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Development of Amperometric Enzyme Biosensor for Determination of Heavy Metal Ions in aqueous samples

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ABSTRACT

An amperometric urease inhibition-based biosensor was developed to detect Pb^{2+} and Cr^{3+} ions in water matrix. The modified GCE/ZnO/urease electrode was developed by immobilizing ZnO nanoparticles and urease using gelatin and glutaraldehyde as crosslinking agent. With urea as a substrate, ZnO catalytic activity was examined through cyclic voltammetry. Working conditions of the biosensing system were optimized in terms of current generated in the presence of substrate (urea) as a function of different variables. Optimal sensor activity (current response) was obtained with a urease loading of 4 µl and ZnONPs concentration of 1.5 mg/L. For solution parameters, optimum pH was in the range of 7.0-7.5, optimum temperature was 35°C, optimal substrate concentration 40 µl. Evaluation of some key biosensing properties revealed relatively stable biosensor activity within the first 7 days, good reproducibility with an RSD of 3.61%. Limits of detection (LOD) for the biosensor ranged from 0.04 - 0.07 while limits of quantitation (LOQ) ranged from 0.22- 0.13 ppm. Applying the developed biosensor to real samples was evaluated by analysing water samples from Kasuwangada River in Mubi North Local Government Area. The system showed good percentage recovery of the added heavy metal standard (0.05 – 0.25 ppm). Percentage recovery within the acceptable range of 98.00% to 103.00% was observed for Pb^{2+}, 92.00% - 102.00 % recovery for Cr^{3+}. Analysis of real samples and assessment of obtained results against a standard method (AAS) showed minimal differences. The developed biosensor showed sufficient sensitivity to the analysed sample and gave quantitative information on the Pb^{2+} and Cr^{3+}content of the sample.

Keywords: Biosensor, Heavy Metals, Urease enzymes, Inhibition studies, Enzyme activity

INTRODUCTION

The accumulation of toxic substances in the environment continuously increases due to diverse pollutants from the industries. Though heavy metals and their ions are naturally ubiquitous, however, the major environmental concern is their occurrence in the environment due to the dispersal of industrial and urban wastes generated by human activities [1]. Controlled and uncontrolled disposal of waste, accidental and process spillage, mining and smelting of metalliferous ores, sewage sludge application to agricultural soils are responsible for the migration of contaminants into non-contaminated sites as dust or leachate and contribute towards contamination of our ecosystem. Heavy metals are highly toxic and tend to remain indefinitely in the environment due to their non-biodegradable nature. According to the 1999 U.S. Environmental Protection Agency (USEPA), Agency for Toxic Substances and Disease Registry Hazardous Substances List, metals account for five of the top twenty hazardous substances, including arsenic (1), lead (2), mercury (3), cadmium (7), and chromium (16). Heavy metal contamination in the natural water environment is particularly of more concern because of the serious threat it poses to human health and the entire ecosystem, [2]. Today, the significance of monitoring various samples for quality assurance, risk assessment, or, more seriously, disease diagnosis cannot be overstated. Several analytical techniques, both simple and sophisticated, have made it possible to achieve the same goal and, as a result, raise human living standards. Although Powerful analytical methods/techniques, such as atomic absorption spectroscopy (AAS), inductively coupled plasma (ICP), Mass spectrometry, Neutron Activation Analysis (NAA), Differential Pulse Polarography, Electrochemical Metal Analyzer and other techniques are widely used for the analysis of heavy metal ions, [3] these techniques exhibit high

sensitivity, selectivity, reliability, and accuracy, but require sophisticated instrumentation inadequate for use outside the laboratory, skilled personnel, complicated sample collection, pretreatment (pre-concentration), and a long measuring period. Consequently, there is an aspiration for portable, reliable, fast, and relatively inexpensive detection techniques. With the advent of biosensor technology, such aspirations have begun to come to fruition. A biosensor that combines the exquisite selectivity of a biological component with the processing power of a transducer presented an attractive choice to the classical methods, due to their less complex instrumentation, minimal operator training, and shorter measuring period for onsite detection of toxic and potential hazardous substances [4].

This work is therefore, aimed at developing an amperometric enzyme biosensor for onsite determination of heavy metal ions in aqueous sample using velvel beans *(mucuna pruriens)* urease enzyme as biorecognition element. The principle in biosensor application is that when target analytes interact with the complementary biorecognition layer, which is either integrated with or intimately associated with a physicochemical transducer, it can rapidly generate a measurable signal. The specific interaction of the immobilized capture reagents with the target analytes causes a physicochemical change in the reaction cell. These changes can be measured and output as a digital reading via the transducer which serves to transfer the output signal generated by biochemical reaction to electrical signal, which can be amplified and processed by appropriate equipment to provide quantitative or semi-quantitative information about the analytes [5].

MATERIALS AND METHOD

Chemicals and Reagents

All reagents used are of analytical grade and were used without further purification these include among others; potasium dihydrogen phosphate ($KH_2PO_4.2H_2O$), potasium hydrogen phosphate ($KHPO_4.2H_2O$), Zinc acetate, glutaraldehyde, Gelatin, Urea, Nesler reagent, Chromium (III) standard solution (1000 ppm), Cd standard solution (1000 ppm), $Pb(NO_3).3H_2O$, Ethanol, Absolute alcohol, $Pb(NO_3).6H_2O$, Ammonium sulphate, Aluminum oxide, Felt paper, Deionized water, Nitrogen gas, KCl, potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$) and potassium hexacyanoferrate (II) trihydrate ($K_4Fe(CN)_6{\cdot}3H_2O$).

Equipment

Electrochemical work station (Autolab), Metrohm dropsens with NOVA Scientific soft ware, Screen Printed Electrodes (Metrohm dropsens Ref-C11L), Glassy Carbon Electrode (GCE), Uv-Visible spectrophotometer Janway model 7315, AAS, Sonicator, pH meter, FT-IR spectrometer, Ultrasonic water bath, Refrigerated centrifuge Hermle Labnet Z 233 MK, Hot plate, weighing balance, X-ray diffractometer, scanning electron microscope, Magnetic stirrer, Votex mixer Clever scientific limited XH-C.

METHOD

Extraction of Bio-recognition Element (urease)

Urease was extracted from velvet beans seeds according to a slightly modified method of [6]. Ten grams of powdered seeds were soaked in 100 ml extraction buffer (0.2 M phosphate buffer pH 8) and incubated at -4°C for 3 – 4 hours. The mixture was sieved through four layers of muslin cloth and the resulting filtrate was centrifuged at 6000 rpm for 15 mm. The clear supernatant was collected and used as the crude urease extract while the pellets was discarded. Determination of enzyme activity and kinetic parameters was conducted in our previous work.

Preparation of gelatin gel and Enzyme immobilization (Bioactive Elements)

Two sets of bio-active elements and one set of blank elements was prepared by entrapment in gelatin gel matrix according to the method described by [7, 8].

1. Biorecognition Element Containing urease (only)
2. Biorecognition Elements Containing Urease and ZnO Nanoparticles
3. Blank components

Gelatin (0.6 g) was dissolved in 10 ml of 50 mM phosphate buffer (pH 7.5) by heating at 50° C with continuous stirring for 5 min. to obtain clear solution. This solution was cooled and the solidified mixture was stored at 4 C°. Prior to immobilization, the mixture was heated to 60 C° and then slowly brought to 27 C° in order to obtain a clear solution.

A clear solution of gelatin (60 mg/ml), 100 µL of urease enzyme and 0.6% (v/v) glutaraldehyde was mixed together and stirred constantly at 27 C° this mixture was drop-casted on to the ZnONPs embedded WE surface and air dried for 30 min. The enzyme electrode was then immersed in a phosphate buffer and kept at 4 °C in a refrigerator for 8 h for complete cross-linking. The immobilized enzyme film was washed thoroughly with 50 mM phosphate buffer

(pH 7.5) to remove any unbound enzyme prior to use [1, 9, 8, 7].

Electrochemical Characterization of immobilized Bioactive Components

Electrochemical behavior of the immobilized enzyme on glassy carbon electrode (GCE) was investigated according to the method described by [9, 7]. The bare glassy carbon electrode was polished with Al_2O_3 powder (0.05 µm), and then ultrasonically washed with 1:1 nitric acid solution, ethanol 70% (v/v), and distilled water successively. The glassy carbon electrode was scanned and activated to stability by cyclic voltammetry (CV) in 0.5 M sulfuric acid solution at 100 mV^{-S} scan rates. The activated glassy carbon electrode was naturally dried at room temperature for later use. The CV technique was used to study the electrochemical behaver of different modified electrodes in 5 M Ferri/Ferro solution ($[Fe(CN)6]^{3-/4-}$), 1M KCl at 50 mV^{-s}.

Optimization of parameters for Biosensor measurements

Optimization of Urease Concentration in the Biorecognition Matrix

Optimization of the biosensor response as a function of urease concentration in the biorecognition component was done by taking biosensor measurements with different concentrations of urease (1-6 U/ml) in the biosensing matrix [10].

Optimization of ZnO Nanoparticles Concentration in the Biorecognition Matrix

Optimization of ZnO Concentration in biosensing matrix was done by taking biosensor responses as a function of varying concentration of the nanoparticles *(0.05 — 2.5 mg/mL)* incorporated in the sensing platforms [10].

Effect of pH on Biosensor Response

The effect of pH on biosensor response was investigated with a view to establishing the optimal working pH. Assay buffer solutions with varying pH (6.0 — 9.0) were used to take biosensor measurements while keeping all other parameters constant [10].

Effect of Temperature on Biosensor Response

The effect of temperature on biosensor activity was determined over a temperature range of 20 °C to 55 °C under the standard conditions of the assay. Temperature adjustments was made by placing the reaction vessel in water bath while monitoring the temperature of reaction media with a thermometer [10].

Effect of Contact Time with Heavy Metal Ions

The effect of contact time on urease inhibition efficiency of the metals Fe^{2+}, Pb^{2+}, Cr^{3+} and Cd^{2+} were tested. The respective metal 'solutions at 1 mg/L concentration was pre-incubated with the biorecognition elements at different time durations ranging from 2 — 20 minutes after which their amperometric responses was taken under the standard assay conditions [11].

Determination of Heavy metals with Biosensor

Inhibition Measurement procedure

Biosensor measurements was done under the pre-determined optimum conditions as described by [9, 8]. In the first step of the test procedure, a biorecognition element was immersed in the assay cocktail (3 ml assay buffer and 2 ml urea) and the amperometric response of the system after 6 mins was recorded as Io. In the second step, a fresh sensing component was immersed in the test solution (containing 20 ml assay buffer and 10 ml metal solution or sample) and left for 4 mins (contact time) to allow sufficient interaction between metal ions and bio-active element. After the 4 minutes contact period, 2 mL of urea solution was added to the reaction media and left for another 2 minutes after which the amperometric response of the system was measured and recorded as Ii. The level of inhibition for each test solution was calculated using the relationship:

$$Inh\,(\%) = \frac{Io - Ii}{Io} \times 100$$

Biosensor Calibration Curves for Determination of Heavy Metals

Standard solutions of the heavy metal ions; Fe^{2+}, Pb^{2+}, Cr^{3+} and Cd^{2+} and mixed metal standards with concentration ranging from 0.05–0.25 ppm were subjected to biosensor measurement. The level of inhibition due to the action of each metal concentration tested was obtained accordingly and the standard calibration curves of current response against concentration were plotted for the single metals and mixed metal standards [9, 8, 12].

Biosensor performance characteristics

Biosensor Storage Stability/Lifetime

Biosensor storage stability was investigated by taking biosensor amperometric responses to urea daily for a period of 20 days under the established optimal assay conditions. The Sensing elements was stored at -4°C (refrigeration temperature) between measurements [12, 13].

Evaluation of Biosensor Reproducibility

Biosensor reproducibility was evaluated by separately preparing five biorecognition elements under similar conditions and taking their respective amperometric responses to the substrate under optimal assay conditions. The relative standard deviation (RSD) for the obtained data (n = 5) was calculated as a measure of biosensor reproducibility [12, 13].

Repeatability of measurements (Precision)

The precision (repeatability) of biosensor measurements was investigated by taking 5 replicate measurements for 1 mg/L concentration of all the heavy metals investigated. The relative standard deviations (RSDs) for the measurements were taken as a measure of repeatability [12, 13].

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limits of detection (LOD) of the biosensor towards each of the investigated metals and the multi-metal systems were determined as 3 x Standard deviation (SD) of lowest concentration sample/slope of the calibration line while limits of quantitation (LOQ) was estimated as 10 x SD of low concentration sample/slope [9, 13].

Application of the Biosensor to Real Samples

Sample Collection

Water sample was collected from Kasuwan gada river (Mubi metropolis).Water sample was collected by the grab method; at the sampling site, the sample was collected by lowering 1 litre pre-cleaned plastic bottles into the water body at a depth of approximately 30 cm and allowing it to fill and overflow before withdrawing the bottles.

Analysis of Water Sample with the Developed Biosensor

Validation of Biosensor Analytical Performance

In order to evaluate the analytical performance of the biosensor, recovery studies were performed with the biosensor. Known concentration of the metal ion solutions was added to samples of water from Kasuwan gada river and analyzed with the biosensor (recovery studies). The percentage recovery of each of the added metal solution was calculated [9, 12]. The determination of heavy metals to tap water samples was achieved using the standard addition method [9, 12]. The pH of the tap water samples was first measured before the analysis was carried out. The tap water sample (10 mL) was spiked with 0.1ppm of each metal solution (Fe^{2+}, Pb^{2+}, Cr^{3+} and Cd^{2+}) followed by amperometric measurements. The water grab samples were analysed with biosensor as described in 3.2.7.1.1 above and the concentration of each sample was estimated.

Accuracy of measurements

Accuracy of measurements was evaluated for all the investigated samples with respect to AAS results (as accepted values). It was obtained quantitatively in terms of percentage error: % Error = [(Accepted value — Measured value) / Accepted values] x 100 % [12, 13].

Results and Discussion

Electrochemical Behavior of Different Modified Electrodes (Response strengths)

Cyclic voltammetry

Cyclic voltammetry is a powerful and popular electrochemical technique commonly employed to investigate the redox processes of molecular species and also to study the electron transfer-initiated chemical reactions. The CV technique was used to analyze the electrochemical behavior of different modified electrodes [9, 7]. Figure 1 showed a comparative analysis of CV curves obtained at Bare-GCE (green), GCE/Urease (red), GCE/ZnONPs (blue) and GCE/Urease/ZnONPs (black) at a fixed scan-rate of 50 mVs^{-1} in 5 mM $[Fe(CN)6]^{3-/4-}$ containing 0.1 M KCl solution pH 7.0. Bare GCE (green) showed no redox peak confirming the low charge transport phenomenon without modification of the GCE while the modified electrodes exhibited well defined redox peaks at 50 mVs^{-1} vs GCE. A pair of well- defined redox peaks was observed when GCE modified with urease (red) was measured which was assigned to the reversible redox catalytic behavior of urease enzymes, similarly, ZnONPs on the surface of GCE using drop-dry method (Blue), showed excellent conductivity and accelerate electron transfer. ZnONPs has stable chemical properties and excellent redox reversibility, which can react with the active

center of biological enzymes and rapidly improve the density of response current [7], as expected, significant increase in redox peaks was also observed after the immobilization of ZnO and Urease onto the GCE surface through drop-dry attachment (Black), indicating a successful immobilization and enhanced activity of ZnONP as seen in cyclic voltammogram of GCE/ZnONP/Urease electrode. Considering the increased current response and enhanced electron transfer of GCE/ZnONP/Urease electrode over GCE/Urease, GCE/ZnONP/Urease was used for further studies. Similar phenomenon was exhibited for a highly sensitive uric acid electrochemical biosensor based on a nano-cuprous oxide/ferrocene/uricase modified glassy carbon electrode as reported by [7].

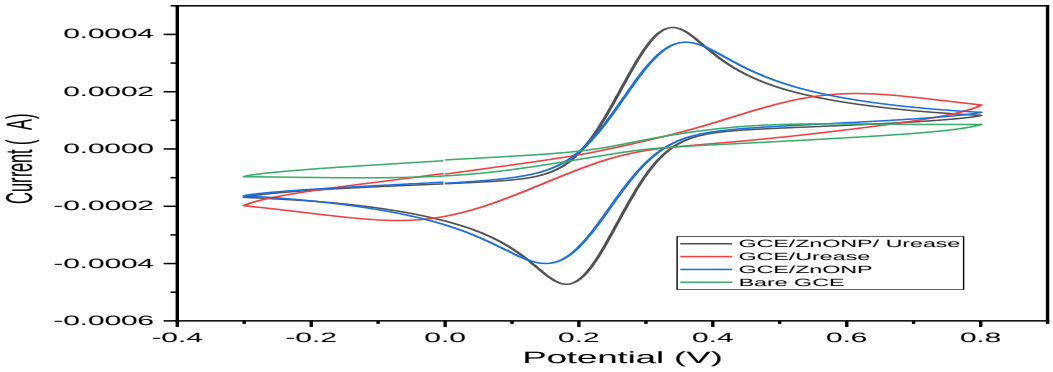

Fig. 1 Cyclic voltamogram of different modified GCE in 5 mM [Fe(CN)6] $^{3-/4-}$ containing 1.0 M KCl at a scan rate of 50 mV^{-s}

Response time of nano enhanced and non-enhanced biosensors

Fig. 2. showed the compared response times for the urease/ZnO biosensor and the urease (only) biosensors. The results revealed a marked decrease (about 4 minutes) in response time when ZnO nanoparticles were added into the biosensor membrane. Highest biosensor response 80.33 µA at 6 minutes was obtained for the biosensor membranes with zinc oxide nanoparticles against 72.67 µA at 10 minutes recorded for the membranes without zinc oxide nanoparticles enhancement. The zinc oxide nanoparticles due to their large surface area and the high surface free energy enhances the activity of the immobilized enzyme by causing rapid contact between the enzyme and its substrate. The nanoparticles also reduce mass-transfer limitations between the reactants and products and provide an environment favorable for promoting electron transfer to the electrodes [14]. The reasons for this trend are similar to those given for the improved signal response (Cyclic voltammograms); ZnO nanoparticles facilitate the electrical communication between the immobilized enzyme and the bulk solution thereby causing the transduction process of the system easier hence, a shorter response time is achieved [9, 14, 7].

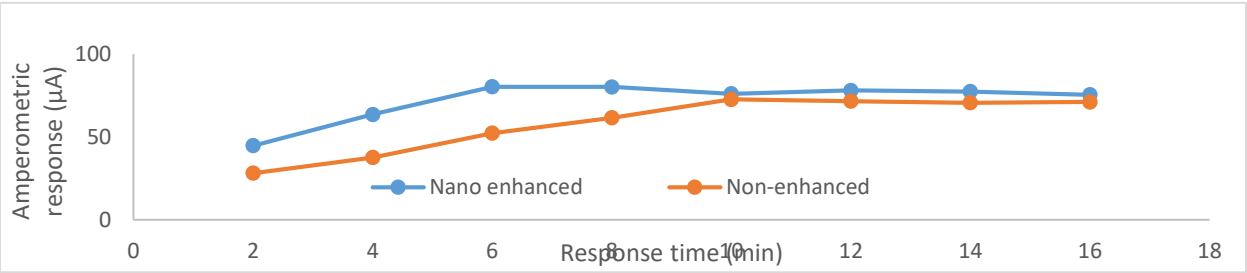

Figure 2 response time of nano enhanced and non-enhanced biosensors

Effect of Urease enzymes concentration in biosensor membrane (enzyme loading)

The effect of urease enzyme loading in biosensor membrane on the amperometric response of the biosensor is depicted on Figure 3. It can be seen that the current response increased significantly from 35.41 µA at 1µL enzyme concentration on the biosensor membrane to a maximum current of 69.21 µA at enzyme concentration of 4.0 µL on the biosensor membrane. Urease enzyme concentration above 4.0 µL indicates significant decrease in current response. [15] also reported 4.0 U/mol of glycine max urease enzyme concentration in biosensor membrane for conductometric enzyme biosensor. The amount of bio-recognition element loaded in a biosensor membrane is a very important factor which determines the performance of the system. Typically, the rate of the enzymatic reaction is directly proportional to the enzyme loading. However, there are several conditions under which this proportionality may not occur. Both low and high enzyme loading have been reported to adversely affect the performance of a biosensor [16, 14]. It was observed that the response decreased above 4.0 µL concentration this leads to the conclusion that at very high urease concentration, enzymatic reactions only occur at the border of the sensing components and many substrate molecules are prevented from diffusing into the sensing elements and further reacting with the enzymes situated within the gel matrix. As a consequence, a low signal is recorded on the other hand, enzyme loading less than 4.0 µL also showed low response. This is because such low concentrations would only be sufficient to convert few substrate molecules to products. [6,17] reported the same conclusions for pure enzyme membranes and for enzymes in algal membranes respectively. Another possible reason for low biosensor responses when large amounts of enzymes are used is the fact that high enzyme loading results in the blocking of the enzyme active sites, especially for enzyme that is located far from the surface of the membrane, thus making some of the enzyme not participating in enzymatic reactions [15]. The optimised enzyme loading of 4.0 µL was used throughout the investigations.

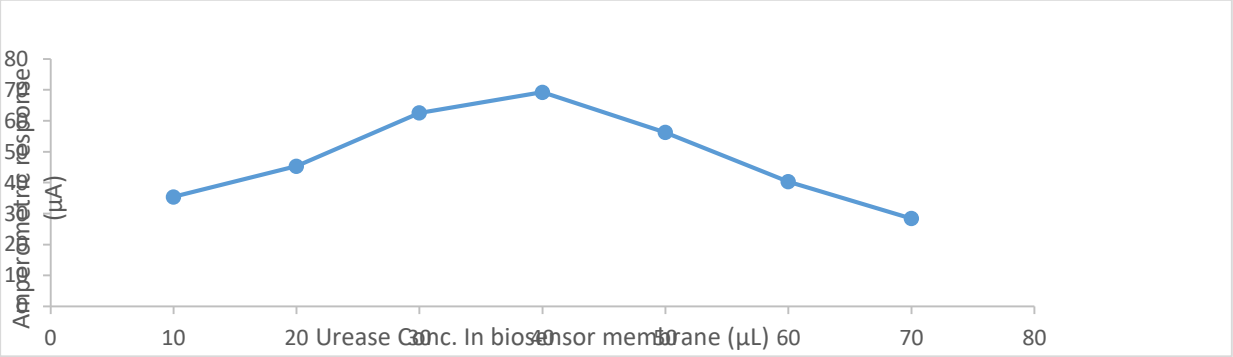

Figure 3 Effect of urease enzyme concentration (enzyme loading) on biosensor response

Effect of ZnO nanoparticles concentration in biosensor membrane on biosensor response

Biosensor response as a function of the amount of ZnO nanoparticles incorporated onto the biosensing platform was investigated and optimized. Fig. 4. showed the effect of ZnO nanoparticles concentration on biosensor response. The Biosensor amperometric response increased with increasing ZnONPs concentration and maximum response was reached 78.64 µA at 1.5 mg/mL. Further increase in the amount of ZnO nanoparticles resulted in decreased biosensor response. Low biosensor response in the presence of high concentration of ZnO nanoparticles has been ascribed to an obstruction of diffusion of the reactants and products [7]. Also, a study by [18] opined that excess nanoparticles concentration may result in brittle and cracked biosensor membrane with resultant loss of bioactivity. Although the optimum loading of nanoparticles was 1.5 mg/mL, the minimum amount that caused a significantly high response (1.0 mg/mL) was selected for this study in order to reduce possible interferences from the nanoparticles to the barest minimum.

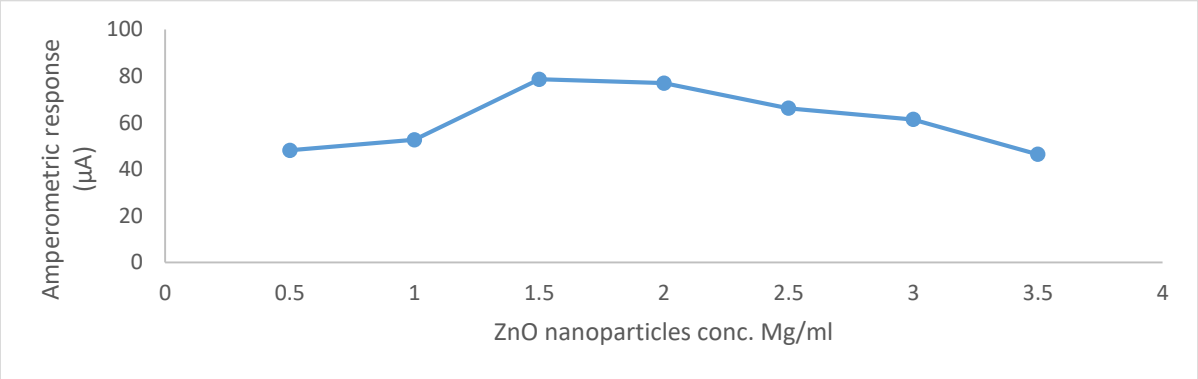

Figure 4. Effect of ZnONPs concentration on biosensor response

Optimization of Substrate concentration in Biosensor Membrane

Fig. 5. showed the effect of varying substrate concentrations on biosensor response. Theoretically, optimal value of substrate concentration is the point of enzyme saturation with the substrate where every enzyme molecule is maximally involved in the process of substrate transformation into the final product [6]. The biosensor enzyme activity followed a classical Michaelis-Menten type behaviour similar to what was observed for the free enzyme. A steady increase in biosensor response was observed up to a substrate concentration of 40.00 µL. Above this concentration, no significant change in the biosensor activity was observed indicating that all the accessible enzyme active sites have been saturated with the substrate. 40.00 µL was selected as optimal substrate concentration and further assays were carried out with this concentration. Optimal urea concentration range between 2.5 to 200 mM have been reported for various urease based biosensors [9, 19, 15]. The value reported in this study is within the range that has been previously reported.

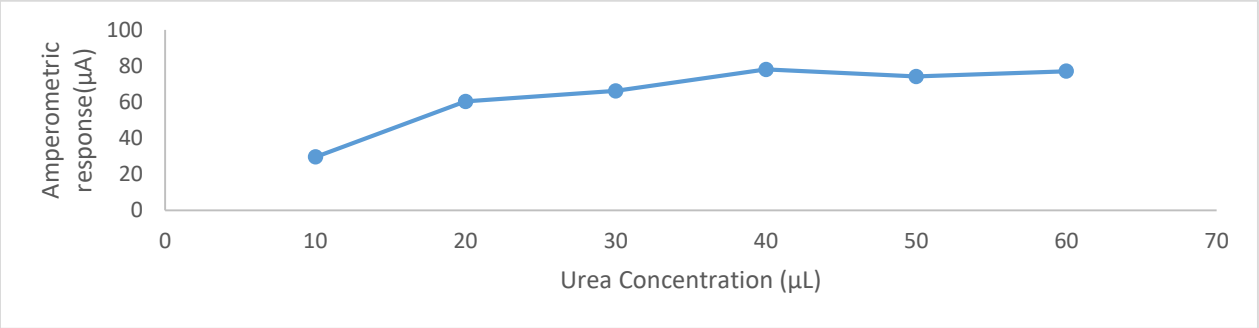

Figure 5. Effect of substrate (urea) concentration on biosensor response

Effect of solution pH on biosensor response

Figure 6. presents the effect of solution pH on biosensor response (Pt/ZnO/urease vs Ag/AgCl) studied over a range of pH 6.5-9.0. Similar to free enzymes in solution, immobilized enzymes also showed maximum and relatively constant signal within the range of 7 - 7.5 after which further increase of pH resulted in rapid decreased responses of the biosensor signal. Enzyme-based biosensors are generally affected by pH because of its effects on the structure and activity of most enzymes [20]. Similar explanations provided for the effect of solution pH on the enzyme activity of free un-immobilized enzymes have been reported my researchers on immobilized enzymes on transducers. pH is known to affect the state of ionization of acidic or basic amino acids (Acidic amino acids have carboxyl functional groups in their side chains while basic amino acids have amine functional groups in their side chains). If the state of ionization of amino acids in a protein is altered, the ionic bonds that help to determine the 3-D shape of the protein would be affected and this can lead to altered protein recognition or inactivation of enzymes. Changes in pH may not only affect the shape of an enzyme but may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot

undergo catalysis [14, 21, 12]. Extremely high or low pH values generally result in complete loss of activity for most enzymes. There is usually a pH of optimal response for enzyme biosensors; ours in this study appears to be from 7.0 - 7.5.

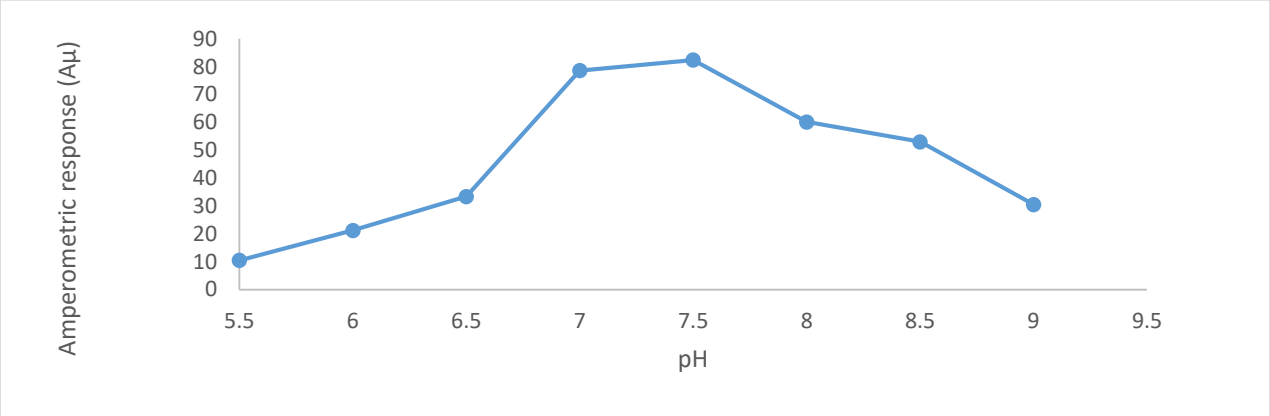

Figure 6. Effect of solution pH on biosensor response

Effect of temperature on biosensor response

Figure 7 depicts the effect of temperature on biosensor signal response. The response of the biosensor (Pt/ZnO/urease vs Ag/AgCl) increased with increasing temperature from 25 to 35°C. There was a slight decrease in the response from 35 to 40°C and a drastic drop (56.42%) in the response was observed at 45°C. Like most chemical reactions, the activities of enzyme-based systems increase with increase in temperature. However, very high temperatures can cause decreased reaction due enzyme denaturation. Loss of biosensor activity at high temperatures can also be attributed to disruption of membrane structure and subsequent leaching of the bio-recognition elements or products of the reaction. Although 35°C was determined to be the optimum temperature for the biosensor activity, all experiments were performed at 30°C in order to minimize the problem of evaporative losses during the course of the reaction and also maintain the integrity of the gel matrix. Our findings here are in agreement with values that have been reported for urease-based biosensors; Studies by [18, 12]. [7] showed maximum biosensor response at 37°C.

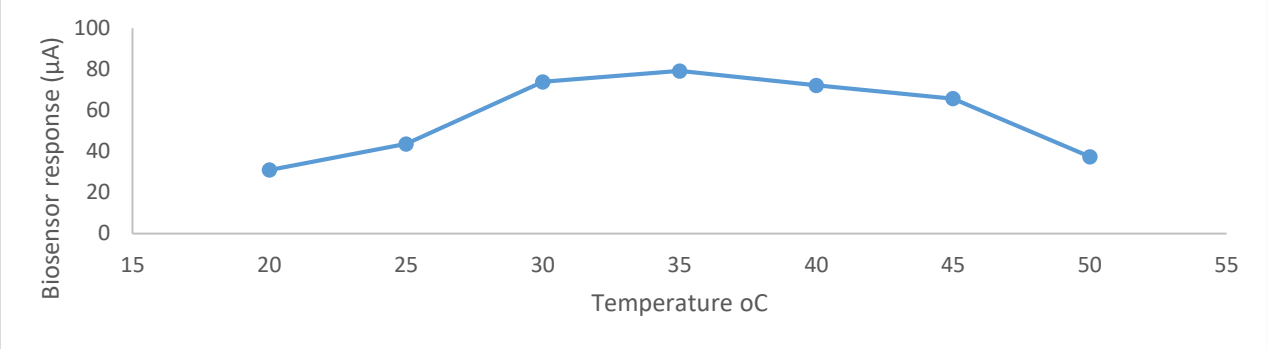

Figure 7. Effect of temperature on biosensor response

Effect of storage time on biosensor response

The storage stability/life-time of the developed biosensor (Pt/ZnO/urease vs Ag/AgCl) is presented in Fig. 8. Two types of stability are most relevant in biosensor development: storage stability and operational stability. The latte is related to the ability of the biosensor enzymes to maintain their activity during use while the former is concerned with biosensor lifetime under storage (shelf life). Only the storage stability of the biosensor is considered in this study because the investigated sensor is a single use type. The biosensor maintained a relatively stable activity within the first 7 days. About 21% loss in activity were recorded on the ninth day. The decline in biosensor activity continued steadily and by 14th

day, only about 58.82% of activity was obtained. Almost total loss of activity was observed on the 20th day. The storage lifetime of the studied biosensor is therefore, around 6 days. Several reports have shown that urease based biosensors can remain stable between three to twelve weeks [18, 9, 15, 12]. It appears therefore that our present biosensor has poor storage stability/life-time. This trend suggests that gelatin immobilization method employed in this study is highly prone to loss of enzyme activity.

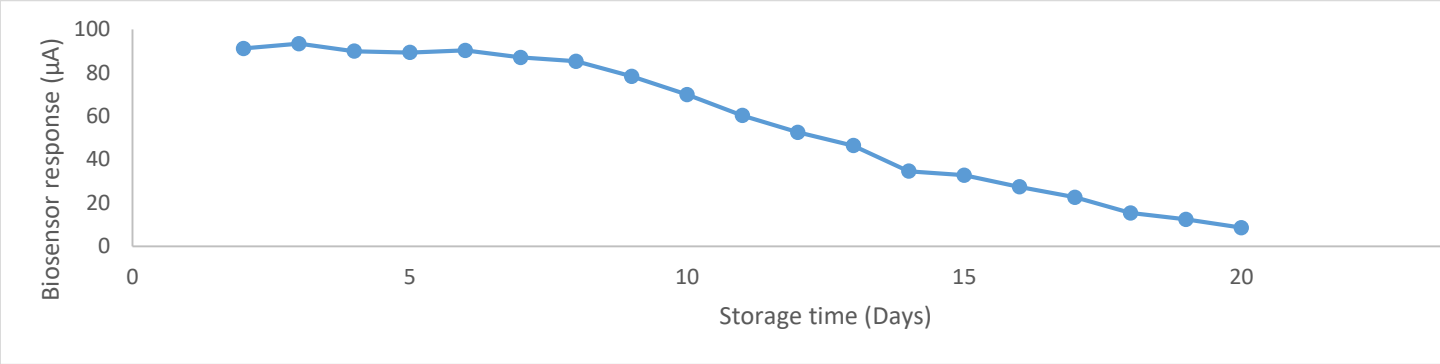

Figure 8: Effect of storage time on biosensor response

Biosensor reproducibility

Reproducibility of biosensor for five (5) independently prepared biorecognition elements (Pt/ZnO/urease vs Ag/AgCl) is depicted in Figure 9. The sensors exhibited good reproducibility with a relative standard deviation (RSD) of 3.61%. Similar urease based biosensors reported in literature showed varying reproducibility trends; a study by [15] showed a relative standard deviation of 4.33%. An RSD of about 3.84% and 4.11% in reproducibility have been reported for two different biosensors based on adsorbed urease and urease immobilized in GA vapour [9, 22]. Biosensor reproducibility with a relative standard deviation of 2.6% and 5.06% has also been reported [12, 23]. A RSD of less than 10% is universally regarded as acceptable for most analytical purposes therefore; the 3.61% showcased in this study indicates acceptable reproducibility.

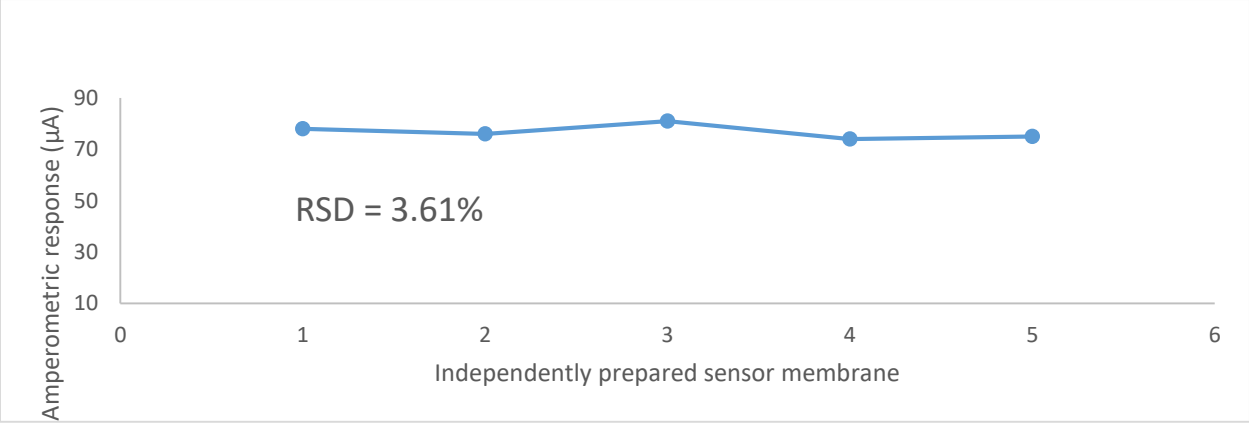

Figure 9. Biosensor reproducibility for five independently prepared biosensor elements

Effect of incubation time with heavy metals

Effect of contact (Incubation) time was investigated for Pb^{2+} and Cr^{3+} metal solutions on Pt/ZnO/urease vs Ag/AgCl at 1.5 mg/L concentration. The result showed that percentage of inhibition increased with incubation time for the investigated metals until maximum inhibition was reached Figure 10. The reason for increased inhibition with time is due to the fact that the longer the contact time, the more the interaction between inhibitor metal and enzyme. However, when the incubation time is insufficient, the enzymatic activity is not totally inhibited and low concentrations of the inhibitors may not be detected. On the other hand, longer exposure time to the inhibitor solution may lead to damage in the structure and the properties of the enzyme [9, 15]. The incubation time must therefore be sufficient to give an

appreciable inhibition but not too long to result in damage to enzyme structure. Long incubation time also means a longer analysis time and a shorter lifetime of a biosensor. 5-6 minutes incubation time was chosen for all the investigated metals. This was selected as a compromise between the percentage of inhibition, analysis time and lifetime of the biosensor. Moreover, the inhibition levels obtained after 5 minute incubation period is considered sufficient because it is higher than 10% and up to 88% of the maximum obtainable inhibition. This is usually taken as an informative indicator of sufficient inhibition [15].

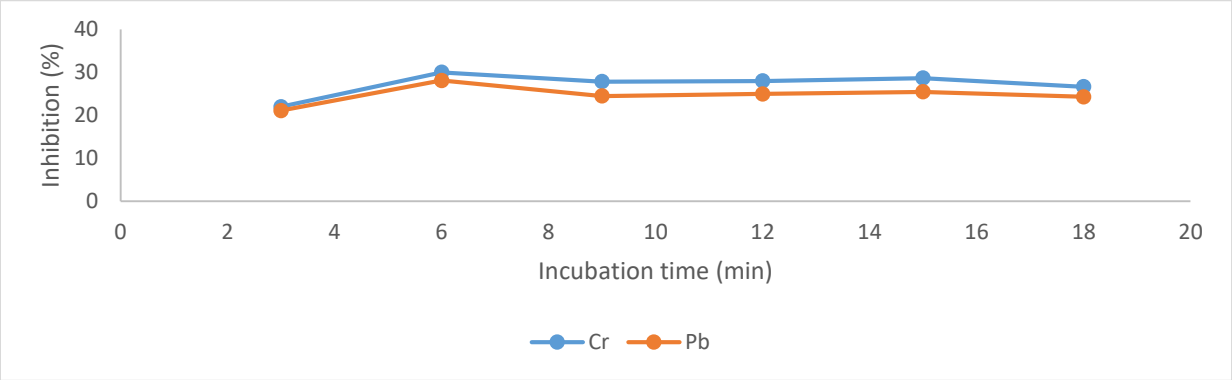

Figure 10 Effect of incubation time with heavy metals

Selectivity (Interferent) study

Selective nature of Pb^{2+} and Cr^{3+} towards Pt/ZnO/urease vs Ag/AgCl electrode was tested in the presence of other metals namely Ca^{2+}, Co^{2+}, Zn^{2+}, K^{+}, As^{3+} and Ni^{2+} (10 ppm), Fig. 11. All these metal ions showed an inhibition of less than 30%, which confirmed the selectivity of the developed sensor towards Pb^{2+} and Cr^{3+} ions. The ultra-low potential of 0, and −0.06, V respectively for Pb^{2+} and Cr^{3+} vs Ag/AgCl offered by Pt/ZnO/urease electrode helped in avoiding cross reactivity. Current research reported by Gumpu *et al.* (2017) also showed the selectivity of Pb^{2+} and Hg^{2+} at 0 and -0.03 V respectively vs Ag/AgCl offered by Pt/CeO_2/urease electrode. Therefore, further determination of the selected heavy metals in synthetic and real samples were performed at the selective potentials of the metal ions (0, − and −0.06 V for Pb^{2+} and Cr^{3+} vs Ag/AgCl).

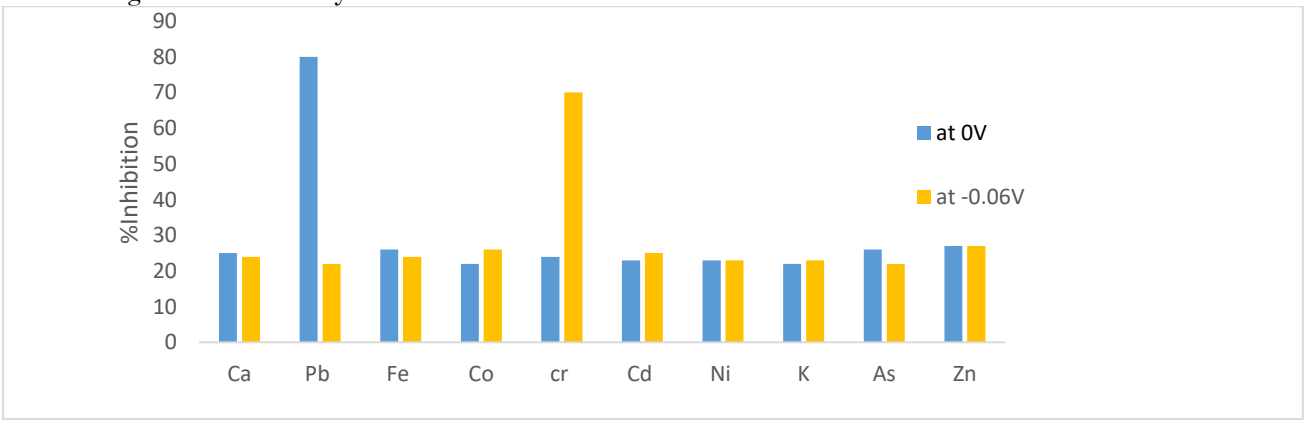

Fig. 11 Selectivity of $Pb^{2+}$and Cr^{3+}towards Pt/ZnO/urease electrode vs Ag/AgCl

Determination of Heavy metals with Biosensor

Biosensor measurements for Pb^{2+} and Cr^{3+} Standards

Biosensor measurements for standard solutions of the investigated metals Pb^{2+} and Cr^{3+}, the results obtained are presented in tables 1 and 2 both Pb^{2+} and Cr^{3+}, showed varying inhibition effects on the immobilized urease within the concentration range of 0.001-10 ppm. Both the metal ions revealed dose dependent inhibition of urease activity as shown by the decreased biosensor response with increasing concentration of the metal ions. Pb^{2+} produced 4.10 - 68.10% inhibition while Cr^{3+}, 5.62-64 % inhibition. Dose dependent inhibition of urease activity has also been reported by other authors [9, 15]. The reasons

for this could be that the immobilization process induced some conformational changes and lowered the accessibility of the essential sulfhydryl groups of urease to the metal ions.

Table 1 Biosensor measurement of Pb^{2+} standard

S/N	Pb^{2+} concentration (ppm)	Biosensor response (µA)	%inhibition
1	0.00 control	84.92 ± 0.11	0.00
2	0.001	81.43 ± 0.04	4.10
3	0.01	73.10 ± 0.23	13.91
4	0.1	70.63 ± 0.01	16.82
5	1	64.43 ± 0.03	24.12
6	10	27.08 ± 0.33	68.10

Table 2 Biosensor measurement of Cr^{3+} standard

	Cr^{3+} concentration (ppm)	Biosensor response (µA)	%inhibition
1	0.00 control	86.76 ± 0.01	0.00
2	0.001	81.90 ± 0.24	5.62
3	0.01	75.94 ± 0.06	12.48
4	0.1	77.91 ± 0.11	15.98
5	1	65.75 ± 0.47	24.00
6	10	31.14 ± 0.08	64.11

Biosensor Calibration Curves for Determination of Heavy metals

Plots of biosensor calibration curves of percentage inhibition against concentration for all the investigated metal solutions are presented in Figures 12 and 13. The calibration curves of $Pb^{2+}$and Cr^{3+} showed linearity within the concentration range of 0.01-10 mg/L. A critical survey of related literatures revealed numerous discrepancies in reported linear ranges for different urease-based biosensors for heavy metal; A study by [11] established a linear range of 10-100 µM (0.34 - 10.66 mg/L) for Cr^{3+} and 7-300 µM (0.76-35.32 mg/L) for Pb^{2+}. [16] demonstrated 0.01 -10 mM as the linear range for Cd^{2+} and 0.1-10 mM for Cu^{2+} and Pb^{2+}. A study by [15] gave the linear ranges for Cu^{2+}, As^{3+}, Cr^{2+} and Cd^{2+} as 0.01-10 mg/L and 0.1-10 mg/L for Zn^{2+} and Pb^{2+} respectively. It is generally opined that the reported discrepancies in biosensor linear ranges emanated due to the facts that the different sensor membranes have different sizes and contain different amounts of the bio-selective element (urease). Another factor that may be responsible for linear range discrepancies is the differences in membrane properties that would consequently result in differences in mass transport of chemical species from the sample to the sensing region. Biosensor linear range could also be a consequence of the range of analyte concentrations under consideration. Assay conditions such as incubation time could also affect the extent of inhibition and consequently, the linear range for detection.

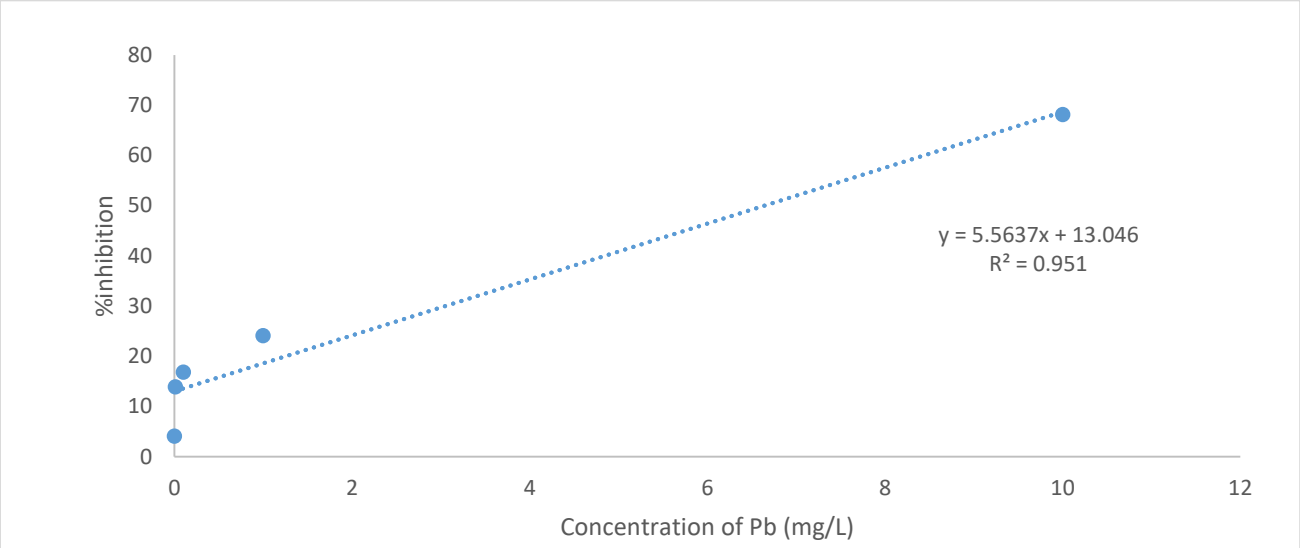

Fig. 12 Standard calibration curve for estimation of Pb^{2+}

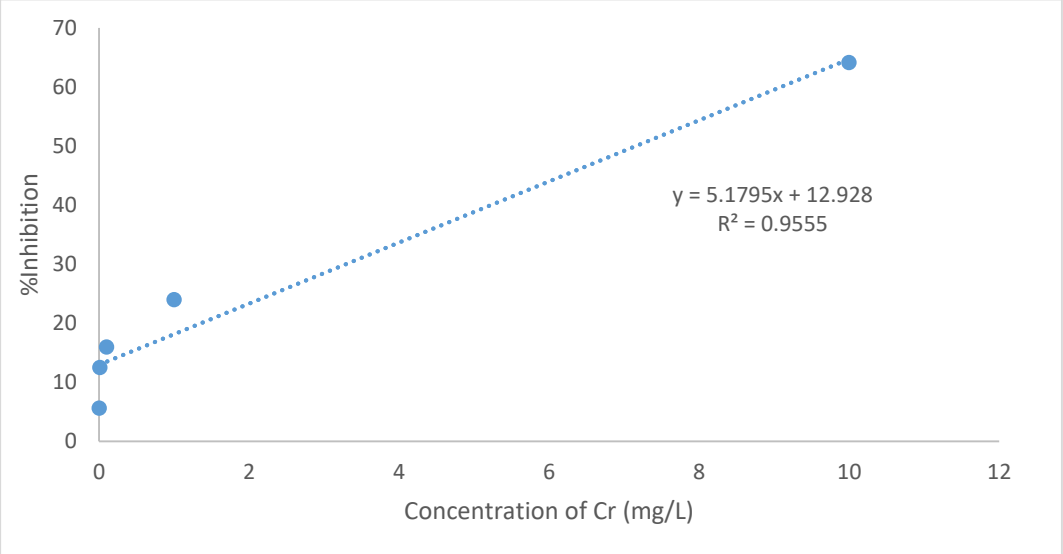

Fig. 13 Standard calibration Curve for determination of Cr^{3+}

Table 3. Summary of Analytical Characteristics of Biosensor Calibration curves for determination Pb^{2+} and Cr^{3+}

Metals	Precision (%RSD)	LOD (ppm)	LOQ (ppm)	Sensitivity (%Inh/ppm)	Regression equation
Pb^{2+}	9.30	0.07	0.22	5.56	y = 5.56x + 13.05
Cr^{3+}	9.40	0.04	0.13	5.18	y = 5.18x + 12.93

Real sample analysis and validation

In Mubi, Kasuwan gada river is considered to be one of the fresh water source covering large area and has many tributaries but in recent years, due to release of household effluent and waste disposal along the river bank, the metal ion concentration has increased significantly. For the purpose of detection of Pb^{2+} and Cr^{3+} ions in samples of Kasuwan gada river water (pH 6.6), standard addition method was adopted. The validation study was carried out using AAS. The amount of $Pb^{2+}$and Cr^{3+} ions were present in 100 µL of 100-fold diluted metal ion samples, the concentration of $Pb^{2+}$and Cr^{3+} ions were estimated using the following equations [9].

$$\frac{Io}{C_{samp.} X V_{std} / V_{samp.}} = \frac{Ii}{C_{samp.} X V_{std} / V_{samp.} + C_{samp.} X V_{std} / V_{samp.}}$$

Where J_o and I_i are the amperometric response for sample and spiked sample, V_{std} and $V_{samp.}$ Are the volumes of spiked standard and that of the sample respectively, while $C_{samp.}$ and C_{std} are the concentration of sample and the spiked standard solutions. Concentration of the analytes in solution is evaluated by making $C_{samp.}$ the subject formula.

Recovery Studies

In other to demonstrate the analytical application of the developed biosensor in real samples, recovery studies was first performed by adding known concentrations of the selected heavy metal solutions in the samples and analyzing them with the developed biosensor. Tables 4 and 5 presents the results for the recovery studies of 0.05 – 0.25 ppm of added heavy metal standard solutions and the corresponding amount recovered as analyzed with the developed biosensor. Percentage recovery within the acceptable range of 98.00% to 103.00% and relative standard deviations of 4.38% to 1.62% was observed for Pb^{2+} table 4. While, 92.00% - 102.00 % recovery and RSD of 0.66% - 3.32% was obtained for Cr^{3+} Table 5The recovery values and the RSD of our developed biosensor is within the values reported by [9, 7], the biosensor developed can therefore, be scored very high due to low RSD which are less than 10% recommended RSDs for good biosensors.

Table 4. Recovery studies of Pb^{2+}

Sample (10ml KG water)	Pb^{2+} Added (ppm)	Pb^{2+} detected (ppm)	Recovery (%)	RSD (%) (n = 5)
1	0.05	0.049 ± 0.01	98.00	4.38
2	0.10	0.100 ± 0.32	100.00	3.16
3	0.15	0.147 ± 0.11	98.00	2.45
4	0.20	0.206 ± 0.10	103.00	1.62
5	0.25	0.251 ± 0.01	100.40	3.16

Table 5. Recovery studies Cr^{3+}

Sample (10ml KG water)	Cr^{3+} Added (ppm)	Cr^{3+} detected (ppm)	Recovery (%)	RSD (%) (n = 5)
1	0.05	0.048 ± 0.22	96.00	2.21
2	0.10	0.110 ± 0.10	101.00	0.66
3	0.15	0.150 ± 0.01	100.00	3.32
4	0.20	0.204 ± 0.01	102.00	1.87
5	0.25	0.230 ± 0.43	92.00	2.18

Determination of $Pb^{2+}$and Cr^{3+} with the developed biosensor and AAS in Kasuwan Gada river water

The validation study was carried out using AAS as standard method; the results are presented in Table 6. From the results obtained, Pb^{2+} was not detected with both AAS and the developed biosensor, however, the amount of Cr^{3+} detected from the sample by the two methods showed good agreement considering the values of the observed absolute errors and the relative errors which are all within the tolerable limits. These results have further justified the biosensor's sufficient sensitivity to the analysed samples and gave quantitative information on the heavy metal content of the sample.

Table 6. Determination of Pb^{2+} and Cr^{3+} with the developed biosensor and AAS in Kasuwan Gada river water

Heavy Metal	Proposed method (ppm)	AAS (ppm)	Absolute error	Relative Error (%)
Pb^{2+}	ND	ND	0.00	0.00
Cr^{3+}	0.31 ± 0.33	0.35 ± 0.11	0.04	12.90

CONCLUSION

This work has shown the feasibility of amperometric biosensor for determination of heavy metal ions using gelatin entrapped Urease/ZnO as bio-recognition component. The result obtained showed convincing

evidence of the great potential of the biosensor in environmental monitoring. Compared to previous reports on urease-based sensors for determination of heavy metals, the present biosensor exhibits several advantages such as easy production of sensor membrane, good sensor-to-sensor reproducibility and an immobilization method that does not require chemical modification of the substrate or enzyme. More so, the use of crude urease from Velvet beans as opposed to the expensive pure enzymes used by previous workers would allow a sensible reduction in costs and ultimately result in a relatively cheap biosensing device.

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