

Development and validation of biosensing method for acetaminophen drug monitoring

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Received: 28 September 2014, Revised: 17 November 2014 and Accepted: 25 November 2014

ABSTRACT

A polyphenol oxidase (PPO) was immobilized by employing magnetic nanoparticles-zinc oxide/zinc hexacyanoferrate (Fe₃O₄NP-ZnO/ZnHCF) hybrid film electrodeposited on the surface of Pt electrode. The surface functionalization of Fe₃O₄NP-ZnO/ZnHCF hybrid film was characterized by cyclic voltammetry (CV), scanning electron microscopy (SEM), atomic force microscopy (AFM) and electrochemical impedance spectroscopy (EIS) before and after immobilization of PPO. The biosensor exhibited optimum response within 4s at pH 7.0 and 35°C and linearity in the range 0.04 to 10000 μM for acetaminophen with a detection limit of 0.04 μM (S/N=3). Accuracy of the proposed sensor was found to be 99%. The use of Fe₃O₄NP/ZnO/ZnOHCF for construction of amperometric acetaminophen biosensor has resulted into relatively rapid response, higher sensitivity, broad linear range, lower detection limit, good reproducibility and long term stability of this biosensor. This sensing interface provides better avenue for the fabrication of various sensor. Copyright © 2015 VBRI press.

Keywords: Drug monitoring; acetaminophen; magnetic nanoparticles; zinc oxide/zinc hexacyanoferrate film; Pt electrode.



Jagriti Narang is currently working as an assistant professor at Amity University, Noida. She completed her PhD in Biochemistry and Genetics at Maharishi Dayanand University. She is running successfully DST young scientist project. Her research interest includes synthesis, characterization and applications of novel nanostructured materials with different dimensions and morphologies (such as: nanoparticles, nanowires, nanocubes, nanorods etc). Enzymatic and non-enzymatic electrochemical nanobiosensor biosensors based on nanostructured materials.



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Introduction

Acetaminophen (*N*-acetyl-*p*-aminophenol or Paracetamol) is an acylated aromatic amide and is the most used medicine after acetylsalicylic acid in many countries as an alternative to aspirin and phenacetin [1]. It is an effective and safe analgesic agent used worldwide for the relief from mild to moderate pain associated with headache, backache, for arthritis pain and postoperative pain [2]. Overdose and under dose of the drug induces adverse effects like renal impairment, hepatic insufficiency or in severe case it may cause death [1–4]. These point the call for the sensitive, accurate, fast, simple and rapid method for drug monitoring. The development of effortless, specific and accurate method for the analysis of this drug is very useful. Earlier techniques used for analysis of acetaminophen are colorimetric method, high pressure liquid chromatography (HPLC) and spectrometric methods. However most of these methods require time consuming pretreatment of sample, bulky apparatus and trained person to operate. Biosensors prove to be best alternative to overcome these problems due to their intrinsic specificity, low costs, fast analyses and minimal requirements for sample pretreatment [5]. Horseradish peroxidase (HRP) [EC 1.11.1.7] catalyzes the oxidation of acetaminophen (paracetamol) into *N*-acetyl-*p*-benzoquinoneimine. Hence, the amperometric biosensors

based on direct enzyme immobilization on the transducer surface are the main analytical strategies used for acetaminophen analysis [6].

Metal hexacyanoferrates have attracted considerable attention, as an excellent surface modifier, ever since reported by Neff [7] as deposition of a thin film of prussian blue (PB) on an electrode surface. The sensing electrodes have been modified with metal hexacyanoferrate through various techniques such as electrodeposition, adsorption, entrapping into a polymer matrix and mechanical attachment [8, 9]. Zinc hexacyanoferrate (ZnO/ZnHCF) has received much attention ever since its use by deposition on a carbon electrode reported by Joseph *et al.* [10]. Metal oxide nanoparticles exhibit higher ratios of surface area to volume than their bulk counterparts and thus provide larger electrochemically active areas for higher detection sensitivities for target molecules [11-16]. Nanostructured magnetic nanoparticles ($\text{Fe}_3\text{O}_4\text{NP}$) based electrochemical transducer surfaces promote the direct electron transfer reactions to amplify and orient the analytic signals of the biorecognition events [17].

In the present report, we first electrodeposited thin film of ZnO/ZnHCF on the surface of sensing electrode (Pt) and then magnetic nanoparticles were physically absorbed to the film modified electrode. Then purified enzyme PPO was immobilized on the surface of sensing electrode. Use of enzyme produces amplified signal as compared to non-enzymatic sensor. Our electrochemical measurements show that the Pt -modified ZnO/ZnHCF film provide excellent matrixes for the immobilization of PPO and that the immobilized PPO exhibits high stability and retains high catalytic activity. The electrochemical response of the fabricated biosensor to acetaminophen shows broad range linearity. In addition, our study reveals that the presence of enzyme significantly enhances the sensitivity of the designed biosensor.

Experimental

Materials

Acetaminophen (Paracetamol) was purchased from SIGMA (St. Louis, MO). Zinc nitrate ($\text{Zn}(\text{NO}_3)_2$), potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) and ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) from Sisco Research Laboratory Pvt. Ltd., Mumbai, India were used. All other chemicals were of analytic reagent grade. Double distilled water (DDW) was used in all experiments.

Apparatus

Cyclic voltammetry (CV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) measurements were performed in a Potentiostat/Galvanostat (Autolab, Eco Chemie, The Netherlands. Model: AUT83785) with a three electrode system consisting of a Pt wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode and modified Pt wire as a working electrode. All the electrochemical experiments were performed at ambient temperature (25 °C).

Synthesis of $\text{Fe}_3\text{O}_4\text{NP}$

$\text{Fe}_3\text{O}_4\text{NP}$ were prepared according to the method of Predoi [18]. The 0.5 M ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) in 2M HCl and 0.5 M ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in DDW were mixed in equal volumes at room temperature. The mixture was dropped into 200 ml of 1.5 M NaOH solution under vigorous stirring for about 30 min. The resulting precipitates were isolated after centrifugation at 8000g and dried at 40 °C.

Preparation of ZnO/ZnHCF film

Prior to film deposition, the Pt was polished with alumina (Diameter 0.05 mm), then ultrasonically cleaned for about a min in DDW and finally washed thoroughly with DDW. The electrochemical deposition of ZnO/ZnHCF film was accomplished by potentiodynamic cycling of the Pt electrode between preset potential range in a suitable aqueous solution of H_2SO_4 (2 M, pH 2.0) containing 10^{-4}M $\text{Zn}(\text{NO}_3)_2$ and 10^{-4}M $\text{K}_3[\text{Fe}(\text{CN})_6]$. After 30 cycles, the electrode was taken out, rinsed thoroughly and used for further characterization. Cyclic voltammetric study of ZnO/ZnHCF film-modified electrode was recorded during electrodeposition of ZnO/ZnHCF hybrid film at a scan rate of +0.4 V to +1.6 V s^{-1} at 50 mVs^{-1} intervals.

Construction of $\text{Fe}_3\text{O}_4\text{NP}$ – ZnO/ZnHCF film modified Pt electrode

$\text{Fe}_3\text{O}_4\text{NP}$ were absorbed on the surface of ZnO/ZnHCF film by physical adsorption. Different durations of physisorption of magnetic nanoparticles would result in different amounts of $\text{Fe}_3\text{O}_4\text{NP}$ being deposited onto ZnO/ZnHCF film, which would eventually generate different catalytic activities towards acetaminophen. So, the optimal time duration was determined and found to be 24h for physisorption of $\text{Fe}_3\text{O}_4\text{NP}$.

Effect of applied voltage

To optimize the applied potential for the acetaminophen determination, the effect of applied potential on the response current was investigated in the range -0.6 to +0.8 V vs Ag/AgCl. The optimal current was measured at +0.25 V vs Ag/AgCl and the same were used in subsequent electrochemical studies.

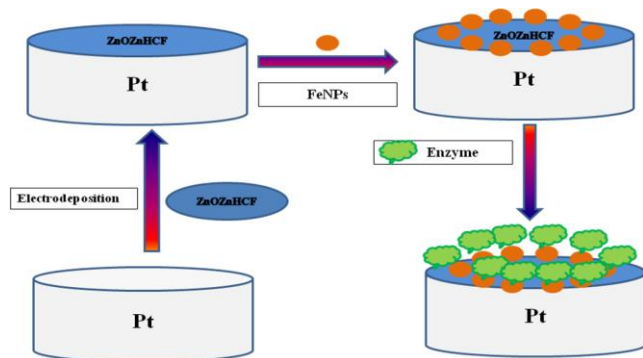
Preparation of enzyme electrode

The purified PPO enzyme was immobilized onto the surface of $\text{Fe}_3\text{O}_4\text{NP}$ – ZnO/ZnHCF film modified Pt electrode by layering 10 μl of enzyme solution (40 mg ml^{-1} protein) in acetate buffer (pH 5.0) and keeping it undisturbed for approximately 12 h at 4 °C. The electrode was finally washed with acetate buffer (pH 5.0) to remove unbound enzyme.

Electrochemical characterization of PPO / $\text{Fe}_3\text{O}_4\text{NP}$ -ZnO/ZnHCF/Pt electrode

Cyclic voltammetry studies was carried out using a three electrode system composed of PPO/ $\text{Fe}_3\text{O}_4\text{NP}$ -ZnO/ZnHCF/Pt electrode as working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode. To

discern the role of individual components, cyclic voltammograms of bare Pt electrode, $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF/Pt}$ electrode, and $\text{PPO /Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF/Pt}$ electrode was recorded in sodium phosphate buffer (0.1 M, pH 7.0) containing 0.1 mM H_2O_2 at a scan rate of 0.0 to +1 V s^{-1} at an interval of 50 mV s^{-1} . The principal of working of this biosensor included PPO as biological component to convert acetaminophen to p-aminophenol, which was monitored amperometrically by oxidation at +0.25 V vs Ag/AgCl and the oxidation current was related to the concentration of acetaminophen. The chemical reactions are shown in **scheme 1**.



Scheme 1. Graphical representation of the stepwise amperometric sensor fabrication process.

Preparation of acetaminophen solution

Paracetamol (acetaminophen) was prepared in phosphate buffer solution (pH 7.0). Solutions of different concentrations of Paracetamol (acetaminophen) ranging from 0.04 to 10000 μM was prepared in 0.1M sodium phosphate buffer (pH 7.0) and stored at 4 $^\circ\text{C}$ until use.

Amperometric determination of acetaminophen

An accurate volume of 1.0 ml of each pharmaceutical product was stirred until complete dissolution and then diluted to 10, 20 and 50 ml with 0.1 mol l^{-1} phosphate buffer solution (pH 7.0), respectively. Finally, an aliquot of 400 μl of each medicine solution was added to the cell containing 10 ml of 0.10 mol l^{-1} phosphate buffer solution. The measurements were performed after successive additions of paracetamol or sample solutions. After each addition, cyclic voltammograms was recorded by cycling the potential between -0.6 and +0.8 V at a scan rate of 100 mV s^{-1} . Acetaminophen content in pharmaceutical product was determined by the present biosensor by replacing acetaminophen with pharmaceutical product and recording the current (mA) under its optimal working conditions. The amount of pharmaceutical product was extrapolated from standard curve between acetaminophen concentrations and current in mA.

Storage stability of PPO/ $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF/Pt}$ electrode hybrid film modified Pt electrode

The long-term storage and stability of the working electrode its amperometric current response to 100 μM of paracetamol, was investigated over a period of 6 months at 4 $^\circ\text{C}$.

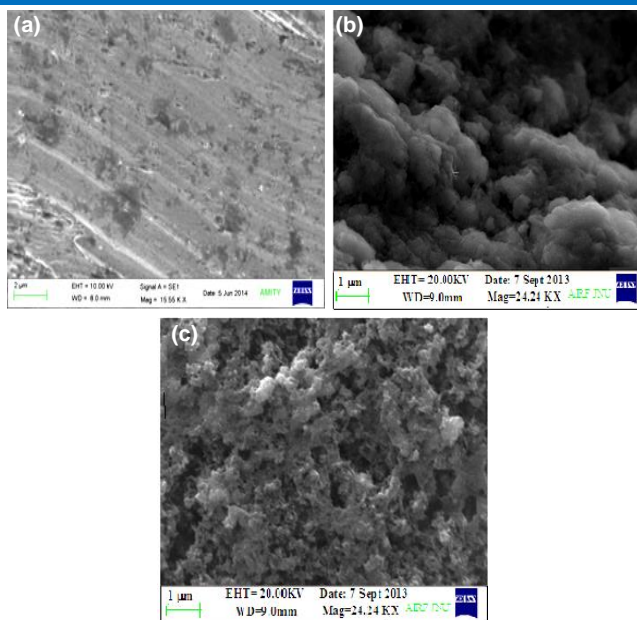


Fig. 1 A. SEM of (i) bare Pt electrode, (ii) $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified Pt electrode and (iii) $\text{PPO/Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified Pt electrode.

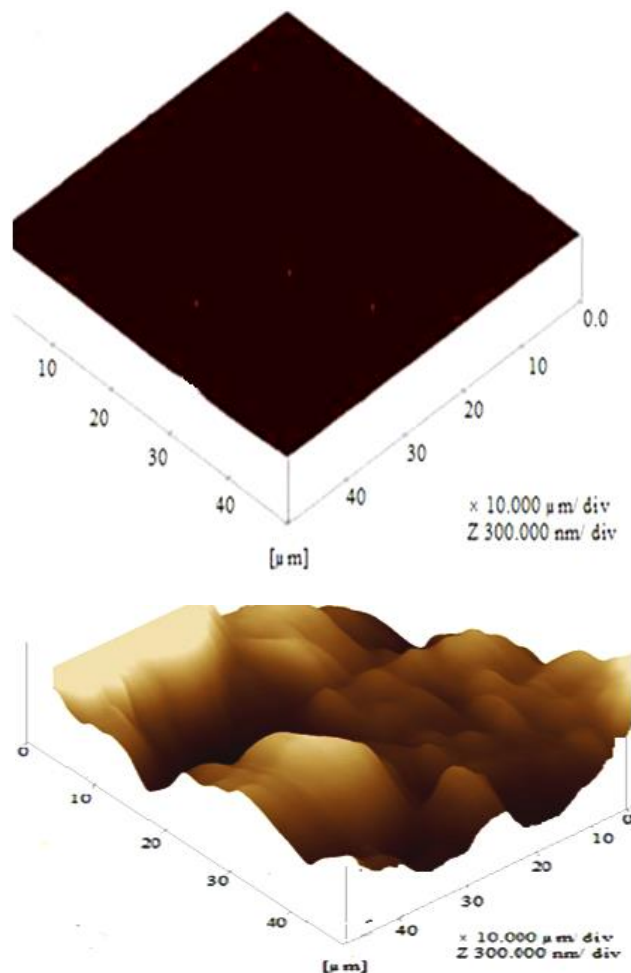


Fig. 1B. Three-dimensional amplification of the AFM image of the bare Pt electrode (i) and $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified Pt electrode (ii).

Results and discussion

Surface morphological characterizations using SEM and AFM studies

The morphologies of (a) bare electrode, (b) $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified Pt electrode and (c) PPO immobilized onto $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film electrode were characterized by SEM studies and AFM studies. The SEM and AFM images of the Pt electrode showed a smooth and featureless morphology (**Fig. 1 A (i) & B (i)**), whereas the granular morphology with heterogeneous roughness of $\text{Fe}_3\text{O}_4\text{NP}$ showed the absorption of nanoparticles on ZnO/ZnHCF hybrid films (**Fig. 1 A (ii)**). Weak interactions and immobilization of PPO onto the Pt electrode showed a globular structure of enzyme (**Fig. 1 A (iii)**) and AFM image of $\text{Fe}_3\text{O}_4\text{NPs}$ were uniformly distributed throughout the film surface (**Fig. 1 B (ii)**).

Electrochemical characterization of ZnO/ZnHCF hybrid film modified Pt electrode

Fig. 2 shows the cyclic voltammogram of Pt electrode during the electrodeposition process. The consecutively increasing currents for both anodic and cathodic peaks demonstrated that ZnO/ZnHCF hybrid film was deposited continuously on the electrode surface. Two redox couples representing ZnHCF and ZnO were noticed. By the reduction of nitrate, ZnO was deposited through the potentiodynamic cycling due to base generation.

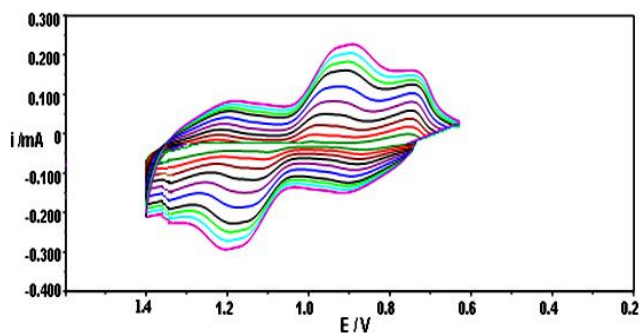


Fig. 2. Cyclic voltammogram of electrochemical deposition of zinc oxide/zinc hexacyanoferrate films at pH 2 of H_2SO_4 solution at a scan rate of $+0.2$ V to $+1.2$ Vs^{-1} with 50 mVs^{-1} .

Construction of PPO/ $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified Pt electrode and cyclic voltammetric measurement

Fig. 3 summarizes reactions involved in fabrication of the acetaminophen biosensor based on immobilization of PPO onto $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF/Pt}$ electrode. In order to evaluate the charge-transfer properties on the surface of the modified electrodes, cyclic voltammetry technique was employed by using potassium ferrocyanide as redox probe. CV recorded in $2.5\text{mM K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ solution and sodium phosphate buffer 0.05 M (pH 7.2) are shown in **Fig. 3**. Small voltammetric response was observed for the

unmodified electrode (**Fig. 3 a**). CV for the electropolymerization of $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film exhibits two oxidation peaks around -0.01 V to $+0.3$ V (**Fig. 3b**). Voltammogram results were recorded after several preliminary scans. The preservation of a quasi reversible electrode process and the large increase in peak currents for the nanocomposite film modified electrode proved that $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film exerted an obvious improvement effect on electric conductivity of enzyme electrode. After immobilization of enzyme, there was slight decrease in current as slow redox process occurred at the electrode surface due to slow electron transfer from the surface showing the immobilization of enzyme (**Fig. 3c**).

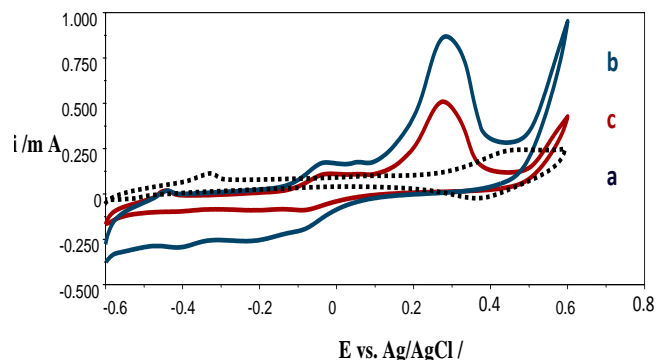


Fig. 3. Cyclic voltammogram of (a) bare Pt electrode, (b) $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF/Pt}$ (c) PPO/ $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified Pt electrode in a $2.5\text{mM K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ solution and sodium phosphate buffer 0.05M (pH 7.2) at a scan rate of 50 mVs^{-1} .

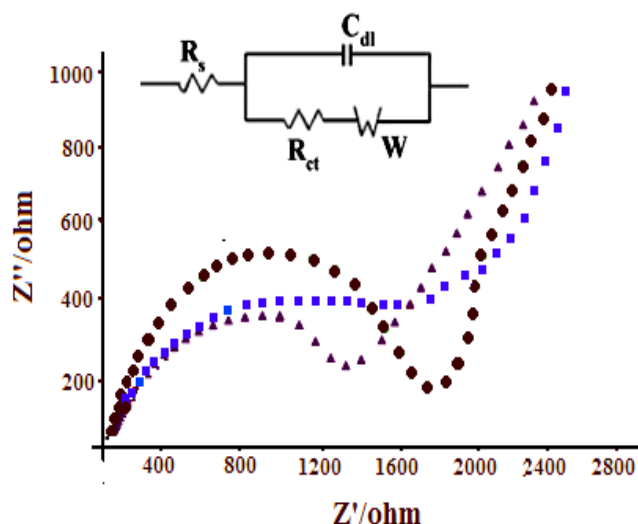


Fig. 4. EIS of (i) bare Pt electrode (ii), $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF/Pt}$ and (iii) PPO/ $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified Pt electrode in a solution containing 1 $\text{mM Fe}(\text{CN})_6^{3-/4-}$ with 0.1 M KCl at 0.20 mVs^{-1} (frequency range of 0.01 Hz -10 kHz).

Electrochemical impedance studies of modified Pt electrode

Fig. 4 shows electrochemical impedance spectra (EIS) of (a) bare electrode (b) $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF/Pt}$ electrode and (c) PPO/ $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified Pt electrode. The charge transfer process in PPO/ $\text{Fe}_3\text{O}_4\text{NP-ZnO/ZnHCF}$ hybrid film modified electrode has been studied by monitoring charge transfer resistance (R_{ct}) at the

electrode and electrolyte interface. The value of the electron transfer resistance (semicircle diameter) (R_{ct}) depends on the dielectric and insulating features at the electrode/electrolyte interface. The decreased R_{ct} value of $Fe_3O_4NP-ZnO/ZnHCF$ hybrid film modified Pt electrode compared to that of bare Pt electrode was due to the adsorption of Fe_3O_4NP onto $ZnO/ZnHCF$ hybrid film. This decrease in R_{ct} could be attributed to the fact that Fe_3O_4NP based electrochemical transducer surfaces promote the direct electron transfer reactions, amplify and orient the analytic signal of the recognition events. Upon immobilization of enzyme the electron transfer via redox couple was hindered by the presence of enzymes on electrode surface. The increased R_{ct} value of $PPO/Fe_3O_4NP-ZnO/ZnHCF$ hybrid film electrode was due to the immobilization of enzymes onto $Fe_3O_4NP-ZnO/ZnHCF$ hybrid film modified Pt electrode.

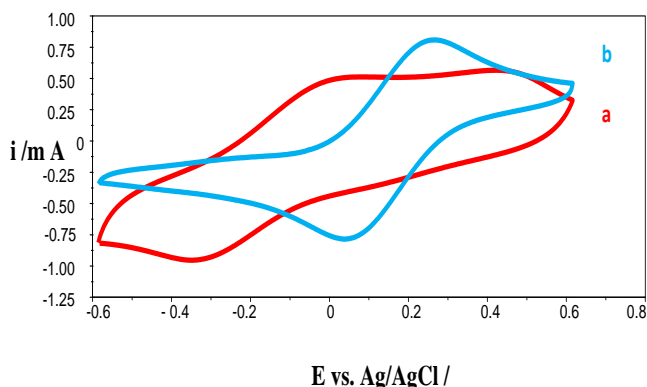


Fig. 5. Cyclic voltammograms (CV) of modified electrode (a) without and (b) with substrate at -0.6 to $+0.8$ V with a scan rate of 50 mVs^{-1} in a $2.5 \text{ mM } K_3Fe(CN)_6 / K_4Fe(CN)_6$ solution and sodium phosphate buffer 0.05 M ($\text{pH } 7.2$) at a scan rate of 50 mVs^{-1} .

Electrocatalytic oxidation of paracetamol at the surface of $Fe_3O_4NP-ZnO/ZnHCF$ hybrid film modified Pt electrode

It is expected that an electrocatalytic mechanism initiated by electrochemical oxidation of the reduced form of the complex exist at the surface of the electrode and then completed by chemical oxidation of paracetamol. To reveal the electrocatalytic activity of $Fe_3O_4NP-ZnO/ZnHCF$ hybrid film towards the oxidation of paracetamol, the voltammetric behaviors of paracetamol were investigated at the surfaces of unmodified and modified electrode. **Fig. 5** shows the cyclic voltammograms of modified Pt electrode in H_2SO_4 solution ($\text{pH } 2$) in the presence of $100 \mu\text{M}$ paracetamol (a) at the surface of the modified electrode and (b) at the surface of the unmodified electrode. At unmodified electrode, the direct oxidation of paracetamol was not significant, and just a small anodic current was observed due to the oxidation of paracetamol (**Fig. 5a**). However if the electrode was modified with $Fe_3O_4NP-ZnO/ZnHCF$ hybrid film, then a large anodic peak was observed with cathodic peak current decreasing accordingly (**Fig. 5b**) indicating the electrooxidation of paracetamol. **Fig. 6** shows square wave voltammogram of enzyme electrode for different concentration of paracetamol (0.04 to $10000 \mu\text{M}$). During response studies of the enzyme electrode, it was found that magnitude of the response

current increased with increasing concentration of paracetamol. Calibration was also performed using cyclic voltammetry with increasing concentration of paracetamol. The peak current is linearly related to the various concentrations of deferiprone (**Fig. 7**) Detection limit of the biosensor was $0.04 \mu\text{M}$ ($S/N=3$). Calibration was also performed using cyclic voltammetry with increasing concentration of paracetamol. The peak current is linearly related to the various concentrations of deferiprone (**Fig. 7**).

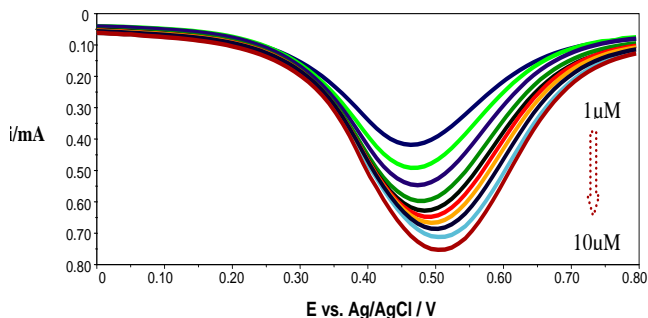


Fig. 6. SWVs of modified electrode for different concentrations of substrate in 0.1 M phosphate buffer/ 0.1 M KCl ($\text{pH } 7.0$) at a potential range of -0.6 V to $+0.8$ V, vs. Ag/AgCl with scan rate 50 mVs^{-1} .

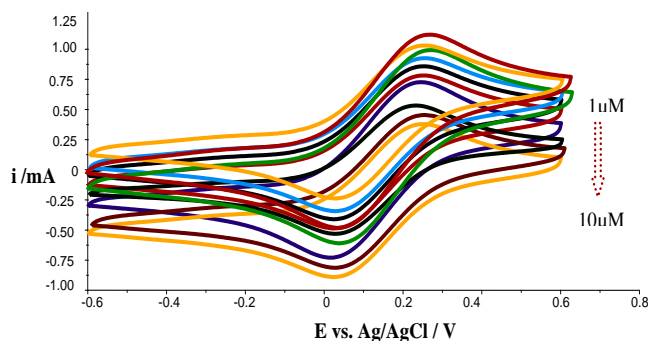


Fig. 7. Cyclic voltammetric responses for different concentrations of substrate in 0.1 M phosphate buffer/ 0.1 M KCl ($\text{pH } 7.0$) at a potential range of -0.6 V to $+0.8$ V, vs. Ag/AgCl with scan rate 50 mVs^{-1} .

Optimization of the biosensor

Effect of pH , response time, working potential and temperature on the biosensor was observed. The formation of $Fe_3O_4NP-ZnO/ZnHCF$ hybrid film on the Pt electrode surface was studied by dipping the modified electrode in H_2SO_4 supporting electrolyte of $\text{pH } 1, 2, 3, 4$ and 5 . The cyclic voltammetric responses of $ZnO/ZnHCF$ hybrid film modified electrodes in H_2SO_4 solution of $\text{pH } 1, 2, 3, 4$ and 5 respectively. There was a little increase in the current response at pH lower than 2 , however, there was a decrease in the current response at pH above than 2 . Thus $\text{pH } 2.0$ was selected for the formation of the film while the effect of pH of the buffer solution on the performance of the biosensor was also demonstrated. The effect of pH (ranging from 3 to 9) onto $PPO/Fe_3O_4NP-ZnO/ZnHCF$ bioelectrode has been investigated using CV technique in PBS (50 mM) containing $5 \text{ mM } [Fe(CN)_6]^{3-/4-}$ at scan rate of 50 mV/s . The pH dependence of the sensor response was evaluated over the pH range from 3 to 9 . The sensor response was found optimum at $\text{pH } 7.0$. (**Fig. 8 a**). The influence of

applied potential on paracetamol detection at hybrid nanocomposite ZnO-ZnHCF modified electrode was studied and an optimal potential of +0.1V vs. Ag/AgCl was found. The working potential was stepped from -0.6 to +0.8 V. This optimum applied potential of +0.25V make certain a minimizing of interference effects. The effect of incubation temperature on the biosensor was examined between 20 to 50 °C. (**Fig. 8b**) The current response of the biosensor increased with increasing temperature and reached a maximum at approximately 35 °C, and then went down as the temperature turned higher. In addition, the current response kept correspondingly steady between 20 to 50 °C, indicating that enzyme bioconjugate with modified electrode had good thermodynamic stability and life span. The effect of time on the enzymes was also determined by recording the current response from 2 to 12 s at an interval of 2 s. The modified electrode showed maximum response at 4s (**Fig. 8c**).

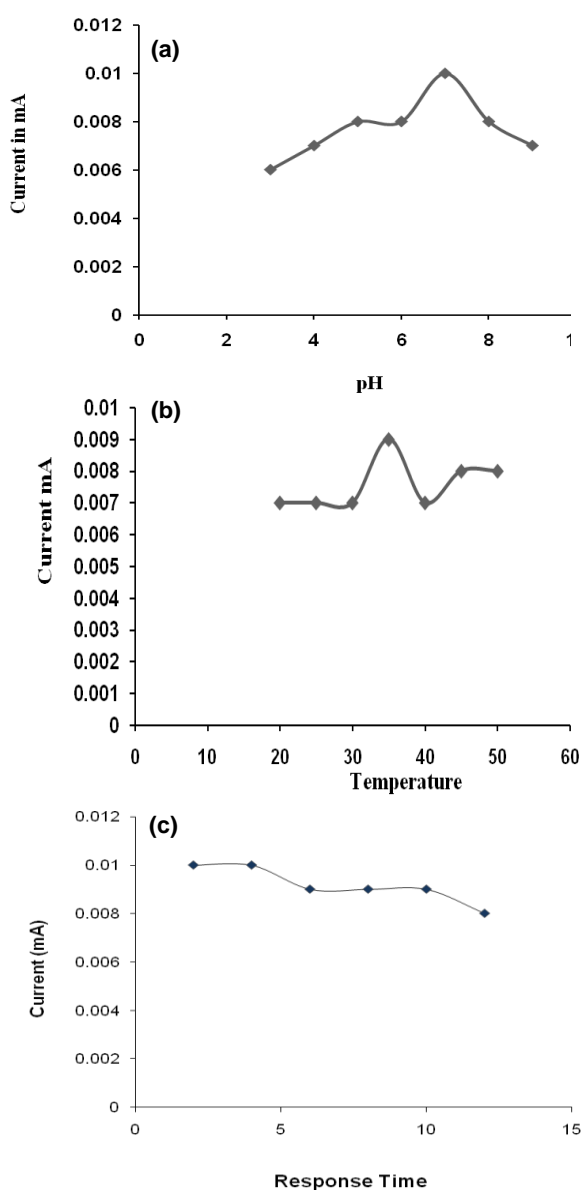


Fig. 8. Effects of pH (a) temperature (b) and response time (c) on the electrochemical response of fabricated acetaminophen biosensor based on HRP/ZrO@ Fe₃O₄NP/ CHIT in 0.1 M sodium phosphate buffer.

Analytic recovery and precision study

The analytic recovery of added acetaminophen was 95-96%. The precision of the present method were determined over seven days. Each day ten replicate quality control samples were analyzed for acetaminophen content. The within and between batch precision were less than 2% and 3% respectively (data not shown). These results demonstrated that the described method was precise and reproducible.

Table 1. Determination of acetaminophen by present sensor based on Fe₃O₄NPs/ZnO-ZnHCF/Pt electrode and standard enzymatic method.

S. No.	Sample	Pharmacopoeia Method (μM)	Present Method (μM)
1.	A	5000	5000
2.	B	2500	2600
3.	C	1000	1020
4.	D	2000	1800
5.	E	3000	3020
6.	F	4000	4000
7.	G	3600	3600
8.	H	5400	5450
9.	I	1450	1520
10.	J	4900	4900

Table 2. Substrate specificity effect on Fe₃O₄NPs/ZnO-ZnHCF/Pt electrode.

Substrate	Relative rate of oxidation (%)
L-DOPA	98.0
Catechol	90.2
Dopamine	98.0
Hydroquinone,	5.2
Isoprenaline	10.3
Terbutaline	10.3

Interference study

Various interfering substances were tested which are used in acetaminophen pharmaceutical formulation such as citric acid, sodium benzoate, stearic acid, sodium metabisulphite and saccharin were evaluated using the proposed biosensor at their formulation concentration, none had practically any significant interference (data not shown). To study the substrate specificity on the electrochemical response of sensor, the following compounds such as L-DOPA, catechol, dopamine, hydroquinone, isoprenaline and terbutaline. All are phenolic compounds which were added in place of paracetamol in the reaction mixture individually at a final concentration of 1.0 mM and the electrochemical response was measured under the similar assay conditions. Results depicted that dopamine, catechol and L-DOPA was utilized as substrate, while hydroquinone and isoprenaline were practically unutilized. This shows the absolute substrate specificity of the developed sensor (**Table 1**).

Determination of acetaminophen in various commercial samples

The proposed procedure was applied to determine paracetamol in pharmaceutical formulations. **Table 2** presents the results obtained for four commercial samples by replacing acetaminophen with samples. To study the accuracy of the present method, acetaminophen level in samples were determined by both the pharmacopoeial method (x) and the present method (y). The values obtained by both the methods matched with each other with a good correlation ($r^2 = 0.99$) (data not shown).

Stability of the enzyme electrode

The shelf life of the PPO/Fe₃O₄NP-ZnO/ZnHCF/PtE was investigated by measuring its electrochemical current response for 6 months at a regular interval of 1 week. The PPO/Fe₃O₄NP-ZnO/ZnHCF/Pt electrode retained about 50% of its initial activity even after 6 months, when stored in dry conditions at 4 °C. A comparison of analytic parameters of various nanoparticles based biosensors for detection of paracetamol with the present biosensor is summarized in **Table 3**.

Table 3. Comparison of present method with other biosensing methods.

Matrix/method	Enzyme	Response time	Detection limit (μM)	Linearity (μM)	Stability	Reference
C-Ni/GCE/DPV	-	-	-	7.8-110	-	[19]
Chronoamperometry MWCNT-film coated electrode	-	-	0.04	0.1-20	-	[20]
Carbon nanoparticles (CNPs)/GCE /Voltammetry	-	-	0.05	0.1-100	-	[21]
Cobalt hydroxyl nanoparticles/Cyclic voltammetry	-	-	10	2.5-1000	-	[22]
Nanogold/ITOE /Cyclic voltammetry	-	-	0.18	0.2-1500	-	[23]
PPO/ZnO/ZnHCF/Fe ₃ O ₄ NP/Pt	PPO	4s	0.04	0.04 to 10000	-	Present

Conclusion

In the present work, we have described a promising electrochemical biosensor for the analysis of acetaminophen based on the sensing interface PPO/Fe₃O₄NP-ZnO/ZnHCF/PtE. The zin oxide film was electrodeposited through potentiodynamic cycling. The decorated film possesses large surface area and good uniformity, ideal matrixes for enzyme immobilization and also increases the electrical activity of the formed. Our electrochemical measurements reveal that the immobilized PPO exhibits high biological activity and stability and that the presence of magnetic nanoparticles further enhances the sensitivity of the designed electrochemical biosensor for analysis of acetaminophen. The developed biosensor

exhibits fast amperometric response, high sensitivity, low detection limit (0.04 μM) and long-time stability.

Acknowledgements

The present work was supported to one of the author (Jagriti Narang) by SERB, Department of Science and Technology (DST), India. Thanks to all scientists referenced throughout the paper whose valuable work has guided the way through to this research work.

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