Journal of Hazardous Materials 279 (2014) 348–355

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

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) immobilised 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).

ARTICLE INFO

Article history:
Received 5 February 2014
Received in revised form 9 July 2014
Accepted 12 July 2014
Available online 21 July 2014

Keywords:
Amperometric biosensor
Horseradish peroxidase
Poly(neutral red)
Cr(III)
Cr(VI)
Enzyme inhibition

ABSTRACT

Amperometric hydrogen peroxide enzyme inhibition biosensors based on horseradish peroxidase (HRP) immobilised 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 immobilisation method, the amount of hydrogen peroxide, applied potential and electrolyte pH were optimised. 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 biosensors and uncompetitive binding of Cr(VI) with I_{50} = 3.9 μM and K_i = 0.78 μM at HRP/CFE biosensors 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).

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 oxidising 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

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http://dx.doi.org/10.1016/j.jhazmat.2014.07.019
0304-3894/

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. Optimisation 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-Haën) and acetic acid (Riedel-de-Haën) 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 $Cr(NO_3)_3 6H_2O$ 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 ± 1 °C).

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 4 °C 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:

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

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 immobilised 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