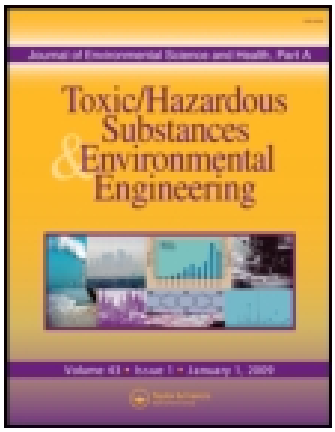

Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lesa20

Amperometric determination of cadmium, lead, and mercury metal ions using a novel polymer immobilised horseradish peroxidase biosensor system

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Published online: 19 Aug 2014.

To cite this article: Bongiwe Silwana, Charlton Van Der Horst, Emmanuel Iwuoha & Vernon Somerset (2014) Amperometric determination of cadmium, lead, and mercury metal ions using a novel polymer immobilised horseradish peroxidase biosensor system, Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 49:13, 1501-1511, DOI: 10.1080/10934529.2014.937169

To link to this article: http://dx.doi.org/10.1080/10934529.2014.937169

Journal of Environmental Science and Health, Part A (2014) **49**, 1501–1511

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934529.2014.937169

Amperometric determination of cadmium, lead, and mercury metal ions using a novel polymer immobilised horseradish peroxidase biosensor system

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This work was undertaken to develop a novel Pt/PANI-co-PDTDA/HRP biosensor system for environmental applications to investigate the inhibition studies by specific heavy metals, to provide data suitable for kinetic studies and further application of the biosensor to environmental samples. The newly constructed biosensor was compared to the data of the well-researched Pt/PANI/HRP biosensor. Optimised experimental conditions, such as the working pH for the biosensor was evaluated. The functionality of the amperometric enzyme sensor system was demonstrated by measuring the oxidation current of hydrogen peroxide followed by the development of an assay for determination of metal concentration in the presence of selected metal ions of Cd^{2+}, Pb^{2+} and Hg^{2+}. The detection limits were found to be 8×10^{-4} μg L^{-1} for cadmium, 9.38×10^{-4} μg L^{-1} for lead and 7.89×10^{-4} μg L^{-1} for mercury. The World Health Organisation recommended that the maximum safety level of these metals should not exceed 0.005 mg L^{-1} of Cd^{2+}, 0.01 mg L^{-1} of Pb^{2+} and 0.001 mg L^{-1} of Hg^{2+}, respectively. The analytical and detection data for the metals investigated were observed to be lower than concentrations recommended by several bodies including World Health Organisation and Environmental Protection Agencies. Therefore the biosensors developed in this study can be used to screen the presence of these metals in water samples because of its low detection limit. The modes of inhibition of horseradish peroxidase by Pb^{2+}, Cd^{2+} and Hg^{2+} as analysed using the double reciprocal plots of the Michaelis–Menten equation was found to be reversible and uncompetitive inhibition. Based on the K_m^{app} and I_{max} values for both biosensors the results have shown smaller values. These results also proved that the enzyme modified electrode is valuable and can be deployed for the determination or screening of heavy metals.

Keywords: Amperometric biosensor, PANI-co-PDTDA conducting polymer, HRP, portable potentiostat, lead, cadmium, mercury.

Introduction

The environmental contamination of the soil and water from toxic heavy metals ions are of worldwide concern and have been reported.[1] These metals are used in various industries from which effluents are consequently discharged into the environment. Introduction of metals in various forms into the environment can produce numerous modifications of microbial communities and affect their activities.[2–4] Common sources of heavy metal pollution include discharge from industries such as electroplating, plastics manufacturing, fertilizer producing plants and wastes left after mining and metallurgical processes.[5] Heavy metals generally exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions or modifying the active conformations of biological molecules.[2]

General consensus holds that the toxicity of heavy metal (HM) ions mainly results from their interactions with proteins (especially various enzymes) or with nucleic acids. Heavy metal ions alter pro-oxidant/antioxidant balance and bind to sulphydryl groups or other ligands in life-relevant species, resulting in inhibition of glutathione metabolism and functions of numerous enzymes and hormones. In addition, HM ions are antagonistic to essential trace elements and usually compete with nutrient elements for binding sites on transport and storage proteins, receptors, and metallo-enzymes, which may result in marked aberrations in the metabolism of carbohydrates, proteins, neurotransmitters, and hormones.

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Received March 10, 2014.
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The strongest interactions of heavy metal ions with enzymes usually take place in the cases of Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, and Ag^{+} ions. Many immobilised enzymes have been used for inhibitive assays of Hg^{2+} and Cu^{2+}, but fewer reports are available on Ag^{+}. The early detection of heavy metal ions, especially bioavailable metal ions, in the environment is very important to safeguard human health. Bioassay and inhibitive enzyme assays are excellent detection methods for bioavailable ions as they are inhibited only by the bioavailable form,[6] whereas instruments, such as atomic absorption and emission spectrophotometry, usually do not discriminate between toxic and non-toxic forms of metal ions.

Taking into consideration the limitations of spectroscopic instruments, there is a need for the development of cheap, simple and portable detector systems for heavy metals. In this regard, the development of electrochemical sensors has been in the forefront. Numerous enzymes such as peroxidase, xanthine oxidase, invertase, glucose oxidase, urease and the proteases papain and bromelain are cheap, do not require costly instruments and are amenable to field testing conditions. In many instances, monitoring is not continuous but requires a number of individual measurements to be performed at different times. In such cases, electrochemical sensors should be manufactured inexpensive so that they may be disposed after a single reading or use.[7,8]

A biosensor is an analytical device which incorporates biological sensing elements such as enzymes, antibodies, receptors, aptamers, nucleic acids, cells, and so forth, on electrode surfaces. Electrode surfaces also determine the sensitivity, selectivity, and reproducibility of the sensor/biosensor, with electronic transducer equipped with an electronic amplifier and was found to have applications in various fields, for example, clinical diagnostics, environmental monitoring, bioprocess monitoring, food, agricultural product processing, etc.[9,10]

The modification of biomolecules or electrode surfaces using novel conducting materials as mediators and design of functional bio-interfaces has become prevalent. Thus, highly conductive organic transducers such as conducting polymers (CPs), nanomaterials, sol-gel films, and self-assembled monolayers, and so forth are gradually emerging for the development of next-generation biosensor design for highly reliable, stable, and robust field-based.[11]

In this study, we have prepared a novel enzyme polymeric electrode based on a co-polymer of aniline and a disulphide derivative of aniline (dithiodianiline) as a mediator for the immobilisation of the enzyme horseradish peroxidase (HRP) in the biosensor construction. The wide scope of tuning different properties of PANI encouraged us to explore the possibility of copolymerizing aniline with dithiodianiline having S-S links in it. This work focused on poly(aniline-co-2,2'-dithiodianiline) [PANI-co-PDTDA)] as a polymer matrix containing HRP for the biosensor analysis of heavy metal ions.

The novel constructed biosensor is denoted as Pt/PANI-co-PDTDA/HRP and has been developed and compared with the most reported Pt/PANI/HRP biosensor for the investigation of the inhibition studies by Cd^{2+}, Pb^{2+}, and Hg^{2+} ions for further determination of the kinetics parameters for environmental analysis. In this current study we have found that the performance factors of the novel biosensor, such as lower detection limit for the detection of Cd^{2+}, Pb^{2+}, and Hg^{2+} metals ions, good stability, repeatability and reproducibility provide good promise compared to the formerly Pt/PANI/HRP biosensor. The study was further conducted to investigate the inhibition of the HRP enzyme by Cd^{2+}, Pb^{2+} and Hg^{2+} metal ions and kinetic studies were also conducted to identify the nature of enzyme inhibition. The Pt/PANI-co-PDTDA/HRP biosensor was further applied to determine trace amounts of heavy metals (Cd^{2+}, Pb^{2+}, and Hg^{2+}) in tap and river water samples.

Materials and methods

Chemicals and reagents

The reagents aniline (99%), 2,2′-dithiodianiline (98%), potassium dihydrogen phosphate (99%), disodium hydrogen phosphate (98%) and diethyl ether (99.9%) were obtained from Sigma-Aldrich, Germany. The enzyme horseradish peroxidase (EC 1.11.1.7 type IV from horseradish, 250–330 units mg^{-1}) was also purchased from Sigma-Aldrich, Germany, while hydrogen peroxide (30–32%) Grade AR was purchased from Merck, South Africa. The standards for cadmium (Cd), lead (Pb) and mercury (Hg) were purchased as atomic absorption standard solutions (1000 mg L^{-1}, AAS) from Sigma-Aldrich, Germany. Phosphate buffer (PB, 0.1 M, pH 6.8 and 7.2) solutions were prepared by mixing stock standard solutions of KH_2PO_4 and Na_2HPO_4, which were then used as supporting electrolytes. Phosphate buffer solutions (PB, 0.1 M) with various pH values were prepared. All other solutions were made up with Millipore water and experiments were performed at room temperature.

Instrumentation

All electrochemical measurements were performed with a PalmSens portable electrochemical potentiostat/ galvanostat, with the PS Trace program and accessories (PalmSens Instruments BV, 3992 BZ Houten, and Netherlands) interfaced to a microcomputer controlled by PS 2.1 software for data acquisition and experimental control. A three-electrode arrangement and setup was employed with the Pt disc as the working electrode, platinum wire as counter electrode and silver/silver-chloride (Ag/AgCl; 3 M NaCl) as the reference electrode. Alumina micro polish and polishing pads (Buehler, IL, USA) were used for electrode

polishing. The pH values of the buffer solutions were measured by means of a microprocessor pH meter with custom buffers (the model HI 221 series, Hanna, instruments.[12]

Electrode surface preparation

The Pt electrode was prepared by mechanically polishing a platinum disc electrode in each of a 1.0, 0.3, 0.05 μm alumina slurry until a mirror finish was obtained. After being rinsed with double-distilled water, the electrode was sonicated in distilled water and ethanol respectively, for approximately 5 min each. The polished electrode was then cleaned electrochemically by cycling the potential between −200 and +1500 mV (vs. Ag/AgCl) in 0.05 M H_2SO_4 solution at the scan rate of 40 mV/s for 10 min or until the CV characteristics for a clean Pt electrode were obtained.[13,14]

Electropolymerisation of polymer films

The polymerisation was done using a reported procedure for the electropolymerisation of PANI on a Pt electrode.[15,16] The potential was cycled repetitively between −200 and +1100 mV (vs. Ag/AgCl) at a scan rate of 60 mV/s for 10 cycles, using a 0.2 M aniline solution dissolved in 1 M hydrochloric acid (HCl).

The preparation of the co-polymer film of polyaniline-co-poly(2,2'-dithiodianiline), abbreviated as PANI-co-PDTDA was done as follows. A 10 mL solution of 0.2 M aniline, plus 0.02 M 2,2'-dithiodianiline and aqueous 5 M H_2SO_4 was prepared in a water bath at 70°C to dissolve the 2,2'-dithiodianiline crystals. This solution was then used in the electropolymerisation of PANI-co-PDTDA on the Pt electrode surface, by repetitive cyclic voltammetric scanning at 50 mV/s from −200 to +1100mV (vs. Ag/AgCl) for 10 cycles.[16,17]

Preparation of polymer-modified enzyme electrodes

Following electropolymerisation, the rinsed electrode was transferred to a cell containing 1 mL PB solution and the polymer surface was reduced at a constant potential of −0.5V (*vs.* Ag/AgCl) until the current signal reached a steady-state value (ca. 1500 s). The optimised amount of the enzyme HRP (50 μL of 2 mg L^{-1} HRP in PB) was immobilised by covalent binding onto the PANI or PANI-co-PDTDA film. This was followed by oxidation at +0.7 V (*vs.* Ag/AgCl) for 1500 s for covalent attachment of the HRP to the polymer film. This enzymatic incorporation was done using the methodology outlined in Mathebe et al. [15]

The prepared biosensor was first rinsed with deionised water and immersed in phosphate buffer solution and kept at 4°C overnight. The biosensor was also stored in buffer at 4°C when not in use.

Biosensor response measurements

Cyclic voltammetry

Cyclic voltammetry (CV) and differential pulse voltammetric (DPV) experiments were performed with the portable PalmSens instrument in a potential range of +0.4 V and −1.0 V (*vs.* Ag/AgCl). The PANI and PANI-co-PDTDA polymer films were used in the following experiments as mediators in HRP immobilised electrodes, having H_2O_2 as the substrate. A reduction potential of −0.25 V (*vs.* Ag /AgCl) was used to monitor the electrocatalytic reduction of H_2O_2, as substrate. The solution was degassed with nitrogen before any substrate was added.

The Pt/PANI/HRP biosensor was immersed in the 0.1 M PB (1 mL) under stirring conditions with direct additions of standard substrate solutions using a micropipette. The signal for the sequential additions was recorded until the current response had reached a steady state. The response of the biosensor towards H_2O_2 was investigated by successively adding aliquots of H_2O_2 to a continuously stirred 0.1 M PB (pH = 6.8 and 7.2) solution under the optimised conditions. The DPV measurement was first obtained in the absence of the substrate H_2O_2, followed by sequential addition of the aliquots of H_2O_2 as substrate.[18,19] In all experiments hydrogen peroxide as substrate was added until a final steady-state peak current was reached.

Differential pulse voltammetric measurements

Differential pulse voltammetric (DPV) analysis of the Pt/PANI/HRP biosensor in 1 mL of 0.1 M PB (pH = 6.8) solution was also performed to compliment the CV measurements. The differential pulse voltammogram (DPV) was collected in the cathodic direction only by applying a linear potential scan between +0.4 V and −1.0 V (vs. Ag/AgCl) at a scan rate of 10 mV/s and pulse amplitude of 20 mV. The pulse width of 100 ms was used. The DPV was first obtained in the absence of the substrate H_2O_2, followed by analysis in the presence of H_2O_2 as substrate.[20,21]

Results and discussion

Optimisation of solution pH

The acidity of the detection solution usually has a large effect on the redox behaviour of enzymes. In the range of pH 4.5–7.5, the influence of pH on the response current was investigated under the potential applied mentioned previously. The Pt/PANI-co-PDTDA/HRP biosensor gave an optimum current signal at the pH = 7.2, compared to the Pt/PANI/HRP biosensor that showed pH = 6.8 as

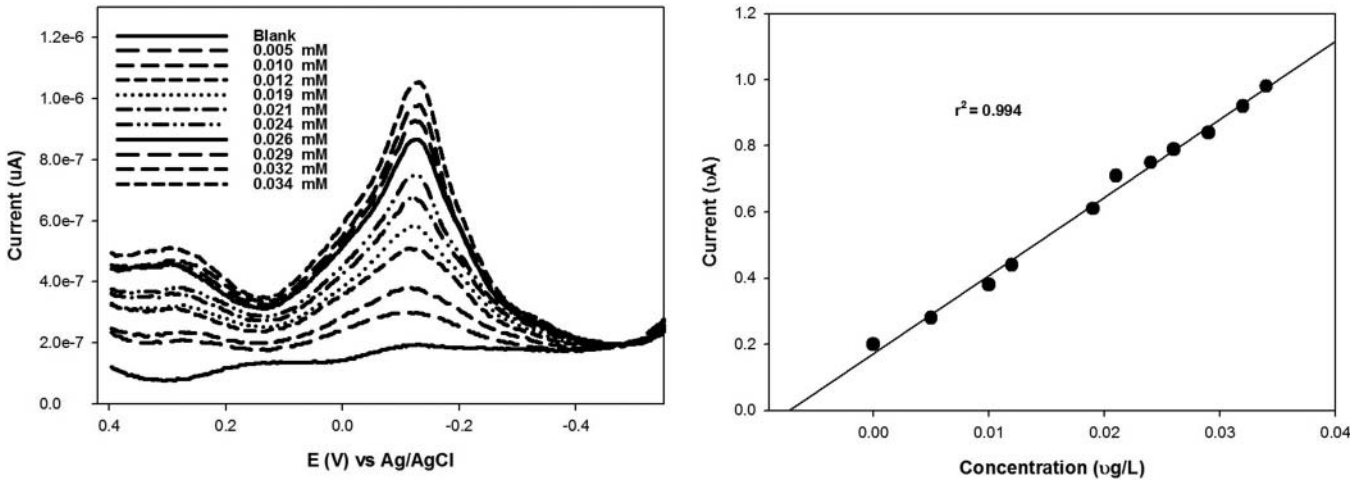

Fig. 1. The cathodic differential pulse voltammograms (DPVs) in (a) of the Pt/PANI-co-PDTDA/HRP biosensor in the presence of different H_2O_2 concentrations evaluated in 0.1 M PB (pH = 7.2) solution is shown, with the calibration plot in (b). Experimental conditions were: amplitude, 20 mV; potential step, 20 mV.

optimum. Further experiments were performed at the newly investigated pH for both biosensors.

Differential pulse voltammetric characterisation of Pt/PANI-co-PDTDA/HRP biosensor

Figure 1 shows the results for the cathodic differential pulse voltammograms (DPVs) of the Pt/PANI-co-PDTDA/HRP biosensor towards the successive additions of 20 μL of 0.1 mM H_2O_2 to 1 mL PB (pH = 7.2) solution under the optimised conditions, plus the calibration plot obtained for the peak current versus increasing H_2O_2 concentrations is also shown.

The working potential of −0.25 V (vs. Ag/AgCl) was then chosen in order to minimise the potential interference from the reduction of the other species in the test medium. As can be seen from the voltammograms (Fig. 1), minimum current response was obtained in the absence of H_2O_2 as substrate at the working potential. The H_2O_2 substrate solution was added in order to check the activity of HRP before addition of heavy metals and the calibration plot obtained from that curve has a linear range of up to 0.034 mM with coefficient $R^2 = 0.994$ ($n = 10$). The detection limit (LOD) of the biosensor was found to be 8.59×10^{-4} mM with a relative standard deviation of 3.73% and the sensitivity of 25.42 μA mM^{-1} were calculated from the initial slope of the calibration curve. The satisfactory DPV results show that the Pt/PANI-co-PDTDA/HRP biosensor operates ideally in the presence of H_2O_2, and the analytical data of this newly constructed sensor compares well to that of the conventional Pt/PANI/HRP biosensor. The results for the comparison of the analytical performance of the evaluated HRP biosensor towards H_2O_2 detection with previously reported modified electrodes are shown in Table 1.

Table 1 shows the analytical performance of proposed HRP biosensors towards H_2O_2 detection with previous reported modified electrode. The results obtained for the new HRP biosensor constructed in this study, showed LOD results of a similar order to that of Du et al.,[22] while

Table 1. Results for the comparison of the analytical performance of the evaluated HRP biosensor towards H_2O_2 detection with previously reported modified electrodes.

Sensor	*Method*	*Linear range (mM)*	*LOD (mM)*	*Reference*
PANI-co-PDTDA/HRP[a]	DPV	0.005–0.034	8.59×10^{-4}	This work
PANI/HRP[a]	DPV	0.043–0.75	3.2×10^{-2}	[15]
HRP/PAni/CS[b]	Amperometry	0.01–1.5	5×10^{-4}	[22]
HRP/PAni/MWCNTCOOH[c]	Amperometry	0.086–10	8.6×10^{-2}	[23]
CS/HRP-p($Ani_{0.6}$-co-o-$Aba_{0.4}$)[b]	Amperometry	0.01–1	1×10^{-3}	[24]
PANi/ASA/HRP [a]	DPV	0.012–0.45	1.2×10^{-2}	[25]
PANI/HRP[a]	Amperometry	0.05–3.17	3.68×10^{-5}	[26]

[a]Platinum electrode (PtE), [b]Glassy carbon electrode (GCE), [c]Gold Electrode (AuE).

Table 2. Performance characteristics of the Pt/PANI-co-PDTDA/HRP biosensor in the presence of selected heavy metals as inhibitor.

Metal ion	$[Cd^{2+}]$, μg/L	$[Pb^{2+}]$, μg/L	$[Hg^{2+}]$, μg/L
Linear range (μg/L)	0–1 × 10^{-2} (n = 5)	0–1 (n = 5)	0–1 (n = 5)
Sensitivity (μA/ppb)	1.07 × 10^{-2}	5.37 × 10^{-2}	1.02 × 10^{-2}
Correlation coefficient (R^2)	0.993	0.949	0.982
LOD (ppb)	8.01 × 10^{-4}	9.38 × 10^{-4}	7.89 × 10^{-4}
LOQ (ppb)	2.67 × 10^{-3}	3.13 × 10^{-3}	2.63 × 10^{-3}

slightly better results was obtained in the study of Nomngongo et al.[26] However, the new biosensor constructed in this study suggests that the linear range and response of the Pt/PANI-co-PDTDA/HRP shows improvement upon the results obtained by previous reported studies.

Differential pulse voltammetric (DPV) evaluation of the Pt/PANI-co-PDTDA/HRP biosensor in the presence of Cd^{2+}, Pb^{2+} and Hg^{2+} metal ions were also performed. The results obtained for the characteristics of the performance of the Pt/PANI-co-PDTDA/HRP biosensor towards the aforementioned metal ions are presented in Table 2.

In the presented results, it was found that the sensitivity, LOD and LOQ was uniform at the same order for each of the Cd^{2+}, Pb^{2+} and Hg^{2+} metal ions, although some variations in the magnitude were obtained. The Pt/PANI-co-PDTDA/HRP biosensor had a linear range between 0 – 0.01 μg L^{-1} for Cd^{2+} and 0 – 1 μg L^{-1} for both Pb^{2+} and Hg^{2+}. The LOD value obtained for the biosensor in the presence of Cd^{2+} was 8.01 × 10^{-4} μg L^{-1}, while for Pb^{2+} and Hg^{2+} it was 9.38 × 10^{-4} and 7.89 × 10^{-4} μg L^{-1}, respectively. The results for the LOQ values was 2.67 × 10^{-3} μg L^{-1} for Cd^{2+}, 3.13 × 10^{-3} for Pb^{2+} and 2.63 × 10^{-3} for Hg^{2+}, respectively. Comparison of the results to that found in other studies have shown that this study has the lowest values of LOD and LOQ, which confirmed good sensitivity of the developed biosensor for determination of heavy metals.[14,26]

Inhibition of Pt/PANI-co-PDTDA/HRP and Pt/PANI/HRP biosensors by heavy metals

To study the inhibition effect of selected heavy metals on HRP as enzyme, the procedure involved the study of the Pt/PANI-co-PDTDA/HRP and Pt/PANI/HRP biosensors, exposed to metal ion concentrations in phosphate buffer (Figs. 2 and 3), respectively. On comparing the inhibitory activities of the two biosensors, several interesting observations were made. Both biosensors gave higher inhibition percentages when tested for Hg^{2+} (Figs 2 and 3) detection, which was expected for Hg^{2+} that is normally regarded as very toxic. In Figure 2 the results obtained for the inhibition studies of the Pt/PANI-co-PDTDA/HRP biosensor in the presence of sequentially added metal ion solutions is shown. Figure 3 shows the results obtained for

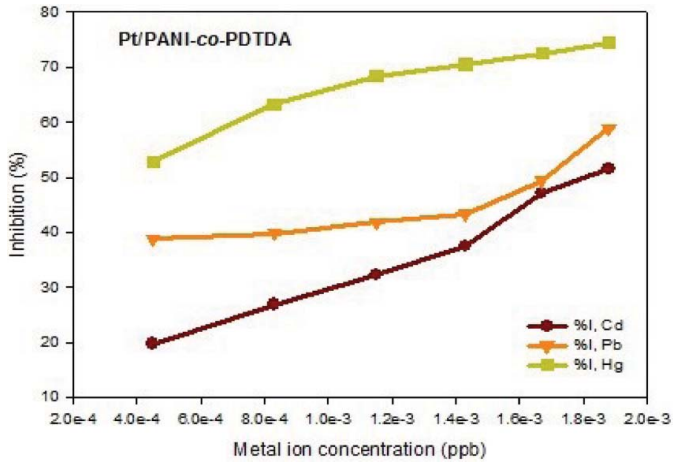

Fig. 2. Results obtained for the inhibition of the Pt/PANI-co-PDTDA/HRP biosensor in the presence of sequential aliquots of 0.005 ppb of Cd^{2+}, Pb^{2+} and Hg^{2+}, respectively. The individual metal aliquots were added sequentially to a 0.1 M PB (pH = 7.2) solution.

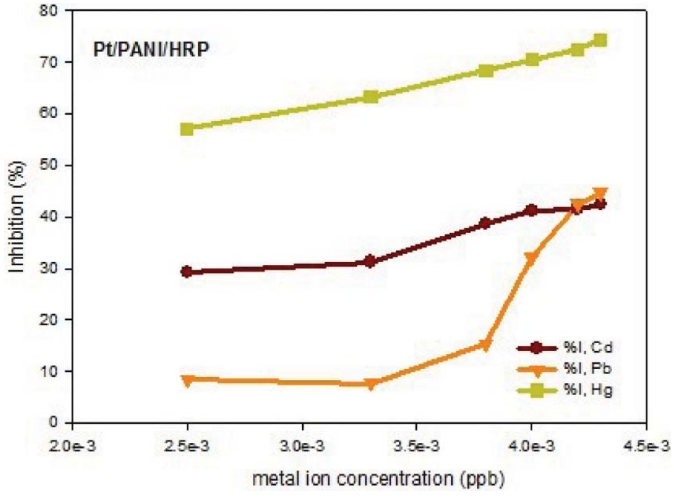

Fig. 3. Results obtained for the inhibition of the Pt/PANI/HRP biosensor in the presence of sequential aliquots of 0.005 ppb of Cd^{2+}, Pb^{2+} and Hg^{2+}, respectively. The individual metal aliquots were added sequentially to a 0.1 M PB (pH = 6.8) solution.

the inhibition studies of the Pt/PANI/HRP biosensor in the presence of sequentially added metal ion solutions.

The method used for the inhibition studies in this report is also referred to as the direct method, since no incubation was involved. The method also included the determination of heavy metal concentration that causes 50% inhibition (IC_{50}), followed by determination of percentage inhibition ($I\%$) using the formula in Eq. 1.[22,26] In Eq. 1 the $I\%$ is the degree of inhibition, wherein I_1 is the steady-state current obtained in buffer solution with no heavy metal ion present, and I_2 is the steady-state current obtained after the biosensor was exposed to sequential additions of the separate heavy metal ions of Cd^{2+}, Pb^{2+}, and Hg^{2+}, respectively.

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

Figures 2 and 3 reports the graphs of percentage inhibition versus inhibitor concentration for Pt/PANI-co-PDTDA/HRP and Pt/PANI/HRP biosensor, respectively, using 0.1 mM as fixed H_2O_2 concentration. Inhibition plots for each of the heavy metals studied (e.g., Cd^{2+}, Pb^{2+}, and Hg^{2+}) were constructed and compared. We found that for the Pt/PANI/HRP biosensor at concentrations higher than 0.004 ppb Cd^{2+}, and Pb^{2+} some interference occurs. The direct method was further employed to establish the metal ion concentration that causes 50% inhibition (IC_{50}).[26]

For Pt/PANI-co-PDTDA/HRP biosensor, the highest inhibition percentages obtained for the individual metal ions were 49.5% for Cd^{2+}, 59% for Pb^{2+} and 78% for Hg^{2+}. For the Pt/PANI/HRP biosensor the highest inhibition percentages obtained for the individual metal ions were 44% for Cd^{2+}, 47% for Pb^{2+} and 73% for Hg^{2+}. The Pt/PANI-HRP biosensor showed increasing inhibition in the concentration range between 0.0005 and 0.0008 μg L^{-1}, with the decreasing inhibition trend observed as $Hg^{2+} > Cd^{2+} > Pb^{2+}$. For the concentrations higher than 0.0008 μg L^{-1}, the inhibition trend observed was $Hg^{2+} > Pb^{2+} > Cd^{2+}$.

Analysis of the results in Figure 2 has also shown that metals were inhibitory in the Pt/PANI-PDTDA/HRP biosensor in order of increasing toxicity, trending as $Hg^{2+} > Pb^{2+} > Cd^{2+}$. These results have clearly indicated the toxicity of the investigated metal ions to HRP as enzyme with Hg^{2+} being the highest inhibitor of these metals tested. The Pt/PANI-co-PDTDA/HRP biosensor has attained the (IC_{50}) values in all of the three metals evaluated at concentrations 49.5% for Cd^{2+}, 59% for Pb^{2+} and 78% for Hg^{2+}, respectively using a direct inhibition method. In the case of the Pt/PANI/HRP biosensor it was difficult to attain the concentration that causes 50% inhibition (IC_{50}) for Cd^{2+} and Pb^{2+} metal ions. In order to establish the type of inhibition involved, Lineweaver-Burk plots (Fig. 4) were constructed and evaluated to ascertain the enzyme activity.

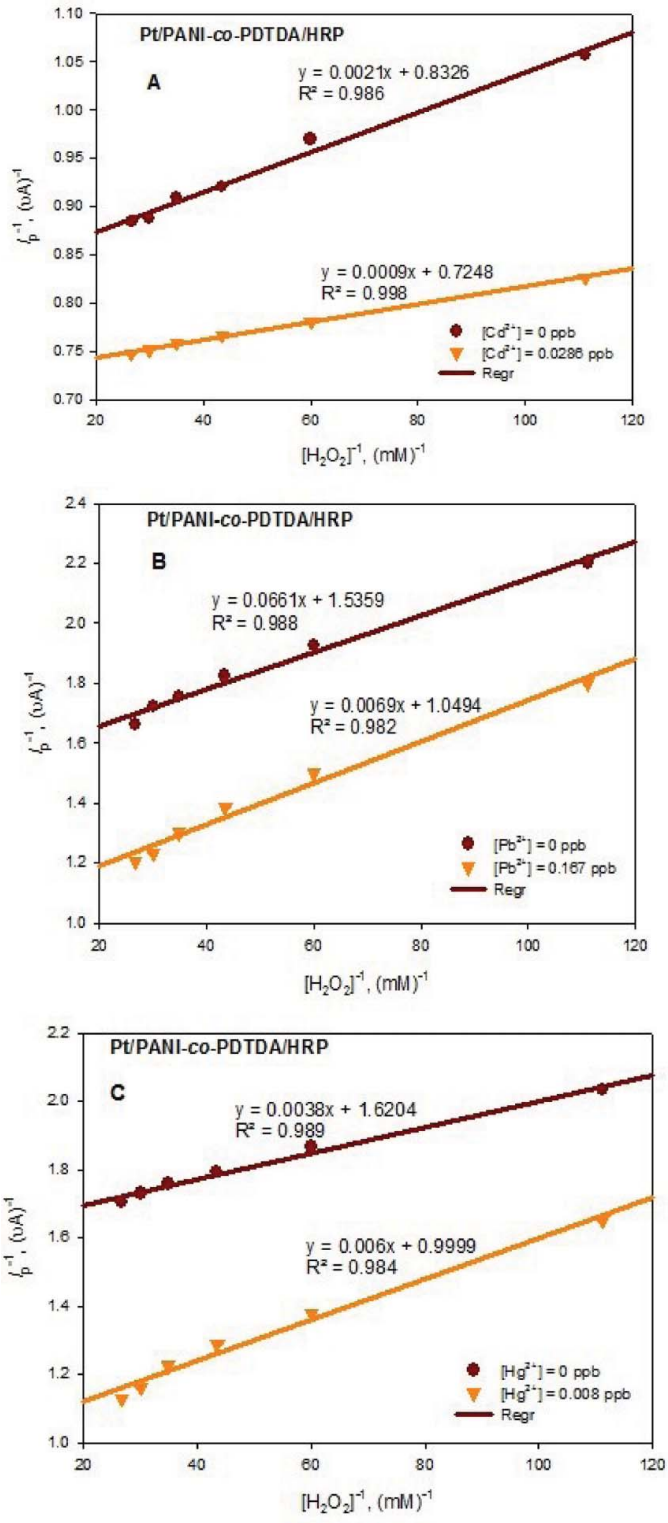

Fig. 4. The Lineweaver-Burk plot results obtained for the Pt/PANI-co-PDTDA/HRP biosensor responses to successive additions of H_2O_2 substrate in the absence and presence of Cd^{2+} in (a), Pb^{2+} in (b) and Hg^{2+} in (c).

Evaluation of biosensor inhibition kinetics

The nature of inhibition of horseradish peroxidase (HRP) activity by heavy metals was predicted from the Lineweaver–Burk plots shown in Figure 4. The graph of current (I_0) was plotted against enzyme concentration $[S]$ and a non-linear curve was obtained. A graph of this type is not useful as the reaction velocity (V_{max}) can never be achieved in practice, thus $[S]$ would theoretically need to reach infinity before the maximum current, I_{max}, is reached. As a consequence, K_m, which is defined as the substrate concentration at half the maximal current, cannot be measured accurately from the graph. To overcome this difficulty, the Michaelis-Menton kinetics at low substrate concentration has been calculated from the electrochemical version of the Lineweaver–Burk equation given in Eq. 2. [12,27]

$$I = \frac{I_{max}[H_2O_2]}{[H_2O_2] + K'_m} \tag{2}$$

This can be simplified to:

$$I = \left(\frac{I_{max}}{K'_m}\right)[H_2O_2] \tag{3}$$

where I is the observed catalytic current after the addition of substrate, $[H_2O_2]$ is the bulk concentration of the substrate and I_{max} is the maximum current measured under saturated substrate condition. The value of the apparent Michaelis–Menten constant, $K^{app}{}_m$, was determined by analysis of the slope and intercept for the plot of the cathodic current (I) versus H_2O_2 concentration.

And when used for the Pt/PANI-co-PDTDA/HRP biosensor,

$$\text{Sensitivity} = \left(\frac{I_{max}}{K'_m}\right) \tag{4}$$

The obtained biosensor data was further used for kinetic studies of the biosensor to determine the type and kinetics of inhibition, as well as to evaluate the affinity of the immobilised HRP towards H_2O_2 catalysis. It is difficult to specify the exact substrate concentration corresponding to $K^{app}{}_m$ from this hyperbolic plot. For this reason, Lineweaver-Burk plots were used since they allow accurate measurements of the values of both $K^{app}{}_m$ and $Vmax$, even at higher substrate concentrations. The Lineweaver-Burk plot indicated several possibilities for the type of inhibition encountered in this study. The $K^{app}{}_m$ values reported for the Pt/PANI/HRP biosensor in this study were in close agreement with the value reported by Yao et al.[28] The results obtained for the Pt/PANI-co-PDTDA/HRP biosensor have shown that the $K^{app}{}_m$ and I_{max} value are 0.7 mM and 0.27 μA, respectively. The $K^{app}{}_m$ for Pt/PANI/HRP was evaluated at 0.6 mM with I_{max} of 1.7 μA, which revealed that that the whole system was controlled by a catalytic kinetic process of the enzyme.

Figure 4 shows a double-reciprocal plot of $\frac{1}{[I_p]}$ vs. $\frac{1}{[H_2O_2]}$. The x-intercept is equal to $\frac{-1}{K_M}$ while the y-intercept is equal to $\frac{1}{V_{max}}$. The slope of the line is equal to $\frac{K_M}{V_{mx}}$. The Lineweaver–Burk plots for the HRP biosensor performance in both biosensors constructed, was used to identify the type of inhibition and the results obtained for the kinetic parameters obtained from the slope and y-intercept values were also calculated. These values were further used to calculate the apparent Michaelis–Menten constants ($K_M{}^{app}$) in the absence and presence of the selected heavy metals as inhibitors.

Analysis of the Lineweaver–Burk plots in Figure 4 indicates that the inhibition obtained for HRP in the presence of the metal ions evaluated in this study, is an indication of uncompetitive inhibition. The Lineweaver-Burk plots obtained for uncompetitive inhibition produces parallel lines when a plot of $\frac{1}{[V]}$ vs. $\frac{1}{[S]}$ is constructed.[29–32] In this study the heavy metals exhibited uncompetitive inhibition for HRP using the H_2O_2 assay, when the plot of $\frac{1}{[I_p]}$ vs. $\frac{1}{[H_2O_2]}$ in Figure 4 is viewed. An uncompetitive mechanism of inhibition implies that an enzyme (HRP in this case) is less catalytically effective in the presence of the metal ions investigated. Further analysis of the plots in Figure 4, also showed that this catalytic inefficiency is not the same for each of the Cd^{2+}, Pb^{2+}, and Hg^{2+} ions evaluated. In the case of the Pb^{2+} ions in Figure 4, this plot is more parallel compared to that obtained for the Cd^{2+} and Hg^{2+} ions.

Comparison between Pt/PANI/HRP and Pt/PANI-co-PDTDA/HRP biosensors

In this section a summary of the comparison of the results obtained for the inhibition kinetics of the Pt/PANI/HRP and Pt/PANI-co-PDTDA/HRP biosensors in the absence and presence of Cd^{2+}, Pb^{2+} and Hg^{2+} as metal ion inhibitors are shown. From the plots in Figure 4, the following kinetic parameter results for $K^{app}{}_m$ and V_{max} were obtained, shown in Table 3.

Comparison and analysis of the results shown in Table 3 indicate a change in apparent values of $K_m{}^{app}$ as well as I_{max} that leads to uncompetitive inhibition for the Pt/PANI-co-PDTDA/HRP biosensor. Furthermore, analysis of the shape of the graphs in Figure 4 shows parallel lines in the absence and presence of inhibitor in all the selected heavy metals (Cd^{2+}, Pb^{2+} and Hg^{2+}) evaluated. The results have also shown that both the $K_m{}^{app}$ and I_{max} values were affected by the addition of the inhibitor, thus yielding the uncompetitive inhibition obtained in both of the sensors. This type of inhibition takes place when an enzyme inhibitor binds exclusively to the complex formed between the enzyme and the substrate (the E-S complex) yielding an inactive enzyme-substrate-inhibitor complex.[32,33]

Table 3. Apparent Michealis-Menten (K_m^{app}) values and I_{max} parameters obtained in the absence of heavy metals and at different concentrations (IC_{50} values) of heavy metals (Cd, Pb and Hg).

	Metal ion inhibitor					
Metal ion	*0 ppb Cd^{2+}*	*0.028 ppb Cd^{2+}*	*0 ppb Pb^{2+}*	*0.167 ppb Pb^{2+}*	*0 ppb Hg^{2+}*	*0.008 ppb Hg^{2+}*
Kinetic parameter			*Slope (uA/mM)*			
PANI-co-PDTDA	2.10×10^{-3}	8.97×10^{-4}	6.6×10^{-2}	6.9×10^{-3}	3.8×10^{-3}	6.0×10^{-3}
PANI	1.4×10^{-2}	1.0×10^{-2}	4.8×10^{-3}	1.3×10^{-2}	4.2×10^{-3}	8.3×10^{-3}
Kinetic parameter			*y-intercept(1/uA)*			
PANI-co-PDTDA	0.833	0.725	1.536	1.049	1.620	1.685
PANI	1.92	1.62	1.03	1.33	1.69	2.75
Kinetic parameter			*K_m^{app} (mM)*			
PANI-co-PDTDA	2.5×10^{-3}	1.2×10^{-3}	4.3×10^{-2}	6.6×10^{-3}	2.3×10^{-3}	6.0×10^{-3}
PANI	7.4×10^{-3}	6.7×10^{-3}	4.7×10^{-3}	9.9×10^{-3}	2.5×10^{-3}	3.0×10^{-3}
Kinetic parameter			*I_{max}*			
PANI-co-PDTDA	120	1.37	0.65	0.95	0.62	1.0
PANI	0.52	0.62	0.96	0.75	0.59	0.36

Results evaluated for both the Pt/PANI-co-PDTDA/HRP and Pt/PANI/HRP biosensors in 0.1 M PB (pH = 7.2; 6.8) solution are shown.

In the case of Pt/PANI/HRP biosensor the results obtained in this study have seen a decrease in I_{max} values and no significant different in K_m^{app} values, which is the indication of non-competitive inhibition. We found that the non-competitive inhibition Cd, Pb and Hg using the Pt/PANI/HRP biosensor is in agreement with the study done by Nomngongo et al.[26] For this study the selected heavy metals have shown relatively small values of K_m^{app}. Hence, the smaller the value of K_m^{app}, the more efficient is the catalyst. The value of K_m^{app} for an enzyme also depends on the particular substrate, the pH of the solution and the temperature at which the reaction is performed.[34–37] For most enzymes K_m^{app} lies between 10^{-1} and 10^{-7} M, depending on the biosensor construction and mediator used. For the results of this study shown in Table 3, the values obtained for K_m^{app} and V_{max} satisfies the aforementioned criteria.

Biosensor performance results

Selective determination of target analyte plays vital role in analytical measurements. To investigate the specificity of the biosensor, we challenged the developed biosensor with other cations and anions that might interfere. Specifically Fe^{2+}, Ni^{2+}, Co^{2+}, sodium (Na^+), sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}) ions were investigated. It is, therefore, necessary to check if the standard addition of one of the metals affects the peak height and therefore the sensitivity of the others. The interfering effect of these cations and anions were studied by mixed method, using the ratio 1:2 for analyte and interferent, respectively. The peaks of heavy metals were monitored and compared to those without any impurities added (figures not shown here).

The results obtained do not cause much decrease in biosensor response. Ten repeat measurement of lowest concentration of H_2O_2 using the same electrode gave a relative standard deviation (RSD) of 3.73%, confirming excellent repeatability. Electrode to electrode reproducibility was also satisfactory. A series of 3 electrodes which were prepared individually gave a RSD of 4, 8% for determination of lowest concentration of H_2O_2 substrate. The storage time of the biosensor was found to be 5–7 days. It can be used to 5 to 7 days for H_2O_2 catalytic responses and only once to measure heavy metal concentrations.

Application of biosensor to real samples

The applicability of the developed biosensor for environmental samples was investigated by analysing tap water and river water with satisfactory recoveries as listed in Table 4. Table 4 shows the analytical results obtained for three independent water samples. The water samples were filtered with quantitative filter papers and spiked with varying concentrations of the selected heavy metals. It was found that the recoveries were in the range 96–132%, which were quantitative assays performed in environmental samples.

The World Health Organisation recommended that the maximum safety level of these metals should not exceed 0.005 mg L^{-1} of Cd^{2+}, 0.01 mg L^{-1} of Pb^{2+} and 0.001 mg L^{-1} of Hg^{2+} in drinking water. Concentrations were determined in μg L^{-1} based on three replicates

Table 4. Results obtained for the recovery studies of heavy metals in water samples, using the Pt/PANI-co-PDTDA/HRP biosensor.

Metal ion	*Added ($\mu g/L$)*	*Found ($\mu g/L$)*	*Recovery (%)*	*RSD ($n = 3$)*
		Tap water		
Pb^{2+}	4.8×10^{-3}	1.1×10^{-2}	104	0.62%
Cd^{2+}	2.5×10^{-3}	4.3×10^{-3}	132	2.11%
Hg^{2+}	2.5×10^{-3}	6.8×10^{-3}	96	4.11%
		River water		
Pb^{2+}	4.8×10^{-3}	1.8×10^{-2}	125	3.82%
Cd^{2+}	2.5×10^{-3}	1.8×10^{-2}	128	3.00%
Hg^{2+}	2.5×10^{-3}	1.2×10^{-2}	112	1.47%

determination. Considering the values of concentration obtained in tap water (Table 4), the tap water was found to be within the allowed maximum contamination level set by WHO.[38] In the case of the river water samples analysed, the results obtained for the Cd^{2+} and Hg^{2+} analysis were within the WHO guideline values. However, the result obtained for the Pb^{2+} analysis, showed the samples exceeding the recommended WHO guideline value. These results therefore confirmed the applicability and sensitivity of the constructed Pt/PANI-co-PDTDA/HRP biosensor for the screening of environmental samples.

Conclusions

The method developed in this study seems very promising as the novel Pt/PANI-co-PDTDA/HRP biosensor constructed in this study was able to the electroanalysis of Cd^{2+}, Pb^{2+} and Hg^{2+} as metal ions. Furthermore, the biosensor was able to obtain IC_{50} values for Cd^{2+}, Pb^{2+} and Hg^{2+} as metal ion inhibitors during inhibition studies. Conducting polymers can play a significant function in adsorption of biomolecules in biosensor construction due to their unique properties, which make them appealing alternatives for specific materials and applications. Our results have also shown the first-time application of the PANI-co-PDTDA conducting co-polymer in the successful immobilisation of HRP on the platinum electrode to enhance the electrochemistry of HRP.

The newly developed biosensor showed excellent electrocatalytic activity for the reduction of H_2O_2 in PB (pH = 7.2) solution and for further inhibition studies of the selected heavy metals. The inhibition results obtained for the Cd^{2+}, Pb^{2+} and Hg^{2+} metal ions were found to be either a non-competitive for the Pt/PANI-co-PDTDA/HRP biosensor and uncompetitive for the Pt/PANI/HRP biosensors, respectively. These results also suggested that the sensitivity of the biosensor can be used for the determination of the concentration of the selected heavy metals. For the Pt/PANI-co-PDTDA/HRP biosensor a detection limit of 8.59×10^{-4} mM was obtained using the equation, $LOD = \frac{3 \times SD}{m}$, where s is the standard deviation and m is the slope of the calibration plot.

The repeatability in the measurement, expressed as relative standard deviation (R.S.D), was 3.73%, obtained by recording lowest concentration of H_2O_2 through 10 successive experiments, while for the Pt/PANI/HRP biosensor a detection limit of 0.032 mM was achieved with a relative standard deviation of 14% under DPV conditions. The detection limits were found to be 8×10^{-4} $\mu g\ L^{-1}$ for cadmium, 9.38×10^{-4} $\mu g\ L^{-1}$ for lead and 7.89×10^{-4} $\mu g\ L^{-1}$ for mercury. The Hg^{2+} ions were noted to have higher degree of enzyme inhibition with a value of 73% and 84% for Pt/PANI/HRP and Pt/PANI-PDTDA/HRP biosensors, respectively. The sequence of inhibition to H_2O_2 activity was $Hg^{2+} > Pb^{2+} > Cd^{2+}$.

This study has further demonstrated that the newly constructed Pt/PANI-PDTDA/HRP biosensor can be successfully applied for the determination of Cd^{2+}, Pb^{2+} and Hg^{2+} metal ions in environmental samples. The World Health Organisation recommended that the maximum safety level of these metals should not exceed 0.005 mg L^{-1} of Cd^{2+}, 0.01 mg L^{-1} of Pb^{2+} and 0.001 mg L^{-1} of Hg^{2+}, respectively. The analytical and detection data for the metals investigated were observed to be lower than concentrations recommended by several bodies including World Health Organisation and Environmental Protection Agencies. Therefore the biosensors developed in this study can be used to screen the presence of these metals in water samples because of its low detection limit.

Funding

This study was financially supported by the Water Research Commission (WRC), Council for Scientific and Industrial Research (CSIR) and the National Research Foundation (NRF) of South Africa. The authors also acknowledge the support from the Sensor Lab, Chemistry Department, University of the Western Cape, Bellville.

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