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Inhibitive Potentiometric Detection of Trace Metals with Ultrathin Polypyrrole Glucose Oxidase Biosensor

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Abstract

A method, based on the inhibition of an ultrathin polypyrrole-glucose oxidase (PPy-GOx) potentiometric biosensor response, is described for the detection of Cu^{2+}, Hg^{2+}, Cd^{2+} and Pb^{2+} ions. Based on experimental conditions (0.2 M pyrrole, 500 U mL^{-1} GOx, and an applied current density of 0.05 mA cm^{-2} and a polymerization period of 500 s) previously published by us, PPy-GOx films of approximately 55 nm thick were used to demonstrate the inhibitive potentiometric detection of selected trace metals down to 0.079 μM Cu^{2+}, 0.025 μM Hg^{2+}, 0.024 μM Pb^{2+} and 0.044 μM Cd^{2+}. Furthermore, good linear concentration ranges were achieved for Cu^{2+} (0.079-16 μM), Hg^{2+} (0.025-5 μM), Pb^{2+} (0.10-15 μM) and Cd^{2+} (0.04-62 μM). The analysis of the nature of the inhibition of glucose oxidase in the PPy-GOx biosensor by these metals was achieved by Dixon and Cornish-Bowden plots. The shapes of the curves (exponential decay, parabolic and linear) obtained for the inhibitors suggest that the inhibition by the metal ions may not be exclusively directed at the essential -SH group, but involve additional binding sites of the enzyme. Dixon and Cornish-Bowden plots suggest that the inhibition is competitive for Cd^{2+}, while non-competitive inhibition was observed for other metal ions. The ultra-thin PPy-GOx

film enabled improved permeability to the metal inhibitors than possible with conventional biosensors with thicker films and, hence, better reflects the actual inhibition effect of the trace metals on the enzyme activity. The use of the ultra-thin film also eliminated the usual need for incubation of the enzyme electrode for a long period in the presence of the inhibitors. Furthermore, a rapid recovery of the enzyme activity was achieved by simply washing the electrode with water and storing in phosphate buffer for 10-15 minutes. The proposed biosensing approach was successfully used for the detection of individual trace metals in tap water, achieving a 98-101% recovery.

Keywords: Ultrathin polypyrrole film; glucose oxidase; biosensor; trace metals; potentiometry; inhibition

1. Introduction

Among the various biosensing approaches for trace metal detection, the inhibition of enzymatic activity has, to date, offered a choice for simple and rapid detection of metal ions due to the selective nature of some inhibitors [1]. In many cases, the inhibition effect may serve as a sensitive screening test for bioavailable metal ions [1]. The achievable detection limit is often much lower than the specified maximum allowable limits in clinical or environmental samples [2, 3].. This is also comparable to those obtained with conventional analytical techniques, such as spectrometry and chromatography [2, 3]. In this context, a high level of interest in using enzyme biosensors for trace metal analyses has been demonstrated by a large number of studies, utilizing various enzymes, such as urease [4, 5], catalase [6], alkaline phosphatase [7], invertase [8] and glucose oxidase (GOx) [9], for inhibitive determination of trace metals. Of these, the most widely reported enzymes for trace metal determination are urease [10] and GOx [11]. GOx, in particular, is not only cheap, but it is also very robust and can identify target molecules quickly and accurately even in complex systems [11]. Due to its high selectivity, there has recently been an increased emphasis on its use as an enzyme that could offer considerable possibilities for designing highly sensitive and selective biosensors for trace metal analyses.

In one study, Donlan et al. [12] proposed a flow system incorporating an amperometric GOx modified electrode which was inhibited only by Hg^{2+}, Cu^{2+} and Ag^{+} out of the 16 metal

ions investigated. A detection limit of 2 mg L^{-1} was achieved for Hg^{2+} by this approach. The feasibility to reactivate the enzyme following Cu^{2+} ion inhibition, and the achievable linear concentration range of 0.25 - 5 mM suggests that there is a reasonable prospect for utilizing a flow system for enzyme inhibitive detection of metals in samples. In another study, Alexander and Rechnitz [13] configured a mediated polyvinylpyridine-GOx membrane, covalently bound to a gold electrode for amperometric detection of Hg^{2+}. The sensor responded selectively to Hg^{2+}, while interferants, such as Zn^{2+}, Cu^{2+}, ascorbic acid, and H_2O_2, had negligible effect. A minimum detectable concentration of 0.2 ppb and a linear range of 1-100 ppb Hg^{2+} were achieved with this method. Another enzyme inhibition detection scheme used GOx immobilized in poly-o-phenylenediamine for detecting metals, such as Hg^{2+} [14]. The lowest concentration of Hg^{2+} measured by this approach was 2.5 µM and the inhibition was reversible by immersion in EDTA. The method has a reasonable response time of <2 min and the biosensor was stable for at least 10 days. However, the inhibition biosensor only enabled determination of total Hg^{2+} concentration, as Cu^{2+} interfered with the Hg^{2+} determination. To circumvent this problem, Guascito et al. [15] measured the direct effect of each inhibitor on H_2O_2 oxidation in the GOx amperometric measurements of Hg^{2+}. Other metal ions such as Ag^{+}, Cu^{2+}, Cd^{2+}, Co^{2+} and Ni^{2+} also interfered in comparable concentrations with Hg^{2+}, but the method gave a fast response time (i.e. 100 s) and was able to discriminate between two different oxidation states of chromium,

with the ability to reject Cr^{3+} and to detect the toxic CrO_4^{2-} species. The electrode had short-time stability (24 h) and was not strictly selective. In another study, Ghica and Brett [9] determined Cd^{2+}, Cu^{2+}, Pb^{2+}, and Zn^{2+} using a glucose biosensor with poly(neutral red) redox mediator, and achieved detection limits down to 1, 6, 3 and 9 μg L^{-1}, respectively. The study revealed that except for Zn^{2+}, which exhibited uncompetitive inhibition, other metal ions exhibited mixed or competitive inhibition.

A number of studies have focused, with considerable success, on developing strategies for achieving enhanced sensitivity and selectivity based on GOx inhibition biosensors for trace metal detection. In some of these studies, the use of non-conducting and conducting polymers, such as polyphenylenediamine [14] and polyvinylpyridine [13] for incorporation of GOx, has resulted in improved inhibitive amperometric biosensing of metal ions. However, the use of conducting polymers, such as polypyrrole (PPy), and the utilization of a potentiometric mode of detection have never been considered for this purpose. Yet, PPy is one of the most widely used conducting polymers for fabrication of biosensors. Unlike other conducting polymers, PPy enables the growth of film from aqueous solutions that are compatible with most biomolecules [16, 17, 18], and also has a perm-selectivity that can eliminate or minimize the interferences at the electrode surface. A unique characteristic of potentiometric detection over amperometric measurement is the greater independence of its sensitivity on the ageing of the biosensor [19], as

well as on interferences [20]. Since there is no need for application of current or potential with potentiometric biosensors, interference caused by the polarizing voltage amperometric detection is eliminated [20].

In most of the reported studies to date, the immobilization of the enzyme was accomplished in the presence of chloride ions, as supporting electrolyte. These were accomplished by covalent attachment, adsorption or cross-linking method and cyclic voltammetric or potentiostatic polymerization. However, the use of galvanostatic method for entrapment of enzymes, which has been proven to be more advantageous [18, 21-24] has rarely been considered. In a recent study [18], we have established that the use of ultra-thin PPy-GOx films formed in the absence of supporting electrolyte enabled rapid and ultra-sensitive potentiometric biosensing of glucose. It has been established in this case that the enzyme itself plays the role of counter-ions and may not be immobilized only by physical entrapment [18]. The cause of the improvement in sensitivity was also identified in that study to be associated with increased conductivity of PPy-GOx film caused by increased GOx loading. It was also reported that the nature and size of doping anions had a great influence on the polymerization process and electrochemical properties of the resulting film [25]. However, no corresponding study has been conducted for galvanostatically grown PPy-GOx film. Yet, it is highly likely that the application of a current during the electropolymerization process may have significant

influence on the morphology and associated electrochemical behaviour. If such differences exist, it may account for the reported improvement in sensitivity for potentiometric detection of glucose with galvanostatically grown PPy-GOx biosensor. Furthermore, the use of ultra-thin PPy-GOx film has not been exploited for inhibitive potentiometric detection of trace metals. Fabrication of ultra-thin PPy-GOx film for inhibitive detection of metal ions may enable improved permeability to the metal inhibitors than possible with conventional biosensors and is more likely to better reflect the actual inhibition effect of the trace metals on the enzyme activity.

In this paper, the fabrication of biosensors based on GOx entrapped in ultra-thin PPy films grown in the absence of supporting electrolyte is investigated for inhibitive potentiometric detection of Hg^{2+}, Cu^{2+}, Cd^{2+}, and Pb^{2+}. Factors to be considered include the degree of inhibition of the activity of the GOx by the metal ions and its possible regeneration with simple reagent, such as a phosphate buffer solution. Other factors considered are operational stability, storage stability and reproducibility. Elucidation of the inhibition mechanism and mode of inhibition will be achieved by constructing Dixon and Cornish-Bowden plots. This will be considered for the kinetics parameters, such as the inhibitory constants (K_i) and dissociation constants (K_i') of enzyme-substrate inhibitor complexes (ESI). Achievable detection limits, linear concentration ranges and sensitivities will also be established.

2. Experimental

2.1 Chemicals and materials.

All chemicals were of analytical grade unless specified otherwise. GOx (Type X-S G7141-50KU, from Aspergillus niger) with an activity of 147000 unit g^{-1} proteins was purchased from the Sigma-Aldrich (Sydney, NSW, Australia). Pyrrole was also purchased from Sigma-Aldrich, but was distilled and stored in the refrigerator in a bottle wrapped with aluminum foil under nitrogen to prevent UV degradation and air oxidation. All solutions were prepared with Milli-Q water. A D (+) -Glucose stock solution from Sigma-Aldrich was prepared and allowed to stay overnight for equilibrium of the α-β optical isomers. The glucose stock solution was stored in the refrigerator and was diluted when necessary to give the required standard concentration. Phosphate buffer solution (pH 7.0; 0.05 M) was prepared with a mixture of sodium dihydrogen orthophosphate monohydrate and anhydrous di-sodium orthophosphate.

2.2 Instrumentation

Electropolymerization of PPy films was carried out in a three-electrode cell, comprising a Ag/AgCl (3 M KCl) reference electrode, a platinum wire auxiliary electrode and a platinum disc working electrode (surface area 2.010 mm^2). All electrodes were purchased from EDAQ Pty Ltd, Sydney, Australia. A potentiostat/galvanostat designed and built in our laboratory was employed for the electrochemical deposition of PPy, as well as for the potentiometric measurements.

Current and voltage signals were monitored as a function of time. A Voltalab 40 (Radiometer, Copenhagen) was used for electrochemical cleaning of working electrodes by potentiodynamic cycling and for cyclic voltammetric measurements.

2.3 Fabrication and morphological characterisation of Pt/PPy-GOx inhibitor electrodes.

The electrode was prepared as described elsewhere [18]. In particular, platinum disc electrode surface was polished with alumina (10 μm) slurry on a polishing pad and rinsed thoroughly with distilled water. The electrode was then sonicated in Milli-Q water for 10 min. The electrodes were also 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 mVs^{-1}, until a constant current-voltage relation was observed. The polymerization solution was purged with nitrogen for 20 min to remove dissolved oxygen before electropolymerization. Electropolymerization parameters, such as enzyme concentration, pyrrole concentration and current density were varied to determine their influence on the performance of the PPy-GOx biosensor. Electropolymerization was carried out galvanostatically in stagnant solutions in a three-electrode cell with Pt auxiliary and Ag/AgCl reference electrodes placed parallel to the working electrode for a uniform current density. The electrode was rinsed several times to remove any loosely bound enzyme and pyrrole after electropolymerization.

PPy-GOx film was also characterized using a JEOL 7001F FEG scanning electron microscope under the following operating conditions: 15 kV accelerating voltage, ca. 9.5 mm working distance 5 and aperture 4 (ca. 37 pA beam current), photo speed 3 (1280 x 960 pixels, 1 m 16 s scan time), SEI mode 3 and 20,000X magnification.

2.4 Potentiometric measurement of metal inhibition

A two-electrode cell was used for all potentiometric measurements which were performed as described previously [18]. 2 mL 0.05 M phosphate buffer was used for all measurements at 20 ^{0}C. The PPy-GOx electrode was maintained at zero current vs Ag/AgCl in the stirred oxygenated buffer solution (pH 7.0) until the background potential stabilized. When the potential between the working and reference electrode has stabilized, an aliquot of D-glucose was added and the corresponding potential change was recorded. When the potential again reached a steady state reading, another addition of glucose addition was made. This sequence was repeated several times. The potentiometric responses for glucose were expressed as potential change with respect to the background. For the inhibitive potentiometric detection of the trace metals, the glucose response was measured in the presence of known concentration of the chosen metal. All potentiometric measurements were performed in triplicates with new PPy-GOx films.

3. Results and Discussion

3.1 Analytical optimization of ultrathin PPy-GOx films

It has been reported that matrices applied to enzyme immobilization can change the efficiency of trace metal inhibition [25]. For this reason, it was necessary that prior to the inhibitive detection of trace metals, the optimal conditions for fabricating ultrathin PPy-GOx films in absence of supporting electrolyte were investigated. The optimum conditions established in our recent study [18] for potentiometric detection of glucose were 0.2 M Py, 500 U mL^{-1} GOx, current density of 0.05 mA cm^{-2} and a polymerization period of 500 s. A film thickness of 55 nm was formed under these conditions and was used in this study for potentiometric detection of Pb^{2+}, Cd^{2+}, Hg^{2+} and Cu^{2+}. A novel aspect of our work lies in the application of the ultra-thin PPy-GOx films formed in the absence of supporting electrolyte to the inhibitive potentiometric biosensing of trace metal ions. For this reason, a comparison of the data obtained in this study will be made to those obtained in other studies.

3.2 *Potentiometric detection of metal inhibition*

Prior to the detection of the trace metals, the potentiometric response (I_o) of 1.2 mM glucose at an ultra-thin PPy-GOx biosensor was measured to be 34 mV over a period of 200 s. This response showed a repeatability of 3.4 mV when the experiment was replicated under the

same conditions using the same biosensor 3 times. This measured potential of 34 mV represents the demonstrated activity of the immobilised GOx for the 1.2 mM glucose. Fig. 1 shows a typical potentiometric responses obtained due to the inhibition of the glucose response of the PPy-GOx electrode by increasing concentrations of Hg^{2+} ions. As can be seen, the GOx biosensor response (*I*, mV) decreased with increasing inhibitor concentration. The decrease in the demonstrated activity of the immobilised GOx due to the inhibition is referred to as the residual activity. For simplicity, this parameter is measured as potential.

The response time of the PPy-GOx biosensor measured for simplicity and in line with many other studies, based on 90% response in presence of 0.25 μM of metal inhibitor was 100 s. However, it is worth noting that the response time varies with the addition of 0.025 to 0.25 μM metal inhibitor from 58-105 s. In comparison, the response time of the PPy-GOx biosensor to glucose in absence of metal inhibitor was 43 s.

Insert Figure 1.

The effects of Cu^{2+}, Hg^{2+}, Pb^{2+} and Cd^{2+} on the activity of glucose oxidase were also investigated in the concentration ranges that had measurable inhibitions. The metal ion inhibitions were investigated in the concentration ranges of 0.079-47.3 μM for copper, 0.025-15 μM for mercury, 0.024-34 μM for lead and 0.044-62 μM for cadmium. The metal ions reacted

with the enzyme primarily in a concentration-dependent manner, and their non-linear calibration plots are shown in Fig. 2a-d. For instance, it is clearly evident that the enzymatic activity of the PPy-GOx electrode decreased significantly from 23 mV to 5 mV with increasing Cu^{2+} concentration from 0.079-47.3 μM. This can be considered as an indirect proof of the interaction between the sulfydryl groups of GOx and the heavy metal ions, as previously postulated [26-30]. As illustrated in Fig. 2, the calibrations plots for all trace metal ions demonstrated exponential decays and the determination of these inhibitors is possible in the μM range. Similar observation has been reported at urease-based biosensors for Cu^{2+} and Ag^{+} [31] and at nitrate reductase-based biosensors for Cd^{2+}, Pb^{2+}, Zn^{2+}, Cr^{3+} and Cr^{6+} [32]. The exponential decay plots showed that the presence of as little as 0.079 μM Cu^{2+}, 0.025 μM Hg^{2+}, 0.024 μM Pb^{2+} and 0.044 μM Cd^{2+} were high enough to inhibit the activity of glucose oxidase, and these represent the minimum detectable concentrations for the trace metals. The use of the term "minimum detectable concentrations" is preferred to the use of detection limits for the inhibitive potentiometric detection as they give the actual minimum metal concentrations that inhibit the glucose response.

Insert Figure 2.

However, the enzyme activity was drastically reduced when metal concentrations greater than 3.0 μM, 1.5 μM, 2.6 μM and 4.8 μM for Cu^{2+}, Hg^{2+}, Pb^{2+} and Cd^{2+}, respectively, were present. This is illustrated by the intersection point of the activity straight line with the abscissa in Fig. 2, as previously reported by Krajewska et al. [33] for inhibition of urease by Hg^{2+}. The intersection point is the point at which a sharp decrease in the residual activity of the GOx occurred. Results reported in Fig 2 also illustrate that, at low concentrations, linear calibration plots were obtained for all metal inhibitors, which is in agreement with previous suggestion made by Guascito et al. [15]. However, by re-plotting the residual enzyme activity against natural logarithm of inhibitors concentration the linear range can be improved, as demonstrated in Fig 3. As can be seen in Figs. 3(a-d), an improved linear concentration range were achieved for Cu^{2+} (0.079-16 μM), Hg^{2+} (0.025-5 μM), Pb^{2+} (0.097-15 μM) and Cd^{2+} (0.04-62 μM) due to the Nernstian dependence of potential difference on logarithm of concentration.

Insert Figure 3.

These achievable linear concentration ranges are better than 0.52-9.2 μM, 0.036-0.14 μM and 0.40–2.2 μM reported by Ghica and Brett and other researchers [9,14,15] for PPy-GOx amperometric determination of Cu^{2+}, Cd^{2+} and Pb^{2+}, respectively, and may be attributable to the use of ultra-thin PPy-GOx film. The minimum detectable concentration observed for Cu^{2+} (0.079 μM) and Pb^{2+} (0.024 μM) with PPy-GOx biosensor in present study compares well, and

the single-step fabrication employed is simpler and direct than those reported by Ghica and Brett [9], whichinvolved multi-step immobilization of GOx on carbon film electrode by cross-linking with glutaraldehyde on top of a film of poly(neutral red) as redox mediator for determination of Cu^{2+} (0.095 μM) and Pb^{2+} (0.014 μM) ions. The analytical characteristics of the ultrathin film PPy-GOx biosensor in terms of minimum detectable concentrations and linear concentration ranges are well within the acceptable levels of 0.79 μM Cu^{2+},0.09 μM Cd^{2+} and 0.24 μM Pb^{2+} for human exposure and 0.05 μM Cu^{2+}, 0.08 μM Cd^{2+} and 0.03 μM P b^{2+} for aquatic life [34]. Since each of the calibrations was done in triplicate and the relative standard deviation of the residual activity of the enzyme are not more than 10%, evidently the reproducibility of the inhibition based sensor is reasonably good. Besides, the biosensor shows negligible loss in activity after repetitive measurements of of 0.5 μM Hg^{2+}, 1.6 μM Cu^{2+}, 0.88 μM Cd^{2+} and 3 μM Pb^{2+} ions. For example, as shown in Fig. 4, after five or six cycles of detection and washing with water and storing in phosphate buffer (0.05 M, pH =7.0) for 10-15 minutes in between usage, a low loss of response (≤10 %) was observed for 1.2 mM glucose. This clearly indicates that the detection step is not destructive to the ultrathin PPy-GOx film and, thus, confirms that the biosensors could be readily re-used.

Insert Figure 4.

Fig. 5 illustrates the percentage inhibition of potentiometric response of 1.2 mM glucose with the PPy.GOx biosensor in the presence of different concentrations of metal inhibitors. The data again revealed an initial rapid inactivation followed by a slow and sustained inhibition, indicating a biphasic concentration-dependent inhibition in the activity. At lower concentration of inhibitor, the inactivation was less and it progressed with increasing level of metal ion. In the presence of Cu^{2+} ions, 50% inhibition (IC_{50}) was observed at 3.1 μM, which increased to about 87% at 47 μM. While in presence of Hg^{2+} ions, 50% deactivation was recorded at 0.25 μM which increased to 95% at 15 μM. In contrast, higher concentrations of Cd^{2+} (4.5 μM) and Pb^{2+} (24 μM) were required to cause 50% inhibition of GOx.

The different inhibitive effect exhibited by each metal could be attributed to the different affinities for binding to the active site, the –SH groups present in the enzyme structure. Interestingly, this observation has been reported for other enzymatic inhibitive detection of heavy metal ions [35-37] with Hg^{2+} and Cu^{2+} ions nearly always listed as the most potent inhibitors [4, 10, 38-41]. Overall, the predominant order of the metal inhibition is $Hg^{2+} > Cu^{2+} > Cd^{2+} > Pb^{2+}$. However, other studies have shown that mercuric ions are more toxic than cupric ions [37,42,43]. Evidently, the results in Fig. 5 indicate that Hg^{2+} ions are likely to interfere in the determination of Cu^{2+} ions because of its stronger inhibitive even at low concentrations. This feature is not unexpected, as similar observation has also been reported for inhibitive

determination of trace metals with glucose biosensors [14, 15] and urea biosensor [10]. Results similar to those obtained for Cd^{2+} were found for Pb^{2+}, as reported in literature [9] and the degrees of their inhibitive effects on GOx were reasonable, indicating that they have moderate affinity for –SH. This suggests that Pb^{2+} and Cd^{2+} may inhibit GOx by different mechanisms to those of Cu^{2+} and Hg^{2+}. Thus, indicating that Pb^{2+} and Cd^{2+} are less inhibitive on GOx than Cu^{2+} and Hg^{2+}.

Insert Figure 5.

The parabolic shape of the curves observed in Fig. 5 for inhibition by all metals has been previously reported for GOx [14, 15] and this indicates that the enzyme activity is not completely eliminated at saturating concentrations of the metal inhibitors. In general, high concentrations of inhibitors were required to cause noticeable inhibition in previous studies. In contrast, much lower concentration of the inhibitors resulted in considerable inhibition of the enzyme activity in our study. For example, the concentration of the inhibitors required to cause 50% (IC_{50}) inactivation of the activity of glucose oxidase in the present study is several orders of magnitude lower than those reported recently by Guascito et al. [15]. However, this disparity was not surprising because the degree of the inhibition depends on the thickness of the film. Consequently, the thicker the film, the more time is required for the inhibitor to penetrate the

film and reach the active site of the enzyme. Interestingly, this observation is consistent with an earlier indication that hindered accessibility of metal inhibitor to the essential –SH groups of the enzyme active site in the immobilized state was responsible for less inhibition at even higher concentration of inhibitors [44]. Therefore, it is clearly obvious that the use of ultra-thin PPy-GOx film in the present study is advantageous in enabling improved permeability to the metal inhibitors than possible with conventional biosensors with thicker films. It can, thus, be concluded that ultrathin films improved on the actual inhibition effects of trace metals on enzyme activity. In addition, the limited diffusion barrier at ultrathin films between an inhibitor and the active site of the enzyme eliminated the previous need for incubation of the immobilized enzyme for a long period in the presence of the inhibitors.

Insert Table 1

The observed exponential decay plots in Fig. 2, linear behaviour in Fig. 3 and parabolic shape in Fig. 5 suggest that the interaction of Hg^{2+}, Cu^{2+}, Cd^{2+} and Pb^{2+} with GOx does not conform to any simple or hyperbolic type of inhibition, competitive or non-competitive [45]. This finding is important because it demonstrates that the inhibition by metal ions may not be exclusively directed at the essential -SH group, but instead may involve additional binding sites [9, 35, 45]. The slope values of 7, 7, 6 and 6 provided in Figures 3 (a-d) indicate that for every

subunit of GOx at least 6 binding sites are involved in the reaction with the metal ions leading to the observed inhibition.

Fig. 6 (a, c, e and g) and Fig. 6 (b, d, f and h) illustrate the Dixon and Cornish-Bowden plots, respectively, for all the four metal ions investigated in this study at three different glucose concentrations. The Dixon plot (Fig. 6a, c, e and g) [46], which is the reciprocal of the glucose response (mV) versus inhibitor concentrations in ppm provides information about the relationship between the inhibitor concentration and the enzyme activity. Thus, for an enzyme-inhibitor complex, this plot enables the determination of the type of enzyme inhibition and the dissociation constant (K_i). Using this approach, the dissociation constant of GOx-M^{++} complex K_i and GOx-M^{++}-glucose complex $K`_i$ could be estimated from the extrapolated straight line intersection.

The data obtained for K_i and K'_I for Hg^{2+}, Cu^{2+}, Cd^{2+} and Pb^{2+} are compared with those obtained in previous studies. As the data in Table 1 reveal, the K_i values obtained in this work is lower than those reported by other researchers. Generally, the lower the K_i values, the higher the inhibition [37]. Thus, confirming that the extent of metal inhibition is higher with the use of ultra-thin PPy-GOx film in this study.

Insert Figure 6.

The analysis of complementary plot such as Cornish-Bowden plot [47] (Figs. 6b, d, f and h) differentiates between the mode of inhibition (competitive and uncompetitive). This plot is also useful when dealing with an enzyme-inhibitor complex for determining the type of enzyme inhibition and the dissociation constant (K_i). In this case, the glucose concentration (mM) divided by the glucose response (mV) is plotted as a function of inhibitor concentration (ppm). Consequently, by analysing this plot together with the Dixon plot, all types of inhibition can be characterized (competitive, mixed and uncompetitive). From the Dixon plot, the crossing lines exclude the occurrence of uncompetitive inhibition. Expressing the same inhibition data by Cornish-Bowden plot conclude that the inhibition is competitive for Cd^{2+} because the three divergent lines on the graph are parallel. Conversely, non-competitive inhibition occurred for Pb^{2+}, Cu^{2+} and Hg^{2+}.

Table 2 provide the results of the individual analysis of each metal in tap water by inhibitive potentiometric detection with ultrathin PPy-GOx biosensor. Evidently, none of the trace metals could be detected in the tap water above their minimum detectable concentrations. Consequently, a recovery study was conducted by spiking the tap water individually with 2.5 µM of each metal. The results of the recovery study for each metal, as given in Table 2, achieved between 98 and 101% recovery. Thus, demonstrating that the biosensor can be reliably used for the detection of the trace metals individually in samples where the concentrations of other metals

are very low. Application of the method to the determination of the metals in contaminated samples or samples where concentrations of all metals were high will be difficult and was not attempted.

Insert Table 2

4. Conclusions

The successful use of an ultrathin film PPy-GOx biosensor have been demonstrated for the inhibitive potentiometric detection of Cu^{2+}, Hg^{2+}, Cd^{2+} and Pb^{2+} ions for the first time. The ultrathin PPy-GOx electrode resulted in considerable improvement in analytical performance, such as good sensitivity, detection limit, linear concentration range, operational stability, and reproducibility. More than 60 - 100-fold improvement in minimum detectable concentrations were achieved for Cu^{2+}, Hg^{2+}, Cd^{2+} and Pb^{2+} ions with the ultra-thin PPy-GOx film compared with the conventional use of a thicker film [15] and carbon paste electrodes (CPE) modified with GOx and mediator [48]. More notably, the use of the ultra-thin film enabled improved permeability to inhibitors and, consequently, better reflects the inhibition effect of the trace metals on the enzyme activity. More so, the minimum detectable concentrations of 0.079 μM Cu^{2+}, 0.025 μM Hg^{2+}, 0.024 μM Pb^{2+} and 0.044 μM Cd^{2+} achieved with galvanostatic entrapment in present study are lower than 15 μM observed for Hg^{2+} and Cu^{2+} with GOx

entrapped in poly(L-noradrenalin) (PNA), using cyclic voltammetric [49] . Rapid recovery of activity by washing with water, followed by storage in phosphate buffer (0.05 M, pH =7.0) for 5 minutes between usage indicates that the inhibition step is not destructive to the PPy-GOx film, and can, thus be re-used. The observed exponential decay plots (Fig. 2), linear (Fig. 3) and parabolic (Fig. 5) behaviors for the metal ions provided evidence that the inhibition by the inhibitors may not be exclusively directed at the essential -SH group, but may involve additional binding sites of the enzyme. The slopes of the calibration plots also suggest that the inhibitors bind to GOx at at least 6 binding sites. The performance characteristics obtained in this study for the PPy-GOx biosensor, in terms of sensitivity, linear range and detection limits, makes it potentially suitable for determination of copper, cadmium, lead and mercury in drinking water. Application of the PPy-GOx biosensor for inhibitive potentiometric detection of each metal individually in tap water proved to be successfully, but the determination of these metals in contaminated samples require further refinement to permit selective determination of some or all of the trace metals. This aspect is currently being considered in our laboratories. This will require further improvement in the response time (240 s) achieved with the PPy-GOx biosensor to permit more rapid determination.

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Figure captions

Figure 1. Typical potentiometric response of the PPy-GOx biosensor to 1.2 mM glucose in a stirred phosphate buffer (pH 7.0, 0.05M) before (blank) and after addition of different concentrations of Hg^{2+} ions.

Figure 2. Inhibitive effect of different concentrations of (a) Cu^{2+}, (b) Hg^{2+}, (c) Pb^{2+} and (d) Cd^{2+} on the PPy-GOx biosensor response for 1.2 mM glucose.

Figure 3. Linear regression of residual enzyme activity vs natural logarithm of (a) Cu^{2+} (b) Hg^{2+} (c) Pb^{2+} and (d) Cd^{2+} in 0.05 M phosphate buffer (pH 7.0).

Figure 4. Operational stability of trace metal responses obtained with the PPy-GOx biosensor for 0.5 µM Hg^{2+}, 1.6 µM Cu^{2+}, 0.88 µM Cd^{2+} and 3 µM Pb^{2+} ions.

Figure 5. Percentage inhibition of glucose potentiometric response obtained with the PPy-GOx biosensor. 1.2 mM glucose in a stirred phosphate buffer (pH 7.0, 0.05M). (a) Cu^{2+}, (b) Hg^{2+}, (c) Pb^{2+} and (d) Cd^{2+}.

Figure. 6 Dixon plots (a, c, e and g) and Cornish-Bowden plots (b, d, f and h) for Cu^{2+}, Hg^{2+}, Pb^{2+} and Cd^{2+} obtained at three different glucose concentrations.

Figure 1.

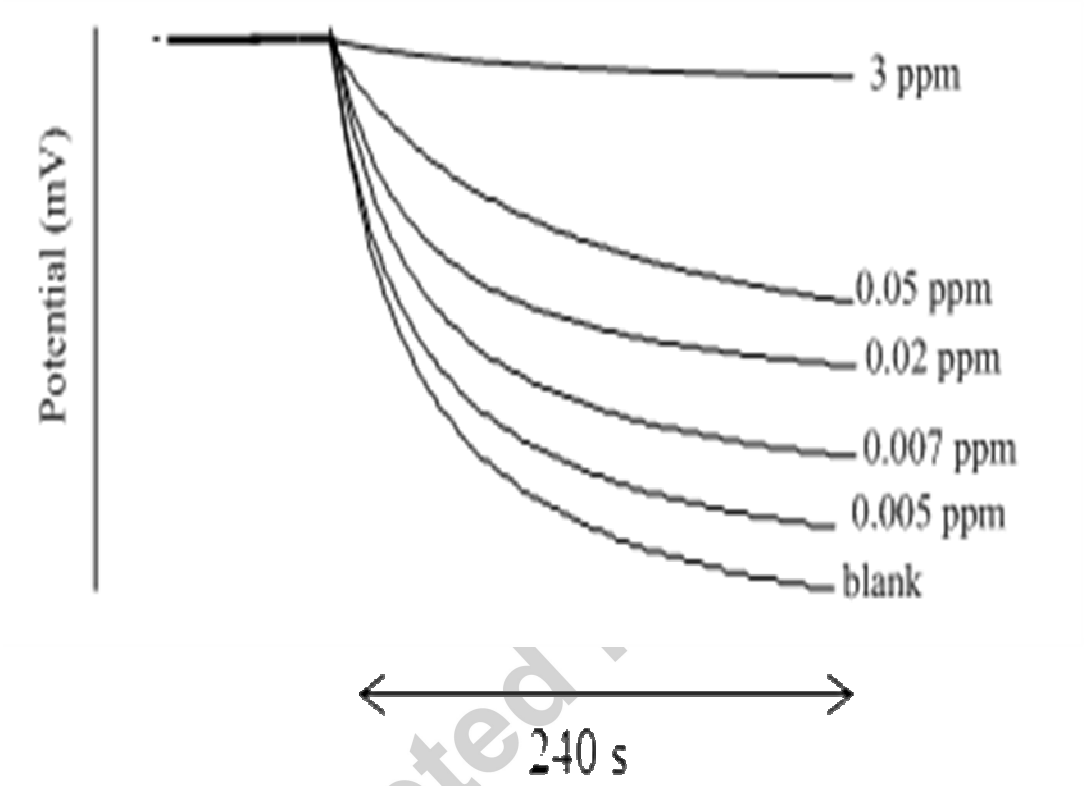
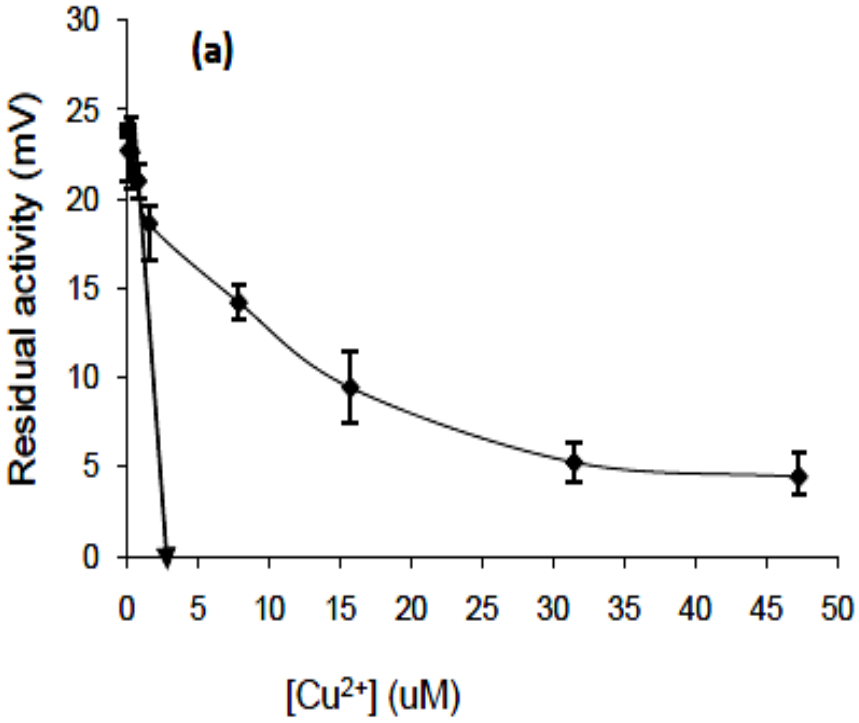
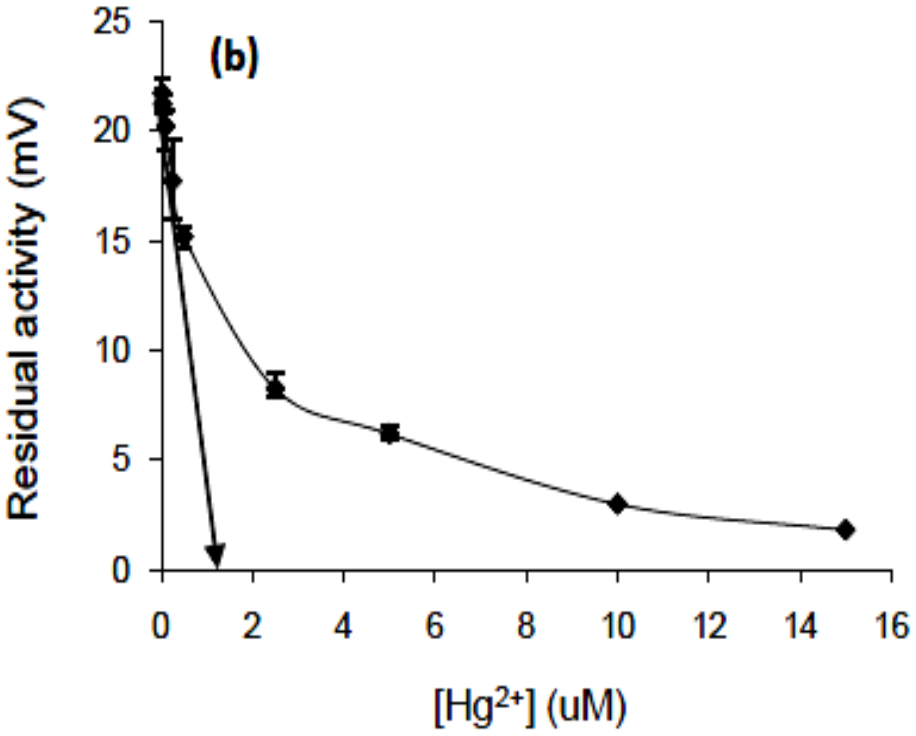
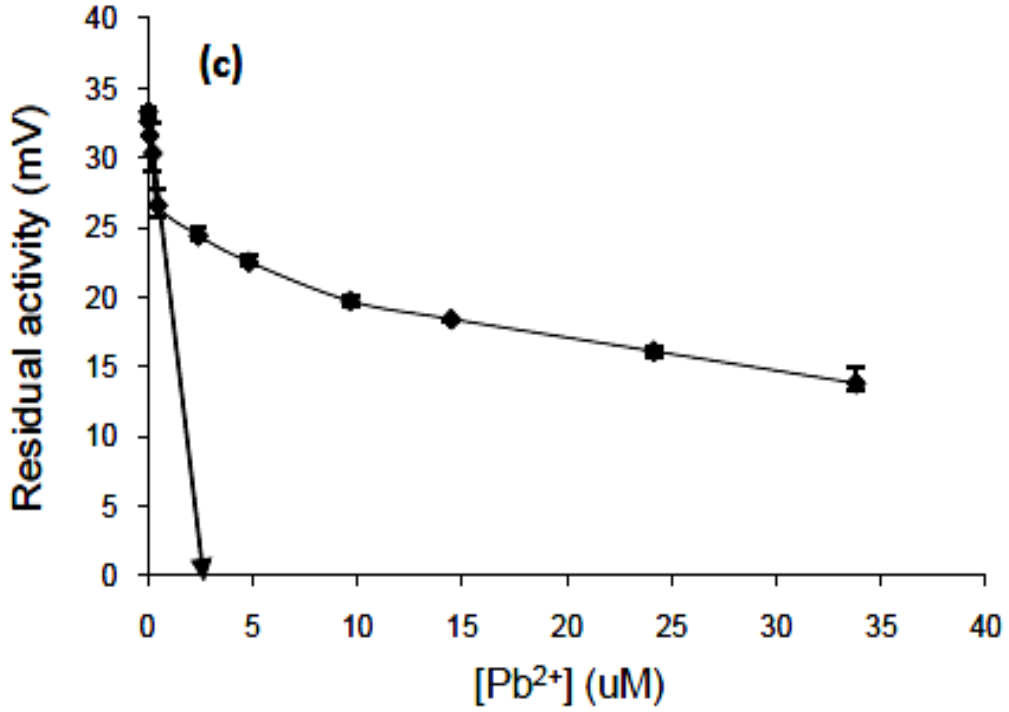


Figure 2.






(c)
Residual activity (mV)
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
[Pb2+] (uM)

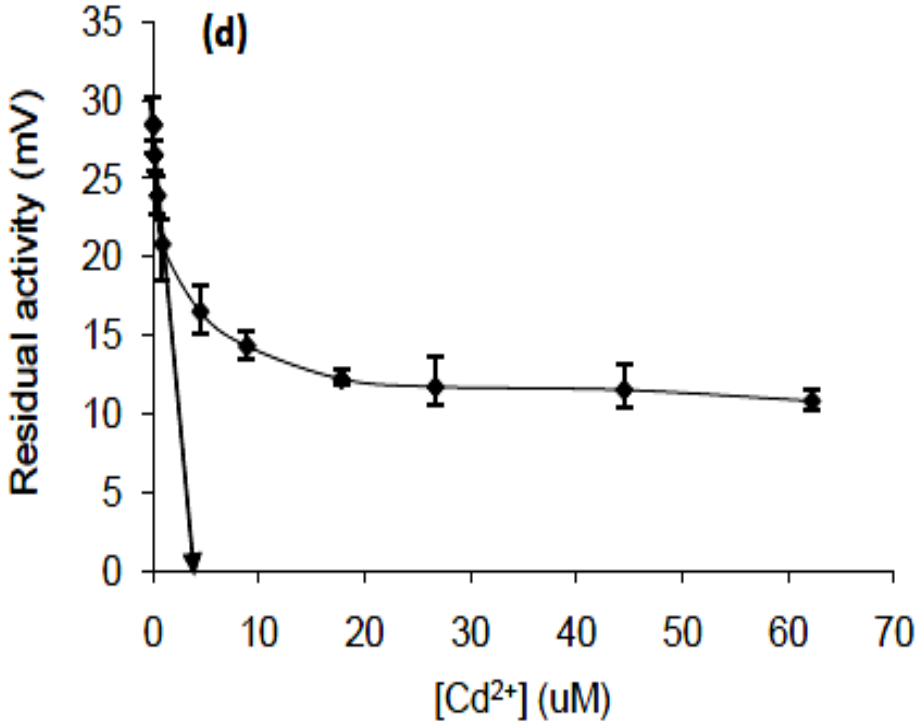
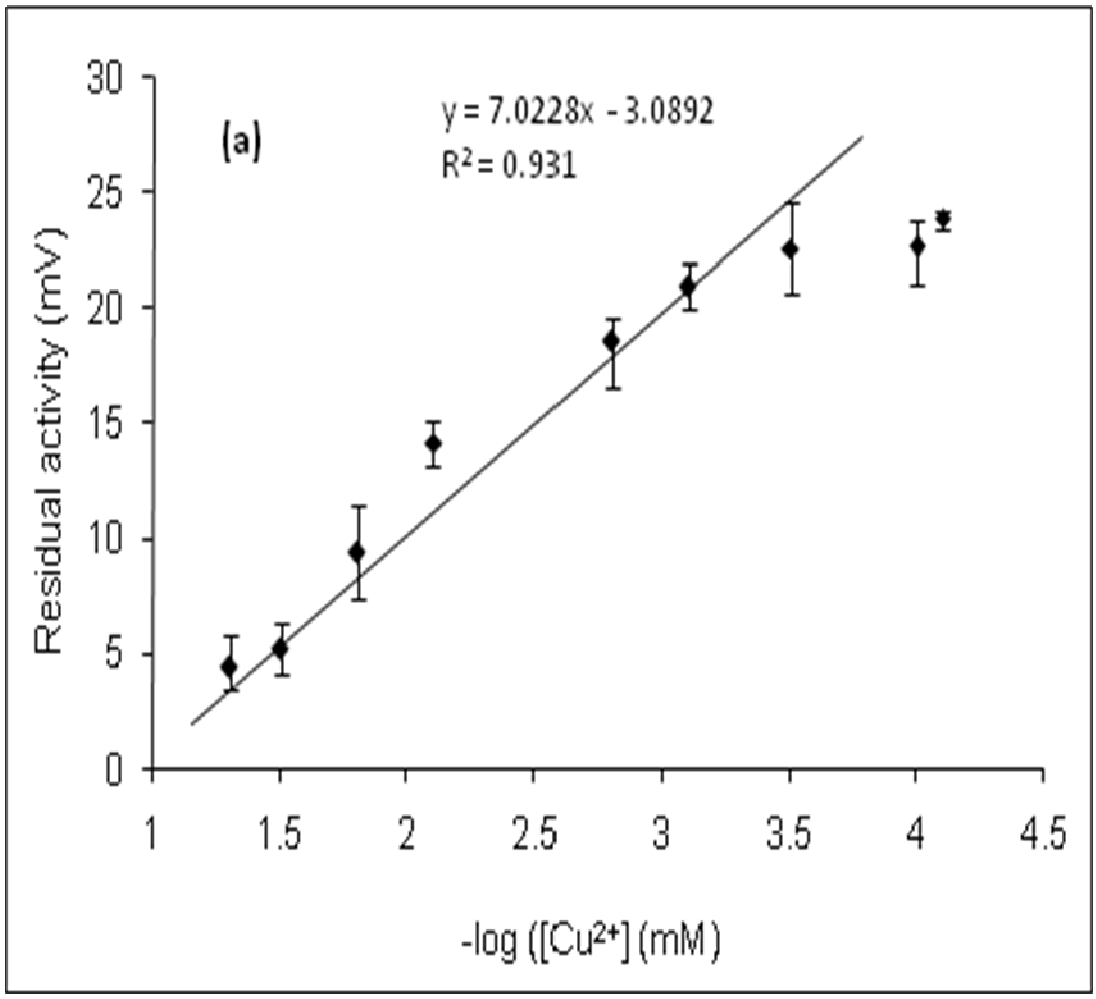
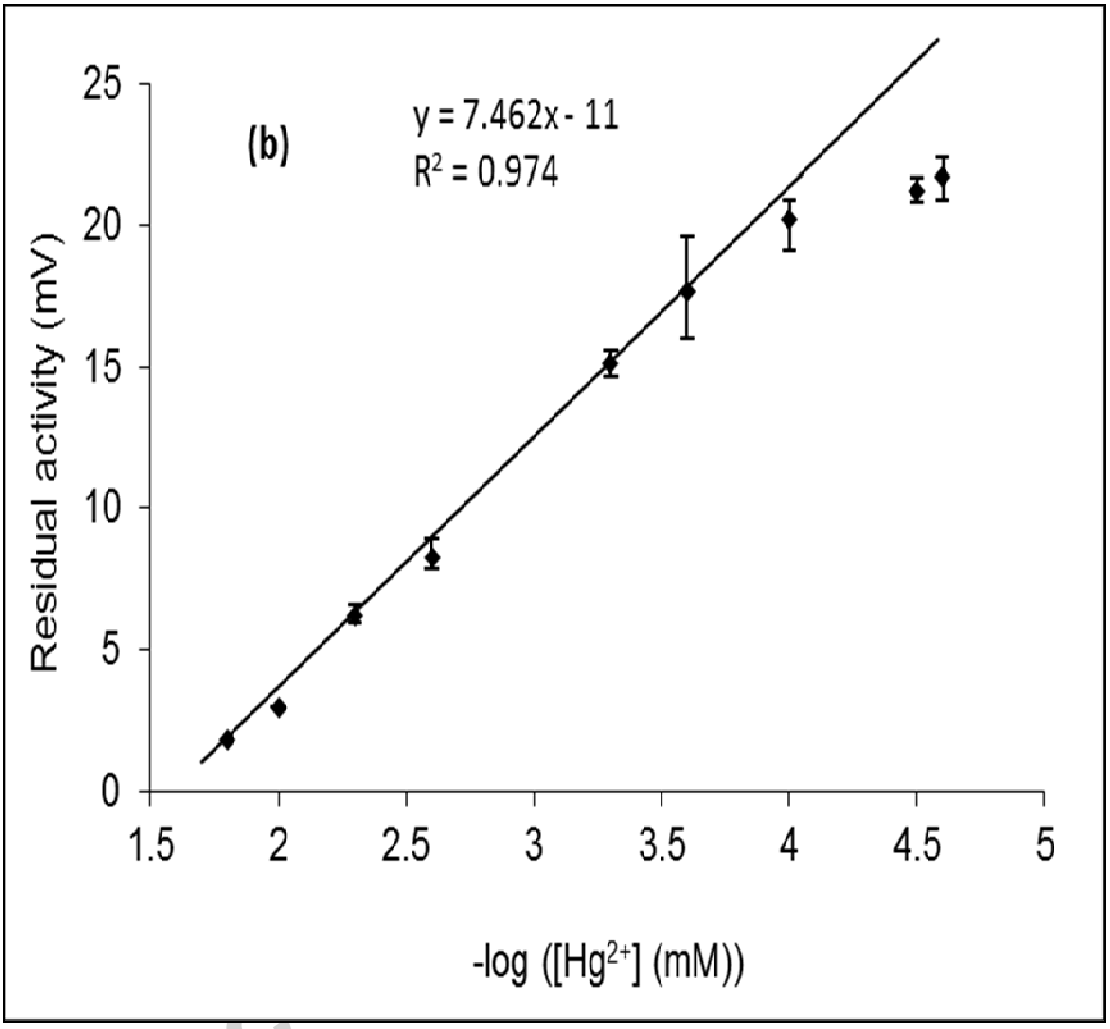
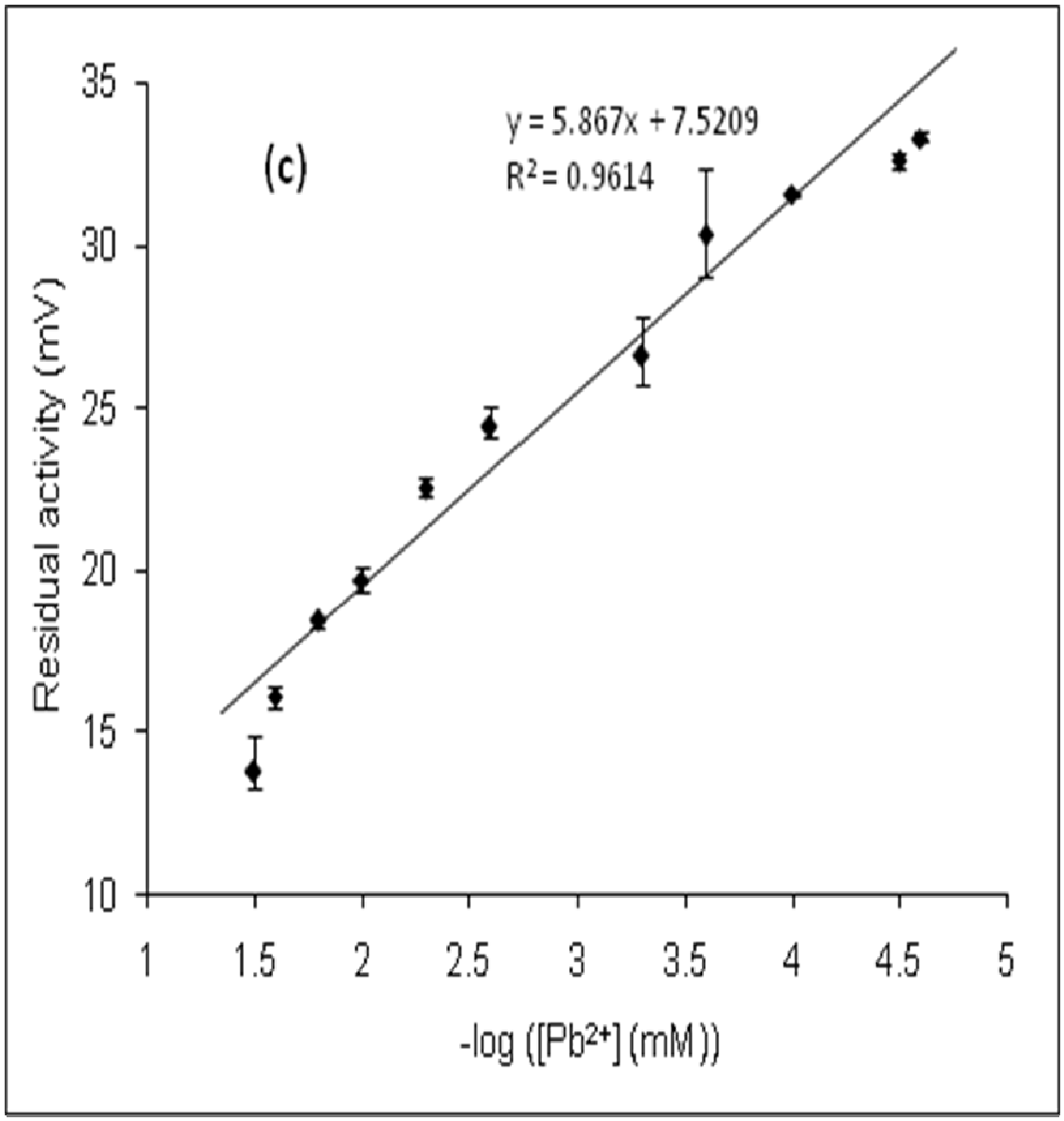


Figure 3.


(a)
y = 7.0228x - 3.0892
R² = 0.931
Residual activity (mV)
-log ([Cu²⁺] (mM)
0
5
10
15
20
25
30
1
1.5
2
2.5
3
3.5
4
4.5


(b)
y = 7.462x - 11
R2 = 0.974
Residual activity (mV)
-log ([Hg2+] (mM))
0
5
10
15
20
25
1.5
2
2.5
3
3.5
4
4.5
5


(c)
y = 5.867x + 7.5209
R² = 0.9614
Residual activity (mV)
-log ([Pb²⁺] (mM))
35
30
25
20
15
10
1
1.5
2
2.5
3
3.5
4
4.5
5

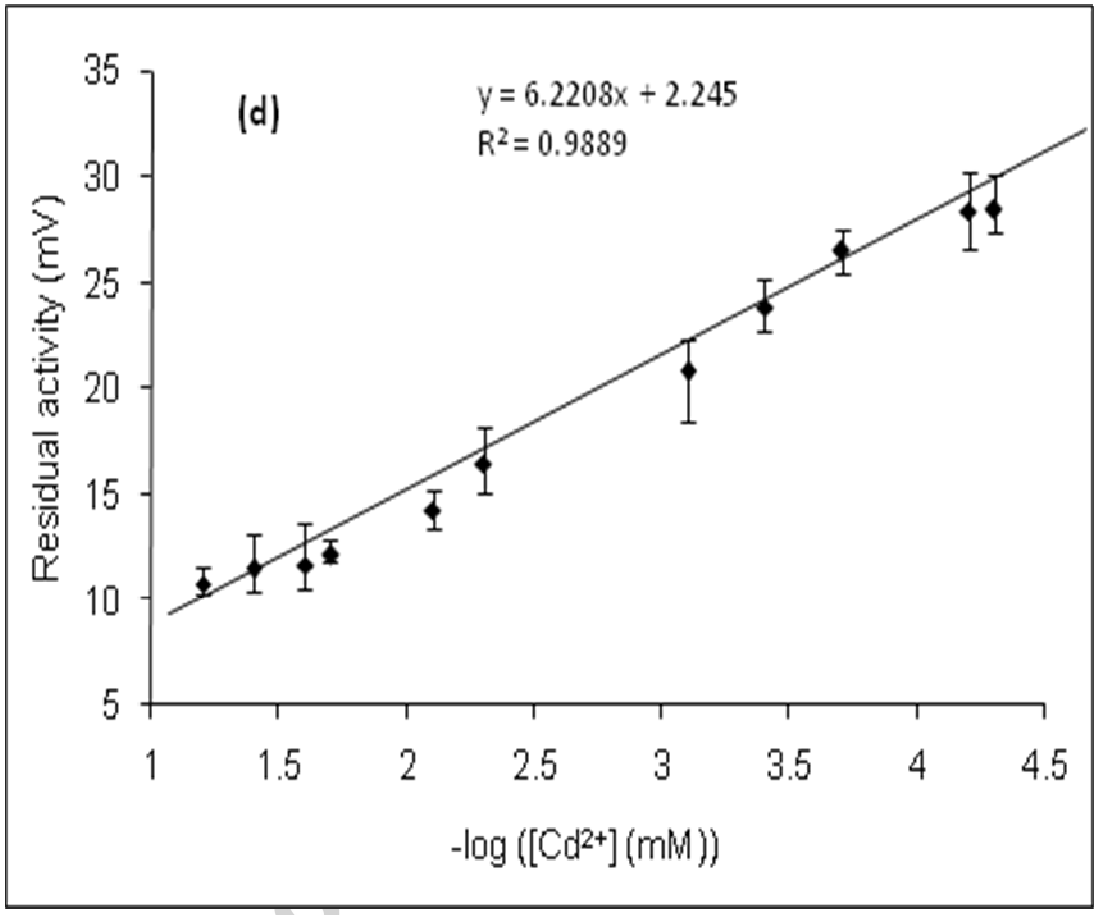

(d)
y = 6.2208x + 2.245
R² = 0.9889
Residual activity (mV)
35
30
25
20
15
10
5
1
1.5
2
2.5
3
3.5
4
4.5
-log ([Cd²⁺] (mM))

Figure 4.

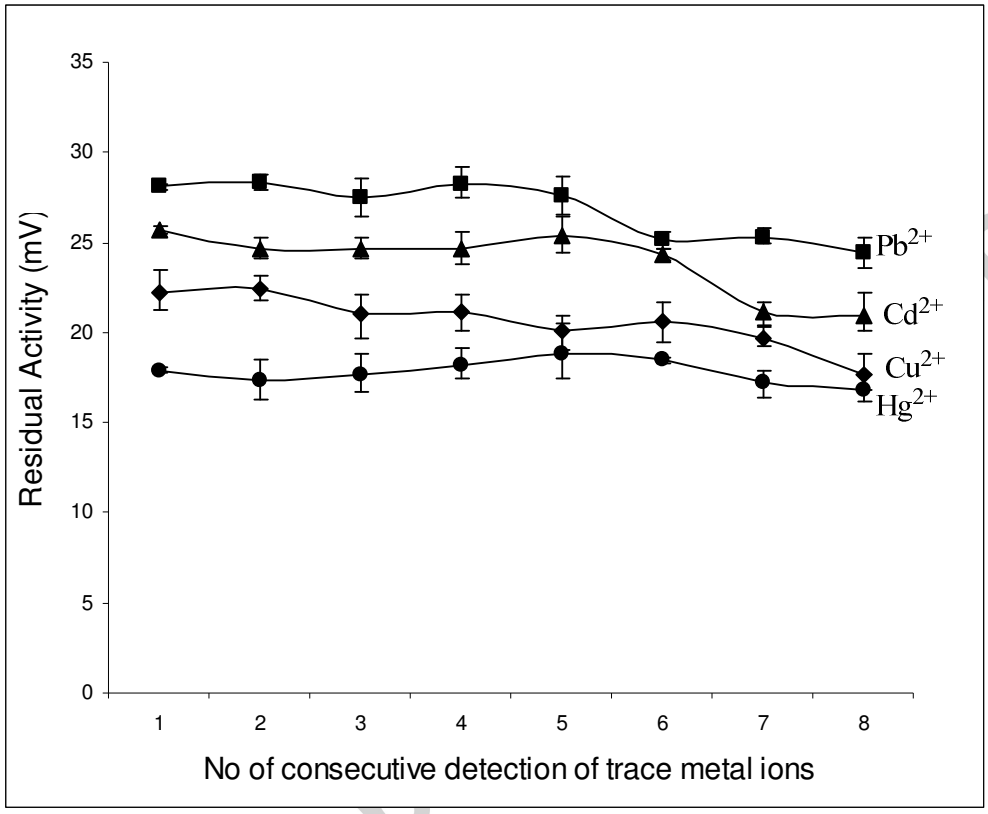
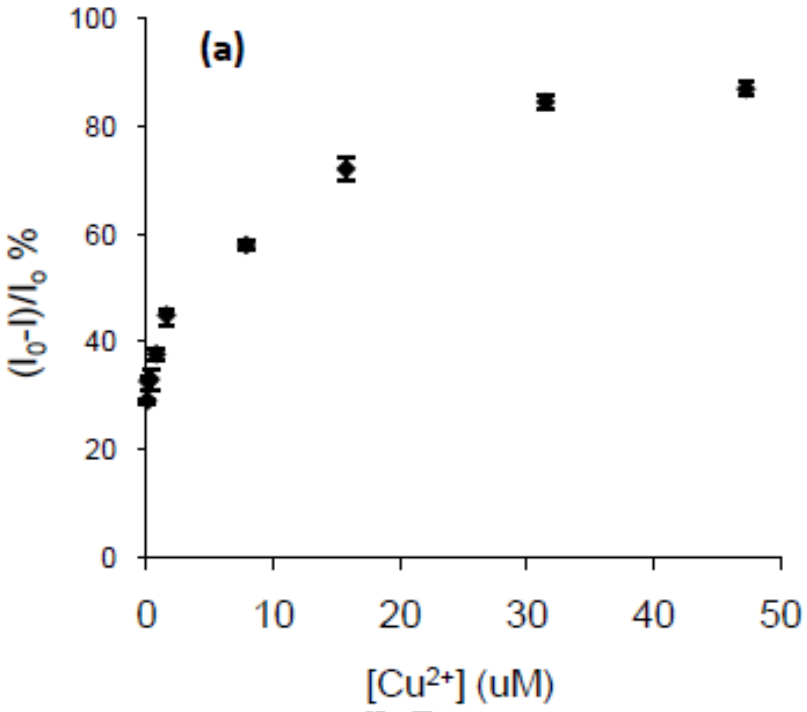
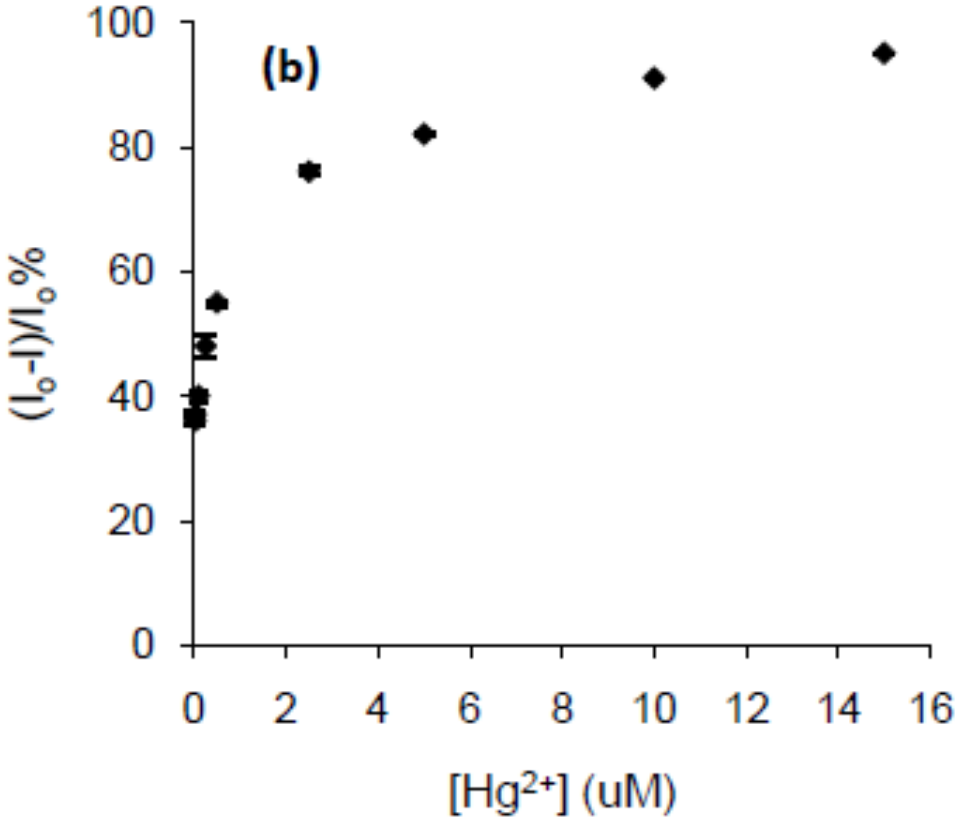
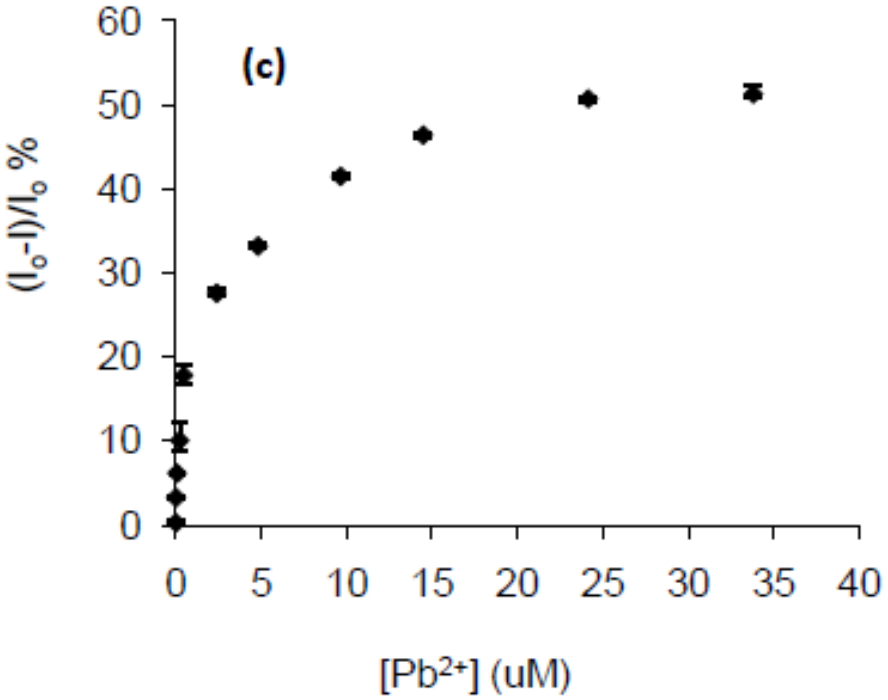


Figure 5.







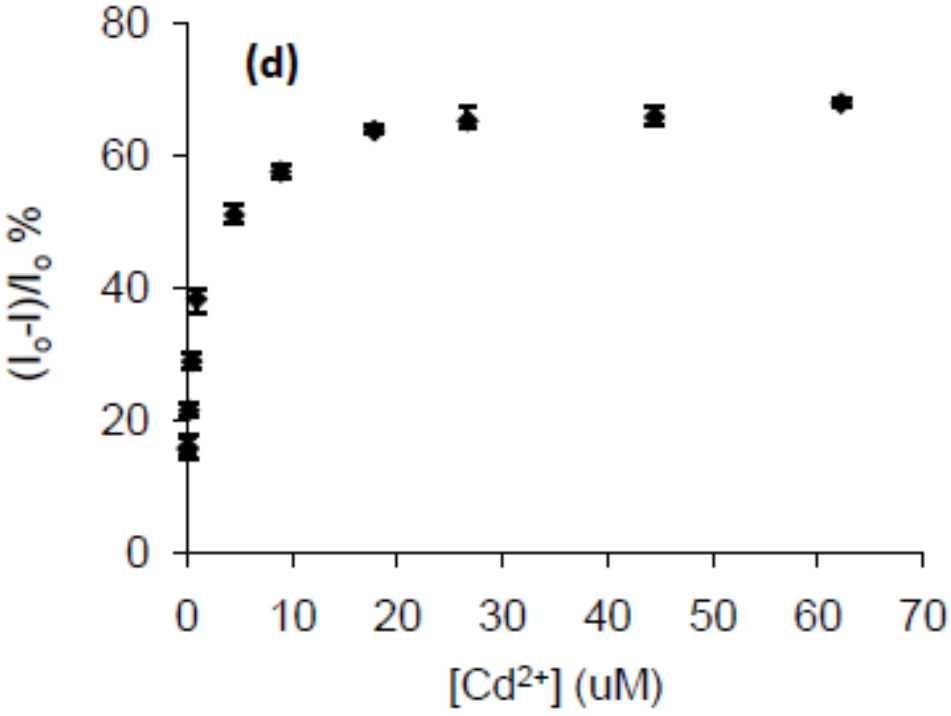
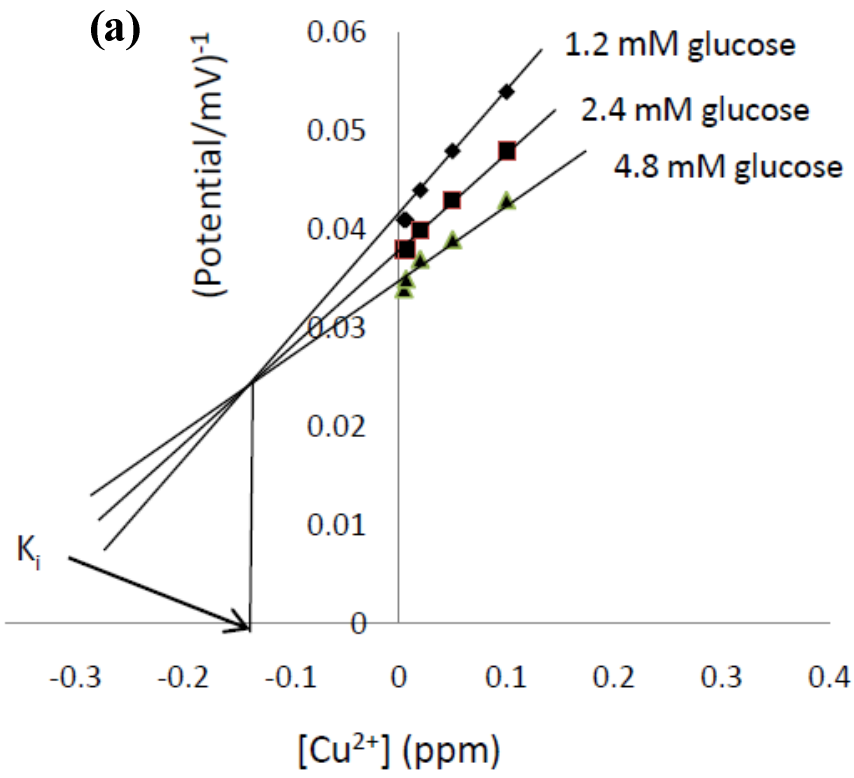
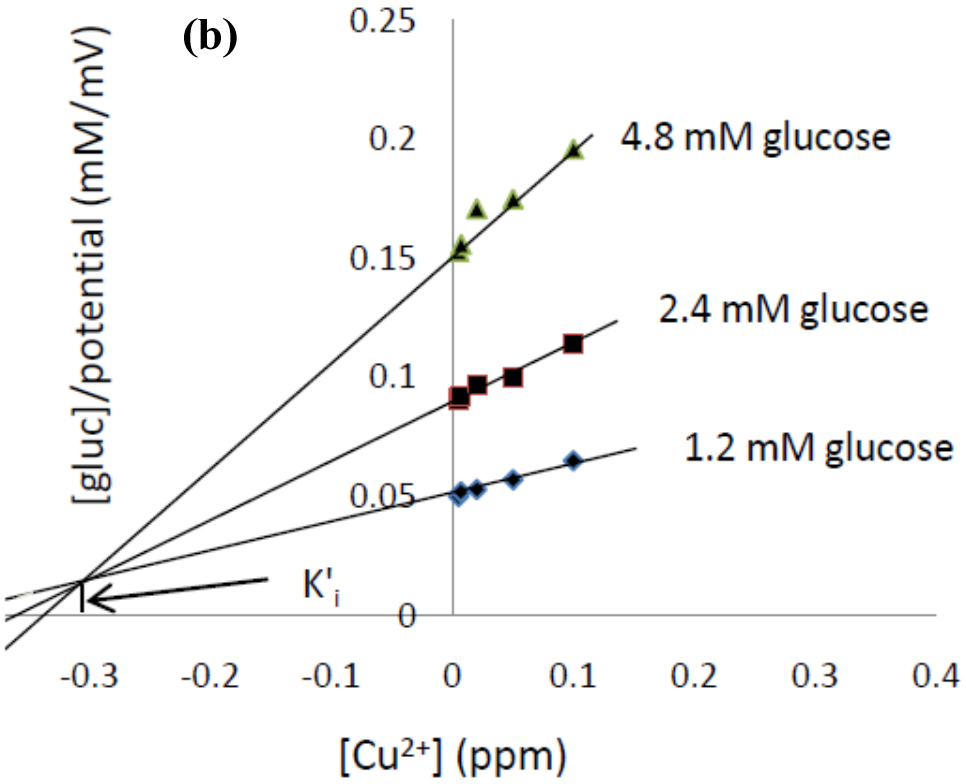
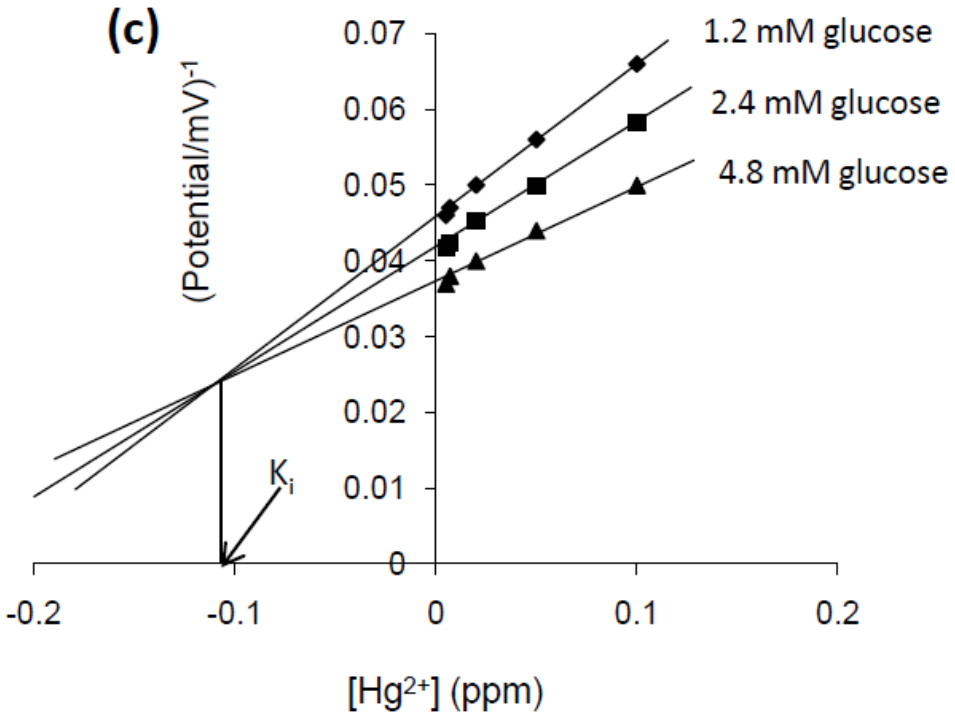
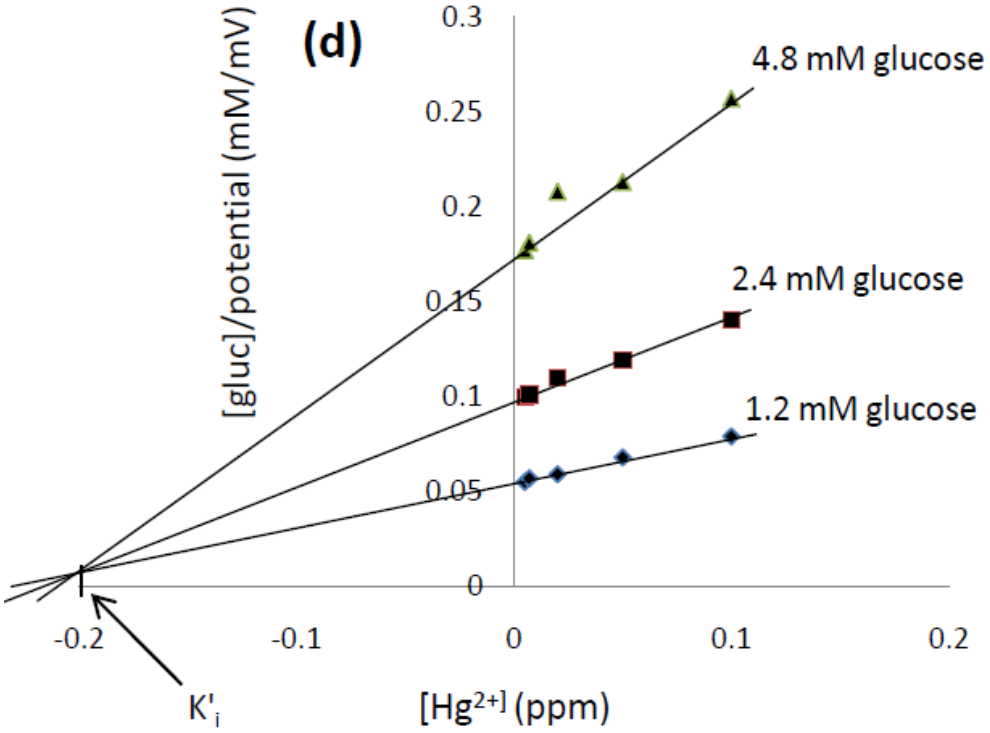


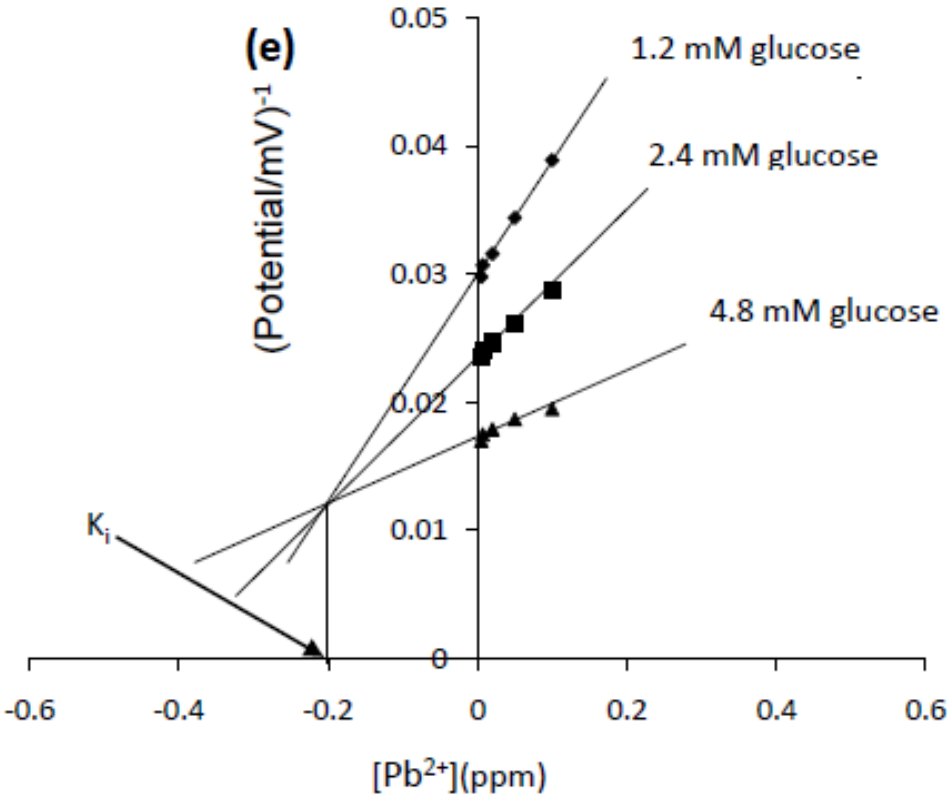
Figure. 6

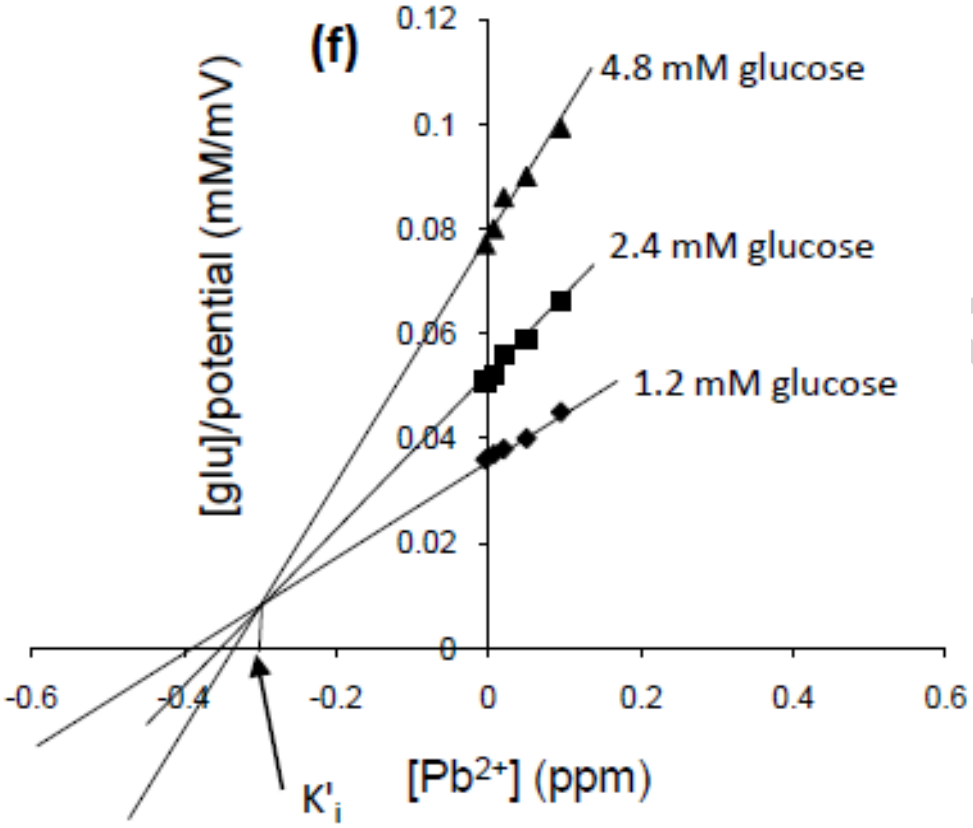


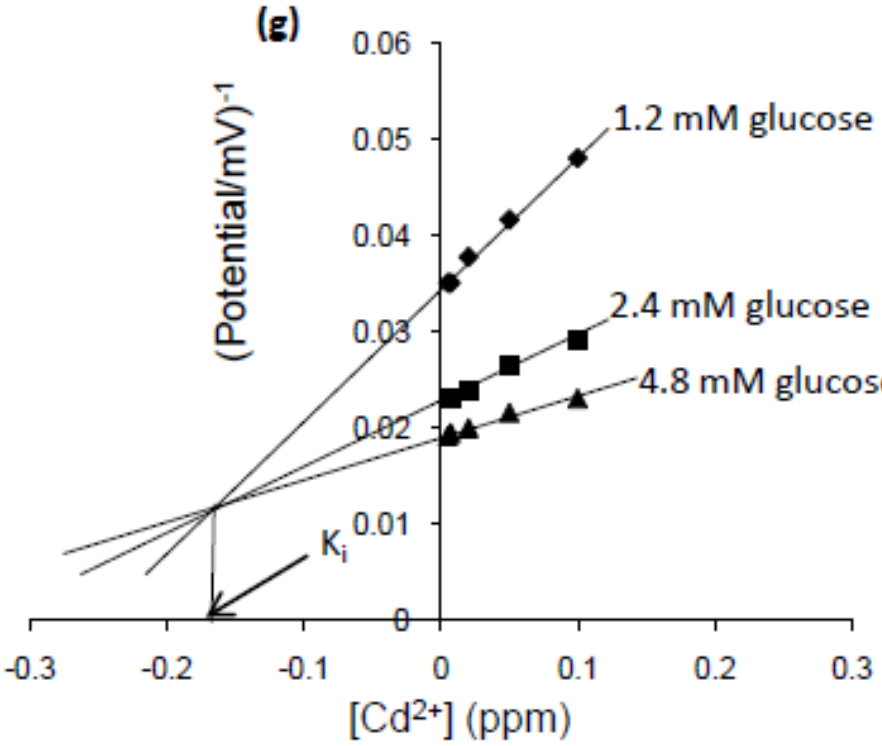

(b)
[gluc]/potential (mM/mV)
0.25
0.2
0.15
0.1
0.05
0
4.8 mM glucose
2.4 mM glucose
1.2 mM glucose
K'i
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
[Cu2+] (ppm)




(d)
[gluc]/potential (mM/mV)
0.3
0.25
0.2
0.15
0.1
0.05
0
4.8 mM glucose
2.4 mM glucose
1.2 mM glucose
-0.2
-0.1
0
0.1
0.2
K'i
[Hg2+] (ppm)







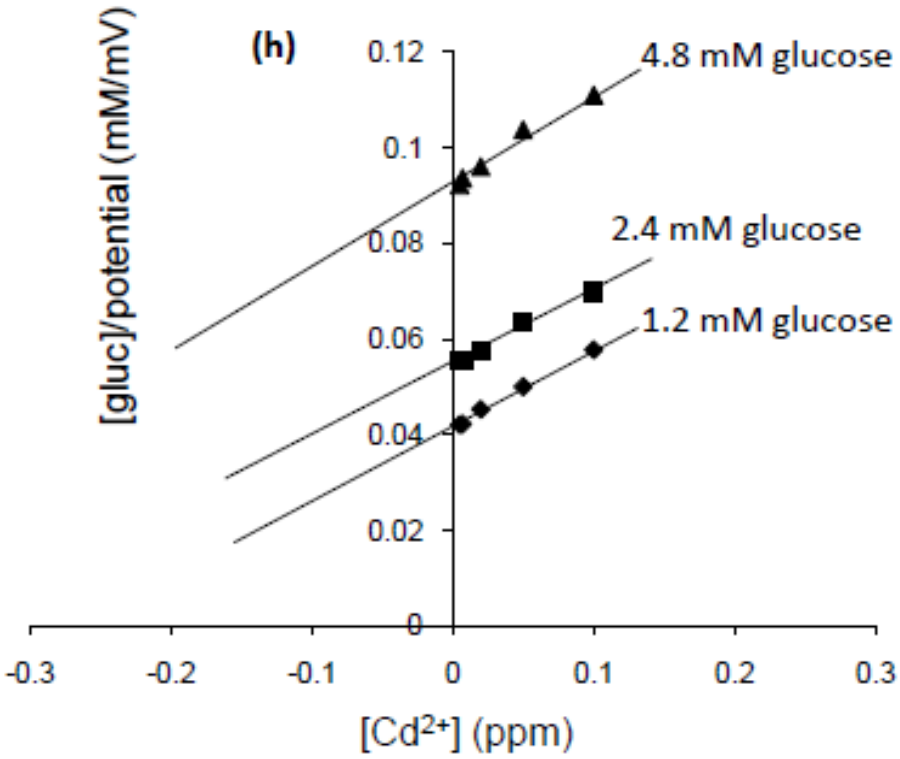


Table captions

Table 1 Comparison of the analytical performances of the PPy-GOx biosensor with those obtained in previous studies for trace metal analysis

Table 2. Recovery of trace metals in tap water samples

Table 1

.

Inhibitor (M^{++})	[M^{++}] that caused maximum inhibition (ppm)	Maximum degree of inhibition attained (%)	Mode of detection	Linear range (ppm)	Minimum detectable conc (ppm)	Sensitivity	IC_{50} (ppm)	K_i (ppm)	K_i' (ppm)	Refs
Cd^{2+},	0.08 (0.711 μM)	27	Amperometric	0.004-0.016 (0.036-0.14 μM)	0.001 (0.0089 μM)[a],	nd,	nd,	0.1	nd,	[9]
Cu^{2+},	23 (362 μM)	41		0.033-0.58 (0.52-9.2 μM)	0.006 (0.095 μM) [a]	nd	nd	3.9	1.1	
Pb^{2+}	15 (75 μM)	20		0.083-0.46 (0.40-2.2 μM)	0.003 (0.014 μM) [a]	nd	nd	4.5	2.8	
Hg^{2+}	40 (200 μM),	40	Amperometric	nd,	0.5 (2.5 μM)	nd	nd	3.2	10.4	[14]
Cu^{2+}	12.6 (198 μM)	50		nd	nd	nd	nd	nd	Nd	
Hg^{2+},	36 (22 μM)	75	Amperometric	1-36 (5-180 μM)	0.50 (2.5 μM)	0.067*	4.4 (22 μM)	nd	nd	[15]
Cu^{2+},	28 (450 μM)	82		0.64-63 (10-100 μM),	0.32 (5.0 μM)	0.11*	4.6 (70 μM)	nd	nd	
				6.4-16 (100-250 μM)	-	0.039*	-			
Cd^{2+}	23 (210 μM)	40		63-158 (20-150 μM)	0.56 (5.0 μM)	0.0072*	24 (210 μM)	nd	nd	
Hg^{2+},	3 (15 μM)	95	Potentiometric	0.005-1 (0.025-5 μM)	0.005 (0.025 μM)	7.46**	0.05 (0.25 μM)	0.11	0.2	Present
Cu^{2+},	3 (47 μM)	87		0.005-1 (0.079-16 μM)	0.005 (0.079 μM)	7.03**	0.2 (3.1 μM)	0.15	0.3	study
$Cd^{2+,}$	7 (62.2 μM)	68		0.005-7 (0.044-62 μM)	0.005 (0.044 μM)	6.23**	0.5 (4.5 μM)	0.17	nd	
Pb^{2+}	7 (24.2 μM)	51		0.024-5 (0.097-15 μM)	0.005 (0.024 μM)	5.87**	5 (24 μM)	0.20	0.3	

* Values are in μA^{-1} μM^{-1}, nd-not determined, ** values in mV mM^{-1}, [a] detection limit (3σ), K_i – Inhibition constants.

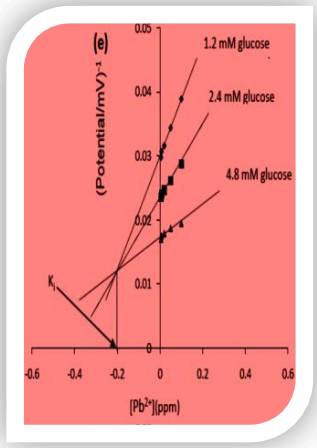
Table 2

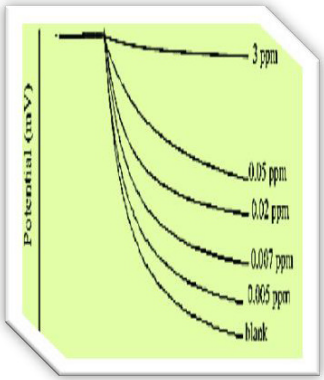
Ion	Conc. Present (μM)	Added Conc. (μM)	Found Conc. (μM)	Recovery (%)
Hg^{2+}	<0.025	2.5	2.47 ± 0.21	99 ± 8
Cu^{2+}	<0.079	2.5	2.52 ± 0.19	101 ± 8
Pb^{2+}	<0.024	2.5	2.43 ± 0.13	98 ± 5
Cd^{2+}	<0.044	2.5	2.49 ± 0.20	100 ± 8

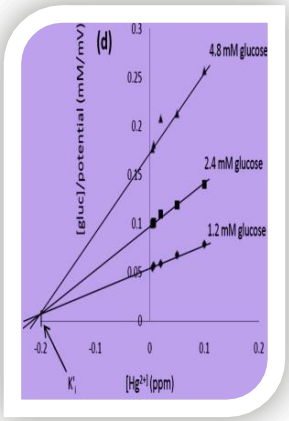
n=3.

Highlights

► Inhibitive potentiometric detection of trace metals with ultrathin PPy-GOx biosensor ► improved permeability to metal inhibitors than with thicker films ► 60 to 100-fold improvement in minimum detectable concentrations ► elimination of need for incubation of biosensor ► more rapid recovery of enzyme activity by washing and storage in phosphate buffer







2