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Optimization of Horseradish Peroxidase Immobilization on Glassy Carbon Electrode Based on Maize Tassel-Multiwalled Carbon Nanotubes for Sensitive Copper(II) Ion Detection

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Enzymatic procedures for measuring trace metal ions, based on the inhibitive action of these metals on horseradish peroxidase (HRP) enzyme activity, have been developed. Glassy carbon electrode (GCE) modified with maize tassel- multiwalled carbon nanotubes (MT-MWCNT) was used as an immobilizing surface of HRP through electrostatic attractions. The voltammetric and amperometric response of HRP was affected by the presence of metal ion, which caused a decrease in the current intensity. The experimental optimum working conditions of MT: MWCNT amount (10 µL, 4:1), enzyme loading (10 µL, 10 mg mL^{-1}), nafion amount (0.5 µL, 0.3%), pH 7, and potential applied (-300 mV) were established. Using Cu^{2+} as a model divalent metal ion, the inhibition rate was proportional to the concentration in the range from 0.068-2.0 mg L^{-1} with a limit of detection of 4.2 µg L^{-1}. Representative Dixon and Cornish-Bowden plots showed that the reaction was reversible and mixed. Under these conditions, repeatability and reproducibility of HRP/MT-MWCNT biosensor was determined, reaching values below 10% in terms of relative standard deviation.

Keywords: Optimization; Horseradish peroxidase; Maize tassel; Multiwalled carbon nanotubes; Biosensor; Copper(II)

1. INTRODUCTION

Trace metals hold a superlative position among the vast number of contaminants in the environment and they have become a public health concern because of their toxicity, non-biodegradability and persistence in the environment [1-3]. The toxicity of these metals is enhanced through bioaccumulation in animal and plant tissues. The assessment of damage caused by these metals has increased in demand in recent years [4]. Copper is an essential element which is required by

all organisms as a catalytic cofactor for biological processes such as respiration, oxidative stress protection and normal cell growth and development [5]. Several manifestations of copper deficiency in animals appear to be related to decreased tissue concentration of copper containing enzymes [6]. In general, a daily copper intake of 1.5-2 mg is essential. However, severe oral intoxication will affect mainly the blood and kidneys. Therefore, the search for portable, rapid and on-site methods for copper monitoring in industrial waste waters before being discharged into natural water bodies is significant.

Several analytical techniques have been developed to detect and quantify trace metals in a variety of matrices, using atomic absorption spectrometry, ultraviolet spectrophotometry, atomic fluorescence spectrometry, x-ray fluorescence, inductively coupled plasma spectrometry and isotope dilution inductively coupled plasma mass spectrometry. However, these techniques are commonly used for measurements of trace metal ions in the laboratory and usually are unfit for field analysis and for rapidly monitoring trace metals in contaminated sites. Expensive instrumentation and complicated sample preparation processes are also required with the aforementioned techniques.

Recently, inhibition based enzyme biosensors are among the biosensors that have gained attention for determining the concentration of inhibitors in the assayed sample by measuring the inhibition degree. Electrochemical biosensors based on the inhibition of enzymes for detection of Cd^{2+}, Cu^{2+}, Cr^{3+}, Zn^{2+}, Ni^{2+} and Pb^{2+} using urease biosensor [7,8]; Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, and Pb^{2+} using alkaline phosphatase [9]; Cd^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Pb^{2+} using glucose oxidase [10,11]; Cr^{6+} using urease [12]; Hg^{2+} using glucose oxidase invertase and mutarose [13]; Cu^{2+}, Cd^{2+}, Mn^{2+} and Fe^{2+} using acetylcholinesterase [14], Cd^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+} using nitrate reductase [15], mercury using glucose oxidase [16]; and Cu^{2+}, $Cd^{2+}$and Pb^{2+} using horseradish peroxidase [17,18] have been reported. The detection principle of the enzyme-based biosensors is based on the target analyte selectively inhibiting the activity of the immobilized enzyme resulting in 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 determination in the environment.

In the present study, we immobilized horseradish peroxidase (HRP) onto maize tassel–multiwalled carbon nanotube (MT-MWCNT/GCE) through adsorption to construct an inhibitor biosensor for the sensing of Cu^{2+} in aqueous solution. The experimental conditions for the analytical performance of HRP enzyme electrode for Cu^{2+} were optimized. The Cu^{2+} ions inhibit the activity of enzyme with an effect of decreasing of H_2O_2 reduction peak current. The mode of inhibition was investigated using the Dixon and Cornish-Bowden plots.

2. EXPERIMENTAL

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-

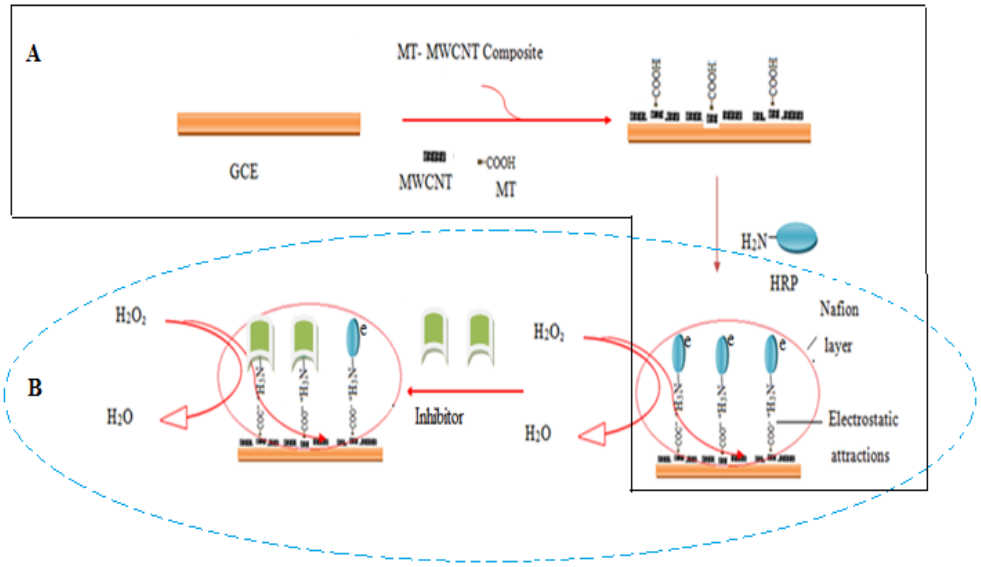
walled carbon nanotube (MWCNT), $Cu(NO_3)_2$, were purchased from Sigma-Aldrich (South Africa). Hydrogen peroxide (H_2O_2, 30% w/w) was obtained from Merck (South Africa) and solutions were freshly prepared before being used. Phosphate buffer solutions with various pH values were prepared by mixing standard 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 $M\Omega cm^{-1}$).

2.2. Apparatus

All electrochemical experiments were performed with a Bioanalytical Systems (USA) CV-50 W conventional three-electrode system. All experiments were carried out at room temperature. The pH measurements were carried out with a Crison 2001 micro pH-meter (Spain).

2.3. Biosensor fabrication procedure

The biosensor was prepared following the steps described in our previous work [19]. Briefly, 10 µL horseradish peroxidase (HRP) solution (10 mg mL^{-1}, dissolved in 0.1 mol L^{-1} pH 7.0 phosphate buffer solution, PBS) and 0.5 µL of 0.3% Nafion to act as a binder was deposited on MT-MWCNT biosensor as shown in process (A) and the process of inhibition (B) is briefly illustrated (see scheme 1).



Scheme 1. Fabrication process (A) Inhibition process (B)

2.4. Detection of trace metal ions

The biosensor was used for the detection of Cu^{2+}. Inhibition plots for the Copper(II) ion detected were obtained using the percentage inhibition method. The process was carried out in a three

step procedure. Briefly, the biosensor was first placed in a stirred 5 mL of 0.1 M PBS and multiple additions of a standard H_2O_2 substrate solution was added until a stable current and a maximum concentration of 0.1 mM were obtained. This steady state current is related to the activity of the enzyme in the biosensor when no inhibitor was present. The electrode was then washed with the same buffer and incubated in anaerobic conditions for 20 min with a standard trace metal ion in PBS. After incubation, steady-state current response was measured following additions of a standard H_2O_2 substrate solution up to 0.1 mM (anaerobic conditions), to a fresh 5 mL of 0.1 M PBS (0.1 M KCl, pH 7.0) solution (anaerobic conditions). The percentage of HRP inhibition ($\%I_{HRP}$) and residual enzyme activity ($\%REA_{HRP}$), was calculated [20] using equation 1 and 2.

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

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

where I% is the degree of inhibition, I_i is the steady-state current obtained in buffer solution, I_F is the steady-state current obtained after the biosensor was incubated for 20 min in phosphate buffer-water solvent mixture.

3. RESULTS AND DISCUSSION

3.1. Biosensor characterization

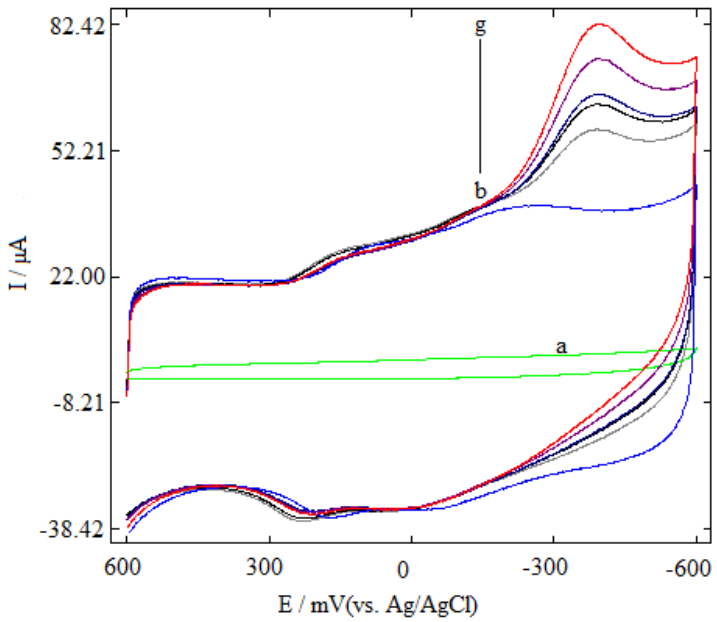


Figure 1. (A) Cyclic voltammograms of GCE (a), HRP biosensor in 0.1 M PBS, pH 7.0 and 0.1 M KCl (b) without substrate, (c–g) with 0.01–0.5 mM substrate.

The enzymatically reduction of H_2O_2 was evaluated by using the fabricated HRP biosensor by cyclic voltammetry in 0.1 M PBS at pH 7.0. At these experimental conditions, a cathodic peak around -320 mV versus Ag/AgCl was obtained. Fig. 1 shows the cyclic voltammograms of the biosensor in the absence (b) and presence of H_2O_2 (0.01–0.5 mM) (c to d) in PBS (pH 7.0) at the scan rate of 100 mV s^{-1}. An increase in cathodic peak current was observed with increase in substrate concentration. For comparison, the GCE (a) was also scanned in 0.1 M PBS and substrate, no significant response was observed. The linear regression equation is Ip/μA = 2.095 (±0.1245) + 0.1985 (± 0.0052) C/mM (R = 0.9969) in the H_2O_2 concentration range from 0.01–0.5 mM. The detection limit of H_2O_2 is 0.85 μM.

3.2. Optimization of experimental conditions of HRP/MT-MWCNT biosensor

To improve the analytical characteristics of the developed biosensor, optimization of experimental conditions such as MT: MWCNT amount, enzyme loading, nafion amount, pH, and applied potential were carried out.

3.2.1 Influence of MT: MWCNT amount on biosensor fabrication

The CVs current responses of the HRP/MT-MWCNT biosensor loading variable amount of MT: MWCNT to 0.1 mM H_2O_2 in 0.1 M PBS (pH 7.0), scan rate, 50 mV s^{-1} were investigated and results are shown in Fig 2.

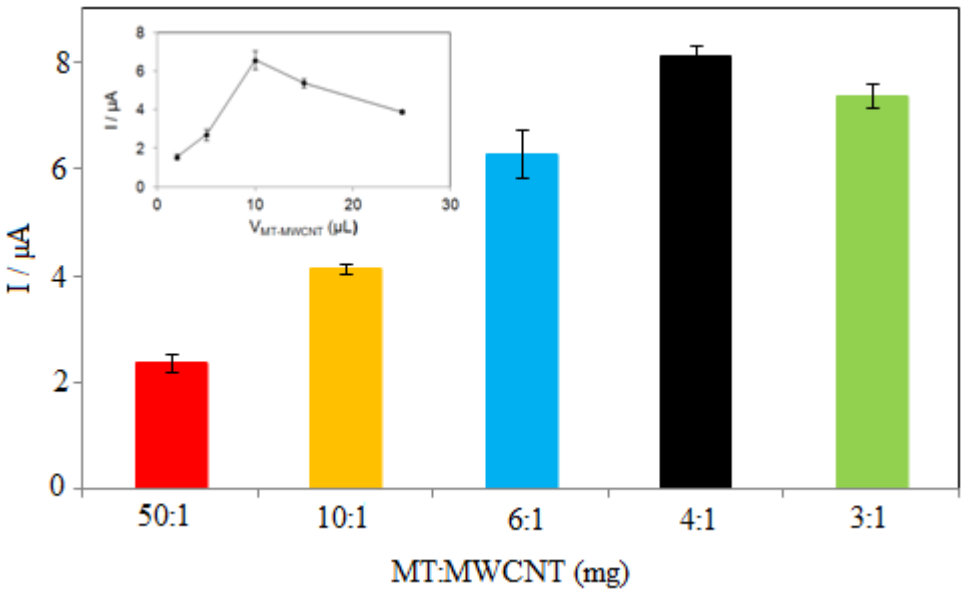


Figure 2. Current responses of the HRP/MT-MWCNT biosensor loading variable amount of MT: MWCNT amount to 0.1 mM H_2O_2 in 0.10 M PBS (pH 7.0). *Inset*: Current responses of volume MT-MWCNT injected on the GCE. Error bar = ± S.D. and *n* = 3.

As shown in Fig. 2, with the mass proportion of MT/MWCNT (MT: MWCNT) changing from 50:1 to 3:1, the reduction current response for H_2O_2 reached its maximum at 4:1. The volume of MT: MWCNT injected from the respective optimized mass proportion onto the GCE to act as an adsorbent/anchorage for HRP was also studied (*see inset*). When the dosage of MT: MWCNT was increased from 2 to 10 µL, the reduction peak current also increased possibly due to the increased amount of composite causing the effective surface area and aggregation effect to increase gradually, thereby increasing the concentration of H_2O_2 on the surface of the electrode, which aids the catalytic reaction. On the other hand, when the volume of MT: MWCNT solution was increased from 10 to 25 µL, the reduction peak current decreased. This might be because the MT: MWCNT film on the electrode surface was so thick that it increased the diffusion distance of H_2O_2 hindering mass transfer and electron transfer [21]. Consequently, 10 µL, mass proportion of 4:1 was adopted for subsequent HRP/MT-MWCNT biosensor fabrication.

3.2.2. Influence of HRP amount on biosensor fabrication

The amount of the enzyme adsorbed on the MT-MWCNT composite is a vital factor affecting the analytical sensitivity of a developed biosensor [22]. The influence of the amount of immobilized HRP on the analytical characteristics of the biosensor was studied using CVs. The effect of the amount of HRP in the MT-MWCNT composite is shown in Fig. 3.

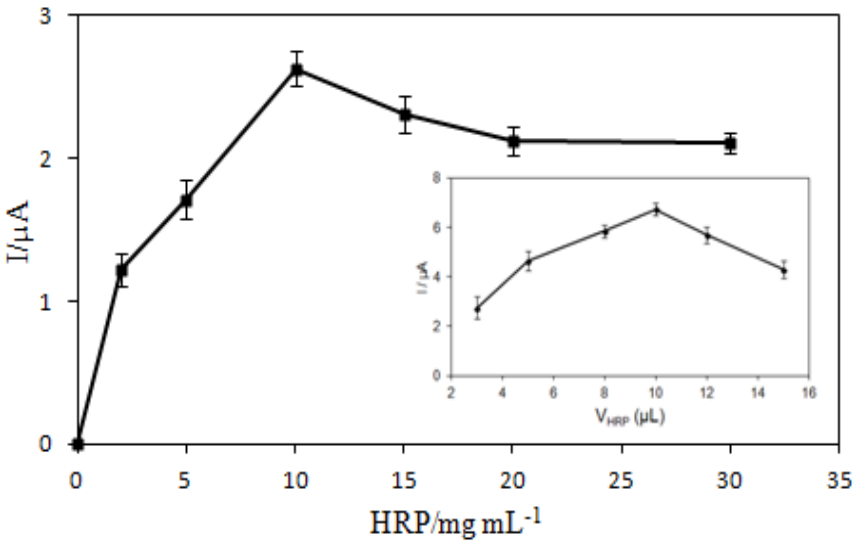


Figure 3. The effect of amount of HRP on the response current of HRP/MT-MWCNT biosensor in 0.1 M PBS (pH 7.0), Scan rate: 50 mV s^{-1}. *Inset*: Current responses of volume HRP injected on the MT-MWCNT/GCE. Error bar = ± S.D. and *n* = 3.

As shown in Fig. 3, the current increases as the amount of enzyme are increased up to a maximum of 10 mg mL^{-1}. For higher amounts than 10 mg mL^{-1}, the biosensor sensitivity decreased perhaps due to diffusion limitation. Furthermore, the volume of HRP injected on the MT-MWCNT composite was also studied (*see inset*). When the amount of HRP was increased from 3 to 10 µL, the

reduction peak current increased since the catalytic reaction of H_2O_2 was facilitated. However, increasing the amount of HRP from 10 to 15 µL, the current steadily decreased. This may be because the increased amount of HRP increased the resistance for interfacial electron transfer. So, an enzyme loading of 10 µL, 10 mg mL^{-1} HRP was selected for further experiments.

3.2.3. Influence of nafion amount on biosensor fabrication

We also investigated the influence of nafion as a binder on the catalytic activity of HRP using CV studies since it can also impede the amount of current flowing through the modified electrode. The range of nafion concentrations tested by the CVs in 0.1 M PBS (pH 7.0) containing 0.1 mM H_2O_2 at the scan rate of 50 mV s^{-1} were 0.10, 0.20, 0.30, 1.0 and 1.25% (Fig. 4).

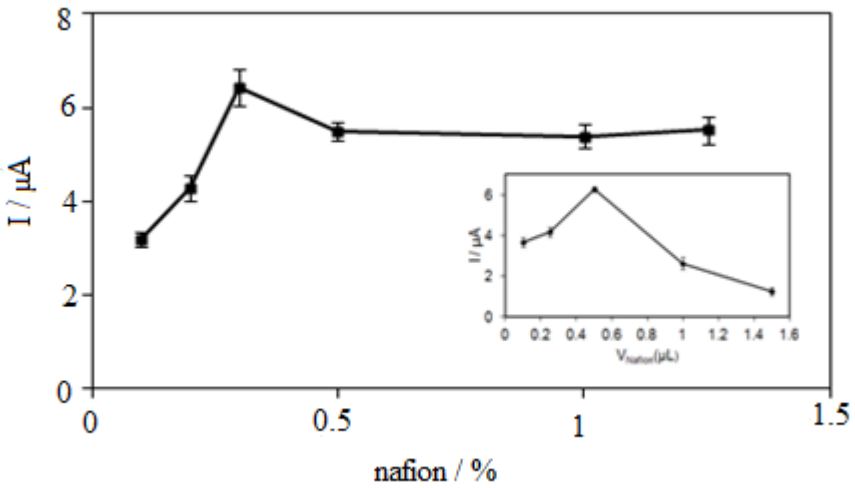


Figure 4. Influence of nafion concentrations on the electrocatalytic response current obtained by HRP/MT-MWCNT biosensor in the presence of 0.1 mM H_2O_2 in 0.1 M PBS (pH 7.0). Error bar = ± S.D. and n = 3.

From Fig.4, it can be seen that the response current increased slightly from 0.10 up to 0.30% nafion, and then decreased to 1.25% nafion. The volume of nafion injected on top of the HRP/MT-MWCNT biosensor was also investigated as shown in Fig 4 (*inset*) and 0.5 µL was found to give maximum current. Therefore, 0.5 µL of 0.3% nafion was used throughout the study.

3.2.4. Influence of pH on HRP/MT-MWCNT biosensor

It is widely acknowledged that pH is one of the critical parameter affecting enzyme activity and its stability in aqueous media [23]. The influence of pH on the electrocatalytic reduction of H_2O_2 at the HRP/MT-MWCNT biosensor was investigated (Fig. 5) by measuring the current response of 0.1 mM H_2O_2 in the pH range from 4.0 to 8.0.

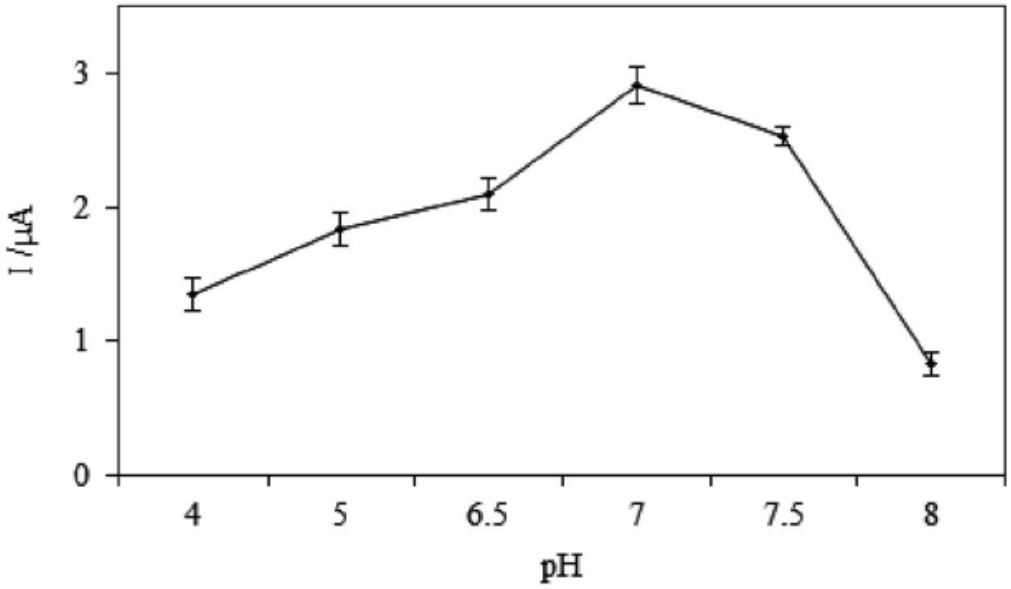


Figure 5. Effects of solution pH on the performance of HRP/MT-MWCNT biosensor in the presence of same concentration of H_2O_2 in 0.1 M PBS. Error bar = ± S.D. and *n* = 3.

As shown in Fig. 5, with solution pH increasing from 4.0 to 8.0, the current response of the HRP/MT-MWCNT biosensor increased and reached a maximum value at pH 7.0. When the pH value is higher than 7.0, a slight decrease of amperometric response is observed, which may be due to the denaturing of the immobilized HRP. Hence, pH 7.0 has been used throughout the experiments which are in agreement with what is reported for the soluble HRP. It can be concluded that the immobilization of HRP on MT-MWCNT composite did not alter the optimal pH value.

3.2.5. Effect of applied potential on the HRP/MT-MWCNT biosensor

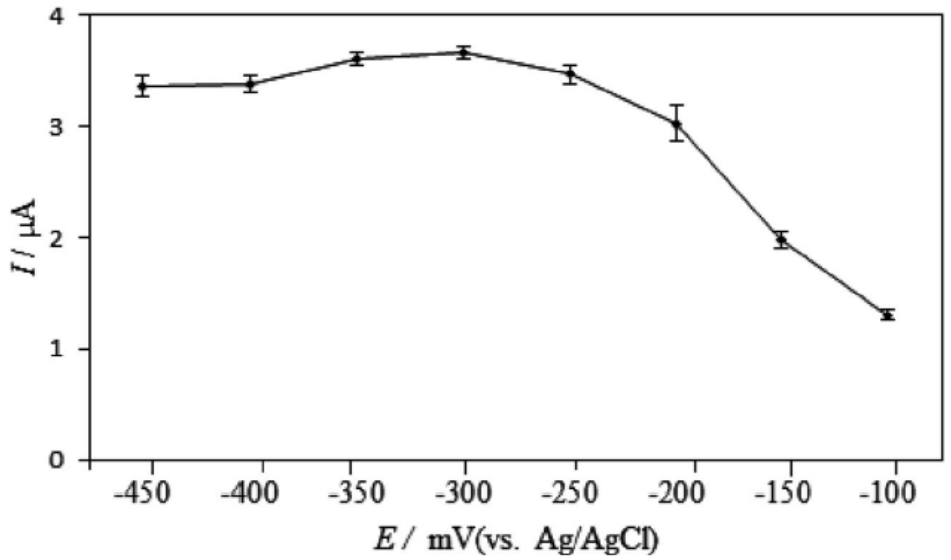


Figure 6. Effects of the applied potential on the performance of HRP/MT-MWCNT biosensor in the presence of same concentration of H_2O_2 in 0.1 M PBS. Error bar = ± S.D. and n = 3.

The biosensor sensitivity and selectivity of the system can be influenced by the applied potential [23]. Hence, the effect of applied potential on HRP/MT-MWCNT biosensor under steady state current was studied and the results are shown in Fig. 6.

In Fig 6, the steady state current increased when the applied potential decreased from -100 mV to -450 mV due to the increased driving force for the fast reduction of 0.1 mM H_2O_2 at the lower potentials and approached a maximum value at -300 mV. In this study, an applied potential of -300 mV was selected for all experiments.

3.3. Inhibition studies

3.3.1. Effect of incubation time

Evaluation of the inhibition time is very important for off-time measurements and on-site analyses [24]. The HRP/MT-MWCNT biosensor was incubated in a Cu^{2+} standard solution of 0.5 mg L^{-1} from 0 to 35 min, and then tested in 0.1 M PBS (pH = 7.0) (Fig. 7).

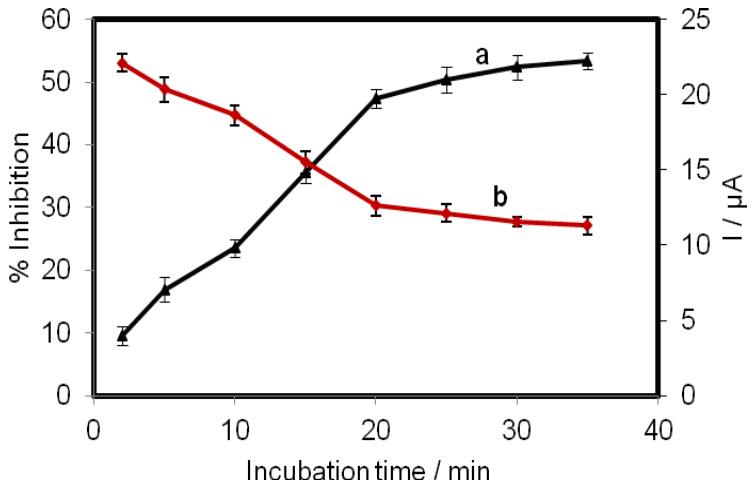


Figure 7. The effect of incubation time on the biosensor response (a) and enzyme activity decay (b) in 0.5 mg L^{-1} Cu^{2+} 0.1 M PBS (pH 7.0) and 0.1 mM H_2O_2, Error bar = ± S.D. and *n* = 3.

The results showed that percentage inhibition increased rapidly for the first 20 min, and then tended to level off because of the saturated formation of Cu^{2+}-HRP complex. A decrease in residual enzyme activity current occurred as the incubation time increased. The change in peak current reflected the alteration of enzymatic activity by the Cu^{2+} 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% is usually preferred in practical analysis in order to obtain low detection limits for different inhibitors. Thereby, incubation time of 20 min was selected used throughout.

Typical percentage inhibition-concentration and percentage residual activity-concentration plots of the HRP/MT-MWCNT biosensor under the optimized experimental conditions for Cu^{2+} are displayed in Fig. 8.

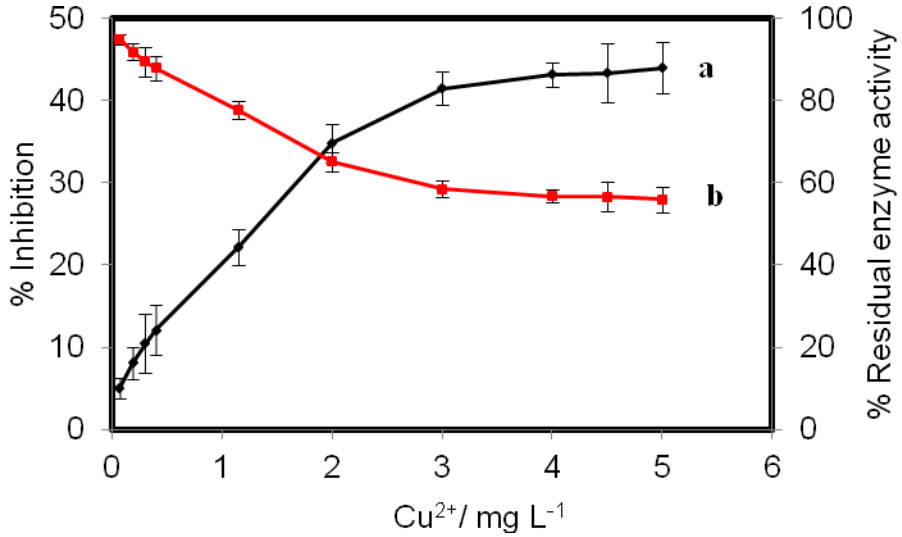


Figure 8. Dose-dependent enzyme inhibition (a) and residual enzyme activity (b) of Cu^{2+} towards HRP-catalyzed H_2O_2, Error bar = ± S.D. and n = 3.

As shown in Fig. 8, this type of inhibition effect exhibited dose-dependent behavior. The percentage inhibition increased with increase in concentrations of Cu^{2+} ions. As can be seen in Fig. 8, 44.0% of the activity of HRP was inhibited by 5 mg L^{-1} for Cu^{2+}. Cu^{2+} had a linear range up to 2.0 mg L^{-1}, and the detection limit was 4.2 µg L^{-1}. The residual enzyme activity decreased with increase in heavy metal ion concentrations.

3. 3.2. Investigation on the type of 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 [25,26]. In this study, the type of inhibition shown by Cu^{2+} over immobilized HRP was studied using increasing concentrations of the trace metals and of the substrate, H_2O_2. Moreover, data modelling using Dixon plot (representation of the inverse of the enzyme activity vs. inhibitor concentration) and Cornish-Bowden plot (the ratio of substrate concentration and enzyme activity vs. inhibitor concentration) was utilized to verify the inhibition mode [27,28]. 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.

In this study, the type of inhibition shown by Cu^{2+} was studied using three different concentrations of H_2O_2 (0.05, 0.2, and 1.0 mM). Representative Dixon and Cornish-Bowden plots are shown in Fig. 9 A, B for Cu^{2+}.

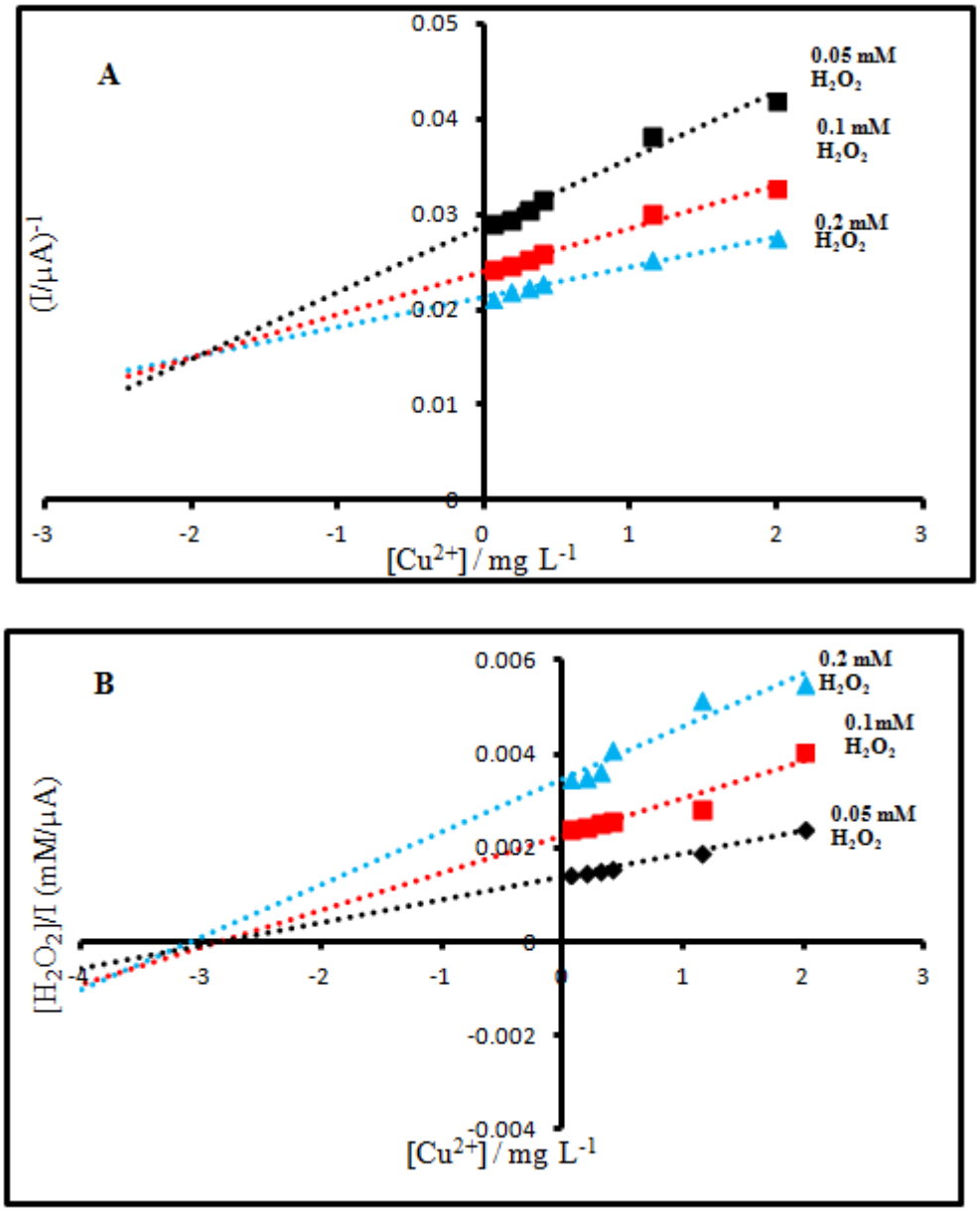


Figure 9. Dixon (A) and Cornish-Bowden (B) plots of the effect of different Cu^{2+} concentrations on HRP.

The pattern of inhibition demonstrated by Dixon plot (Fig 9.A) and confirmed by Cornish-Bowden plot (Fig 9.B) is consistent with the inhibition of HRP by Cu^{2+} ions through a reversible, mixed inhibition since the 3 lines intercept at a single point in the second quadrant above x-axis in the Dixon´s coordinates giving an inhibition constant, K_i (Cu^{2+}) = 1.8 mg L^{-1}) and intercept at a single point in the second quadrant below the x-axis in the Cornish-Bowden giving, K_i' (3.1 mg L^{-1}) [27,28].

Also, $K_i < K_i'$ (where 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). It is widely acknowledged that other HRP inhibitors induce comparable inhibition types. Shaolin and Jinqing (1995) reported that Cu^{2+} inhibited glucose in a non-competitive way [29]. The inhibition by Cu^{2+} for glucose was reversible and mixed [10] and competitive inhibition was observed for Cu^{2+} using L-lactate dehydrogenase [30]. In conclusion, mixed inhibition process is where 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.

3.3.3. 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. In studying the long-term stability, the HRP/MT-MWCNT biosensor was stored in 0.1 M PBS at 4^oC for 18 days and the biosensor response was tested on different days after incubation in the inhibitor. The biosensor did not show a bigger decrease of its initial response for 0.1 mM H_2O_2 after incubation in standard Cu^{2+} ion solution for the different days studied. The repeatability of the HRP/MT-MWCNT biosensor was investigated for fixed Cu^{2+} ion concentrations. Relative standard deviations (*RSD*) of 5.8% were obtained for Cu^{2+}. Five modified biosensors were made independently and were investigated for the determination of the same concentrations of Cu^{2+}. The modified biosensors showed a relative standard deviation (*RSD)* of Cu^{2+} (6.2%).

3.3.4. Selectivity of HRP/MT-MWCNT biosensor

Selectivity is an important parameter in the performance of an HRP/MT-MWCNT inhibition based biosensor. The addition of the following interferents; cations such as Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and anions: F^{-}, CN^{-}, SO_4^{2-}, CO_3^{2-} were studied by the mixed method, using the ratio of 1:2 for analyte and interferents, respectively.

Table 1. Possible interference tested with the HRP/MT-MWCNT biosensor

Possible interference	Ratio	% decrease in biosensor response
		Cu^{2+}
Ca^{2+}	1:2	6.68
Mg^{2+}	1:2	7.25
Na^{+}	1:2	4.35
K^{+}	1:2	4.58
F^{-},	1:2	5.54
CN^{-},	1:2	16.23
SO_4^{2-},	1:2	5.89
CO_3^{2-}	1:2	3.25

From the results in Table 1, the cations and anions do not cause much decrease in biosensor response, except CN^- anions as also reported [31].

3.3.5. Application

To demonstrate the feasibility of the fabricated enzyme inhibition biosensor for possible environmental applications, preliminary application of the biosensor was examined by determination of Cu^{2+}, in tap water by standard addition method. The results are given in Table 2. The recoveries were in the range of 96.0-101.0%, which indicated the efficacy of the biosensor for practical analysis.

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

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

The validation of the HRP/MT-MWCNT biosensor measurements against the ICP-OES technique verified the suitability of biosensor for rapid analysis of trace elements in natural water standard reference material®, 1640a. The concentration of Cu^{2+} (3.98 µg L^{-1}) in the natural water standard reference material® from National Institute of Standard and technology

(NIST) were calculated from the calibration curves. The obtained results after analysis for the trace metal presented in Table 3, corroborated well with those obtained by ICP-OES, with relative error values lower than 10%.

Table 3. Evaluation of the HRP/MT-MWCNT biosensor

Standard reference material ® 1640a- trace elements in natural water			
Cation	HRP/MT-MWCNT biosensor	ICP-OES	E^a/%
Cu^{2+}	83.58 ±0.570	85.75 ± 0.51	2.53

Concentrations were determined in µg L^{-1}; ± S.D. based on three replicates (n = 3) determinations; E^a: HRP/MT-MWCNT biosensor versus ICP-OES (HRP/MT-MWCNT biosensor-ICP-OES method / ICP-OES method) × 100%.

The allowed MCLs by USEPA [32] in drinking water is 1 300 µg L^{-1} for Cu^{2+}. The World Health Organization [33] on the other hand has given the guideline values for Cu^{2+}, in drinking water as 2 000 µg L^{-1}. Based on this, it can be suggested that the HRP/MT-MWCNT biosensor could be used as a management tool for determining the quality of water for the presence of trace metals

4. CONCLUSIONS

We have optimized the HRP/MT-MWCNT biosensor and demonstrated its use for the determination of metals through inhibition studies. It was deduced that HRP was inhibited by Cu^{2+} ion. The highest inhibition obtained for the HRP/MT-MWCNT biosensor was 44.0% for Cu^{2+}. The metal ion was measured with a detection limit of 4.2 μg L^{-1} and a sensitivity of 7.41 x 10^{-3} μA/μg L^{-1}. By modelling the data using the Cornish-Bowden together with Dixon plots, the inhibition was determined to be reversible and mixed. The inhibition constants, K_i (Cu^{2+} = 1.8 mg L^{-1}), and K_i' (Cu^{2+} = 3.1 mg L^{-1}) was deduced. The proposed biosensor does not require any complicated immobilization procedure for the construction and has been shown to sense low concentration in samples.

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