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Biosensors

COMPARISON OF COBALT HEXACYANOFERRATE AND POLY(NEUTRAL RED) MODIFIED CARBON FILM ELECTRODES FOR THE AMPEROMETRIC DETECTION OF HEAVY METALS BASED ON GLUCOSE OXIDASE ENZYME INHIBITION

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Electrochemical biosensors have been developed for the determination of cadmium, cobalt, and copper cations through their inhibitory effect on glucose oxidase activity, using cobalt hexacyanoferrate or poly(neutral red) as redox mediators. Cobalt hexacyanoferrate and poly(neutral red) were used to modify carbon film electrodes by electrodeposition and glucose oxidase was then immobilized on the electrode by crosslinking with glutaraldehyde. A comparison between the two redox mediated biosensors for the determination of the metal ions was performed under the same experimental conditions. In fixed-potential amperometry at −0.35 V vs. the saturated calomel electrode, the detection limits for the metal cations studied were in the low micromolar range, lower at glucose oxidase\|cobalt hexacyanoferrate\|carbon film electrode, and the lowest achieved with glucose oxidase inhibition measurements. The linear dynamic ranges were wider at the glucose oxidase\|poly(neutral red)\|carbon film electrode for all three metal ions. The 10% and 50% inhibition values (I_{10} and I_{50}) for the metal cations were calculated and the degree of inhibition at the biosensors was compared. Copper ions inhibited glucose oxidase to the highest degree for both biosensors. The application for the analysis of tap water was demonstrated.

Keywords: Carbon film electrodes; Electrochemical biosensor; Enzyme inhibition; Heavy metal cations; Redox mediators

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INTRODUCTION

Environmental pollution by heavy metals is increasingly becoming a global challenge and represents a serious threat to public health, especially in developing countries (Yang et al. 2013). Many biosensors used in environmental monitoring are based on enzyme activity inhibition, which are mainly focused on the detection of pollutants, such as pesticides, heavy metal ions, and other toxic compounds at low concentrations and can be used for in situ analysis and low-cost screening procedures (Bagal-Kestwal et al. 2008; Ghica et al. 2013). Heavy metals in the environment have the potential to contaminate soil and water, and they are characterized by relatively high stability and solubility in atmospheric precipitations (Lim et al. 2008). Their high nonbiodegradability and accumulation in human and animal organisms results in various diseases, genetic disorders, and deleterious ecological effects (Gammoudi et al. 2010). Substances such as cadmium have been classified as "priority hazardous substances" (Tekaya et al. 2013) for which necessary measurements should be implemented. In the light of the aforementioned issues, quality control of aquatic ecosystems and foodstuff is extremely important for the protection of nature and the improvement in quality of life (Tekaya et al. 2013). Conventional techniques for heavy metal analysis are based on chromatography and spectrometry; however, these techniques require highly-skilled personnel, long analysis time, as well as complex and expensive equipment (Bagal-Kestwal et al. 2008). Thus, the development of an effective, selective, rapid, and economical method to monitor environmental quality and determine the concentration of hazardous metals is an important challenge. Electrochemical sensors and biosensors are an alternative, or at least a complementary choice, for the determination of hazardous substances (Sbartai et al. 2012), as they offer a promising approach to solve the aforementioned problems. At present, the use of a mediator is a good means for improving sensor performance for a wide variety of enzymes, even in enzyme-immobilized electrode systems (Cass et al. 1984). Inhibition-based biosensors represent a good alternative for the determination of the concentrations of toxic compounds such as heavy metals ions (Bagal-Kestwal et al. 2008; Ghica et al. 2013; Amine at al. 2014).

In this work, carbon film electrodes were used as substrates for biosensor construction, on which one of two redox mediators, cobalt hexacyanoferrate or poly(neutral red), was deposited, followed by immobilization of glucose oxidase. The amperometric measurement of the concentration of cadmium, cobalt, and copper ions was performed through their inhibitory effect on the immobilized glucose oxidase. This work follows previous results obtained with the two mentioned redox mediators based on glucose biosensors (Ghica et al. 2013; Ghica and Brett 2008) for the determination of metal cations by inhibition measurements. The study uses previously optimized conditions and compares the performances of the two biosensors (glucose oxidase/poly(neutral red)/carbon film electrode and glucose oxidase/cobalt hexacyanoferrate/carbon film electrode; a major accomplishment is the improvement in limits of detection and sensitivity.

EXPERIMENTAL

Reagents and Solutions

Sodium phosphate buffer solution (0.10 M, pH 7.0) was prepared from NaH_2PO_4/Na_2HPO_4 Riedel-de-Haen) and 0.05 M NaCl (Riedel-de-Haen) using

Millipore Milli-Q nanopure water (resistivity >18 MΩ cm) and was used as supporting electrolyte for all the electrochemical measurements. Glucose oxidase (E.C. 1.1.3.4, from Aspergillus Niger, 24 U/mg) was from Fluka, α-D(+)-glucose, glutaraldehyde (25% (v/v) in water), and bovine serum albumin were obtained from Sigma. For the inhibition studies, copper(II) chloride, cobalt(II) chloride, and cadmium sulfate were from Merck. Potassium hexacyanoferrate was from Merck, and neutral red from Aldrich. For cobalt hexacyanoferrate electrodeposition, 0.05 M NaCl (Riedel-de-Haën) at pH 3.0 was used. Poly(neutral red) films were formed by electropolymerization of neutral red in potassium phosphate buffer at pH 5.5, which was prepared using 0.025 M K_2HPO_4/KH_2PO_4 from Panreac and 0.10 M KNO_3 from Riedel-de-Haën. All chemicals were of analytical-reagent grade and were used without further purification. Experiments were performed at room temperature, 25 ± 1°C.

Electrochemical Cell and Instruments

Electrochemical measurements, cyclic voltammetry, and amperometry were carried out by using an Ivium CompactStat (The Netherlands) potentiostat and a conventional three-electrode system with a carbon film modified electrode as the working electrode, a platinum wire as the counter electrode, and a saturated calomel electrode (SCE) as the reference electrode. All fixed-potential amperometric experiments were performed in a stirred solution of phosphate buffer (pH 7.0) at the previously optimized potential of −0.35 V vs. SCE (Ghica and Brett 2008; Ghica et al. 2013).

Preparation of the Carbon Film Electrode Substrate

The electrodes used were made from carbon film electrical resistors of 2 Ω nominal resistance; the preparation protocol is described in detail elsewhere (Filipe and Brett 2004). The resistors, fabricated from ceramic cylinders coated with a carbon film obtained by pyrolysis, have a length of 6 mm and external diameter of 1.5 mm with two metal caps and connecting wires as external contact placed over each end. To prepare the electrodes, one of these metal caps is removed and the other insulated in plastic and afterwards protected by epoxy resin (Brett, Angnes, and Liess 2001). The final exposed carbon film electrode geometric area is ∼0.20 cm^2 (Gouveia-Caridade et al. 2008).

The electrodes were electrochemically pretreated by cycling the applied potential between −0.1 and +1.0 V vs. SCE, at a scan rate of 100 mV s^{-1}, for twenty cycles in 0.10 M sodium phosphate buffer pH 7.0 prior to electrodeposition of cobalt hexacyanoferrate, and in 0.025 M potassium phosphate buffer pH 5.5 plus 0.10 M KNO_3 solution prior to electropolymerization of neutral red. This conditioning treatment is essential to ensure a good performance (Filipe and Brett 2004; Brett, Angnes, and Liess 2001) and a reproducible electrode response (Ghica et al. 2013).

Cobalt Hexacyanoferrate Deposition

A freshly prepared solution containing 5.0 mM $CoCl_2$, 2.5 mM $K_3Fe(CN)_6$, and 0.05 M NaCl at pH 3.0 was used for the electrochemical deposition of cobalt

hexacyanoferrate on the carbon film electrode (Florescu and Brett 2004). The electrode was cycled 25 times between −0.2 and +0.9 V at a scan rate of 50 mV s^{-1} and was stabilized for 1 h in 0.05 M NaCl, pH 3.0 and then kept in the dark until use.

Neutral Red Electropolymerization

Poly(neutral red) films were formed on the carbon film electrode by electrochemical polymerization. The carbon film electrode was immersed in a solution containing 1.0 mM of neutral red monomer, 0.025 M potassium phosphate buffer pH 5.5, and 0.10 M KNO_3 (Ghica and Brett 2006). The potential was scanned between −1.0 and +1.0 V at 50 mV s^{-1} for 15 complete cycles.

Enzyme Immobilization

Glucose oxidase was immobilized on the electrode surface using a procedure previously reported (Crespilho et al. 2006) by crosslinking with glutaraldehyde and bovine serum albumin. This procedure leads to a friendly environment for enzyme immobilization which helps to prevent enzyme activity loss. A mixture of glucose oxidase with bovine serum albumin and glutaraldehyde was prepared. The enzyme mixture contained 10 μL enzyme solution (40 mg mL^{-1} bovine serum albumin and 10 mg mL^{-1} glucose oxidase in phosphate buffer) and 5 μL glutaraldehyde (2.5% in water). A volume of 7 μL of this mixture was dispersed over the surface of the cobalt hexacyanoferrate or poly(neutral red)-modified carbon film electrodes and was dried at room temperature for two hours. The electrodes were kept in phosphate buffer solution pH 7.0 at 4°C until use.

Enzyme Inhibition Studies

The glucose oxidase/cobalt hexacyanoferrate/carbon film and glucose oxidase/poly(neutral red)/carbon film modified electrodes were dipped into a stirred phosphate buffer solution (pH 7.0) and the baseline current at the fixed potential to be used left to stabilize. A chosen amount of glucose was added to give a final concentration of 0.2 mM or 0.5 mM, and the steady-state current after injection was recorded, I_0. Solutions of increasing heavy metal cation concentration (Co^{2+}, Cu^{2+}, or Cd^{2+}) were then added separately to inhibit the enzyme activity and the current I_1, was recorded, the decrease ($I_0 - I_1$) being proportional to the concentration of inhibitor in solution. The percentage inhibition (I, %) due to the heavy metal cations was calculated using the relationship (1):

$$I\ (\%) = \frac{I_0 - I_1}{I_0} \times 100. \quad (1)$$

RESULTS AND DISCUSSION

Deposition of Redox Mediators

Cobalt hexacyanoferrate or poly(neutral red) films were deposited on the carbon film electrode substrate by potential cycling, as described in the experimental

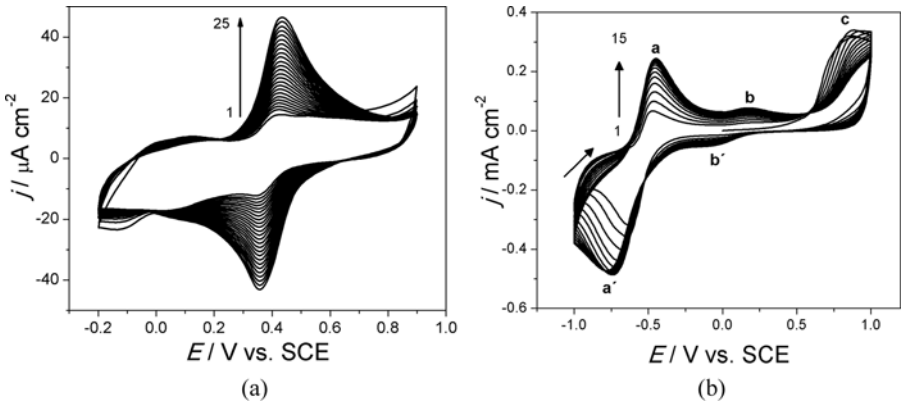

Figure 1. Cyclic voltammetry at the carbon film electrode showing (a) deposition of cobalt hexacyanoferrate between −0.2 and +0.9 V from 5.0 mM $CoCl_2$ + 2.5 mM $K_3Fe(CN)_6$ + 0.05 M NaCl, pH 3.0 and (b) electropolymerization of 1.0 mM neutral red in 0.025 M potassium phosphate buffer +0.10 M KNO_3, pH 5.5. The scan rate was 50 mV s^{-1}.

section. Figure 1 shows cyclic voltammograms obtained during redox mediator deposition; in both cases the increase in peak current with number of cycles is due to an increased amount of mediator film formed. Cyclic voltammograms obtained during cobalt hexacyanoferrate film growth are exhibited in Figure 1a. The electrochemical profile shows one pair of redox peaks with formal potential 0.394 V which might correspond to both processes $Co^{II} \rightleftarrows Co^{III}$and $[Fe^{III}(CN)_6]^{3-} \rightleftarrows [Fe^{II}(CN)_6]^{4-}$ occurring simultaneously. The cyclic voltammograms recorded during neutral red electropolymerization on the carbon film electrode in the potential range −1.0 to +1.0 V are shown in Figure 1b. Two redox couples (peaks a, a′ and b, b′) are visible; the first related to oxidation–reduction of the polymer, also overlapping with monomer oxidation–reduction, and the second due to doping/de-doping of the polymer. Peak (c) in the high positive potential region (0.8–1.0 V) corresponds to irreversible neutral red oxidation and radical formation that initiates polymerization of the monomer (Pauliukaite and Brett 2008).

Amperometric Response to Glucose

Typical current-time response of the biosensors covering a range of glucose (substrate) concentrations from 0.05 mM to 7.0 mM was obtained by successive additions of glucose into a stirred buffer at both glucose oxidase/poly(neutral red)/carbon film electrodes and glucose oxidase/cobalt hexacyanoferrate/carbon film electrodes at −0.35 V vs. SCE. Figure 2 shows that with increasing concentration of glucose, the amperometric response increased linearly from 0.05 mM to 0.85 mM and then reached a saturation value (I_{max}), indicative of a typical Michaelis–Menten process. The apparent Michaelis constants, $K_{M,app}$, which is the concentration of substrate corresponding to $I_{max}/2$ deduced from these response curves, was 0.29 mM and 1.25 mM for the glucose oxidase/poly(neutral red)/carbon film electrode and glucose oxidase/cobalt hexacyanoferrate/carbon film electrode

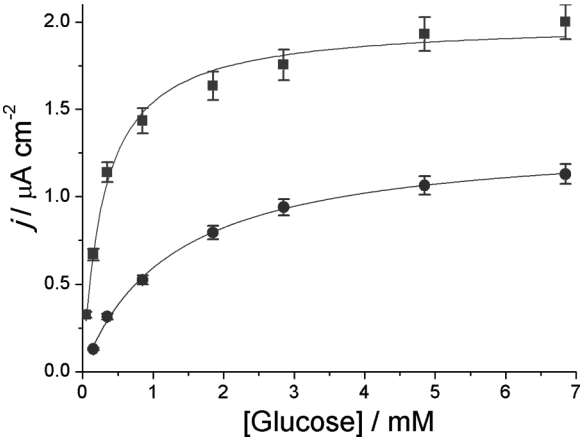

Figure 2. Calibration curves for glucose obtained with (●) the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and (■) the glucose oxidase/poly(neutral red)/carbon film electrode biosensors in 0.10 M sodium phosphate buffer at pH 7.0 at −0.35 V vs. SCE.

biosensors, respectively. The sensitivity was approximately three times higher at the biosensor using poly(neutral red) as redox mediator (Figure 2).

Determination of Heavy Metal Ions

Both cobalt hexacyanoferrate (Florescu and Brett 2004; Wang et al. 2009) and poly(neutral red) (Ghica and Brett 2006; Choi et al. 2011) show excellent properties as redox mediators in glucose oxidase biosensors. The main objective of using these mediators is to obtain a high sensitivity response and to decrease the applied potential necessary for signal transduction. Detection of heavy metals at metal hexacyanoferrate (Ghica et al. 2013) and poly(neutral red) (Ghica and Brett 2008) based biosensors has been previously carried out. The aim here is to compare the performance of the two mediators for inhibition measurements, as well as to provide an alternative for screening heavy metals at low concentrations. For these reasons, amperometric determination of cadmium, cobalt, and copper was carried out under the same experimental conditions at both cobalt hexacyanoferrate and poly(neutral red) based glucose biosensors.

The applied potential in inhibition measurements has to be chosen in such way that the change in response on injecting metal cations is in the opposite direction to that of the enzyme substrate, as discussed in other enzyme inhibition studies (Malitesta and Guascito 2005; Guascito et al. 2008). The fixed applied potential selected was −0.35 V, since in previous optimization studies (Ghica et al. 2013), it was the less negative potential where inhibition was observed for the cobalt hexacyanoferrate based glucose biosensor and it is also suitable for the poly(neutral red) based inhibition based biosensor (Ghica and Brett 2008).

Amperometric inhibition measurements (not shown) for cobalt, copper, and cadmium ions at glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and glucose oxidase/poly(neutral red)/carbon film electrode biosensors were carried

out by successive injections of the heavy metal ions in a stirred phosphate buffer solution pH 7.0 in the presence of glucose. In the first step, following stabilization of the baseline, a defined volume of concentrated stock solution of glucose was added, causing a fast and stable oxidation response corresponding to predominantly flavin adenine dinucleotide (FAD) regeneration (Ghica et al. 2013). In the second step, inhibitor solution was injected to evaluate the decrease of enzyme activity. Calibration curves for the determination of cobalt, copper, and cadmium in the presence of 0.2 mM glucose are shown in Figure 3. The linear ranges for Co^{2+} were up to 36 μM at the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and 85 μM at the glucose oxidase/poly(neutral red)/carbon film electrode; for Cu^{2+}, these linear range upper limits were 1.3 μM and 7.2 μM, and for Cd^{2+} they were 5.4 and 30 μM, respectively. For cobalt and copper ions, a higher response was obtained when using cobalt hexacyanoferrate as redox mediator, and for cadmium ions the response was higher using poly(neutral red).

The detection limits obtained at glucose oxidase/cobalt hexacyanoferrate/carbon film electrode biosensors for these ions are the lowest reported in biosensors based on the inhibition of glucose oxidase, particularly significant for cadmium and copper ions, as can be seen in Table 1. Lead and zinc ion measurements under the same conditions have been also performed in the range 2.0 to 100 μM, but no response was obtained at the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode, which is in agreement with previous results (Ghica et al. 2013).

Effect of the Concentration of Glucose on Heavy Metal Inhibition

The analytical performance for inhibition may vary considerably depending on the concentration of the enzyme substrate. Therefore, two different concentrations of glucose (0.2 mM and 0.5 mM) were used for the evaluation of Cu^{2+}, Co^{2+}, and Cd^{2+} inhibition. The sensitivity values for cobalt and copper obtained in the presence of 0.5 mM glucose (data not shown) were lower than those obtained in

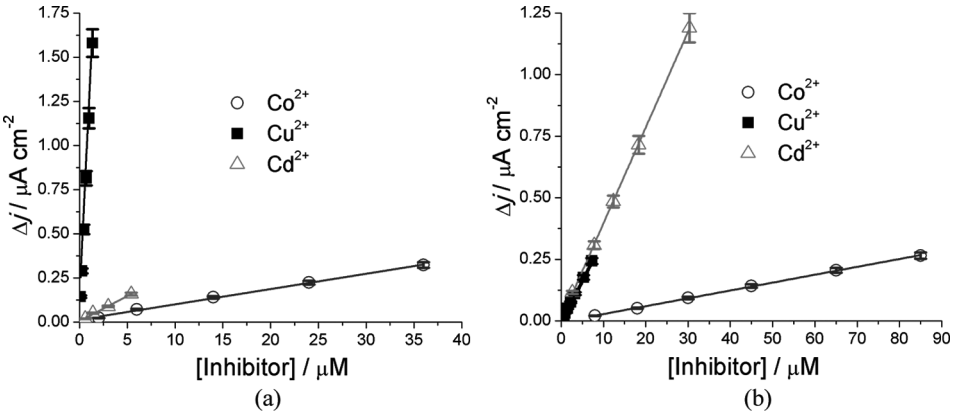

Figure 3. Calibration curves for Co^{2+}, Cu^{2+}, and Cd^{2+} at (a) the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and (b) the glucose oxidase/poly(neutral red)/carbon film electrode biosensors in the presence of 0.2 mM glucose in 0.10 M sodium phosphate buffer pH 7.0 at −0.35 V vs. SCE.

Table 1. Principal analytical data referring to the calibration curves for Co^{2+}, Cu^{2+}, and Cd^{2+}– reported in Figure 3 for the amperometric response at glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and glucose oxidase/poly(neutral red)/carbon film electrode biosensors in the presence of 0.2 mM glucose in phosphate buffer pH 7.0

Biosensor	Metal ion	I_{10} (LOD)/ μM	I_{50}/ μM	$K_{i,app}$/ μM	Sensitivity/ nA cm^{-2} μM^{-1}	Reference
GOx/CoHCF/CFE	Co^{2+}	2.10	13.5	5.700	0.75	(Ghica et al. 2013)
	Cu^{2+}	0.20	1.4	0.032	27.40	
	Cd^{2+}	2.40	17.0	11.000	3.75	
GOx/PNR/CFE	Cu^{2+}	4.70	[a]	34.600	9.57	(Ghica and Brett 2008)
	Cd^{2+}	10.60	[a]	1.500	3.95	
GOx/PPD/Pt	Co^{2+}	8.00	380.0	[b]	47.70	(Guascito et al. 2008)
	Cu^{2+}	5.00	70.0	[b]	3566.00	
	Cd^{2+}	5.00	210.0	[b]	229.00	
GOx/PPD/Pt	Cu^{2+}	10.00	40.0	[a]	[a]	(Malitesta and Guascito 2005)
GOx/PNA/Au	Co^{2+}	30.00	1700.0	[b]	[c]	(Chen et al. 2011)
	Cu^{2+}	20.00	100.0	[b]	[c]	
GOx/GA/PA/Fc/Pt	Cu^{2+}	<7.80	[a]	[a]	[a]	Liu et al. 2009
	Cd^{2+}	<4.40	[a]	[a]	[a]	
GOx/CoHCF/CFE	Co^{2+}	1.70	21.2	5.000	8.50	This work
	Cu^{2+}	0.09	0.5	0.140	1153.00	
	Cd^{2+}	0.30	2.7	[a]	28.50	
GOx/PNR/CFE	Co^{2+}	18.60	55.9	38.000	3.10	This work
	Cu^{2+}	1.20	4.3	3.700	34.00	
	Cd^{2+}	7.90	41.0	[a]	39.00	

GOx: glucose oxidase; CoHCF: cobalt hexacyanoferrate; CFE: carbon film electrode; PNR: poly(neutral red); PPD: poly(p-phenylenediamine); Pt: platinum electrode; PNA: peptide nucleic acid; Au: gold electrode; GA: glutaraldehyde; Fc: ferrocene.

[a]value not determined.

[b]value not specified.

[c]the units provided are not comparable with those here.

the presence of 0.2 mM glucose and for Cd^{2+} no inhibition response in the presence of 0.5 mM glucose was obtained.

According to a previous report in the literature, the sensitivity for inhibition can be calculated from the slope of the linear part of the curve obtained from $\Delta I = I_0 - I_1$, vs. the inhibitor concentration (Shan, Mousty, and Cosnier 2004). The biosensor sensitivity for the tested heavy metal ions in the presence of 0.2 mM glucose, follows the sequence: $Cu^{2+} > Cd^{2+} > Co^{2+}$ for glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and $Cd^{2+} > Cu^{2+} > Co^{2+}$ for glucose oxidase/poly(neutral red)/carbon film electrode (Table 1). The results at the cobalt hexacyanoferrate-based biosensor agree with those reported previously (Ghica et al. 2013; Ghica and Brett 2008; Guascito et al. 2008).

The I_{10} value obtained for Cu^{2+} (0.09 μM, Table 1) at the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode biosensor in the presence of 0.2 mM of glucose is low enough to ensure that this biosensor can be successfully employed for monitoring Cu^{2+} in aqueous solution with no interfering effects from other analyzed metals. The value obtained is much lower than that reported by Ghica et al. (2013) in the presence of 0.15 mM of glucose ($I_{10} = 0.2$ μM) with a similar biosensor.

In order to characterize the inhibitor efficiency, it is important to determine the concentration of inhibitor corresponding to 50% inhibition, or I_{50} (also designated: IC_{50}, $i_{0.5}$), a parameter usually used in pharmacology (Kosterin et al. 2005). The lowest I_{50} was obtained for copper at both biosensors (Table 1), meaning that this metal is the one inhibiting to the highest extent, as can be verified in Figure 4. The inhibition data indicate that Cu^{2+}, Co^{2+}, and Cd^{2+} have a greater inhibition effect in the presence of 0.2 mM, rather than 0.5 mM glucose. In particular, the concentration to obtain 50% inhibition was around four and seven times higher in the presence of 0.5 mM glucose for Co^{2+} and Cu^{2+}, respectively, when using glucose oxidase/cobalt hexacyanoferrate/carbon film electrode, and around two times higher when using glucose oxidase/poly(neutral red)/carbon film electrode for Co^{2+} and Cu^{2+}.

Determination of the Mechanism of Inhibition

To assess the type of inhibition by heavy metal ions, the amperometric data were analyzed according to the Dixon method (Dixon 1953). The apparent binding inhibition constants, K_i, were determined from Dixon plots at glucose concentrations corresponding to 0.2 mM and 0.5 mM. The glucose oxidase inhibition caused by Cu^{2+} and Co^{2+} is a competitive process, as observed for the same metal cations at glucose oxidase/cobalt hexacyanoferrate/carbon film electrodes (Ghica et al. 2013). The K_i values at glucose oxidase/cobalt hexacyanoferrate/carbon film electrodes were 0.14 μM for Cu^{2+} and 5.0 μM for Co^{2+}; and they increased to 3.7 and 38 μM when using the glucose oxidase/poly(neutral red)/carbon film electrode biosensor.

For cadmium ions, no inhibition response was obtained in the presence of 0.5 mM glucose. This behavior may be due to competitive inhibition, since if it is the case then at high substrate concentration the inhibition effect is greatly diminished, because the substrate competes with the inhibitor (Amine et al. 2006). Previously, cadmium ions were found to cause competitive inhibition at the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode (Ghica et al. 2013) but mixed

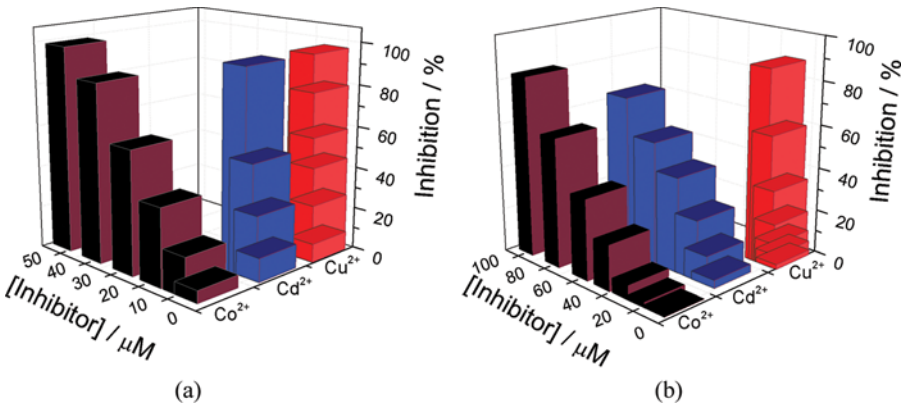

Figure 4. Inhibition of Co^{2+}, Cu^{2+}, and Cd^{2+} at (a) the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and (b) the glucose oxidase/poly(neutral red)/carbon film electrode biosensors in the presence of 0.2 mM glucose in 0.10 M sodium phosphate buffer pH 7.0 at −0.35 V vs. SCE.

Table 2. Recovery of Co^{2+}, Cu^{2+}, and Cd^{2+} from tap water using the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode

Metal	Added/μM	Found/μM	Recovery (%)
Co^{2+}	0.500	0.480 ± 0.022	96
Cu^{2+}	0.100	0.113 ± 0.005	113
Cd^{2+}	0.100	0.094 ± 0.003	94

(competitive and uncompetitive) inhibition at the glucose oxidase/poly(neutral red)/carbon film electrode (Ghica and Brett 2008). The sensitivity at glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and glucose oxidase/poly(neutral red)/carbon film electrode biosensors toward Co^{2+}, Cu^{2+}, and Cd^{2+} decreases with increase in glucose concentration. The I_{50} values also increased at higher glucose concentrations, in agreement with the competitive inhibition mechanism predicted from the Dixon plot.

Further experiments showed that after enzymatic inhibition reactions, the glucose oxidase modified electrode could be reused after simple rinsing with phosphate buffer. This behavior suggests that the inhibition mechanism of glucose oxidase by Co^{2+}, Cu^{2+}, and Cd^{2+} is reversible. Indeed, the restoration of 100% of enzyme activity after washing with buffer, indicates that the biosensors could be used for continuous measurement of these metal ions. Several analyses could be performed during one day, and use in flow injection analysis may also be possible. Furthermore, in the case of reversible inhibition, no time for incubation of enzyme with metal ion inhibitors is required; therefore, the total time of analysis is greatly reduced (Arduini and Amine 2014). As the inhibition by Co^{2+}, Cu^{2+}, and Cd^{2+} of the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and glucose oxidase/poly(neutral red)/carbon film electrode is competitive, a low concentration of glucose is recommended in order to observe inhibition.

Analysis of Tap Water

To apply the enzyme-inhibition sensor to determine heavy metal ions (Co^{2+}, Cu^{2+}, and Cd^{2+}) in tap water, known concentrations (0.5 μM of Co^{2+} and 0.1 μM of Cu^{2+} and Cd^{2+}) were added separately to three distinct tap water samples from different locations. Enzyme inhibition fixed potential amperometric measurements were performed using the biosensor that provided the best results, i.e., with the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode. Heavy metal ion recovery rates were estimated by comparing the metal ion concentrations recovered from the tap water with the respective concentrations of the added standard solutions. The average recovery rates were 96% for Co^{2+}, 113% for Cu^{2+}, and 94% for Cd^{2+} as shown in Table 2.

CONCLUSIONS

A comparative study of inhibition based biosensors with cobalt hexacyanoferrate and poly(neutral red) as redox mediators has been carried out. The method showed high potential for the rapid assay of micromolar concentrations of copper,

cobalt, and cadmium ions. The heavy metals were determined with higher sensitivity at glucose oxidase/cobalt hexacyanoferrate/carbon film electrode biosensors than at glucose oxidase/poly(neutral red)/carbon film electrode biosensors. The lowest detection limits reported for cobalt, copper, and cadmium in glucose oxidase inhibition were obtained at the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode biosensor. The metal ion which inhibited the most was copper for both biosensors and its detection limit was the lowest, permitting its determination without interferences from the other two cations. The mechanistic interpretation of the amperometric response to Co^{2+}, Cu^{2+}, and Cd^{2+} at the glucose oxidase/cobalt hexacyanoferrate/carbon film electrode and glucose oxidase/poly(neutral red)/carbon film electrode biosensors, recorded in the presence of two different concentrations of glucose, allowed identification of a competitive type inhibition process.

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