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Rapid amperometric detection of trace metals by inhibition of an ultrathin polypyrrole-based glucose biosensor

Joseph G. Ayenimo, Samuel B. Adeloju *

NanoScience and Sensor Technology Research Group, School of Chemistry, Monash University, Clayton, VIC 3800, Australia

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ABSTRACT

A sensitive and reliable inhibitive amperometric glucose biosensor is described for rapid trace metal determination. The biosensor utilises a conductive ultrathin (55 nm thick) polypyrrole (PPy) film for entrapment of glucose oxidase (GOx) to permit rapid inhibition of GOx activity in the ultrathin film upon exposure to trace metals, resulting in reduced glucose amperometric response. The biosensor demonstrates a relatively fast response time of 20 s and does not require incubation. Furthermore, a complete recovery of GOx activity in the ultrathin PPy-GOx biosensor is quickly achieved by washing in 2 mM EDTA for only 10 s. The minimum detectable concentrations achieved with the biosensor for Hg^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+} by inhibitive amperometric detection are 0.48, 1.5, 1.6 and 4.0 μM, respectively. Also, suitable linear concentration ranges were achieved from 0.48–3.3 μM for Hg^{2+}, 1.5–10 μM for Cu^{2+}, 1.6–7.7 μM for Pb^{2+} and 4–26 μM for Cd^{2+}. The use of Dixon and Cornish-Bowden plots revealed that the suppressive effects observed with Hg^{2+} and Cu^{2+} were via non-competitive inhibition, while those of Pb^{2+} and Cd^{2+} were due to mixed and competitive inhibition. The stronger inhibition exhibited by the trace metals on GOx activity in the ultrathin PPy-GOx film was also confirmed by the low inhibition constant obtained from this analysis. The biosensor was successfully applied to the determination of trace metals in tap water samples.

1. Introduction

Trace metals are ubiquitous in nature due to various anthropogenic activities which often result in environmental contamination. Some of the most toxic trace metals that can have serious impact on the environment include Hg^{2+}, Pb^{2+} and Cd^{2+} [1–4] and numerous studies have been reported about their toxicities and contamination [5–8]. Various health problems such as mammalian cancers, respiratory diseases, organ failures and intellect retardation have been traced to human poisoning by these metals [9–11]. These toxic effects have been attributed in some studies to the binding of metals to the sulfhydryl –SH groups and displacement of the essential metal cofactor of the enzyme. The associated mechanism involved in the exertion of toxicity by the thiol-binding metals is generally described as “the consequence of interaction with and inhibition of essential thiol groups of enzymes and proteins” [12].

Due to the environmental and toxicological risks of trace metals, there has been a concerted effort and interest in applying various analytical methods to their determination in various samples [13]. Unfortunately, most of these methods are very expensive and, sometimes, require a complicated sample treatment, which limits their amenability for in-field and/or for rapid instantaneous measurement of trace metals in various sample matrices. However, these limitations can now be overcome by using biosensors that do not require complex and labour-intensive handling of the samples.

Recent advances in the development of biosensors for detection of trace metals involve incorporation of various biomolecules, such as amino acids [14], plant peptides [15–17], microbes [18], nucleic acids [19,20], DNAzymes [21,22], whole cells [23,24] and enzymes [25–30]. Among these approaches, the inhibition of enzymatic activity or reaction has, to date, offered the best choice for simple and rapid detection of metal ions due to minimum sample pretreatment, more rapid analysis, high sensitivity and selectivity of some enzymes to metal inhibitors [31–33]. In many cases, the inhibition effect may serve as a sensitive screening test for bioavailable metal ions [31,32]. A number of examples, involving different configuration of enzyme biosensors for metal analysis have been reported [34–39]. A review [40] on this subject revealed that most of these studies focused on developing strategies for achieving enhanced sensitivity and selectivity with enzyme inhibition biosensors for trace metal detection. For example, Liu et al. [41] developed a novel inhibitive amperometric glucose

* Corresponding author.
E-mail address: Sam.Adeloju@monash.edu (S.B. Adeloju).

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oxidase (GOx) biosensor for Hg^{2+} determination in a compost extract. In that study, GOx was cross-linked with glutaraldehyde on a polyaniline membrane in the presence of ferrocene. A detection limit (DL) of 0.49 μg L^{-1} and linear concentration ranges of 0.49–783.21 μg L^{-1} and 0.78–25 mg L^{-1} were achieved for Hg^{2+}. It was noted that the interference of other metal ions with mercury determination was minimal. Another study conducted by Alexander and Rechnitz [42] used an amperometric GOx biosensor with the self-assembly of 2-amino-ethathiol on a gold electrode coated with polyvinyl-pyrrolidone (PVP) membrane and with sulfhydryl (–SH) as mediator to achieve a lower detection limit of 0.2 μgL^{-1} for Hg^{2+}. However, the inhibition of the amperometric response in this case was attributed to a reaction between Hg^{2+} and –SH group on 2-amino-ethanethiol [42]. By using a glucose biosensor with poly(neutral red) (PNR) redox mediator at a carbon fibre electrode (CFE) (GOx/PNR/CFE), Ghica and Brett [35] achieved detection limits down to 1, 6, 3 and 9 μg L^{-1} for Cd^{2+}, Cu^{2+}, Pb^{2+}, and Zn^{2+}, respectively. All the metals investigated exhibited mixed or competitive inhibition, except for Zn^{2+} which involved non-competitive inhibition. The practical DL (concentration that gives 10% inhibition, I_{10}) and sensitivity achieved with this biosensor, were 10.6, 4.7 μM and 3.95, 9.57 nA cm^{-2} μM^{-1} for Cd^{2+} and Cu^{2+}, respectively. Recently, the same group [27] achieved a remarkable improvement in the analytical performance of the biosensor by substituting PNR mediator with hexacyanoferrate (HCF) mediators. The DL achieved with GOx/CoHCF/CFE and GOx/CuHCF/CFE configurations were 2.4 and 0.2 μM for Cd^{2+} and Cu^{2+}, and 1.2 and 0.5 μM for Cd^{2+} and Cu^{2+}, respectively. These values were much lower than those obtained with GOx/PNR/CFE for Cd^{2+} (10.6 μM), Cu^{2+} (4.7 μM). This was also confirmed by a recent study which demonstrated that trace metals determined with GOx/CoHCF/CFE film electrodes showed lower DL of 0.09 and 0.30 μM for Cu^{2+} and Cd^{2+} than those obtained with GOx/PNR/CFE electrodes [29]. Malitesta and Guascito [25] also employed an inhibition detection scheme based on immobilization of GOx into poly-o-phenylenediamine for detection of metals, such as Hg^{2+}. The investigated enzymatic inhibition can be reversed with EDTA treatment and responded to the lowest Hg^{2+} concentration of 2.5 μM with a long amperometric response time (< 2 min) and a stability period of at least 10 days. Evidence provided from the observed decrease in current response indicated that the GOx activity is potentially inhibited by Hg^{2+}, Cu^{2+}, Pb^{2+}, Ag^{+} and Cd^{2+}, with response times ranging from 5 to 30 min. These response times are obviously too long for achieving rapid determination of trace metals, and this highlights the need for further development of new improved inhibitive biosensors with more rapid response times.

Most of the studies performed to date on inhibition of GOx by trace metals are based on the immobilization of the enzyme on the electrode surface by adsorption or covalent attachment or cross-linking with glutaraldehyde on polymers, such as poly-o-phenylenediamine, polyvinyl-pyrrolidone and polyaniline membranes. A major disadvantage of these approaches is that the electrode fabrication process is complex and usually takes several hours or days for complete preparation. We demonstrated in our previous study [30] that this problem may be overcome by using an ultrathin polypyrrole (PPy)-GOx film for potentiometric inhibitive detection of trace metals [30]. However, a somewhat long response time of 100 s was achieved with the biosensor. There is, therefore, still a need for further development that will enable a more rapid and reliable detection of trace metal ions.

In this study, we explore the use of an ultrathin PPy-GOx film for fabrication of an amperometric glucose biosensor capable of rapid inhibitive detection of Hg^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+}. Generally, amperometric biosensors based on galvanostatic entrapment of enzyme on electrodes have remarkable characteristics, such as low response time, ease of fabrication, high control of enzymatic layer thickness coupled to interferent rejection and long life-time [25,43]. To our knowledge, amperometric glucose biosensors based on entrapment of GOx in ultrathin PPy film has never been employed for development of an inhibitive biosensor for detection of trace metal ions. The development of the inhibitive amperometric biosensor involves consideration of the degree of inhibition achieved for different trace metals, nature of inhibition, reversibility of metal inhibition, storage stability and its application to the determination of some trace metals in water samples.

2. Experimental

2.1. Reagents.

GOx (Type X-S G7141-50KU, 147,000 unit g^{-1}) derived from Aspergillus niger, D (+)-glucose and pyrrole were purchased from the Sigma-Aldrich (Sydney, NSW, Australia). Before use, pyrrole was distilled under vacuum and stored in the refrigerator in a bottle wrapped with aluminium foil under nitrogen to prevent UV degradation and air oxidation. Phosphate buffers of different pH and concentrations were prepared with a mixture of sodium dihydrogen orthophosphate monohydrate and anhydrous di-sodium orthophosphate. A 1000 ppm D (+)-glucose stock solution was prepared and allowed to stand overnight to mutarorate. The stock solution was then appropriately diluted to prepare the desired concentration. Also, the appropriate volume of the stock solution (1000 ppm) of the inhibitor was diluted to give desired concentrations. All solutions were prepared with Mili-Q water (Millipore, North Ryde, NSW, Australia).

2.2. Preparation of PPy-GOx electrode

Fabrication of ultrathin PPy-GOx biosensors and amperometric measurements were performed with a potentiostat/galvanostat. Platinum electrodes (2.010 mm^2) were polished prior to use with 50 nm alumina on a polishing pad and then rinsed with Milli-Q water, acetone and again with Milli-Q water. The electrodes were sonicated for 10 min in Milli-Q water to remove any residual alumina particles and finally dried in air. The electrodes were cleaned electrochemically by cycling the electrode potential between −200 and +1450 mV versus Ag/AgCl in 1 M H_2SO_4 for 10 min at a sweep rate of 75 mV s^{-1} in a 1.0 M H_2SO_4 solution, until a constant current–voltage response was obtained.

The electropolymerization of monomer solutions was performed galvanostatically in a three-electrode cell consisting of a platinum working electrode, platinum auxiliary and reference electrodes. The ultrathin PPy-GOx biosensors were prepared galvanostatically, as described previously [44], from a deoxygenated solution containing 0.2 M pyrrole, 300 U mL^{-1} GOx, by applying a current density of 0.05 mA cm^{-2} for 500 s [44]. The monomer solution was deoxygenated for 10 min prior to galvanostatic polymerization. The PPy-GOx electrode was rinsed with Milli-Q water after preparation and stored in a pH 7.0 phosphate buffer (0.05 M) at 4 °C when not in use.

2.3. Amperometric measurements of metal inhibition

All measurements of glucose response, with and without inhibitor in solution, were carried out in in a pH 7.0 phosphate buffer at an applied potential of 700 mV at room temperature, with stirring, in a three-electrode cell consisting of a PPy-GOx working electrode, a platinum auxiliary and a Ag/AgCl reference electrodes (3 M KCl). In the first step of the inhibitive amperometric measurement, the biosensor response to 8 mM glucose in the absence

of an inhibitor was measured and the steady-state current was recorded. Then, increasing metal concentration was added successively to inhibit the GOx activity and, consequently, suppress the glucose response in proportion to the inhibitor concentration. The percentage inhibition was evaluated using the equation: $(I_0-I)/I_0 \times 100$, where I_0 and I are the biosensor responses to 8 mM glucose without and with different inhibitor concentrations, respectively. All measurements of glucose response were determined in triplicate unless stated otherwise.

3. Results and discussion

3.1. Trace metal inhibition at an ultrathin PPy-GOx biosensor

The optimisation of an ultrathin PPy-GOx biosensor has been described in our previous study for potentiometric detection [44]. In the present study, the amperometric measurement of 8 mM glucose at an ultrathin PPy-GOx biosensor was initially used to establish the current magnitude in absence of an inhibitor, I_0, which was 36 ± 2.2 μA. With the addition of Hg^{2+}, which inhibit GOx activity, the glucose response decreases, as illustrated in Fig. 1 with increasing Hg^{2+} concentrations. The effect of other metal ions, Pb^{2+}, Cd^{2+} and Cu^{2+}, on GOx activity was also investigated. All experiments were carried out in a pH 7.0 phosphate buffer (0.05 M) at an applied potential of 700 mV. This was based on our previous study [44] which demonstrated the determination of glucose at a PPy-GOx biosensor between pH 5 and 7 in real samples, which also agrees with previous studies [35,45,46].

The decline in GOx activity and, hence, the glucose response (I_0, μA) with increasing Hg^{2+} concentration shown in Fig. 1 is due to the interaction between the thiol group of the active site of GOx and Hg^{2+} ions. It is also obvious from Fig. 1 that the ultrathin PPy-GOx biosensor has a relatively fast response time of 20 s which is much lower than the 100 s achieved with our previous inhibitive potentiometric biosensor for trace metal detection [30], and is also lower than the 100 s reported by Malitesta and Guascito [25] for amperometric detection of Cu^{2+} and Hg^{2+} ions. Furthermore, it is lower than the 80 s reported in another study for Hg^{2+} determination in compost extract with an inhibition-based GOx biosensor [41]. These observations demonstrate that the use of the ultrathin PPy-GOx film is beneficial for improving the performance of the

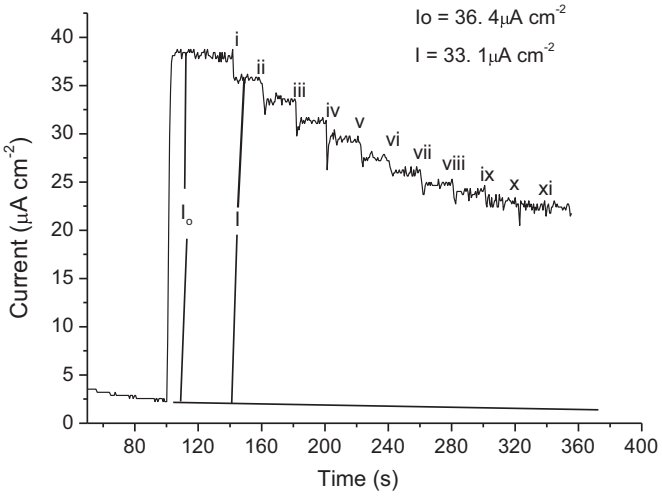

Fig. 1. Effect of increasing addition of Hg^{2+} ions on the amperometric response of ultrathin PPy-GOx biosensor to glucose. (i) +0.48, (ii) +0.91, (iii) +1.3, (iv) +1.7, (v) +2, (vi) +2.3, (vii) +2.6, (viii) +2.9, (ix) +3.1, (x) +3.3, and (xi) +4.1 μM. Applied potential +700 mV vs. Ag/AgCl; 8 mM glucose in a stirred phosphate buffer (pH 7.0, 0.05 M).

inhibitive amperometric biosensor for detection of Hg^{2+}. This is due to the limited diffusion barrier between the inhibitor and the active site of the enzyme. For this reason, it was also not necessary to incubate the enzyme with the inhibitor for a long time, as usually required [41].

3.2. Degree of inhibition

Fig. 2 shows a comparison of the degree of inhibition achieved with the four different metal ions. Evidently, the suppression of GOx activity was less at lower inhibitor concentrations, but increased with increasing concentrations of the metal ions. As shown in Fig. 2(a), the presence of 0.48 μM Hg^{2+} ions resulted in a 9% decrease in the glucose response. The required concentration of Hg^{2+} ions for 50% inhibition (I_{50}) of GOx was 1.5 μM. Maximum inhibition (I_{max}) of 45% was achieved when the metal concentration (I_{cmax}) was increased to 4.1 μM Hg^{2+} (Table 1). Further increase of the metal ion concentration above 4.1 μM Hg^{2+} did not cause any significant change in the glucose amperometric response. In the case of Cu^{2+} (Fig. 2b), the presence of 1.5 μM of the metal ion caused 3.6% suppression in the current response, but increased progressively to 38% I_{max} at I_{cmax} of 13 μM. This result demonstrated that Cu^{2+} has a comparable inhibitive effect on GOx as with Hg^{2+}. This also indicates that Cu^{2+} will interfere with the determination of Hg^{2+} if present together and vice-versa. This is in agreement with previous use of inhibitive biosensors for determination of glucose [25,34] and urea [38]. However, the Hg^{2+} concentration required to inhibit glucose response is lower than those observed with Cu^{2+}. This, therefore, indicates that the former is more inhibitive and, hence, more toxic than the latter. This is due to the ability of Hg^{2+} ion to form highly stable mercaptides [37], but in contrast Cu^{2+} belongs to the group of borderline metal ions which have lower affinity to sulphur.

Weak inhibition ($< 1\%$) was observed with 4.0 μM Cd^{2+}, as shown in Fig. 2c. Beyond this concentration, the inhibition becomes stronger with increasing Cd^{2+} concentration. The metal ion concentration which resulted in at least 4–6% of inhibition is usually taken as the lower detection limit or minimum detectable concentration [47]. Consequently, the minimum detectable concentration for Cd^{2+} was 4.0 μM which caused 4% inhibition of the enzyme and the I_{50} was 14 μM. From Fig. 2d, it is obvious that an appreciable inhibition of the enzyme was not attained until the Pb^{2+} concentration increased from 1.6 to 2.9 μM with increasing inhibition from 1% to 6% and finally reaching 27% with increasing Pb^{2+} concentration up to 11 μM. The I_{50} for this metal ion was achieved at 4.9 μM with 14% inhibition. This observation is much better than reported by a spectrophotometric method, which suggested that there is no considerable inhibition of GOx by Pb^{2+} ions at concentrations below 260 mg L^{-1} (898.8 μM) [48] and, therefore, further indicates that the ultrathin PPy-GOx biosensor has far superior sensitivity to metal inhibition.

As expected, Cd^{2+} and Pb^{2+} exhibited similar and moderate inhibitive effects on GOx activity [49]. In agreement with our previous inhibitive potentiometric detection [30], the relative effectiveness of the metal ions as inhibitor is: $Hg^{2+} > Cu^{2+} > Cd^{2+} > Pb^{2+}$. This trend is also in agreement with other reported results for urease [50] and GOx [34,51]. Overall, the results demonstrate that enzymatic inhibition is the dominant process responsible for the observed inhibition of glucose response in the presence of the metal ions and, thus, confirms their strong affinity with GOx.

3.3. Determination of the nature and type of metal inhibition

To enable an analysis of the mode of interaction between the metal ions and glucose oxidase, the inhibition constants were

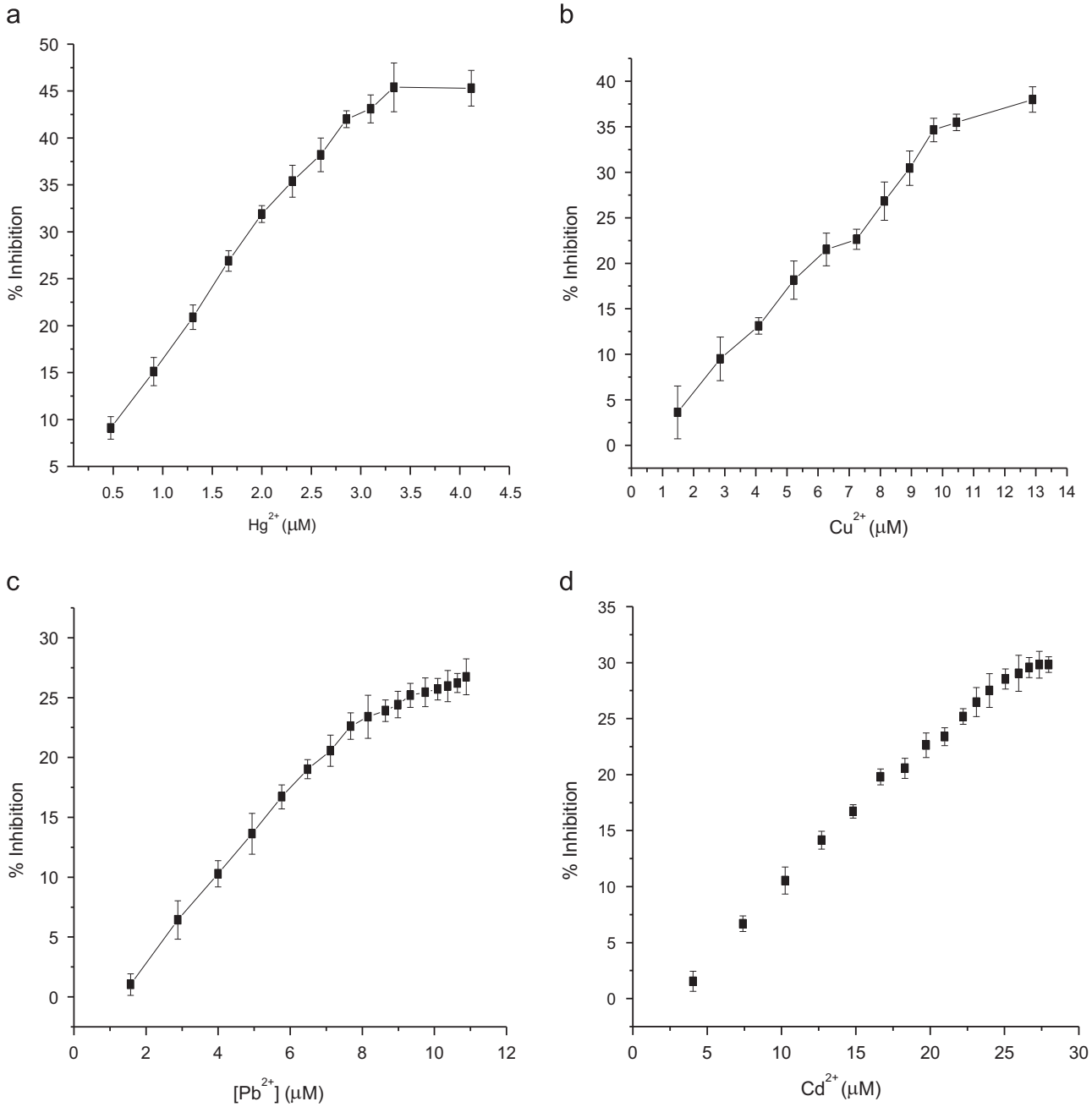

Fig. 2. Influence of inhibitor concentration on percentage inhibition. 8 mM glucose in 0.05 M phosphate buffer (pH 7.0, 0.05 M).

estimated from Dixon plots [52] which are based on the plot of $1/I$ vs. M^{2+} concentration (where I denotes current response), which is commonly employed for reversible inhibition by using at least two different enzyme–substrate concentrations. To obtain a more accurate estimation of the constants, three different glucose concentrations: 4, 8 and 16 mM were employed for construction of the Dixon plots, as shown in Fig. 3(a, c, e and g). The extrapolated abscissa intercept of each linear plot corresponds to the inhibition constants (K_i). In this way, K_i values of 3.6, 14, 13 and 23 were estimated for Hg^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+}, respectively. However, this inhibition parameter alone is not sufficient to fully elucidate the type of inhibition because the Dixon plots for mixed and competitive inhibition are similar [35]. By using Cornish-Bowden plots (glucose concentration/I vs. M^{2+} concentration) [53] shown in Fig. 3b, d, f and h, as a complementary confirmation, it was possible to differentiate between these two types of inhibition. Using this approach, the dissociation constant of the enzyme–inhibitor complex (K_i) and the dissociation constant of the enzyme–substrate–inhibitor complex (K_i') were estimated from the extrapolated abscissa intercept of each linear plot.

From the Dixon plot, the crossing lines exclude the possibility of uncompetitive inhibition for all the tested metal ions. Expressing the same inhibition data by using Cornish-Bowden plot, it was deduced that non-competitive inhibition occurred with Hg^{2+} and Cu^{2+} because the centre of rotation or intersection points of both plots are above the abscissa axis. Mixed inhibition occurred with Pb^{2+} due to the occurrence of the intersection point of the Cornish Bowden plot below the abscissa axis. On the other hand, Cd^{2+} exerted competitive inhibition as the straight lines drawn through the experimental points are parallel and this is consistent with observations from previous studies [35,51].

Table 1
Comparison of the performance characteristics of the ultrathin PPy-GOx biosensor with those other biosensors.

Biosensor	*Metal ion*	*Mode of detection*	*Linear range* (μM)	*Sensitivity* ($\mu A\ cm^{-2}\mu M^{-1}$)	*MDC* (μM)	K_i (μM)	K_I (μM)	I_{cmax} (μM)	I_{max} (%)	I_{C50} (μM)	Refs
CoHCF-CFE-GOx	Cd^{2+}	Amperometric	1.5–6.0	0.004	nd	11	nd	nd	nd	17	[27]
	Cu^{2+}		0.2–3.0	0.027		0.03	nd			1.4	
CuHCF-CFE-GOx	Cd^{2+}	Amperometric	1.5–6.0	0.018	nd	2.0	nd	nd	nd	5.8	[27]
	Cu^{2+}		0.2–3.0	0.031		0.04	nd			2.2	
Ultrathin PPy-GOx	Hg^{2+}	Potentiometric	0.1–15		0.025	0.55	1	15	95	0.25	[30]
	Cu^{2+}		0.32–47		0.079	2.4	4.7	47	87	3.1	
	Pb^{2+}		0.1–24		0.024	0.97	1.5	24	51	24	
	Cd^{2+}		0.04–62		0.04	1.5	ND	62	68	4.5	
PPDA-GOx	Hg^{2+}	Amperometric	5–180	0.067	2.5	nd	nd	22	75	22	[34]
	Cu^{2+}		10–100	0.11	5.0	nd	nd	450	82	70	
			100–250	0.04							
	Cd^{2+}		20–150	0.007	5.0	nd	nd	210	40	210	
PNR-CFE-GOx	Cd^{2+}	Amperometric	0.04–0.14	nd	0.009	0.89	nd	0.71	27	nd	[35]
	Cu^{2+}		0.5–9.1	nd	0.095	61	nd	362	41	nd	
	Pb^{2+}		0.4–2.3	nd	0.014	23	nd	75	20	nd	
Ultrathin PPy-GOx	Hg^{2+}	Amperometric	0.48–3.3	4.0	0.48	3.6	4.0	4.1	45	1.5	Present study
	Cu^{2+}		1.5–11	1.2	1.5	14	8.5	13	38	5.2	
	Pb^{2+}		1.6–7.7	1.0	1.6	13	22	12	27	4.9	
	Cd^{2+}		4–26	0.5	4.0	23	nd	27	30	14	

I_{cmax} – concentration of metal required for maximum inhibition, I_{max} – maximum inhibition, concentration of metal that caused 50% inhibition, (I_{C50}), nd – not determined.

3.4. Calibration plots for Hg^{2+}, Pb^{2+}, Cd^{2+} and Cu^{2+} ions

The calibration plots obtained for the four metal ions at the ultrathin PPy-GOx biosensor are shown in Fig. 4. These results demonstrated unambiguously that all the metal ions exerted an inhibition effect on the enzymatic process and that the inhibition increased with increasing inhibitor concentration, and they are in agreement with previously reported data [34,35]. It can be seen from Fig. 4a that a linear range was obtained for Hg^{2+} ions from 0.48 to 3.3 μM. The minimum detectable concentration was 0.48 μM, which is the minimum Hg^{2+} concentration which caused significant inhibition of the enzyme activity.

For Cu^{2+}, as shown in Fig. 4b, a linear concentration range was obtained from 1.5 to 11 μM and a minimum detectable concentration of 1.5 μM was achieved. Fig. 4c shows that the Cd^{2+} response was linear up to 26 μM. The analytical performances of the ultrathin PPy-GOx biosensor for inhibition of glucose response by the four metals are summarised in Table 1. Evidently, the detection limits and linear ranges of the proposed biosensor are compatible with typical trace metal concentrations in real-life samples [54–56] and meet the general requirements formulated by EPA [47], as well as with previously reported results [34,35,40,57–62]. For lead, the response was only linear up to 7.7 μM. These results demonstrate that the catalytic action of GOx was markedly inhibited by Hg^{2+} and Cu^{2+} ions, in agreement with previous studies [51,63]. These metal ions are always reported as the most potent inhibitors for GOx and other enzymes [25,49,59].

Notably in Table 1, the ultrathin PPy-GOx biosensor gave much higher sensitivities for metal inhibition than those reported in other studies for amperometric detection of metal ions. However, the achieved minimum detectable concentrations for the four metals were not as low as those reported in our previous study for potentiometric detection of metal ions [30] and for amperometric detection of Cu^{2+}, Cd^{2+} and Pb^{2+} reported by Ghica and Brett [35]. Nevertheless, the metal ion concentrations required to achieve maximum inhibition of the amperometric responses obtained with the ultrathin PPy-GOx biosensor were much lower than obtained using potentiometric detection, and also lower than that of previously reported amperometric detection [34,35]. For example, as indicated in Table 1, the metal concentrations required to achieve maximum inhibition with amperometric detection in this study were 4.1, 13, 12 and 27 μM for Hg^{2+}, Cu^{2+}, Pb^{2+}, and Cd^{2+}, respectively. In contrast, the corresponding concentrations of the four metal ions used to achieve maximum inhibition with potentiometric detection in our previous study [30] were 15, 47, 24.2 and 62.2 μM, respectively.

3.5. Reversibility, reproducibility and storage stability

The use of the ultrathin PPy-GOx films for inhibitive detection of metal ions is particularly advantageous, as no incubation is required for the metal ions to exert their inhibitive effects on the –SH group of the enzyme. Also, after each inhibition, no metal-chelating agent, such as ethylenediaminetetratacetic acid (EDTA) or triton-X, is needed for reactivation of GOx. For example, with inhibition of the PPy-GOx response obtained for 1.5 mM glucose with the addition of 0.5 μM Hg^{2+}, it was possible to achieve 80–90% recovery of its original response by simply washing for 10–15 min in phosphate buffer (pH 7). This clearly indicates that the inhibition of GOx activity in the ultrathin PPy-GOx film is reversible. However, a more rapid and complete restoration of enzyme activity would be beneficial and the possibility of achieving this was evaluated by washing the electrodes with different EDTA concentrations. Fig. 5 illustrates the three-step process of metal inhibition and restoration of GOx activity employed for this purpose. In the first step, 1.5 mM glucose was added to obtain baseline amperometric response. This was followed by the addition of

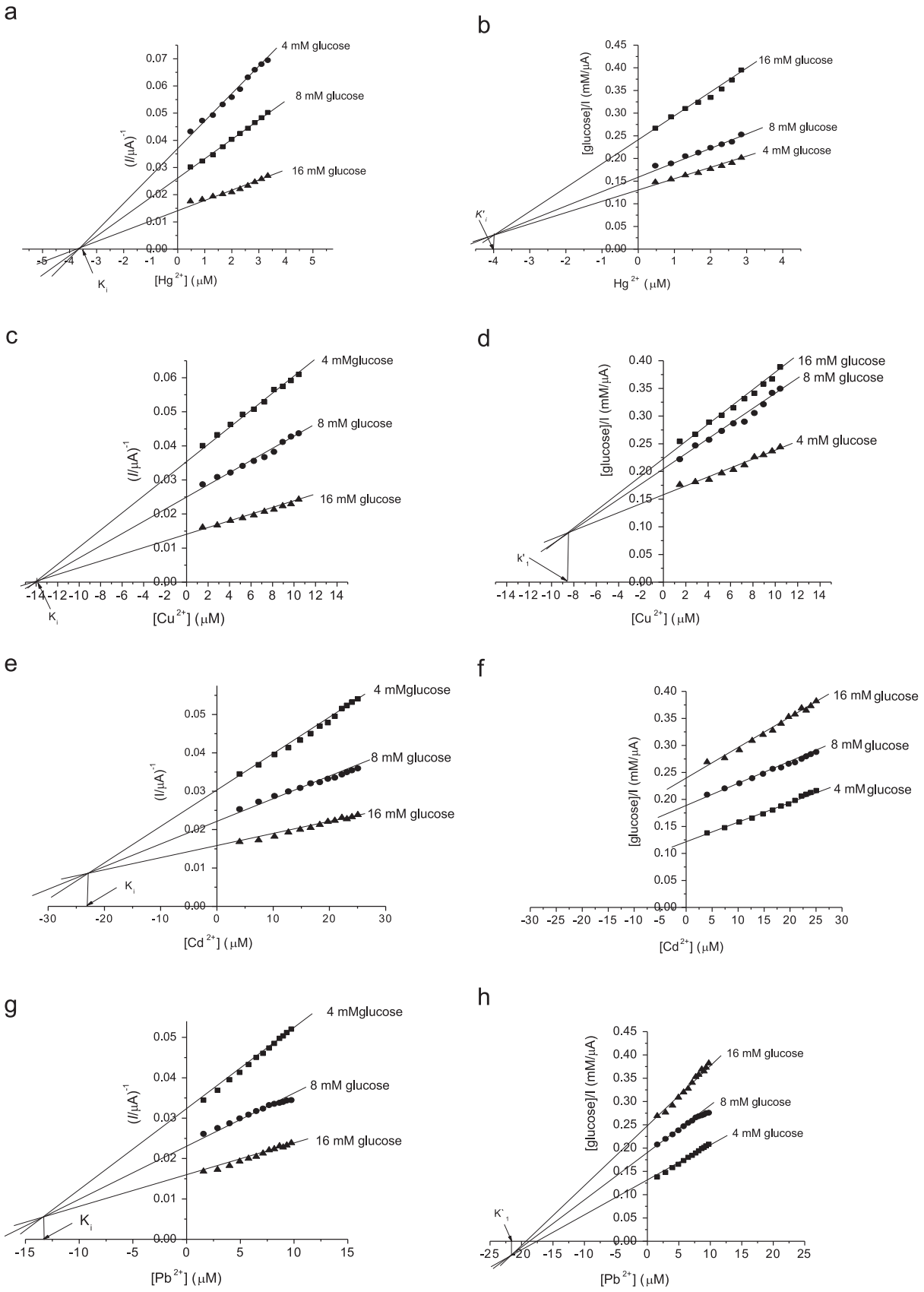

Fig. 3. Dixon plots (a, c, e and g) and Cornish-Bowden plots (b, d, f and h) for Hg^{2+}, Cu^{2+}, Cd^{2+} and Pb^{2+} at three different glucose concentrations.

0.5 μM Hg^{2+}, which resulted in a rapid depression of the glucose response from 6.0 ± 0.8 to 3.0 ± 0.2 μA cm^{-2} ($n=6$). By washing with 0.5 mM EDTA, a 30% recovery was achieved. A gradual increase of the EDTA concentration in the washing solution up to 2 mM resulted in a 98% recovery of GOx activity, almost back to the original response level. It is also important to note that the

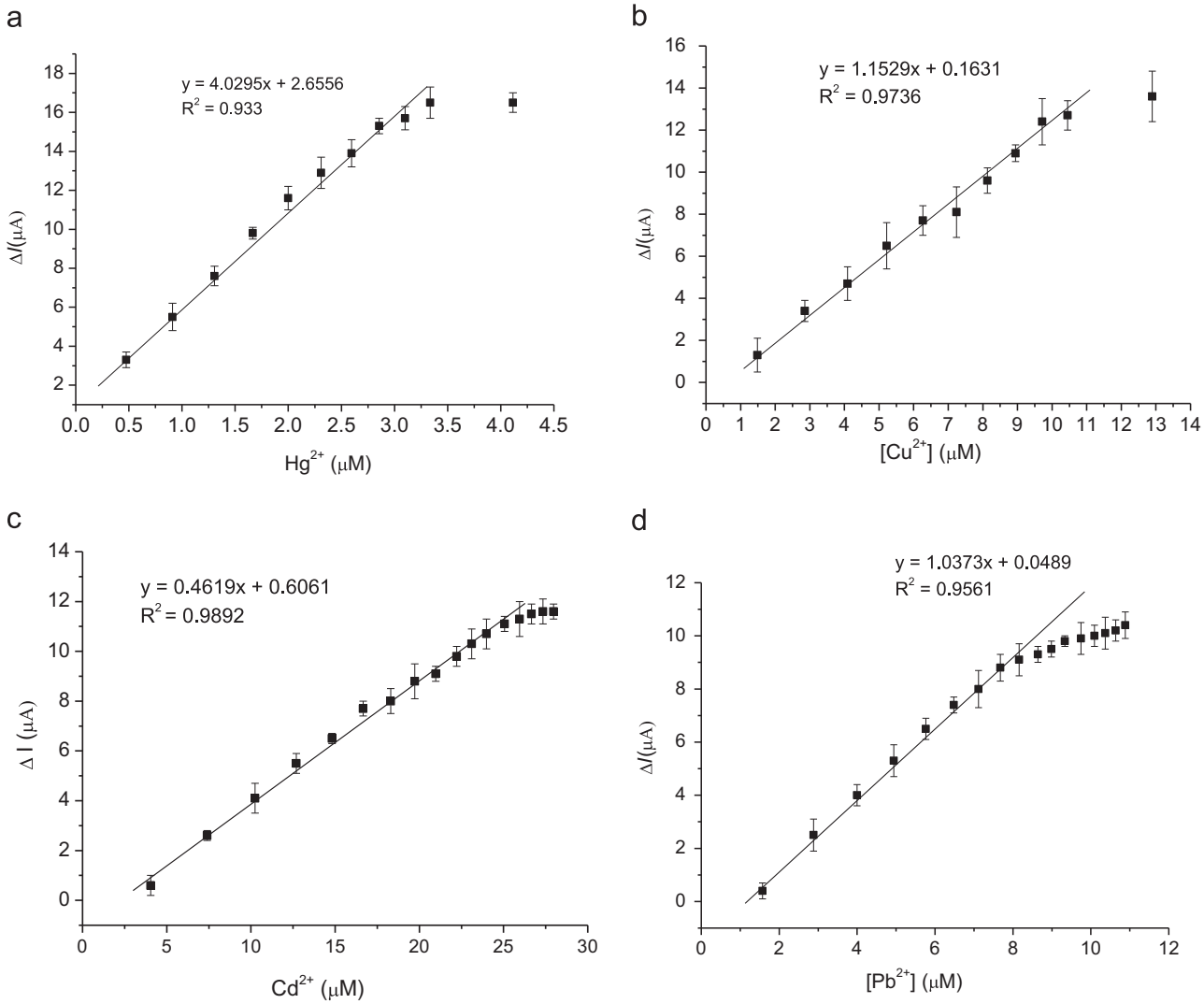

Fig. 4. Calibration curves for determination of (a) Hg^{2+}, (b) Cu^{2+}, (c) Cd^{2+} and (d) Pb^{2+} obtained by inhibition of glucose oxidase in 0.05 M phosphate buffer (pH 7.0, 0.05 M) in the presence of 8 mM glucose.

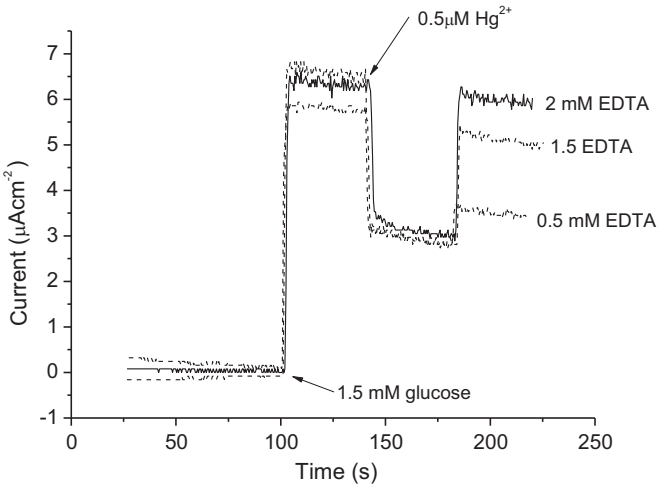

Fig. 5. Glucose response inhibition induced with the addition of 0.5 μM Hg^{2+} in the presence of 1.5 mM glucose and response recovery after washing with different EDTA concentrations.

reversibility of the inhibition was very rapid and occurred within 10 s, which is 10–20 times faster than 100–200 s reported in other studies [34,35].

The reproducibility of the ultrathin PPy-GOx biosensor was also investigated by performing 5 successive measurements with addition of 0.5 μM Hg^{2+} using the same electrode and washing with 0.05 M phosphate buffer for 10–15 min after each measurement. The relative standard deviation (R.S.D) of the current response to 1.5 mM glucose is less than 15%, while that of deactivation of GOx activity by 0.5 μM Hg^{2+} was 9.6%. The storage stability of the biosensor in 0.05 M phosphate buffer (pH 7.0) after 10 consecutive inhibition measurements was evaluated by checking the current response for 1.5 mM glucose intermittently. No significant loss ($<10\%$) in the glucose response was observed after storing the electrode in the buffer solution for 15 min. The response of the ultrathin PPy-GOx biosensor to glucose remains stable and reproducible for almost 3 weeks. After this period, the biosensor lost 60% of its response. The loss in the response may be due to the increased porosity of the ultra-thin film, which may have caused some of the GOx to diffuse out of the film.

Table 2
Recovery of trace metals in tap water samples.

Metal ions	Levels present (μM) AAS	Added (μM)	Recovered (μM) biosensor	% Recovery biosensor	Recovered (μM) AAS	% Recovery AAS	T_{cal}	P_{cal}
Hg^{2+}	ND*	0.5 (n=4)	0.46 ± 0.03	92 ± 7	0.44 ± 0.08	88 ± 18	0.4682	0.6562
		1 (n=3)	1.1 ± 0.11	105 ± 10	0.99 ± 0.05	94 ± 5	0.8601	0.4382
		2 (n=6)	1.9 ± 0.27	96 ± 14	2.1 ± 0.10	107 ± 5	1.9567	0.0789
Cu^{2+}	ND	1 (n=3)	0.96 ± 0.06	96 ± 6	1.1 ± 0.04	105 ± 4	2.1617	0.0967
		2.5 (n=3)	2.7 ± 0.20	108 ± 7	2.5 ± 0.16	99 ± 7	1.5554	0.1948
		5 (n=3)	4.8 ± 0.17	97 ± 4	4.9 ± 0.12	97 ± 3	0.3224	0.7554
Pb^{2+}	ND	2 (n=3)	2.0 ± 0.05	102 ± 3	1.9 ± 0.15	93 ± 8	1.9718	0.1199
		3 (n=5)	4.0 ± 0.12	100 ± 3	4.1 ± 0.1	102 ± 3	0.7682	0.4715
		8 (n=3)	7.6 ± 0.1	95 ± 1	7.8 ± 0.33	97 ± 4	1.0046	0.3719
Cd^{2+}	ND	5 (n=4)	4.9 ± 0.05	98 ± 1	5.0 ± 0.1	99 ± 2	0.8944	0.4055
		10 (n=4)	8.9 ± 0.15	89 ± 2	9.1 ± 0.2	91 ± 1	2.0800	0.0827
		15 (n=4)	15 ± 0.27	101 ± 2	15 ± 0.57	99 ± 4	0.6976	0.5115

ND-not detectable.

* Cold vapour AAS[64].

3.6. Analytical application

The application of the ultrathin PPy-GOx biosensor to the determination of metal ions in tap water was investigated by spiking with known concentrations of Hg^{2+}, Pb^{2+}, Cd^{2+} and Cu^{2+} ions. These samples were also analysed by atomic absorption spectrometry (AAS) for comparison and the results obtained are summarised in Table 2. Evidently, the biosensor achieved excellent recoveries of 92–105% for Hg^{2+}, 96–108 for Cu^{2+}, 95–102 for Pb^{2+} and 89–101 for Cd^{2+}. Also, as demonstrated by the data in Table 2, the concentrations and recoveries obtained for the four metals in the tap water sample by AAS were in reasonably good agreement with those achieved with the ultrathin PPy-GOx biosensor as revealed by Welsh student t-test (http://www.graphpad.com/quickcalcs/ttest1.cfm). The difference in the results obtained by the two methods is statistically not significant since the calculated two-tailed P value (P_{cal}) > 0.05 and $T_{cal} < T_{tab}$ at 95% confidence limit.

4. Conclusion

We have successfully demonstrated that ultrathin PPy-GOx film can be used for fabrication of an effective and reliable glucose biosensor for more rapid inhibitive amperometric detection of trace metals. The use of Dixon and Cornish-Bowden plots revealed that the suppressive effects observed in the presence of Hg^{2+} and Cu^{2+} were via non-competitive inhibition, while those of Pb^{2+} and Cd^{2+} were due to mixed and competitive inhibition. The achieved detection limits for Hg^{2+}, Cu^{2+}, Cd^{2+} and Pb^{2+} by this method were 0.48, 1.5, 4 and 1.6 μM, respectively, while useful linear concentration ranges were obtained from 0.48–3.3, 1.5–11, 4–26 and 1.6–7.7 μM for the four metals, respectively. More notably, the ultrathin PPy-GOx biosensor demonstrated a relatively fast response time of 20 s and the unique characteristics of not requiring washing with EDTA for its regeneration, unless more rapid restoration of the enzyme activity is required. The biosensor was successfully applied to the determination of Hg^{2+}, Cu^{2+}, Cd^{2+} and Pb^{2+} in spiked tap water with very good to excellent recoveries.

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