electrodes are reported below, and in Table 2, in which the limits of detection for Cr(III) and Cr(VI) were calculated based on a signal-to-noise ratio of 3. I_{10} values, as well as I_{50}, corresponding to the concentrations of chromium species that lead to 10 % and 50 % degree of inhibition, respectively, were also estimated.

3.4.1 Cr(III) measurements

Cr(III) was measured in the presence of 1.0 mM H_2O_2 with the two biosensors (HRP/CFE and HRP/PNR/CFE). The curve in Fig. 5a illustrates the time-dependent response of the HRP/PNR/CFE electrode to Cr(III). After an increase in amperometric response when 1.0 mM H_2O_2 is added, the response decreases due to the successive addition of Cr^{3+} into the

buffer solution, clearly indicating that chromium (III) inhibits the activity of HRP immobilized in the HRP/PNR/CFE electrode. The signal corresponding to the initial response to H_2O_2 is equal to 22.4 ± 0.5 μA cm^{-2}, in which the SD represents the noise level. Thus, the detection limit is calculated to be the inhibitor concentration leading to a change in current response of 3xSD = 1.5 μA cm^{-2}. From Fig.5, this corresponds to 6.7 % inhibition. or 0.27 μM of Cr(III). In a similar way, the detection limit of Cr(III) at HRP/CFE was determined to be 1.15 μM, corresponding to 5.2 % inhibition. The value of LOD obtained at HRP/PNR/CFE was lower than that obtained at a glassy carbon electrode modified with electropolymerised pyrrole, in which tyrosinase was immobilized, tyrosinase-Ppy/GCE biosensor (0.5 μM) [5].

A linear response was obtained between 0.2 μM and 5.1 μM with the HRP/PNR/CFE electrode (Fig.5b), and 1.0–8.0 μM with the HRP/CFE electrode. The I_{10} of the HRP/PNR/CFE biosensor was 0.4 μM which is lower than that obtained using the HRP/CFE sensor (2.2 μM) (Table 2) and lower than those reported for glucose oxidase with electropolymerized aniline at a platinum electrode with ferrocene as redox mediator, GOx/PANI/Fc/Pt biosensor (9.6 μM) [30] or glucose oxidase immobilized on a carbon paste electrode modified with manganese dioxide, GOx/MnO_2/CPE (1009 μM) [31]. The inhibition sensitivity obtained with the HRP/PNR/CFE electrode was 58.5 % μM^{-1} for Cr(III), whereas for HRP/CFE the sensitivity was 4 times lower, 14 % μM^{-1}. Cr(III) was shown to also inhibit tyrosinase [5], glucose oxidase [30,31] and NADPH-cyt P450 reductase [32], but no sensitivity values for comparison purposes are specified in these studies, and in [5] the value is not comparable with that obtained here.

3.4.2 Cr(VI) *measurements*

The amperometric response of the HRP/CFE biosensor when injecting different concentrations of Cr(VI) ranging from 0.05 to 1.5 μM is shown in Fig. 6a. The degree of

inhibition as a function of Cr(VI) concentration is shown in the typical inhibition calibration curve in Fig. 6b, in which the concentration of substrate (H_2O_2) is fixed at 1.0 mM. Both HRP/CFE and HRP/PNR/CFE biosensors provided a linear response to the increase of Cr(VI) concentration over the range of 0.05-0.35 μM. For Cr(VI), the sensitivity was 5 times higher at HRP/CFE than at HRP/PNR/CFE biosensor. Results reported in Table 2 showed that the I_{10} of the HRP/CFE biosensor was 0.1 μM, which is lower than that obtained using the HRP/PNR/CFE sensor (2.5 μM) and lower than that reported for a GOx/PANI/Fc/Pt biosensor (0.15μM) [30].

The detection limit was calculated to be 0.09 μM, based on a signal-to-noise ratio of 3 by Cr(VI) using the HRP/CFE biosensor and 1.6 μM using the HRP/PNR/CFE biosensor, both values being much lower than the 48 μM obtained by inhibition of glucose oxidase immobilised on a poly-o-phenylenediamine platinum electrode, GOx/PPD/Pt [33] and the 3.8 μM obtained by inhibition of Cyt c_3 immobilised on a GCE [34]. A lower detection limit (0.009 μM) than here was achieved by the use of GOx/PANI/Pt, but this electrode is less selective than those developed in this study, since several metal ions such as Cu^{2+}, Cd^{2+} and Co^{2+} interfered with their chromium determination [30].

3.5 Kinetic study and mechanism of inhibition

Because the preparation procedure of a sensor affects the substrate as well as the inhibitor kinetics of the enzyme, the type of HRP inhibition that chromium causes was investigated. Amperometric measurements for Cr(III) using HRP/PNR/CFE and Cr(VI) using HRP/CFE were carried out in the presence of 1.0 mM and 5.0 mM H_2O_2, with subsequent additions of chromium species into supporting electrolyte at pH 6.0. An apparent inhibitor binding constant (K_i = 11.3±0.7 μM, the dissociation constant of the enzyme-inhibitor complex) was determined for Cr(III), in the presence of the two different H_2O_2 concentrations, using Dixon

plots (Fig. 7a1) [35]. In these plots, the reciprocal value of the steady-state current is plotted against inhibitor concentration at different substrate concentration values. For reversible binding a series of straight lines is obtained that intersect at K_i, as occurs here.

The value of I_{50}, the concentration of inhibitor producing 50 % of the inhibition signal was calculated to be 36.8 μM in the presence of 1.0 mM H_2O_2, approximately half of the value (I_{50}= 71 μM) determined for the same electrode in the presence of 5.0 mM H_2O_2. This doubling of the I_{50} value indicates a decrease in the enzyme/substrate interaction and also illustrates the characteristic behaviour of a competitive inhibitor. In the case of competitive inhibition, at high substrate concentrations, the inhibition effect is not observed since the substrate dominates the response [12], thus masking competitive inhibition [36]. There is only one study on the inhibition mechanism of Cr(III) in which it was found that Cr(III) inhibits NADPH-cyt P450 reductase non-competitively [32].

In the present study, the type of inhibition for Cr(III) was found to be mixed, a mixture of competitive and uncompetitive inhibition. This conclusion was reached by analysing Dixon (Fig. 7a1) and Cornish-Bowden (Fig. 7a2) plots which show intersection to the left side of the y-axis, above (for Dixon) and below (for Cornish-Bowden) the inhibitor axis.

In relation to Cr(VI), the Dixon plot (Figure7b1) showed parallel lines, indicating uncompetitive inhibition. In order to confirm this, a Cornish-Bowden [37] plot was drawn (Fig. 7b2) and showed intersection of the lines on the left side of the y axis, above the inhibitor axis, in agreement with uncompetitive inhibition. The value of K_i', the dissociation constant of the enzyme-inhibitor-substrate complex, was determined as 0.78±0.05 μM. To our knowledge, no study about the type of inhibition of Cr(VI) has been previously performed.

3.6 Selectivity and stability

Selectivity is an important parameter in the performance of a biosensor. In order to demonstrate the selectivity of the biosensor, the potential interference from other metals was examined using 1.0 mM of H_2O_2. Several possible interferents, i.e. Zn(II), Cu(II), Cd(II), Co(II), Ni(II), Hg(II) and Pb(II), were selected to investigate whether they have any influence on the determination of chromium. No noticeable inhibition effect was detected in the presence of Zn(II), Cu(II), Cd(II), and Pb(II), which were tested in the concentration range up to 120 μM. However, Ni(II), Co(II) and Hg(II) were found to have some influence, causing inhibition of the enzymatic activity of HRP, see Table 3. The HRP/PNR/CFE biosensor was able to detect Ni(II) but with an I_{10} value of 10.3 μM, higher than Cr(III) and Cr(VI) which were 0.4 and 2.5 μM respectively. The I_{50} value for Ni(II) was 47 μM. The 10 % inhibition value in the presence of Co(II), 11.6 μM, was also significantly higher. However, the presence of 1.0 μM Hg(II) induced a strong and irreversible inhibitory effect on the biosensor response. It should be noted here that inhibition by chromium species is reversible and thus washing the biosensors restores the full activity of the enzyme. On the other hand, the mercury inhibition is irreversible and residual inhibition can be observed after washing the biosensors with buffer. Thus, any interference of mercury can be recognized and distinguished from chromium inhibition during measurements.

Selective determination of Cr(VI) and Cr(III) in mixtures can be performed by determining Cr(VI), then oxidizing chromium(III) into chromium(VI) to determine total chromium and calculating the amount of Cr(III) in the original sample by subtraction.

The developed biosensor exhibited good stability; it can be used for chromium speciation for at least 3 weeks after which a decrease of 10 % from the initial response was noticed.

Conclusions

An enzymatic biosensor for Cr(III) and Cr(VI) detection has been developed for the first time based on the chromium inhibitor effect on the activity of horseradish peroxidase (HRP), which was immobilized by cross-linking with glutaraldehyde on CFE and PNR/CFE electrode. The HRP/PNR/CFE and HRP/CFE electrodes described represent an environmentally friendly method for the analysis of chromium. Under the optimum experimental conditions, the apparent inhibition binding constant was determined from Dixon and Cornish-Bowden plots and the inhibition mechanism was found to be mixed for Cr(III) and uncompetitive for Cr(VI). The developed electrodes allow the selective and sensitive amperometric determination of Cr(III) and Cr(VI) in the presence of several other heavy metal ions, offering a method for chromium speciation analysis.

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Tables

Table 1 Performance characteristics of HRP/CFE, PNR/CFE and HRP/PNR/CFE biosensors obtained from H_2O_2 calibration curves

Biosensor	Linear range (mM)	LOD (μM)	Sensitivity ($\mu A\ \mu M^{-1}$)
HRP/CFE	up to 0.8	0.5	0.26
PNR/CFE	up to 1.8	0.6	22
HRP/PNR/CFE	up to 0.8	0.03	154

Table 2 Principal analytical data referring to the calibration curves for the amperometric response of HRP/CFE and HRP/PNR/CFE biosensors and comparison with chromium inhibition biosensors in the literature

Biosensor		LOD (μM)	I_{10} (μM)	I_{50} (μM)	Ref
Tyrosinase-PPy/GCE	Cr(III)	0.5	*	*	[5]
GOx/PANI/Fc/Pt	Cr(III)	*	9.6	*	[30]
	Cr(VI)	0.009	0.15	*	
GOx/MnO_2/CPE	Cr(III)	*	1009	*	[31]
	Cr(VI)	4807	*	*	
NADPH-cyt P450 reductase	Cr(III)	*	*	24	[32]
GOx/PPD/Pt	Cr(VI)	48	*	1450	[33]
Cyt c_3/GCE	Cr(VI)	3.8	*	*	[34]
HRP/PNR/CFE	Cr(III)	0.27	0.4	3.9	This work
	Cr(VI)	1.6	2.5	63	
HRP/CFE	Cr(III)	1.15	2.2	37	This work
	Cr(VI)	0.09	0.1	3.8	

*-not specified

Table 3 Inhibition effect of Cr(III), Cr(VI) and possible interfering cations tested with the HRP/PNR/CFE biosensor

Metal ion species	Concentration (μM)	% Inhibition
Cr(III)	0.4	10
Cr(VI)	2.5	10
Ni(II)	10.3	10
Co(II)	11.6	10
Hg(II)	1.0	100

Figure captions

Fig. 1. Cyclic voltammograms at (a) PNR/CFE and (b) HRP/PNR/CFE electrodes (--) without and (—) with 20 mM H_2O_2 in pH 6.0, 50 mM acetate buffer, scan rate 50 mV s^{-1}.

Fig. 2. Calibration curves obtained with (▲) PNR/CFE, (■) HRP/CFE and (●) HRP/PNR/CFE modified electrodes in 50 mM acetate buffer pH 6.0 at -0.5 V vs. Ag/AgCl. Inset shows a typical response to successive H_2O_2 injections.

Fig. 3. Effect of the applied potential on the enzyme electrode response to (a) 1.0 mM H_2O_2 and (b) 4.0 μM Cr(III). Supporting electrolyte 50 mM acetate buffer pH 6.0.

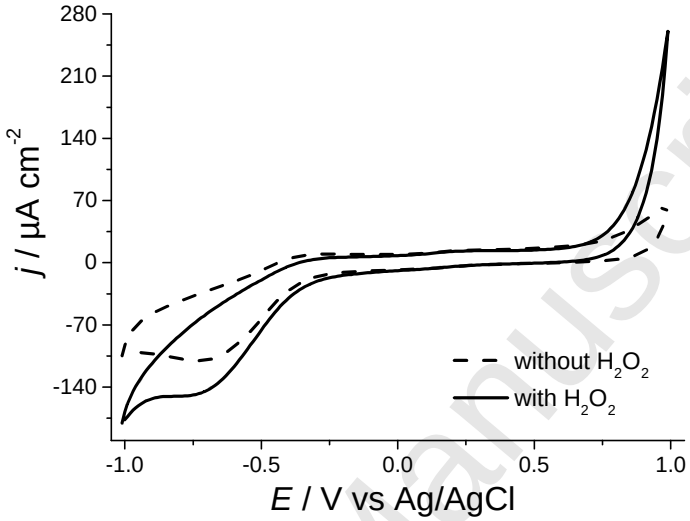
Fig. 4. Effect of pH on (a) degree of inhibition of 1.0 μM of Cr(III) and (b) chromium concentration corresponding to 10% of inhibition (I_{10}). Applied potential -0.5 V vs Ag/AgCl. Supporting electrolyte 50 mM acetate buffer pH 6.0 containing 1.0 mM H_2O_2.

Fig. 5 (a) Response to Cr(III) at HRP/PNR/CFE and (b) calibration curve obtained with (○) HRP/PNR/CFE and (■) HRP/CFE at -0.5 V in 50 mM acetate buffer pH 6.0 in the presence of 1.0 mM H_2O_2.

Fig. 6 (a) Response to Cr(VI) at HRP/CFE and (b) calibration curve obtained with (■) HRP/CFE and (○) HRP/PNR/CFE at -0.5 V in 50 mM acetate buffer pH 6.0 in the presence of 1.0 mM H_2O_2.

Fig. 7. Analysis of the type of inhibition of HRP by Cr(III) and Cr(VI) in the presence of 1.0 mM and 5.0 mM H_2O_2; other experimental conditions as Fig.6. (a) Dixon plot (a1) and Cornish-Bowden plot (a2) for Cr(III) at HRP/PNR/CFE. (b) Dixon plot (b1) and Cornish-Bowden plot (b2) for Cr(VI) at HRP/CFE.

(a)



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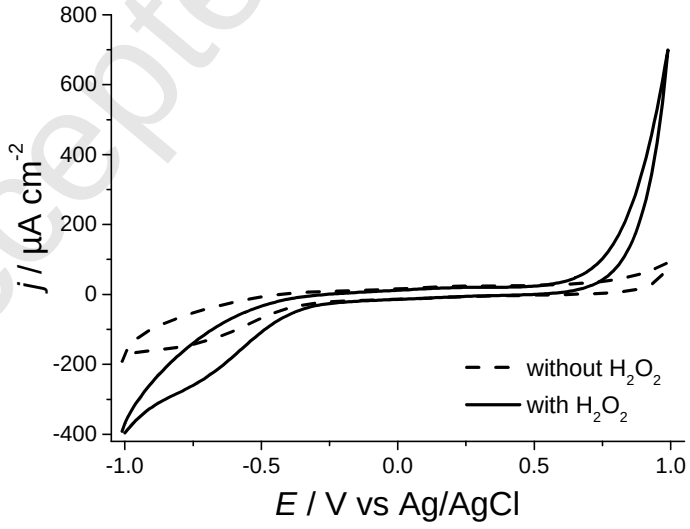

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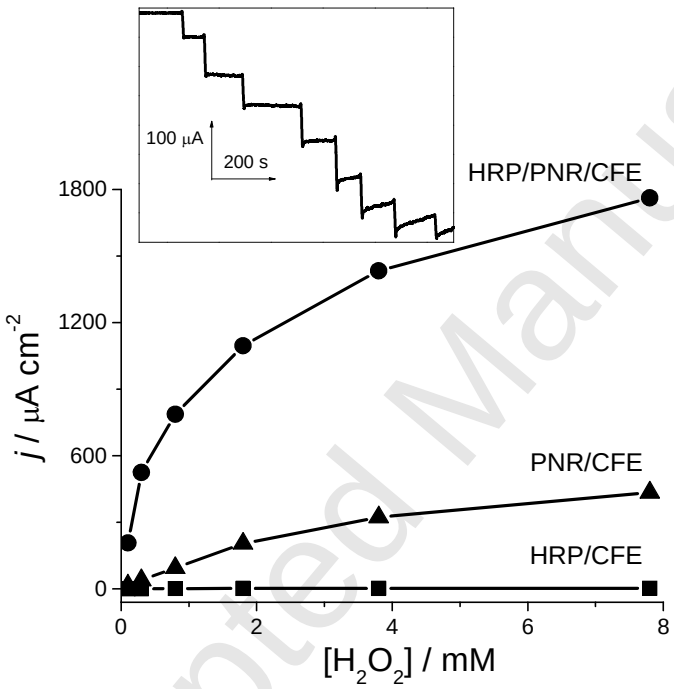

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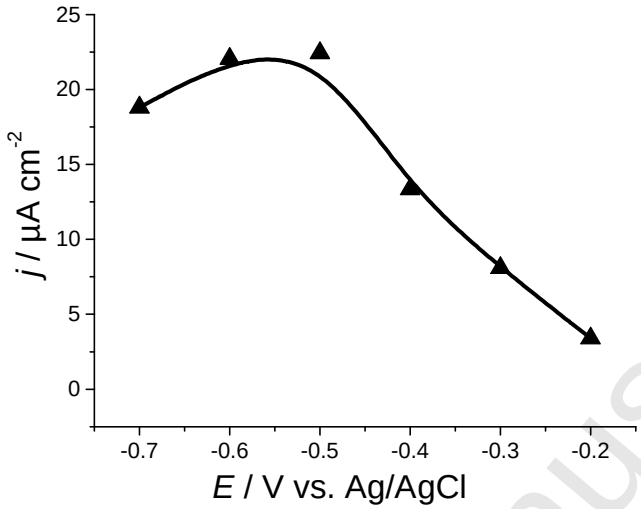

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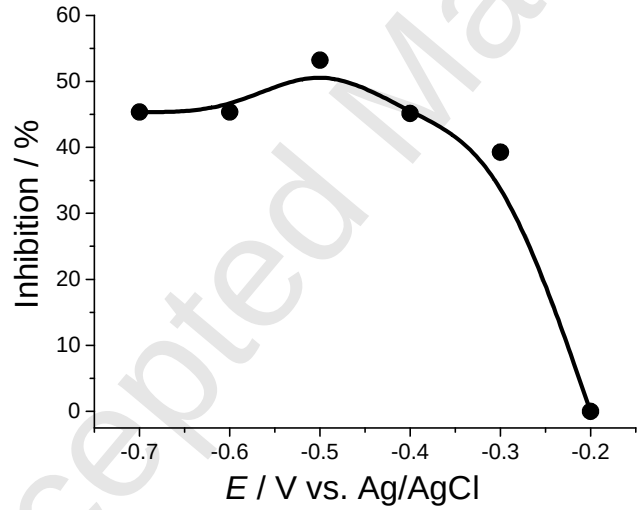

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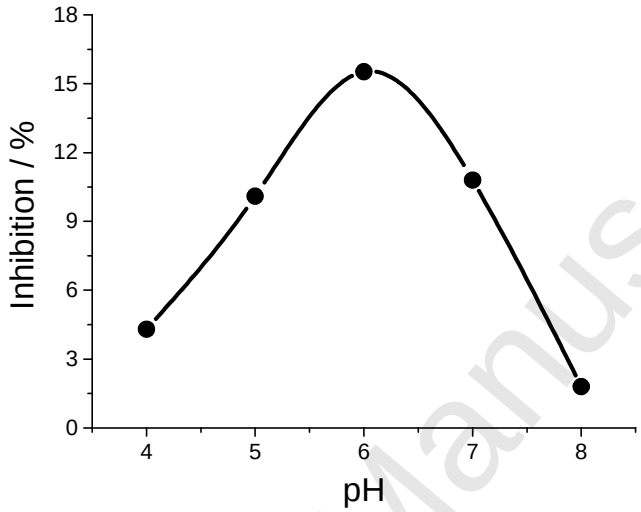

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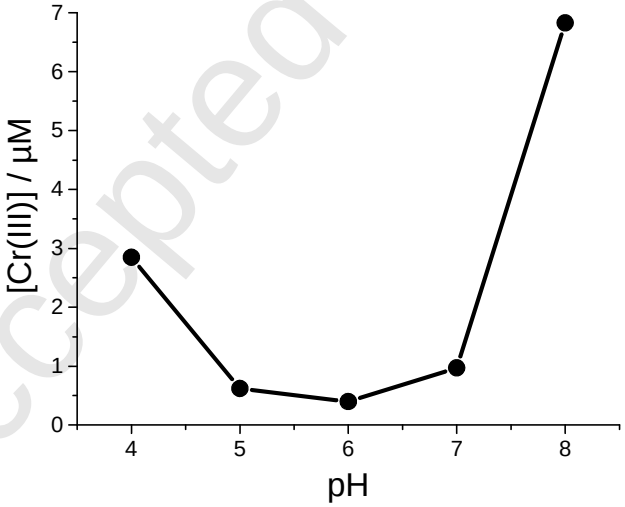

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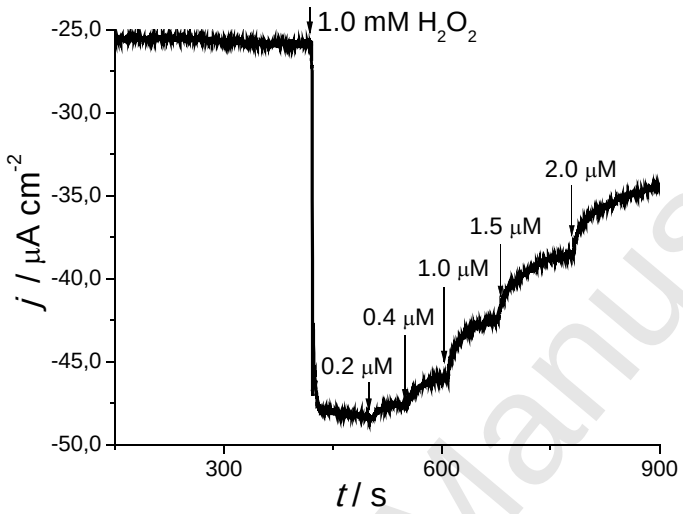

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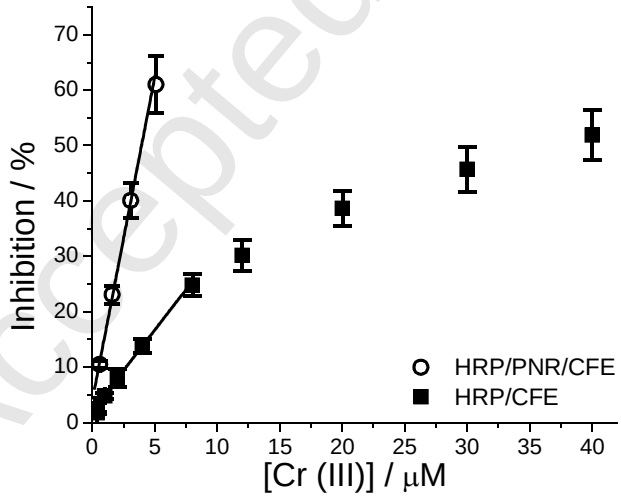

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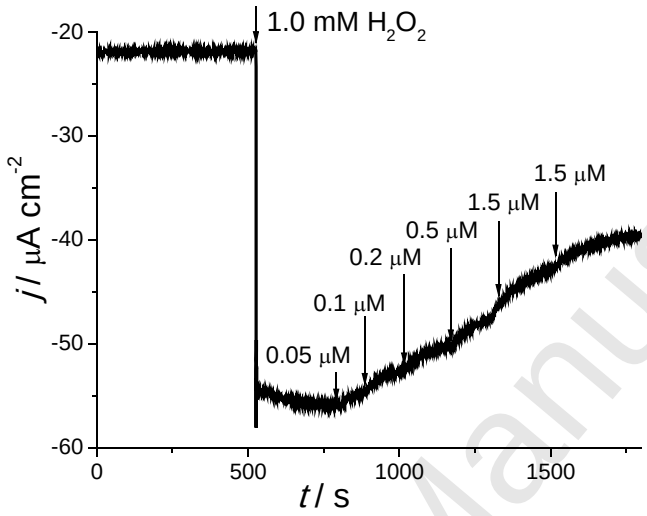

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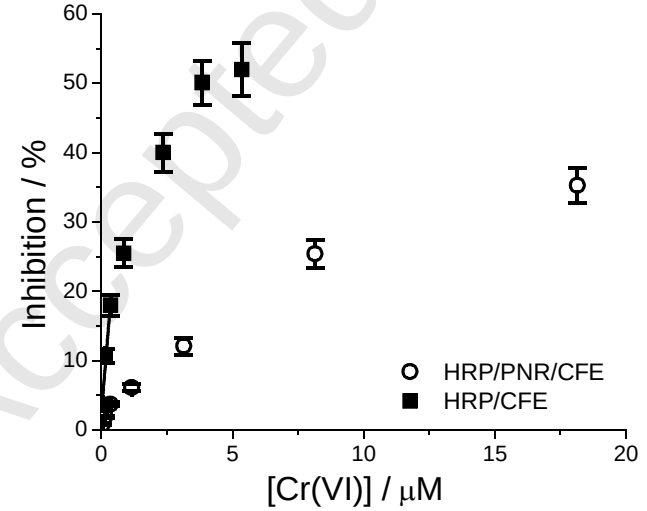

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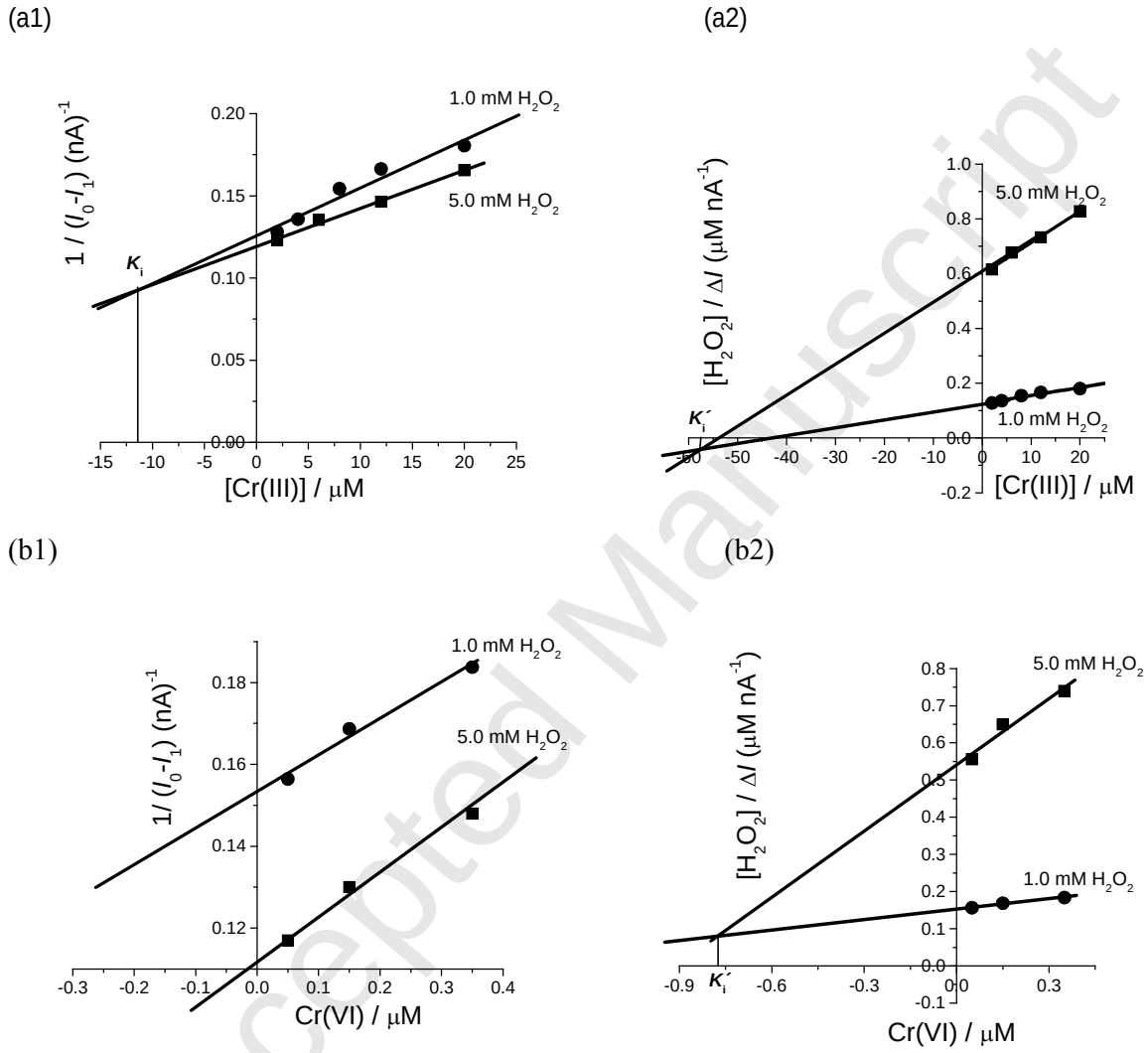

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