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Glucose oxidase enzyme inhibition sensors for heavy metals at carbon film electrodes modified with cobalt or copper hexacyanoferrate

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ABSTRACT

Electrochemical enzyme sensors prepared from cobalt or copper hexacyanoferrate modified carbon film electrodes plus glucose oxidase (GOx) immobilised by crosslinking with glutaraldehyde were successfully applied to the determination of heavy metal cations using fixed potential amperometry. Sensor performance was optimised with respect to the applied potential and influence of pH of the electrolyte solution. Cadmium, cobalt, copper and nickel ions were detected in the presence of fixed amounts of glucose, and the response to glucose was tested in the absence and presence of a fixed concentration of inhibitor. Electrochemical impedance spectroscopy was used for the first time to characterise the response of glucose biosensors in the presence of the inhibitors. The enzyme inhibition mechanism is reversible and competitive and 10% enzyme inhibition was achieved with submicromolar or micromolar concentrations of the metal cations.

1. Introduction

Environmental contamination from toxic heavy metal (HM) ions is a worldwide concern. Following their accumulation in the atmosphere they can transfer to the food chain and lead to serious health consequences for humans, animals and plants. The toxicity of HM results mainly from their interaction with proteins (especially enzymes) by binding to the sulfhydryl groups, leading to inhibition of glutathione metabolism and of the function of numerous enzymes and hormones. They may compete with nutrient elements for binding sites causing aberrations in the metabolism of carbohydrates, proteins, neurotransmitters and hormones [1].

Heavy metals are often needed at ultratrace levels for the essential functions of living organisms and humans, but become toxic if the tolerance values for the respective organism are exceeded [2]. Due to the high toxicity that HM can cause, there is an obvious need to determine them rapidly at trace levels. The necessity for faster and more cost-effective methods for environmental monitoring has led to a variety of field analytical methods, such as miniaturised laboratory methods, field test kits and chemical (bio)sensors.

In environmental analysis, heavy metals are generally detected by using biosensors based on enzyme activity inhibition, which are able to detect single elements and/or species at low concentrations, and can be used for in situ analysis and/or low-cost screening procedures [3]. Biosensors based on inhibition use enzymes such as urease (the most used) [2,4], horseradish peroxidase [5,6], alcohol oxidase [7], choline oxidase [8], and the most determined metals are mercury [7,9,10], copper [5,7,9], cadmium [2,5] and lead [2,5].

Glucose oxidase (GOx) is an ideal enzyme for studies of inhibition due to its low cost, good stability and high specific activity; however, its use in enzymatic inhibition biosensors is not widespread. Nevertheless, there are a few reports on the heavy metal inhibition of GOx immobilised by different methods using amperometry [1,9,11,12]. With the addition of inhibitors to the electrolyte solution, the response of the GOx biosensor to its substrate, glucose, decreases.

The objective of this paper is the investigation of glucose oxidase-based biosensor platforms with cobalt or copper hexacyanoferrate redox mediator for the quantitative determination of heavy metal cations by inhibition. The enzyme was immobilised with glutaraldehyde (GA) and bovine serum albumin (BSA), to form a biologically sensitive membrane. The response of the sensor to varying concentrations of heavy metal ions, namely cadmium, cobalt, copper and nickel, was evaluated by measuring the activity of glucose oxidase after adding different concentration of metal ions. The response of the biosensor to glucose in the presence and absence of HM was also estimated. The inhibition of heavy metals

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using different biosensor configurations was evaluated and compared with other sensors from the literature, especially regarding the concentration leading to 10% and 50% enzyme inhibition. Electrochemical impedance spectroscopy was used for characterisation of the modified electrodes and, for the first time, to monitor the behaviour of an enzymatic biosensor in the presence and absence of heavy metal ions.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade and were used without further purification. Glucose oxidase (GOx, E.C. 1.1.3.4, from *Aspergillus Niger*, 24 U/mg) was acquired from Fluka. α-D(+)-glucose, glutaraldehyde (GA) (25% (v/v) in water) and bovine serum albumine (BSA) were purchased from Sigma. Potassium hexacyanoferrate $K_3Fe(CN)_6$, copper chloride $CuCl_2$, cobalt chloride $CoCl_2$, nickel chloride ($NiCl_2$), cadmium sulphate (Cd_2SO_4) were from Merck.

All solutions were prepared using Millipore Milli-Q nanopure water (resistivity > 18 MΩ cm). The supporting electrolyte for sensor/biosensor evaluation was sodium phosphate buffer saline (NaPBS) (0.1 M NaH_2PO_4/Na_2HPO_4 + 0.05 M NaCl), pH 7.0, all reagents were Riedel-de-Haën. For the deposition of cobalt hexacyanoferrate (CoHCF), 0.05 M NaCl (Riedel-de-Haën), pH 3.0 was used and copper hexacyanoferrate (CuHCF) films were prepared in 0.1 M KCl (Fluka), pH 5.5.

2.2. Electrochemical measurements and apparatus

A three-electrode electrochemical cell of 10 mL volume was used, containing the carbon film electrode (CFE) as working electrode, a platinum wire as counter electrode and a saturated calomel electrode (SCE) as reference.

The electrochemical measurements, cyclic voltammetry and amperometry, were performed using a computer-controlled BAS CV-50W analyser (Bioanalytical Systems, West Lafayette, IN). Electrochemical impedance spectroscopy (EIS) was carried out with a Solartron 1250 Frequency Response Analyser, coupled to a Solartron 1286 Electrochemical Interface (Ametek, UK) controlled by ZPlot software (Scribner, USA). The voltage perturbation was 10 mV rms over a frequency range from 65 kHz to 0.1 Hz with 10 frequencies per decade, and integration time 60 s. Equivalent circuit fitting was done with ZView software (Scribner, USA).

The pH measurements were carried out with a Crison 2001 micro pH-meter (Spain) at room temperature.

2.3. Carbon film electrode preparation

2.3.1. Electrode pre-treatment

Electrodes were made from carbon film resistors (2 Ω nominal resistance), 6 mm in length and 1.5 mm in diameter [13]. Since carbon film electrode surfaces cannot be renewed by polishing or other mechanical methods, electrochemical pre-treatment was used in order to obtain a reproducible response. This conditioning pre-treatment, cyclic voltammetry in the buffer solution between −1.0 and +1.0 V vs. SCE, at a scan rate of 100 mV s^{-1}, until a stable voltammogram was obtained, was always performed before electrode use.

2.3.2. Cobalt hexacyanoferrate deposition

Cobalt hexacyanoferrate films were made by electrochemical deposition. For this purpose, the electrode was cycled 25 times between −0.2 and +0.9 V at a scan rate of 50 mV s^{-1} in a freshly prepared solution containing 5 mM $CoCl_2$, 2.5 mM $K_3Fe(CN)_6$ and 0.05 M NaCl at pH 3.0 (adjusted with HCl) [14]. After deposition, the electrodes were stabilised for 1 h in 0.05 M NaCl, pH 3.0 and then kept in the dark until use.

2.3.3. Copper hexacyanoferrate deposition

For deposition of copper hexacyanoferrate, a chemical method was used, since it was observed in previous work that this leads to more robust films [15]. The freshly prepared deposition solution contained 10 mM $CuCl_2$, 10 mM $K_3Fe(CN)_6$ and 100 mM KCl (pH 5.5). The electrodes were immersed in this solution and left under continuous stirring during 50 min. After deposition, films were dried in hot air for 5 min and were then aged for at least 24 h in the dark.

2.3.4. Enzyme immobilisation

Glucose oxidase (GOx) was immobilised by the crosslinking method using glutaraldehyde (GA) and bovine serum albumin (BSA). For the drop-coating technique [16] 1 mg of GOx and 4 mg of BSA were dissolved in 100 μL of 0.1 M NaPBS (pH 7.0). A volume of 10 μL of this solution was then mixed with 5 μL of GA (2.5%) and 7 μL of this mixture was spread over the electrode surface (previously modified with CoHCF or CuHCF). For comparison, an enzyme electrode without mediator was also prepared in a similar way. When not in use, enzyme electrodes were kept at 4 °C in phosphate buffer electrolyte, pH 7.0.

2.4. Determination of metal cations

Heavy metal ions were detected by injecting different concentrations of each in the presence of fixed amounts of glucose. The inhibition was evaluated by determining the response to glucose in the absence and presence of the metal cation and applying the expression:

$$I(\%) = 100\frac{(I_1 - I_2)}{I_1} \quad (1)$$

where I is the degree of inhibition, I_1 is the response to glucose in the absence of metal and I_2 is the response in the presence of metal.

In another series of experiments, calibration curves for glucose were constructed in the absence and presence of different concentrations of heavy metals and the inhibition was evaluated using the same expression.

Electrochemical impedance spectra were recorded at different stages in the two types of procedure.

3. Results and discussion

3.1. Deposition and characterisation of hexacyanoferrate modified electrodes

3.1.1. Cyclic voltammetry

The modification of carbon film electrodes by cobalt hexacyanoferrate (CoHCF) was carried out by potential cycling between −0.2 and +0.9 V vs. SCE at 50 mV s^{-1} during 25 cycles [14]. Successive, continuous cyclic voltammograms are shown in Fig. 1A and film growth is demonstrated by the increase in current density with each cycle. Copper hexacyanoferrate (CuHCF) films were deposited chemically using the procedure optimised in [15], and described in Section 2.

Both types of hexacyanoferrate modified electrode were characterised in phosphate buffer pH 7.0, see the voltammograms in Fig. 1B. The current peaks are higher for the CuHCF film, the charge accumulated was 137 μC cm^{-2} for CoHCF and 250 μC cm^{-2} for CuHCF. This difference, and thence thicker film, is most probably due to the higher reagent concentrations used for CuHCF deposition: 2 and respectively 4 times more than for CoHCF. In agreement with [16] for CuHCF deposition (using the same concentration of

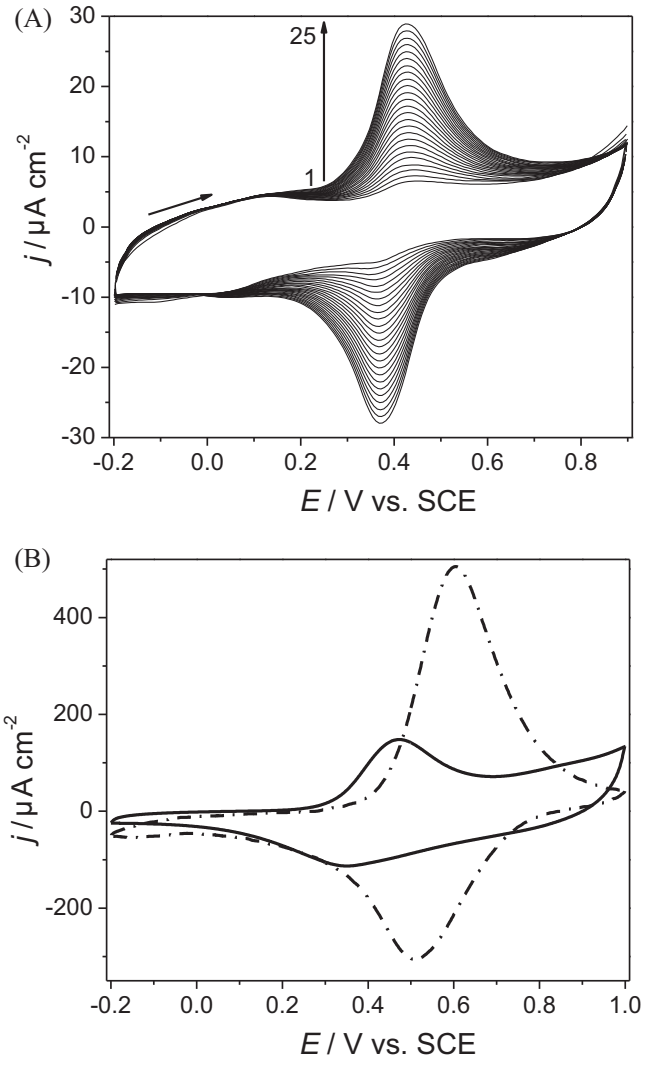

Fig. 1. Cyclic voltammetry of (A) deposition of CoHCF between −0.2 and +0.9 V at 50 mV s^{-1} from 5 mM $CoCl_2$ + 2.5 mM $K_3Fe(CN)_6$ + 0.05 M NaCl (pH 3.0) and (B) electrodeposited CoHCF (–) and chemically deposited CuHCF (–·–·) in 0.1 M NaPBS, pH 7.0.

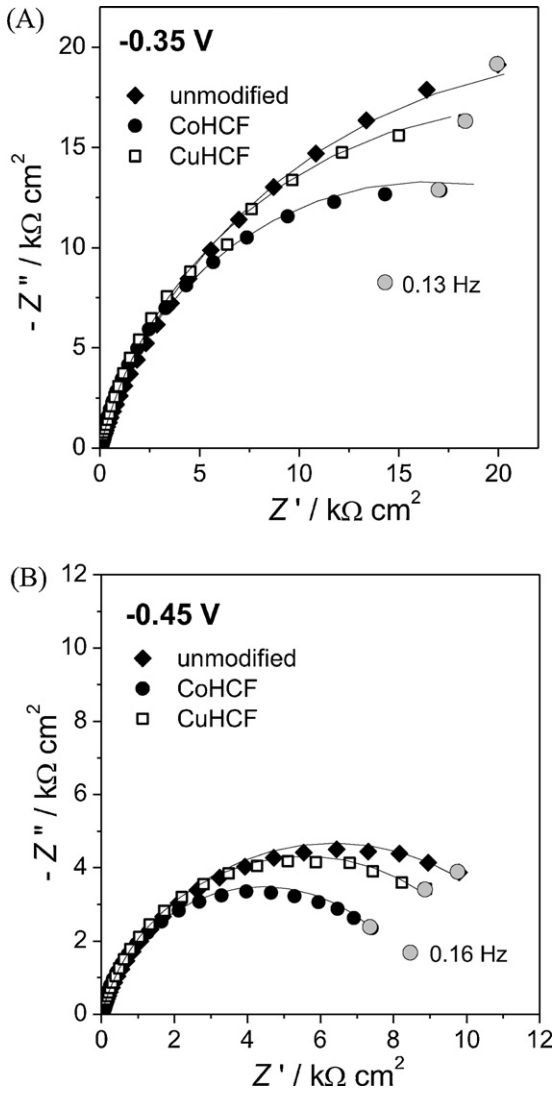

Fig. 2. Complex plane impedance spectra of (♦) unmodified, (□) CoHCF and (●) CuHCF modified electrodes in 0.1 M NaPBS at (A) −0.35 V and (B) −0.45 V. Lines show equivalent circuit fitting.

reagents), it was observed that a thicker film is obtained by the chemical than by the electrochemical method.

3.1.2. Electrochemical impedance spectroscopy

The unmodified electrodes and those modified with cobalt or copper hexacyanoferrate were characterised by electrochemical impedance spectroscopy (EIS). Measurements were performed in 0.1 M phosphate buffer (NaPBS) pH 7.0 at −0.35 and −0.45 V vs. SCE, the potentials used in fixed potential amperometry, to be described below. All spectra were fitted with the same electrical equivalent circuit consisting of the cell resistance, R_Ω, in series with a parallel combination of a constant phase element, CPE, and a charge transfer resistance, R_{ct}. The CPE is modelled as a non-ideal capacitor according to the relation $CPE = -1/(C\,i\omega)^\alpha$, where C is the capacitance (describing the charge separation at the double layer interface), ω is the angular frequency and α is the roughness factor (due to heterogeneity of the surface).

Spectra are exhibited in Fig. 2 and the results of the fitting are shown in Table 1. Values of α are 0.82 at unmodified electrodes and 0.85 for both types of modified electrode; the small increase of the α exponent suggests that the modified electrodes may have a slightly smoother and more uniform surface. At both −0.35 V and −0.45 V vs. SCE the values of the capacitance increase for the modified electrodes, due to an increase of the double layer charge accumulation; on the other hand, the charge transfer resistance values decrease with modification, indicating that electron transfer is facilitated.

3.2. Optimisation of inhibition biosensors

Electrodes modified with CoHCF or CuHCF were further modified by the enzyme layer to form the biosensor by immobilisation of glucose oxidase (GOx) with glutaraldehyde (GA) and bovine serum albumin (BSA), as described in Section 2. The biosensors were then

Table 1
Equivalent fitting circuit parameters for unmodified and CoHCF and CuHCF modified carbon film electrodes at −0.35 and −0.45 V in 0.1 M NaPBS, pH 7.0.

Electrode	R_{ct} (kΩ cm^2)		C (µF cm^{-2} $s^{\alpha-1}$)	
	−0.35 V	−0.45 V	−0.35 V	−0.45 V
Unmodified	51.5	12.7	30.1	36.0
CoHCF	33.2	8.8	37.1	41.0
CuHCF	42.9	11.1	34.5	38.1

applied to the determination of glucose by fixed potential amperometry, without and with inhibition.

3.2.1. Influence of applied potential

The detection principle in hexacyanoferrate-based biosensors with oxidase enzymes is normally hydrogen peroxide detection, which allows operation near 0.0 V. In inhibition enzyme biosensors, the change in the current response from the enzyme substrate due to inhibition is measured; the decrease in current dictating the degree of inhibition of the enzyme activity. In order to observe an inhibitive response, it is thus necessary that the change in response on injecting metal cations is in the opposite direction to that of the enzyme substrate, as reported in previous enzyme inhibition studies [9,12]. Since, in this work, the response to the metal cations is a current change in the cathodic direction, it is necessary to choose a potential where the response to glucose is in the anodic direction. This occurs at −0.35 V for CoHCF and at −0.45 V for CuHCF-based electrodes or at more negative potentials – at more positive potentials close to 0 V, the response to glucose is in the cathodic direction.

The reason for this can be explained as follows. At less negative potentials, hydrogen peroxide reduction occurs and at more negative values FAD regeneration (oxidation reaction) takes place as well as peroxide reduction, as in [17]. Consequently, at less negative potentials where FAD regeneration cannot occur, on adding glucose a cathodic change in current is observed, due to hydrogen peroxide reduction, and with the addition of metal ions the change in current is also cathodic. At more negative potentials, FAD regeneration predominates over peroxide reduction, so that injecting glucose leads to a net anodic current and injection of metal ions causes inhibition of the enzyme activity and a cathodic change. The least negative potential for this to happen is −0.35 V for CoHCF and −0.45 V for CuHCF-mediated electrodes. Unless otherwise specified, experiments were conducted at these potentials for the two types of biosensor.

Direct amperometric measurements of the metal cations at redox-mediator modified carbon film electrodes at these potentials give only a very small response, mainly from copper ions, as would be expected. When there is inhibition (inhibition of FAD regeneration) this occurs principally close to the enzyme/electrolyte interface and the substrate (glucose) or inhibitor (metal ions) binds to the enzyme active sites. Thus, only the products of the enzyme reaction will pass through the enzyme layer to the mediator layer. The experimental results are in agreement with this observation.

3.2.2. Influence of buffer pH

The pH of the solution can influence the overall enzyme activity since, like natural proteins, enzymes have a native tertiary structure that is sensitive to pH; denaturation of enzymes can occur at extreme values of pH. It is well known that enzyme activity is highly pH dependent and the optimum pH for an enzymatic assay must be determined experimentally. It is best to choose a plateau region so that the pH does not have any effect on enzyme activity and will not interfere with the results obtained in relation to enzyme inhibition. For glucose oxidase from *A. Niger*, the enzyme used here, the greatest activity is observed in the pH range 5.5–9.0, being highest at pH 6.5 [18]. For this reason, the influence of the buffer pH on the inhibition response in 0.1 M phosphate buffer in the pH range from 6.0 to 8.0 was studied. Phosphate buffer was chosen for practical convenience, because it is the electrolyte normally used for glucose biosensor studies and covers the optimum enzyme pH range. The determination of metals in the presence of fixed concentrations of glucose (different for each metal in order to ensure a good inhibition response) was carried out at pH 6.0, 7.0 and 8.0. Fig. 3 shows the response for different metal ions at CoHCF (−0.35 V) and CuHCF (−0.45 V) based biosensors as a function of pH. The behaviour was similar for all the metals, the response increasing with increase

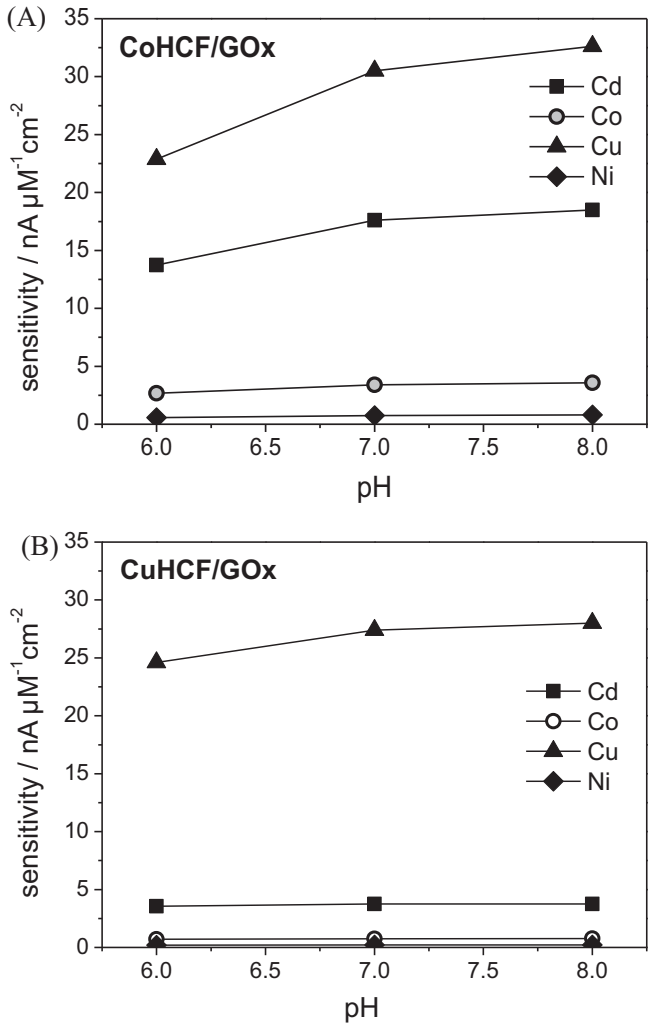

Fig. 3. Influence of the pH in 0.1 M NaPBS buffer solution on the determination of metal cations at (A) CoHCF/GOx at −0.35 V for Cd^{2+} and Co^{2+} (presence of 50 μM glucose) and for Cu^{2+} and Ni^{2+} (150 μM glucose) and (B) CuHCF/GOx at −0.45 V for Cd^{2+} and Co^{2+} (300 μM glucose) and for Cu^{2+} and Ni^{2+} (100 μM glucose). The sensitivity was calculated from the calibration curves for each metal ion.

in pH value. A significant increase of the response of each cation occurs from pH 6.0 to pH 7.0 and a much smaller increase from pH 7.0 to pH 8.0. Based on these results, and taking into account the optimal enzyme activity, it was decided to perform all further experiments in 0.1 M phosphate buffer pH 7.0. Other enzyme inhibition biosensors for metal determination use phosphate buffer at pH 7.0 [1,12,19] or citrate–phosphate buffer, pH 7.0 [20], whilst acetate buffer was used in others [21,22]. However, acetate buffer might form complexes with heavy metals, as observed in [22] for lead, which does not allow determination at trace levels.

3.2.3. Influence of redox mediator

Redox mediators are normally used in order to decrease the applied potential necessary for signal transduction and thence reduce interferences; the optimum applied potential depends on the substrate to be detected. Normally, for glucose a potential closer to 0.0 V would be better (to reduce interferences), but in the present work the concern was also the determination of heavy metals by inhibition.

In order to compare the response for glucose and especially for the metallic cations under these circumstances a biosensor without mediator was prepared. The amperometric responses to cobalt and copper ions at −0.45 V for the three biosensors: no mediator,

CuHCF and CoHCF mediators were compared. For cobalt ion determination, an increase in response by a factor of 17 was observed using CuHCF mediator compared to the biosensor without mediator and by a factor of 7 with CoHCF. For copper, the response increase was 6 (for CuHCF) and 5 (for CoHCF) fold, respectively. At the same time, the response to glucose is about 2 times higher with these redox mediators than the biosensor without them. These results are convincing evidence that hexacyanoferrate redox mediators improve the sensitivity for the determination of metal cations. Measurements of metal cations were also carried out at carbon film electrodes modified only with redox mediator and a very small response was obtained. However, this does not influence the determination of metal ions by enzyme inhibition, since this is achieved by measuring the decrease in glucose response when adding the metal cation.

3.3. Determination of enzyme inhibition by fixed potential amperometry

In general, inhibition studies can be done with or without incubation of the toxic species. The influence of incubation was investigated, but no differences in response were obtained after different incubation times of 5, 15, 30 min. These results are in agreement with a competitive inhibition mechanism, for which incubation is not needed. Hence, all further experiments were carried out without incubation.

For the amperometric determination of heavy metal cations, two different strategies were evaluated. The reason for using these two strategies was to ascertain whether there is competitive inhibition – if there is, then the order in which inhibitor or glucose is injected will influence the degree of inhibition. In both cases, the inhibition of enzyme activity was evaluated by formula (1), see Section 2, with I_1 the response in the absence of inhibitor and I_2 in its presence.

In the first strategy, following baseline stabilisation, a known amount of stock glucose solution was added to the buffer solution, and the response was recorded (I_1). Inhibitor solution was then injected and the response (I_2) measured, lower because of the decrease of enzyme activity.

In the second strategy, first the biosensor response to glucose without any inhibitor was recorded (I_1). The biosensor was then transferred to fresh buffer solution without glucose, a known amount of inhibitor was added to the buffer, followed by injections of glucose as previously, and the response was recorded (I_2). The degree of inhibition was calculated in the same way.

Fig. 4 illustrates results from both kinds of experiment for cadmium ions at the two biosensors at −0.45 V: for the CuHCF/GOx biosensor the response to different concentrations of Cd^{2+} in the presence of 300 μM glucose (strategy 1 – Fig. 4A) and for the CoHCF/GOx biosensor, typical responses to glucose in the absence and presence of 60 μM Cd^{2+} (strategy 2 – Fig. 4B).

Using the first strategy with injection of increasing concentrations of inhibitor, the analytical parameters can be obtained. The linear range for cadmium determination with both biosensors was 1.5–6.0 μM in the presence of 300 μM glucose with CuHCF/GOx and 50 μM glucose with the CoHCF/GOx biosensor. The sensitivity was higher in the case of the CuHCF/GOx biosensor, mainly due to the high loading deposited chemically (Fig. 1B), as happened for all four metal ions investigated. The analytical parameters are summarised in Table 2 which also includes a comparison with other biosensors based on GOx inhibition for heavy metal detection.

Comparing with the literature, cadmium was measured in previous work by the inhibition of lactate dehydrogenase coupled with lactate oxidase bound to a Clark electrode [23] after 5 min of incubation; the concentration of this metal necessary to give 50% of inhibition, I_{50}, was 10 μM. In the present work 50% inhibition

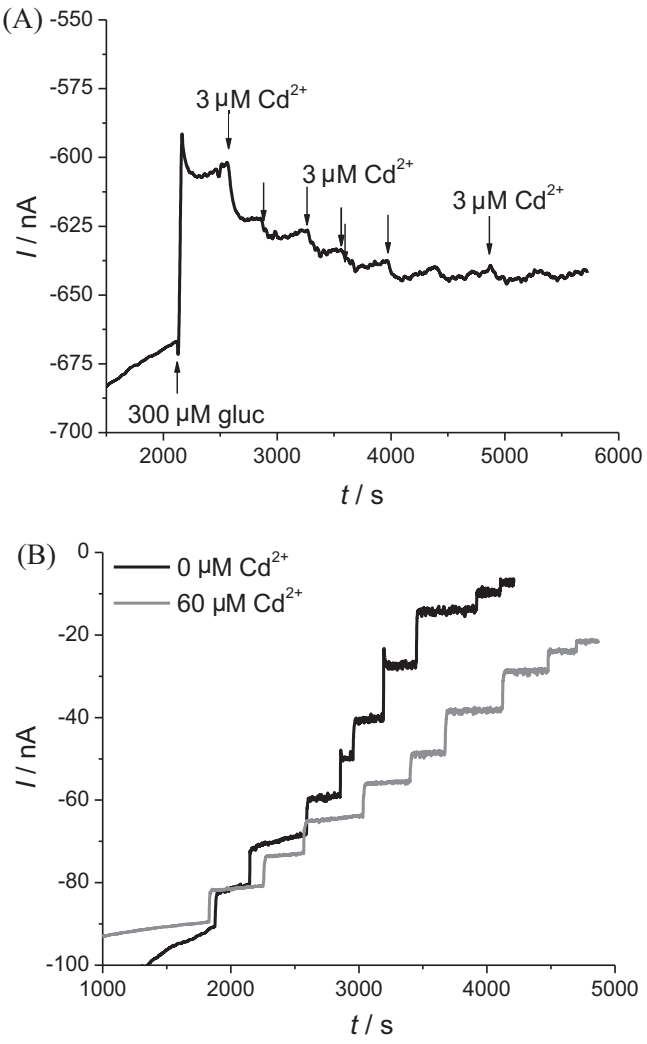

Fig. 4. Biosensor current–time responses in 0.1 M NaPBS pH 7.0; applied potential −0.45 V: (A) response to 300 μM glucose, followed by injections of 3 μM Cd^{2+} at CuHCF/GOx electrode and (B) typical response of glucose in the absence (–) and presence (__) of 60 μM Cd^{2+} at CoHCF/GOx electrode.

of glucose oxidase was achieved for 17 μM of cadmium with CoHCF/GOx biosensor in the presence of 50 μM glucose and 5.8 μM of cadmium with the CuHCF/GOx biosensor in the presence of 300 μM glucose, see Table 2. In [12], cadmium was measured by inhibition of GOx at a platinum electrode modified with poly(o-phenylenidiamine) (GOx/PDDA/Pt) with a linear response between 20 and 150 μM (higher concentrations than in the present work), the detection limit was 5 μM (higher than I_{10} obtained with both biosensors in the present work) and I_{50} was 210 μM. In [19] the I_{10} for cadmium was also higher than here.

The same experiment was repeated for the other metal ions: cobalt, copper and nickel and the results (linear range, sensitivity and detection limit) are also presented in Table 2. Cobalt was measured in the presence of 300 μM glucose with both biosensors and a linear response was obtained between 5.0 and 35 μM with CoHCF/GOx electrode (I_{50} = 13.5 μM for 50 μM glucose) and 2.0–40 μM with CuHCF/GOx electrode (I_{50} = 19 μM for 150 μM glucose). In [12] cobalt was determined in the linear range 35–440 μM – higher concentrations than in the present work, the detection limit was much higher (8 μM) and I_{50} was 380 μM. Cobalt also inhibited glucose oxidase at a poly(N-noradrenaline) modified gold electrode, GOx/PNA/Au, between 30 and 340 μM with a high detection limit of 30 μM [1].

Table 2
Analytical parameters for the determination of metal cations at CoHCF and CuHCF based biosensors and comparison with other biosensors based on glucose oxidase inhibition. See text for detailed experimental conditions and abbreviations.

Biosensor	Heavy metal ion	Linear range (μM)	Sensitivity (nA cm^{-2} μM^{-1})	LOD (I_{10}) (μM)	I_{50}(μM)	K_i (μM)	Ref.
GOx/PNA/Au	Co^{2+}	30–340 340–2500	[b]	30	1700	[a]	[1]
	Cu^{2+}	20–90 90–300		20	100		
GOx/PPDA/Pt	Cd^{2+}	20–150	2.29	5	210	[a]	[12]
	Co^{2+}	55–400	41.8	8	380		
	Cu^{2+}	10–100 100–250	3566 1242	5	70		
	Ni^{2+}	35–440	50.9	4.8	349		
GOx/PNR/CFE	Cd^{2+}	0.035–0.142	3.95	10.6	None	1.5	[19]
	Cu^{2+}	0.51–9.1	9.57	4.7		34.6	
GOx/MnO_2/CPE	Cd^{2+}		[a]		<4% (3.9 mM)	[a]	[20]
	Cu^{2+}				<4% (2.2 mM)		
GOx/PANI/Fc/Pt	Cd^{2+}		[a]		1.56% (7.9 μM)	[a]	[21]
	Cu^{2+}				6.17% (4.4 μM)		
GOx/CoHCF/CFE	Cd^{2+}	1.5–6.0	3.75	2.4	17	11	This work
	Co^{2+}	5.0–35	0.75	2.1	13.5	5.7	
	Cu^{2+}	0.2–3.0	27.4	0.2	1.4	0.032	
	Ni^{2+}	20–120	0.22	3.3	21.8	22.7	
GOx/CuHCF/CFE	Cd^{2+}	1.5–6.0	17.6	1.2	5.8	2.0	This work
	Co^{2+}	2.0–40	3.4	0.9	19	1.9	
	Cu^{2+}	0.2–3.0	30.5	0.5	2.2	0.039	
	Ni^{2+}	20–120	0.75	4.8	50.1	39.8	

[a] Not specified.
[b] The values indicated cannot be used for comparison.

Copper was detected between 0.2 and 3.0 μM with both types of biosensor i.e. CoHCF/GOx (I_{50} = 1.4 μM for 150 μM glucose) and CuHCF/GOx (I_{50} = 2.2 μM for 100 μM glucose). In [12] copper was sensed with two linear ranges 10–100 and 100–250 μM (both higher than found in the present work), the detection limit was higher than here at 5 μM and I_{50} was 70 μM. In [7] with alcohol oxidase on a platinum electrode was inhibited by copper between 7.8 and 156 μM, I_{50} = 110 μM and the detection limit was 31.4 μM (much higher than the I_{10} achieved here). In other studies, 7.9 μM copper inhibited glucose oxidase by just 1.5% [21] and 3.9 mM of copper ions inhibited GOx less than 4% in [20].

Nickel determination was carried out from 20 to120 μM in the presence of 300 μM glucose with CoHCF/GOx electrode and 200 μM glucose for CuHCF/GOx electrode. The I_{50} value corresponded to 50.1 μM nickel for the CuHCF/GOx biosensor (in the presence of 50 μM glucose) and to 21.8 μM for the CoHCF/GOx (150 μM glucose) biosensor. In [12] the linear range for nickel determination was wider, 35–440 μM but I_{50} was higher at 349 μM although the detection limit was similar to the I_{10} obtained in the present work. Nickel was also measured with sarcosine oxidase immobilised on a platinum electrode [7] between 8.5 and 85 μM, the detection limit was 8.5 μM (higher that I_{10} obtained with both biosensors here) and I_{50} was 64.7 μM.

A representation of the pattern of inhibition response obtained with the four cations is illustrated in Fig. 5 in 3D plots. As can be seen, the inhibition response is similar at both types of modified electrode, except that the maximum inhibition reached is higher at CuHCF/GOx, for reasons which are not entirely clear. It was found that for all four metal cations, on decreasing the glucose concentration the maximum percentage inhibition increased, indicating competitive inhibition of glucose oxidase.

A practical detection limit can be defined as the concentration that gives 10% of inhibition, I_{10}. Values of I_{10} are in the micromolar range (see Table 2). In [12] cadmium, cobalt, copper and nickel were measured using glucose oxidase inhibition biosensor developed on a poly-o-phenylenediamine platinum disk electrode. The detection limits obtained in [12] of 5 μM (Cd^{2+}), 8 μM (Co^{2+}), 5 μM (Cu^{2+}) and 4.8 μM (Ni^{2+}) were higher than the I_{10} values obtained with the proposed biosensors using hexacyanoferrate mediators, except for Ni^{2+} at CuHCF/GOx electrodes, which is the same.

The discrepancy between reports in the literature with respect to the metal ion concentration required to produce 50% of enzyme inhibition could be due to varying amounts of enzyme used in each case, since the higher the enzyme activity in the assay, the less the inhibition effect for the same metal concentration. The use of phosphate buffer by some authors could also be related to the higher I_{50} values, since the presence of phosphate could bind the metals or inactivate the enzyme [24].

Following the second strategy, as seen for cadmium ions in Fig. 4B, it is possible to see the decrease in glucose response in metal ion-containing buffer solution. In the absence and presence of metal ions, the glucose biosensor exhibited a linear response to glucose up to 1.3 mM. The sensitivity determined at −0.45 V both with CuHCF/GOx and CoHCF/GOx electrodes was 1.54 μA cm^{-2} mM^{-1} and 2.34 μA cm^{-2} mM^{-1}, with similar detection limits of 33 μM and 35 μM, respectively. In the presence of heavy metals the sensitivity decreased and the inhibition was 17% for 60 μM Cd^{2+}, 11% for 200 μM Ni^{2+}, 12% for 200 μM Co^{2+} and 6.0% for 20 μM Cu^{2+} in the case of CoHCF/GOx electrode. For the same concentration of metals the inhibition was lower with the CuHCF/GOx electrode: 12%; 7.2%; 6.5% and 3.2%, respectively. In all cases less inhibition was obtained by this second strategy than by adding inhibitor to a glucose-containing solution: cadmium ion inhibited glucose oxidase to the greatest extent, followed by cobalt, nickel and, finally, copper.

Lead and zinc ions were also tested as possible inhibitors, but no response was obtained at the CoHCF/GOx and CuHCF/GOx biosensors. The absence of inhibition by Pb^{2+} and Zn^{2+} was also observed in [12] at an amperometric glucose biosensor with polyphenyldiamine at 0.7 V vs. SCE.

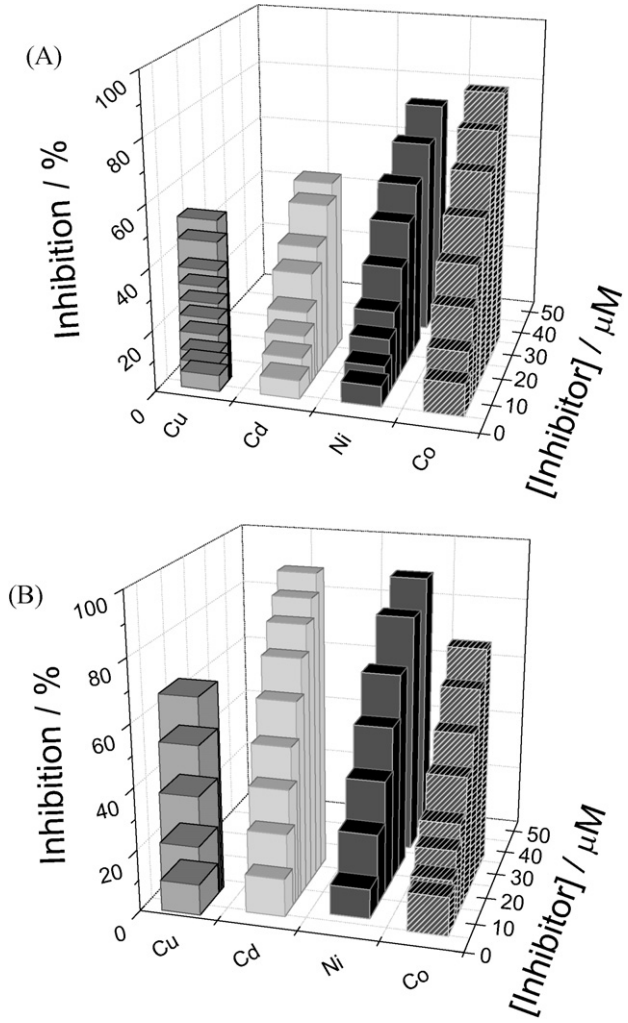

Fig. 5. 3D plot of the inhibition caused by the heavy metal ions at (A) CoHCF/GOx at −0.35 V and (B) CuHCF/GOx at −0.45 V based biosensor in 0.1 M NaPBS, pH 7.0.

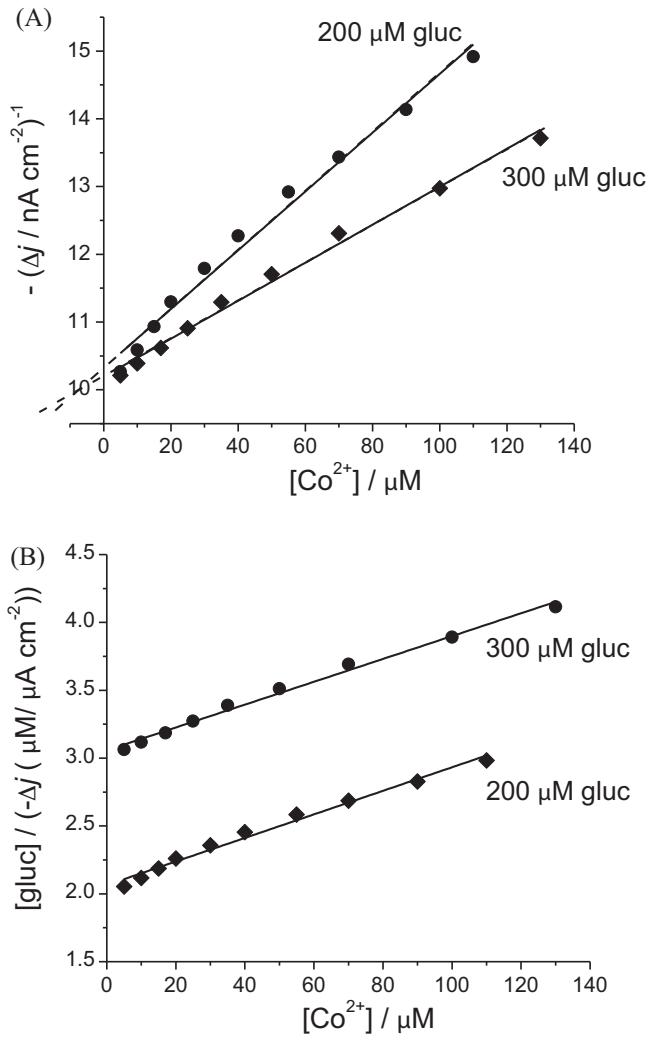

Fig. 6. (A) Dixon and (B) Cornish-Bowden plots for Co^{2+} obtained for two different glucose concentrations: 200 and 300 μM.

3.4. Determination of the type of enzyme inhibition

In order to determine the type of enzyme inhibition Dixon [25] and Cornish-Bowden [26] plots were used. The Dixon plot (representation of the inverse of the enzyme activity vs. inhibitor concentration) by itself cannot clearly distinguish between competitive and mixed inhibition. In the Cornish-Bowden plot, the ratio of substrate concentration and enzyme activity is plotted vs. inhibitor concentration, cannot always distinguish between mixed and uncompetitive inhibition. By analysing these two plots together, all types of inhibition can be characterised.

Dixon and Cornish-Bowden plots (see Fig. 6 for cobalt) were made for two different glucose concentrations, 200 and 300 μM. From the Dixon plot it can be deduced that the inhibition is competitive or mixed, since there is an interception of the two lines in the left part of the *y* axis. Using this information, the Cornish-Bowden plot clearly shows that the inhibition is competitive because the straight lines drawn through the experimental points are parallel. From the Dixon plot, the inhibition constant, K_i, can be estimated by drawing a line parallel to the *y* axis from the point where the straight lines cross down to the *x* axis. For cobalt the values were 5.7 μM for CoHCF/GOx and 1.9 μM for CuHCF/GOx electrodes. The K_i values for all the metals are given in Table 2. The plots for the other metal ions were similar, so that it can be concluded that cadmium, cobalt, copper and nickel all inhibit glucose oxidase activity in a reversible and competitive way. This was confirmed by the fact that when increasing the substrate concentration (glucose) the inhibition decreased for all the metals tested, because the substrate competes with the inhibitor. In [12], competitive inhibition of glucose oxidase by copper ions was also obtained. On the other hand, in [27] Cu^{2+} was found to inhibit GOx in a non-competitive way, whilst in [19] the inhibition of GOx by copper was reversible and mixed. Regarding cadmium, the results are in agreement with those in [19], where the inhibition was also competitive. For cobalt and nickel, only [12] reports competitive inhibition for GOx and no other studies with GOx were found to compare with. When using other enzymes, such as acetylcholinesterase [24] and amperometric detection, reversible and non-competitive inhibition was found for cadmium and copper and, using L-lactate dehydrogenase [28], competitive inhibition was observed for copper.

These observations can be rationalised as follows. For biosensors based on reversible inhibition, a parameter that needs to be carefully considered is the type of immobilisation. The effect of the enzyme immobilisation technique on inhibition can influence the response of biosensor in different ways: (1) decreasing the sensitivity of the inhibition biosensor and (2) changing the type of inhibition [29]. A change in the type of inhibition was reported and investigated in [24] for acetylcholinesterase inhibition by heavy metals. Similarly, in [30] which employs inhibition of polyphenol oxidase, it was demonstrated that immobilising the enzyme

Table 3
Equivalent fitting circuit parameters for CoHCF/GOx (−0.35 V) and CuHCF/GOx(−0.45 V) biosensors in 0.1 M NaPBS, pH 7.0, adding metal cation after glucose and adding glucose after metal cation.

Inhibitor	R_{ct} (kΩ cm^2)		C (μF cm^{-2} s$^{\alpha-1}$)	
	CoHCF/GOx	CuHCF/GOx	CoHCF/GOx	CuHCF/GOx
Glu + M^{2+}				
Cd^{2+}	32.4	14.9	33.9	39.0
Co^{2+}	37.0	14.1	27.8	39.3
Cu^{2+}	36.0	15.9	28.1	40.7
Ni^{2+}	40.2	11.4	38.3	30.3
M^{2+} + Glu				
Cd^{2+}	35.6	15.3	31.4	37.1
Co^{2+}	39.1	15.5	26.4	37.3
Cu^{2+}	35.5	13.4	32.6	37.3
Ni^{2+}	34.9	14.3	26.1	37.5

in polymeric films of different characteristics changes the inhibition type and the inhibition constant (affinity of the inhibitor for the enzyme). Therefore, the immobilisation procedure could be an explanation for obtaining different types of inhibition for the same metal ion, even when using the same enzyme.

3.5. Electrochemical impedance spectroscopy of inhibition biosensors

Conductometric biosensors for heavy metal determination have been previously used [31]. However, to our knowledge impedance spectroscopy has never been previously employed to investigate heavy metal enzyme inhibition sensing. Using EIS experiments, the change in the charge transfer resistance was monitored as being a diagnostic of heavy metal inhibition, although it cannot yet be used quantitatively to calculate the degree of inhibition.

The CoHCF/GOx and CuHCF/GOx biosensors were characterised by electrochemical impedance spectroscopy (EIS) in 0.1 M NaPBS pH 7.0 at the potentials applied for amperometric detection: −0.35 V for CoHCF and −0.45 V for CuHCF based electrodes. Two different sets of spectra were recorded, corresponding to the conditions used in the amperometric experiments:

(1) Spectra recorded in buffer, then after addition of glucose and finally a known concentration of metal ion was added.
(2) Spectra recorded in buffer, then metal ions were added and finally glucose.

All spectra were fitted to the same equivalent circuit used above for the characterisation of modified and unmodified carbon film electrodes (Section 3.1.2). The values from the fitting circuits are presented in Table 3. For the two biosensors, the R_Ω values were around 9 Ω cm^2 and the α values were 0.87 for CoHCF/GOx and 0.85 for CuHCF/GOx electrodes.

From the first set of spectra (Fig. 7A) it is seen that the addition of glucose to buffer leads to a decrease of the charge transfer resistance (from 12.8 to 10.8 kΩ cm^2 in the case of CuHCF/GOx and from 34.4 to 32.0 kΩ cm^2 in the case of CoHCF/GOx), meaning that electron transfer is easier in the presence of glucose. The capacitance of the CPE, C, also decreases from 38.4 μF cm^{-2} s$^{\alpha-1}$ to 31.5 μF cm^{-2} s$^{\alpha-1}$ (for CuHCF/GOx) and from 27.8 to 24.6 μF cm^{-2} (for CoHCF/GOx), respectively. With the addition of metal ions the value of the charge transfer resistance increases again (see Table 3), so that electron transfer is hindered by the presence of the metal ion in solution. This is evidence of enzyme activity inhibition by heavy metals.

The second set of spectra (Fig. 7B) shows that with the addition of metal ion to the buffer almost no difference in the charge transfer resistance, R_{ct}, occurs, so that the biosensor assembly itself, in the absence of enzyme substrate, is not sensitive to the metal ions. However, when glucose is then added an increase of R_{ct} occurs, in agreement with amperometric data, again showing that enzyme activity is inhibited. Furthermore, it is convincing extra evidence that the inhibition is competitive (the metal which is added first binds to the active centre of the enzyme, impeding glucose, the natural substrate, to bind afterwards). Using as parameter the variation of the charge transfer resistance, the greatest inhibition by both biosensors was shown by cadmium, followed by cobalt, then nickel and finally copper. These results are in agreement with those obtained by amperometry.

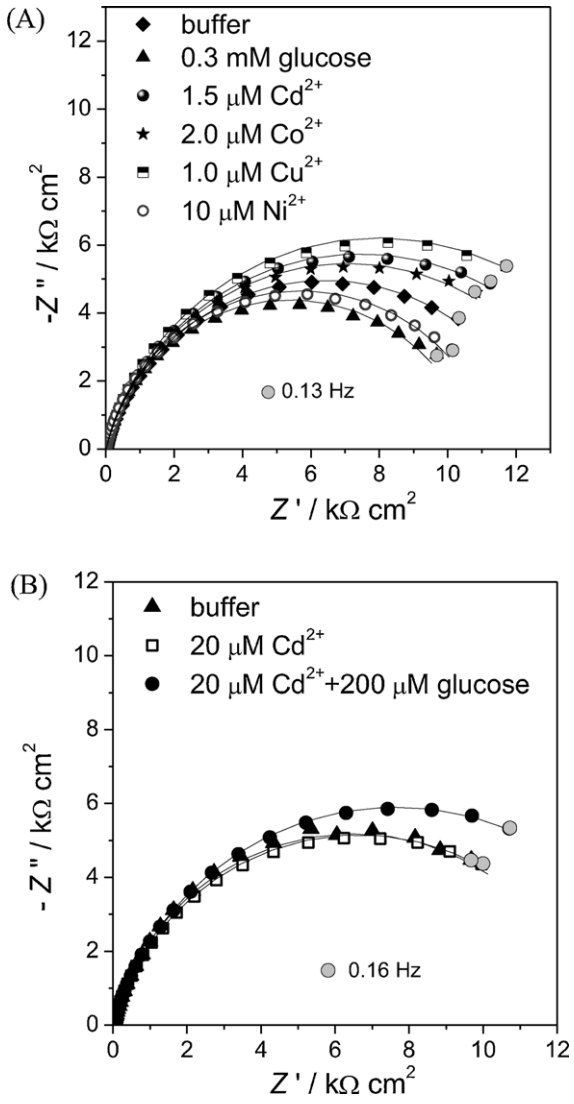

Fig. 7. Complex plane impedance spectra of CuHCF/GOx biosensor in pH 7 0.1 M NaPBS (A) with addition of glucose followed by addition of metal ions and (B) with addition of 20 μM Cd^{2+} followed by addition of 200 μM glucose. Lines show equivalent circuit fitting.

4. Conclusions

A novel electrochemical enzyme inhibition biosensor for inhibition assays, based on glucose oxidase immobilised on cobalt or copper hexacyanoferrate-modified carbon film electrodes, has been developed, characterised and evaluated. Electrochemical impedance spectroscopy showed that modification with metal hexacyanoferrates facilitates the electron transfer process and the effects of inhibition were also seen in the spectra. Enzyme

inhibition was quantitatively evaluated by fixed potential amperometry, with 10% inhibition at the submicromolar or micromolar level – enzyme activity was suppressed more in the presence of copper, followed by cobalt, then cadmium and finally nickel ions for both types of mediated biosensor. The type of inhibition observed was reversible and competitive for all metal ions studied.

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