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An amperometric biosensor based on horseradish peroxidase immobilized onto maize tassel-multi-walled carbon nanotubes modified glassy carbon electrode for determination of heavy metal ions in aqueous solution

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ABSTRACT

A biosensor for trace metal ions based on horseradish peroxidase (HRP) immobilized on maize tassel-multiwalled carbon nanotube (MT-MWCNT) through electrostatic interactions is described herein. The biosensor was characterized using Fourier transform infrared (FTIR), UV–vis spectrometry, voltammetric and amperometric methods. The FTIR and UV–vis results inferred that HRP was not denatured during its immobilization on MT-MWCNT composite. The biosensing principle was based on the determination of the cathodic responses of the immobilized HRP to H_2O_2, before and after incubation in trace metal standard solutions. Under optimum conditions, the inhibition rates of trace metals were proportional to their concentrations in the range of 0.092–0.55 mg L^{-1}, 0.068–2 mg L^{-1} for Pb^{2+} and Cu^{2+} respectively. The limits of detection were 2.5 μg L^{-1} for Pb^{2+} and 4.2 μg L^{-1} for Cu^{2+}. Representative Dixon and Cornish-Bowden plots were used to deduce the mode of inhibition induced by the trace metal ions. The inhibition was reversible and mixed for both metal ions. Furthermore, the biosensor showed good stability, selectivity, repeatability and reproducibility.

1. Introduction

The increasing levels of contaminants in the environment from rapid urbanization and industrialization have become a source of concern because of their health implications. Among the contaminants, heavy metals such as arsenic, copper, cadmium, lead, zinc and mercury have received much attention and have been implicated in soil, air and water contaminations in many parts of the world [1–3]. These heavy metals are non-degradable, cannot be detoxified biologically and can accumulate in the biosphere and transfer to the alimentary chain, thereby giving arise to potential serious health consequences for human beings, animals and plants [4,5].

In recent times, various laboratory-based instrumental techniques such as atomic absorption spectroscopy [6,7], isotope dilution inductively coupled plasma mass spectrometry [8] inductively coupled plasma optical emission spectrometry [9], voltammetric methods [10] have been used for determination of heavy metal ions. Although the aforementioned techniques are highly sensitive and selective, most are cumbersome, time consuming for sample pre-treatment, and require expensive equipments, qualified and experienced persons to operate. Thus, an expanding need for the development of a quick, easy, and low cost technique is still important for simple, rapid and reliable detection of trace metal ions.

Over the last decade, enzyme-based amperometric biosensors have emerged as alternatives or complementary devices for toxicity analysis and environmental monitoring when compared to some conventional techniques. They possess advantages such as minimum sample pre-treatment, less time of analysis, sufficient sensitivity and selectivity [11–15]. Of particular interest is the determination of pollutants such as heavy metal ions. In this area efforts have shifted toward inhibition-based enzyme biosensors to determine the concentrations of heavy metal ions in an assayed sample through either direct or indirect inhibition methods with lower detection limits [5,15–27]. The detection principle of enzyme-based biosensors is based on the target analyte selectively inhibiting the activity of the immobilized enzyme resulting in

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a decrease in voltammetric or amperometric signal that is proportional to the amount of target analyte present in the test solution. The concept of enzyme inhibition involving immobilizing enzymes on electrodes is believed to broaden the possible applications of biosensors and offers alternative methods for heavy metal ion sensing in the environment.

Maize tassel (MT), an inexhaustible and non-edible natural and renewable polymeric material resource, is the male part of the maize plant which is discarded as waste [28]. It is a lignocellulosic material possessing several desirable characteristics such as being mesoporous, high adsorption capacity and the presence of a number of surface active functional groups such as —OH, —NH_2, C=O, and —COOH [29]. Hence, MT might be used as an immobilization matrix after mixing it with non functionalized multi-walled carbon nanotubes (MWCNTs) to make a MT-MWCNT composite.

In the present study, we immobilized HRP onto MT-MWCNT/GC electrode through adsorption to construct an inhibitor biosensor for the determination of Pb^{2+} and Cu^{2+} in aqueous solution. The measurement was performed with Pb^{2+} and Cu^{2+} inhibiting the catalytic activity of horseradish peroxidase (HRP) enzyme to reduce H_2O_2. The decrease in the reduction current is expected to be proportional to the concentration of either Pb^{2+} or Cu^{2+} in aqueous solution. The mode of inhibition was investigated using the Dixon and Cornish-Bowden plots.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade and were used without further purification. Horseradish peroxidase (HRP, 250 U mg^{-1}), N,N-dimethylformamide (DMF), Nafion (5% ethanol solution), multi-walled carbon nanotube (MWCNT), $Pb(NO_3)_2$, and $Cu(NO_3)_2$, were purchased from Sigma–Aldrich (South Africa) and used as received. Hydrogen peroxide (H_2O_2, 30%, w/w) was obtained from Merck (South Africa) and solutions were freshly prepared before being used. Phosphate buffer solutions with various pH values were prepared by mixing standard stock solutions of 0.10 M Na_2HPO_4 and 0.10 M NaH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or NaOH from Merck, South Africa. All solutions were prepared using Milli-Q water (resistivity 18 MΩ cm^{-1}).

2.2. Apparatus

All electrochemical experiments were performed with a Bioanalytical Systems (USA) CV-50W conventional three-electrode system. All experiments were carried out at room temperature. FTIR analyses were carried out on a Bruker Tensor 27 spectrophotometer. A UV-1800 spectrophotometer (Shimadzu, Japan) was used to record absorption spectra. The pH measurements were carried out with a Crison 2001 micro pH-meter (Spain).

2.3. Preparation of the maize tassel

The maize tassel powder was prepared using a reported procedure [28]. Briefly, maize tassel was plucked off the woody parts of the maize plant, thoroughly washed with water and air-dried at room temperature. The material was then milled and fractionated to obtain particles of diameter range of 50–100 μm which were washed twice with 0.01 M hydrochloric acid (HCl) in order to remove any impurities that might be on its surface. The acid-washed biomass was then washed twice with high purity water prior to composite preparation.

2.4. Horseradish peroxidase immobilization

Prior to modification, the GC electrode (ϕ = 4 mm) was polished to a mirror finish by use of the BASi polishing kit with 1.0, 0.3 and 0.05 μm diamond slurry in sequence, rinsed thoroughly with ultra-pure water, then ultrasonically rinsed with ethanol and ultra-pure water for 10 min in sequence, in order to remove any adsorbed substances on the electrode surface. The cleaned GC electrode was dried in air. The composite was prepared by dispersing MT-MWCNT (4:1, w/w) with the aid of ultrasonic agitation for 1 h in 10 mL of N,N-dimethylformamide (DMF) to give a 0.1 mg mL^{-1} yellow-black solution. A 10 μL drop of this dispersion was cast on to the surface of a GC electrode and, after drying in air, the electrode was then coated with 10 μL HRP solution (10 mg mL^{-1}, dissolved in 0.1 mol L^{-1} pH 7.0 phosphate buffer solution) and 0.5 μL of 0.3% Nafion to act as a binder. The electrode was covered with a beaker so that water can evaporate slowly in air and a uniform film can be formed. The resulting electrode was marked as HRP/MT-MWCNT/GC electrode and was stored at 4 °C in a refrigerator when not in use.

2.5. Response measurement of HRP/MT-MWCNT/GC electrode

The electrochemical measurements; linear sweep voltammetry, cyclic voltammetry, and amperometry were performed with a Bioanalytical Systems (USA) CV-50W conventional three-electrode system with the HRP/MT-MWCNT/GC electrode as the working electrode, a platinum wire as the auxiliary electrode and an Ag/AgCl (saturated 3 M KCl) electrode as the reference electrode, against which all potentials were quoted. Phosphate buffer solution (0.1 M PBS, pH 7.0) was used as supporting electrolyte and kept in thoroughly anaerobic conditions by purging it with high-purity nitrogen for at least 15 min before and continuously during the experiments. Linear sweep voltammetry and cyclic voltammetry were scanned from +0.6 to −0.6 mV. Amperometric measurements were carried out at an applied potential of −0.30 V while stirring.

2.6. Analytical application

2.6.1. Inhibition by trace metal ions

The detection principle used in this study was based on the inhibitory effect of different concentrations of trace metal ions on HRP immobilized on the MT-MWCNT composite as shown in Scheme 1. The determination on the decrease in the current obtained for the reduction of H_2O_2 by HRP using voltammetry was used as basis of evaluation. The process was carried out in a three step procedure. The biosensor response was first measured in 0.1 M PBS, (0.1 M KCl, pH 7.0) and 0.1 mM H_2O_2 to have steady-state current before inhibition (I_i). The electrode was then washed with the same buffer and incubated in solution containing known concentrations of Pb^{2+} (0.092–2.5 mg L^{-1}) or Cu^{2+} (0.068–5 mg L^{-1}). After incubating for 20 min, the modified electrode response was measured and this corresponded to steady-state current after inhibition (I_F). The percentage of HRP inhibition (%I_{HRP}) and residual enzyme activity (%REA_{HRP}) were calculated using Eqs. (1) and (2) [30,31] respectively:

$$\%\,\text{Inhibition}\,(I\%) = \frac{(I_i - I_F)}{I_i} \times 100 \quad (1)$$

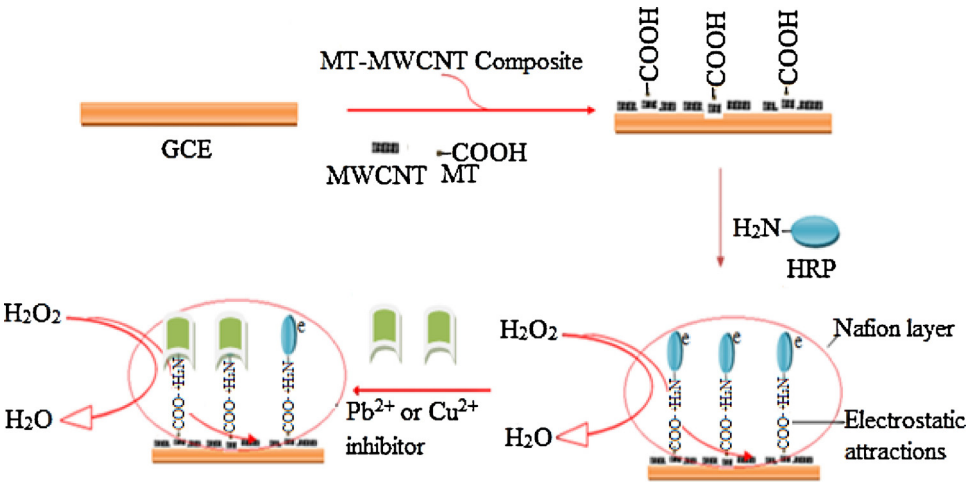
$$\%\,\text{Residual enzyme activity}\,(REA\%) = \frac{I_F}{I_i} \times 100 \quad (2)$$

3. Results and discussion

3.1. Spectrometric characterization of HRP/MT-MWCNT composite

Spectrometric characterizations to demonstrate the HRP stability in the composite film were conducted using FTIR and UV–vis spectroscopy. Detailed information on the secondary structure of the polypeptide chain is provided by the shape of the amide I (1700–1600 cm^{-1}) and amide II (1620–1500 cm^{-1}) using FTIR spectroscopy. As can be seen, Fig. 1A (curve a), the amide I band of HRP, which is caused by C=O stretching vibrations of peptide linkages, appeared at 1641 cm^{-1}. The peak at 1516 cm^{-1} was assigned to strong N—H bending vibration of amide II [32–34]. In Fig. 1A (curve b) the FTIR spectrum of HRP immobilized on the surface of MT-MWCNT is shown. It can be seen in Fig. 1A (curve b) that the amide I and II bands in FTIR spectrum exhibited similar shapes to that of free HRP in the FT-IR spectrum (curve a) except that the bands only shifted slightly to 1636 and 1514 cm^{-1}, respectively. The FTIR spectrum of MT-MWCNT did not show observable peaks in the range of amides II (curve a).

The Soret band may provide useful information on the biological activity of HRP before and after immobilization on MT-MWCNT composite by monitoring the absorption band in the prosthetic heme-group region using UV–vis spectroscopy [35,36]. As shown in Fig. 2B, there was a pronounced absorption band for native HRP at 402 nm (a); while the absorption band for HRP entrapped in the MT-MWCNT film (b) was observed at 403 nm. The slight shift in band and the decrease in absorbance might be due to the interaction between MT-MWCNT and enzyme for the surface potential energy and adsorption. Such interactions allowed the HRP entrapped in the MT-MWCNT composite film to possibly retain its structure. The spectrum of MT-MWCNT (c) does not show any absorption around the wavelength of HRP, thus the band of HRP/MT-MWCNT at 403 nm is entirely due to the



Scheme 1. Schematic illustration of the stepwise amperometric biosensor fabrication process and immobilized horseradish peroxidase inhibition in trace metal ion solution.

HRP Soret band. The UV–vis results further confirmed that the structure of HRP remained almost unchanged in MT-MWCNT composite film.

3.2. Response measurements of the biosensor toward H_2O_2

Fig. 3 shows the linear sweep voltammograms (LSV) for the HRP/MT-MWCNT/GC electrode in PBS (pH 7.0) in the absence H_2O_2 (a) and presence of increasing concentrations of H_2O_2 (b–e). When different concentrations of H_2O_2 were added into the PBS solution, the biosensor showed remarkable increase in reduction peak currents indicating that the immobilized HRP retained its bioelectrocatalytic activity and was not denatured. Cyclic voltammograms (CVs) (*inset*) in the absence of H_2O_2 (a) and presence of 0.05 mM H_2O_2 (b) were also used to illustrate the electrocatalytic activity of the immobilized HRP.

The amperometry technique was performed in order to further illustrate the relationship between the electrocatalytic reduction current and the concentration of H_2O_2. As shown in Fig. 4, the steady-state responses of the biosensor toward H_2O_2 were studied, and the linear response of the biosensor to H_2O_2 concentration ranged from 9 μM to 1 mM with the regression equation of: I_p (μA) = 0.4741 + 2.6634 C (unit of C is mM) and a correlation coefficient of 0.9978. The detection limit was 4.0 μM (S/N = 3). High bioelectrocatalytic activity of the biosensor was certainly facilitated by the presence of the MWCNTs in the composite.

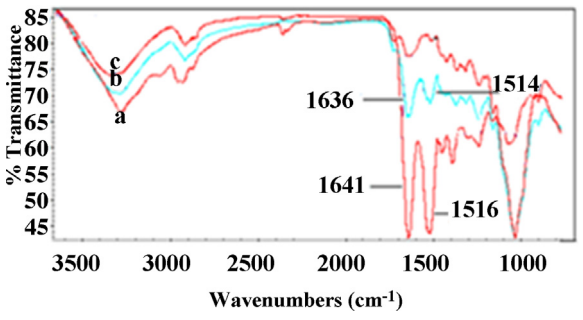


Fig. 1. FTIR spectra of the free HRP (a), HRP/MT-MWCNT film (b), and MT-MWCNT film (c).

3.3. Application of HRP/MT-MWCNT/GC electrode for inhibition

3.3.1. Principle

The possible bioelectrocatalytic mechanism of immobilized HRP on an electrode can be expressed as follows [37,38]:

$$\text{HRPFe(III)} + H_2O_2 \rightarrow \text{CompoundI(FeIV = O)} + H_2O \quad (3)$$

$$\text{CompoundI(FeIV = O)} + 2e^- + 2H^+ \rightarrow \text{CompoundII} + H_2O \quad (4)$$

$$\text{Netreaction}: H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O \quad (5)$$

When an inhibitor is introduced in solution, it can coordinate with compound I resulting in the decrease of the reduction current [39]. The reduction of HRP activity by different inhibitors leads to a decrease in signal production that is directly proportional to the concentration of the inhibitor in the test solution. In general, the percentage of inhibited enzyme that results after exposure to the inhibitor is quantitatively related to the inhibitor concentration and the incubation time [40].

3.3.2. Effect of incubation time

Evaluation of the inhibition time is very important for off-time measurements and on-site analyses [26]. Investigation of the aforementioned parameter on the response of the developed biosensor was carried out and the percentage inhibitions corresponding

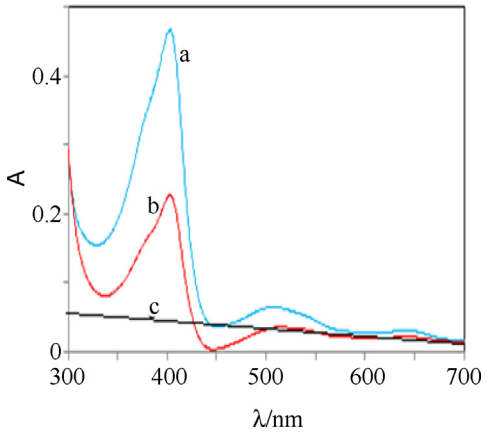


Fig. 2. UV–vis spectra of HRP (a), HRP/MT-MWCNT (b), and MT-MWCNT (c).

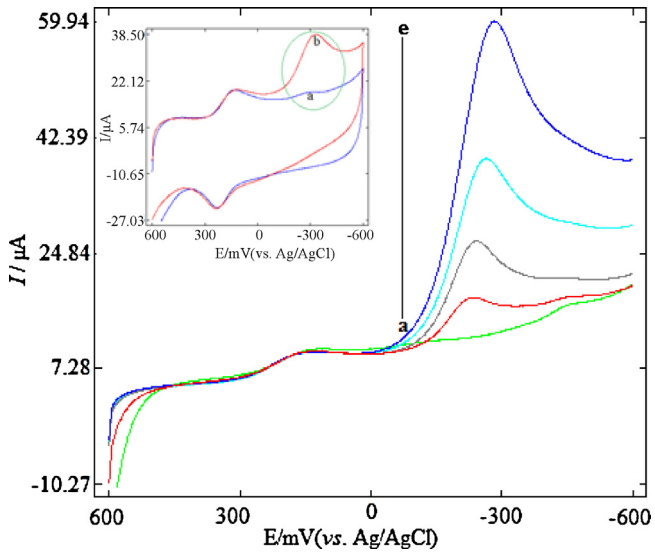


Fig. 3. LSVs of HRP/MT-MWCNT/GC electrode in the presence of different concentration of H_2O_2 (a–e): 0, 0.01, 0.05, 0.2, and 0.5 mM in 0.1 M PBS (pH 7.0) at the scan rate of 100 mV/s. *Inset*: CVs of HRP/MT-MWCNT/GC electrode (a and b): 0 and 0.05 mM in 0.1 M PBS (pH 7.0) at scan rate of mV/s.

to different incubation times were calculated using Eq. (1). The biosensor signal results are depicted in Fig. 5.

As shown in Fig. 5, an increase in incubation time caused an increase in the percentage of inhibition whilst a decrease in residual enzyme activity occurred as the incubation time increased. The explanation for this trend may be due to more interaction between the active site of the enzyme and the inhibitor which occurred with increase in incubation time. The change in peak current reflected the alteration of enzymatic activity by the heavy metal ion, which resulted in the change in the interactions with its substrate (H_2O_2). Incubation time that gives a percentage inhibition greater than 10%

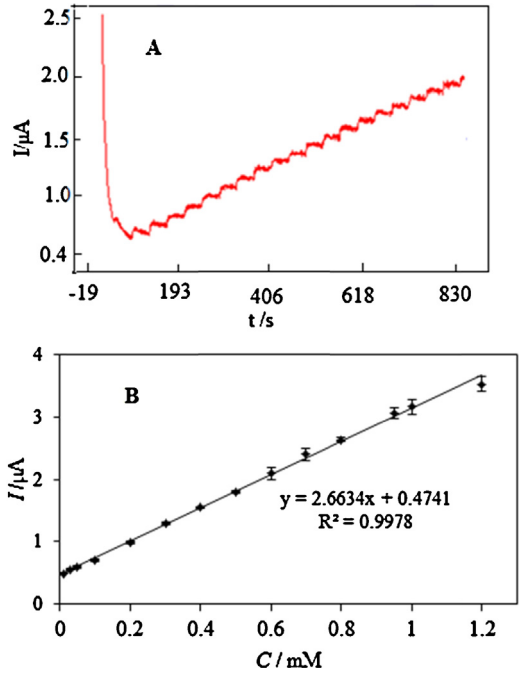


Fig. 4. (A) Amperometric response of the HRP/MT-MWCNT/GC electrode at −0.3 V (*vs.* Ag/AgCl) upon successive addition H_2O_2 in 0.1 M pH 7.0 phosphate buffer solution after every 25–30 s. Rotation speed: 800 rpm. (B) Catalytic current (I/μA) as a function of concentration of H_2O_2 (C/mM). Error bar = ±SD and n = 3).

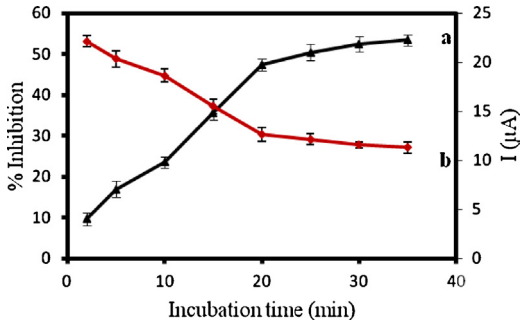


Fig. 5. The effect of incubation time on the biosensor response (a) and enzyme activity decay (b) in 0.5 mg L^{-1} Pb^{2+} 0.1 M PBS (pH 7.0) and 0.1 mM H_2O_2. Error bar = ±SD and n = 3.

is usually preferred in practical analysis in order to obtain low detection limits for different inhibitors. In this study, an incubation time of 20 min was selected for further experiments for the metals tested, although it is clearly dependent on the specific metal ion and its concentration. The same incubation time was used so that its influence on the response of the biosensor when applied to determination in natural matrices is evaluated.

3.3.3. *Inhibition studies of Pb^{2+} and Cu^{2+}*

Typical percentage inhibition-concentration and percentage residual activity-concentration plots of the biosensor under the optimized experimental conditions for both Pb^{2+} and Cu^{2+} are shown in Fig. 6. As shown in Fig. 6A and B, this type of inhibition effect exhibited dose-dependent behavior.

The percentage inhibition increased with increase in concentrations of Pb^{2+} and Cu^{2+}. As can be seen in Fig. 6A and B, 37.8 and 44.0% of the activity of HRP was inhibited by 2.5 mg L^{-1} and 5 mg L^{-1} for Pb^{2+} and Cu^{2+}, respectively. The linear response and detection limit of the biosensor for Pb^{2+} and Cu^{2+} is shown in Table 1. On the other hand, the residual enzyme activity decreased with increase in Pb^{2+} and Cu^{2+} concentrations.

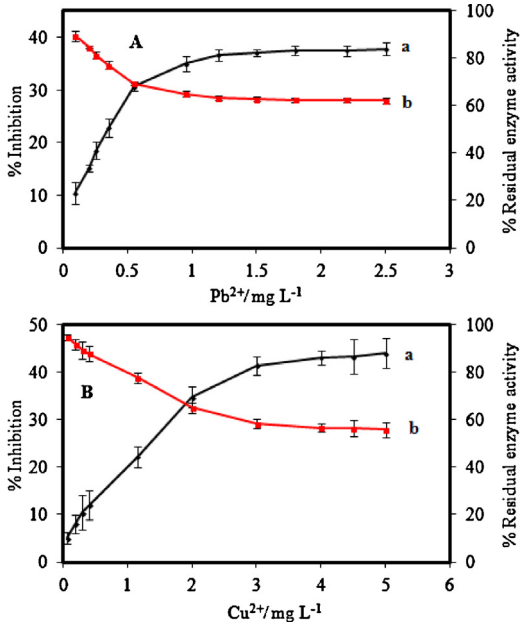


Fig. 6. Dose-dependent enzyme inhibition (a) and residual enzyme activity (b) of (A) Pb^{2+} (B) Cu^{2+} toward HRP-catalyzed H_2O_2. Error bar = ±SD and n = 3.

Table 1
A summary of analytical characteristics for determination of heavy metal ions.

Metal ion	Concentration range (mg L^{-1})	Linear range (mg L^{-1})	LOD (μg L^{-1})	R^2	K_i (mg L^{-1})	K'_i (mg L^{-1})
Pb^{2+}	0.092–2.5	0.092–0.55	2.5	0.9937	2.6	3.8
Cu^{2+}	0.068–5.0	0.068–2.0	4.2	0.9954	1.8	3.1

3.3.4. Investigation on the type of HRP inhibition

The mode of enzymatic reversible inhibition is variable from one inhibitor to another, and may be competitive, non-competitive, and uncompetitive or mixed inhibition [41,42]. In this study, the type of inhibition shown by Pb^{2+} and Cu^{2+} over immobilized HRP was studied using increasing concentrations of the trace metals and of the substrate, H_2O_2. Dixon plots and Cornish-Bowden plots were utilized to verify the inhibition modes [43,44]. During inhibition, it should be noted that the different types of inhibition can be characterized by analysing these two plots together. The Dixon plot by itself cannot clearly distinguish between competitive and mixed inhibition and on the other hand, the Cornish-Bowden plot cannot always distinguish between mixed and uncompetitive inhibition.

Representative Dixon and Cornish-Bowden plots are shown in Figs. 7A and B and 8A and B for Pb^{2+} and Cu^{2+} respectively.

The pattern of inhibition demonstrated by Dixon plot and confirmed by Cornish-Bowden plot is consistent with the inhibition of HRP by Pb^{2+} and Cu^{2+} ions through a reversible, mixed inhibition. In both cases, the 3 lines intercept at a single point in the second quadrant above *x*-axis in the Dixon' coordinates and intercept at a single point in the second quadrant below the *x*-axis in the Cornish-Bowden plot [43]. Furthermore, the values for K_i (the inhibition constant, is the dissociation constant of the enzyme-inhibitor complex) and K'_i (is the dissociation constant of the enzyme substrate-inhibitor complex) 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. The values of K_i and K'_i for two metal ions are given in Table 1.

The results obtained in this paper were compared to other enzymes reported in literature such as glucose oxidase [5,19,22]. The inhibition by Cu^{2+} was competitive, reversible and mixed. Competitive inhibition was observed for Cu^{2+} using L-lactate dehydrogenase [45]. The inhibition was reversible and mixed when Pb^{2+} was determined using glucose oxidase [19]. In mixed inhibition, the inhibitor binds at a site other than the active site (enzyme or enzyme-substrate) and causes changes in the overall 3-dimensional shape of the enzyme that leads to a decrease in activity.

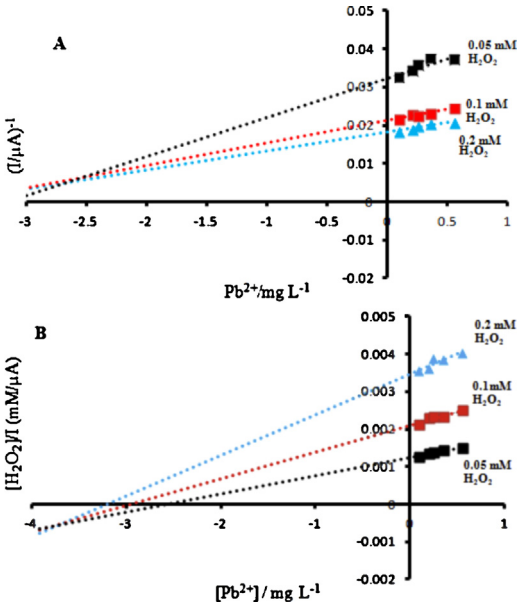


Fig. 7. Dixon (A) and Cornish-Bowden (B) plots of the effect of different Pb^{2+} concentrations on HRP.

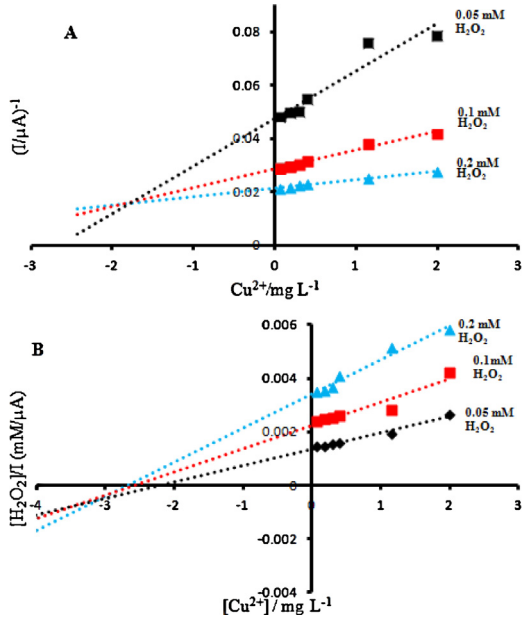


Fig. 8. Dixon (A) and Cornish-Bowden (B) plots of the effect of different Cu^{2+} concentrations on HRP.

3.4. Stability, repeatability and reproducibility

The stability of the biosensor was first examined in the presence of 0.1 mM H_2O_2 concentration in 0.1 M PBS (pH 7.0). For the same metal concentration, it was observed that after 10 successive series of measurements, the biosensor lost about 30% of the initial sensitivity for the two metal cations studied. In studying the long-term stability, the HRP/MT-MWNT/GC electrode biosensor was stored in 0.1 M PBS at 4 °C for 10 days, the biosensor maintained 82% and 78% of its initial response for 0.1 mM H_2O_2 after incubation in Pb^{2+} and Cu^{2+}, respectively. The repeatability of the HRP/MT-MWCNT/GC electrode biosensor was investigated for fixed heavy metal ion concentrations. A relative standard deviation (RSD) of 5.2% and 5.8% was obtained for Pb^{2+} and Cu^{2+} respectively. Five modified biosensors were made independently based on the same bare GC electrode, and were investigated for the determination of the same metal concentrations. The modified biosensors showed a relative standard deviation (RSD) of Pb^{2+} (6.8%) and Cu^{2+} (6.2%).

3.5. Interference study and preliminary application of the biosensor

The addition of the following interferents such as Ca^{2+} (3.0 mg L^{-1}), Mg^{2+} (3.0 mg L^{-1}), Na^{+} (3.0 mg L^{-1}) and K^{+} (3.0 mg L^{-1}), were studied by the mixed method, using the ratio of 1:2 for analyte and interferents, respectively. From the results in Table 2, the cations do not cause much decrease in biosensor response.

Table 2
Interference study for the determination of 1.5 mg L^{-1} Pb^{2+} and 1.5 mg L^{-1} Cu^{2+} with the HRP biosensor.

Possible interference	Ratio	% decrease in biosensor response			
		Pb^{2+} (without interferant)	Pb^{2+} (with interferant)	Cu^{2+} (without interferant)	Cu^{2+} (with interferant)
Ca^{2+}	1:2	5.24	5.23	6.69	6.68
Mg^{2+}	1:2	6.54	6.53	7.26	7.25
Na^{+}	1:2	4.33	4.32	4.36	4.35
K^{+}	1:2	5.35	5.34	4.59	4.58

Table 3
Recovery test for Pb^{2+} and Cu^{2+} in tap water.

Heavy metal ion	Added (mg L^{-1})	Found (mg L^{-1})	Recovery (%)
Pb^{2+}	0.50	0.52	104.0
	0.55	0.53	96.4
Cu^{2+}	0.50	0.48	96.0
	1.00	1.01	101

To demonstrate the feasibility of the fabricated enzyme inhibition biosensor for possible environmental applications, preliminary application of the biosensor was examined by determination of Pb^{2+} and Cu^{2+} in tap water by standard addition method. The results are given in Table 3. The recoveries were in the range of 96.0–104.0%, which indicated the efficacy of the biosensor for practical analysis.

4. Conclusions

In this paper, we have described the use of a HRP/MT-MWCNT/GC electrode biosensor for the determination of Pb^{2+} and Cu^{2+} through inhibition studies. The proposed biosensor does not require any complicated immobilization procedure for the construction. The metal ions were measured with low detection limits of 2.5 μg L^{-1} and 4.2 μg L^{-1} for Pb^{2+} and Cu^{2+}, respectively. All cations studied inhibited HRP, with Cu^{2+} (44.0%) showing the highest degree of inhibition followed by Pb^{2+} (37.8%). Inhibition constants were determined by using Dixon plots. By modeling the data using the Cornish-Bowden plots together with Dixon plots, the inhibition was determined to be reversible and mixed for both metal cations. This work based on immobilized enzyme inhibition coupled to an electrode surface significantly broadens the possible applications of biosensors and offers alternative methods for detection of other heavy metal ions as a measure of toxicity in environmental samples.

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