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# Monitoring analgesic drug using sensing method based on nanocomposite

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**Subject category:** Enzymatic assays and analysis

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**Abstract**

This work shows rapid, reliable and sensitive electrochemical method for acetaminophen determination, which is a safe analgesic drug. Most of the methods used till now for its therapeutic drug monitoring required a pre-treatment of the sample. Biosensors steer clear of this kind of drawbacks. A horseradish peroxidase (HRP) was immobilized using core-Shell ZrO@ $Fe_3O_4$ nanoparticles on chitosan hybrid film electrodeposited on the surface of Au electrode. The surface functionalization of core-Shell ZrO@ $Fe_3O_4$NPs on chitosan hybrid film was characterized by cyclic voltammetry (CV), scanning electron microscopy (SEM), and electrochemical impedance spectroscopy (EIS). Experimental variables that can affect acetaminophen amperometric response, like pH, temperature and applied potential have been optimized in order to perform a selective acetaminophen determination. An average limit of detection of 0.01 μM (S/N=3) was obtained. The biosensor had been finally applied to the determination of acetaminophen in complex matrices, such as pharmaceutical drugs.

*Keywords:* Acetaminophen, core-Shell ZrO@ $Fe_3O_4$ nanoparticles, chitosan film, Au electrode

## 1. Introduction

APAP (*N*-acetyl-*p*-aminophenol or paracetamol) is an analgesic and one of the most commonly taken drugs [1]. It is an effective mild analgesic and antipyretic agent extensively used at therapeutic dosage for the safe relief of mild to moderate pain [2-4] but acute overdoses can cause serious hepatic damage which may result in death [5]. Its overdoses causes 2600 hospitalizations, >56,000 emergency room visits, and approximately 458 acute liver failures each year in the United States [6]. So, extensive effort should be made to detect the cause for its toxicity and its mitigation but for that, its exact level determination would require an accurate method. Various methods are available for determination of acetaminophen, like raman spectrometry [7], liquid chromatography (LC)[8] chemiluminescence[9] , and spectrometric methods[10] . However, these methods have some setbacks which make them unsuitable for routine analyses such as high costs, time consuming and pretreatment of sample, skilled person to operate and even low sensitivity and selectivity in some cases. Thus there should be reliable and precise method for measuring APAP drug to maintain a constant concentration, thereby optimizing individual dosage regimen. The use of biosensors is a method that overcomes these problems due to their intrinsic specificity, low costs, fast analyses and minimal requirements for sample pretreatment[11]. Hence, the amperometric biosensors based on direct enzyme immobilization on the transducer surface are the main analytical strategies used for acetaminophen analysis [12].

Nanoparticles exhibit very unique electrical and magnetic properties which are distinct from their bulk counterparts. Among these, magnetic nanoparticles are biocompatible and potentially non-toxic for biosensors applications [13-15]. Immobilization of enzymes on magnetic nanoparticles has advantage of distinctive characters like enhancing their activity, mediating rapid contacting between the enzyme and its substrate, and reducing mass-transfer limitations [16, 17].

Magnetic nanoparticles provide large surface area and biocompatible micro-environment to the immobilized enzyme which helps in providing close proximity to the analyte and sensing element and prove to be best sensing interface for the fabrication of biosensor. Magnetic nanoparticles have a large surface area which can be easily oxidized to form aggregates but this can change their original structure and unique properties. To prevail over this difficulty, the surface of NPs has been coated with protective layer of various materials [18, 19] Here, $ZrO_2$ (Zirconium oxide) has become a favored coating material due to its good insulating property, simple synthetic procedure & chemical functionality, chemical inertness and wear resistance [20]. It is believed that magnetic nanoparticles can avoid being oxidized and maintain

their magnetic properties (such as coercivity or blocking temperature) by the $ZrO_2$ coating. Thus, the magnetic cores can be protected from oxidation and corrosion [21].

Chitosan (CHIT) is an extensive biopolymer for immobilization of biomolecules, due to its excellent film-forming ability, high permeability, mechanical strength, non-toxicity, biocompatibility, low cost and easy availability [22]. It was chosen as the orientation directing matrix because large quantities of amino and hydroxyl groups are present on the CHIT units to amplify binding ability to enzyme [23–27].

We describe herein the therapeutic drug monitoring of acetaminophen using biosensor based on Core-Shell ZrO@ $Fe_3O_4$ Nanoparticles on chitosan hybrid film.

## 2. Materials and methods

### *2.1. Materials*

Acetaminophen (Paracetamol) was purchased from Sigma (St. Louis, MO). Iron(III) chloride hexahydrate (98%), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$) sodium borohydride powder (98%) and zirconium(IV) tert-butoxide were obtained from Sisco Research Laboratory Pvt. Ltd., Mumbai, India. All other chemicals were of analytic reagent grade. Double distilled water (DW) was used throughout the experiments.

### *2.2. Apparatus and methods*

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed on 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 Au wire as a working electrode. All the electrochemical experiments were performed at an ambient temperature (25 ◦C). Fourier transform infrared (FTIR) spectroscopy was performed on FTIR spectrometer (Make: iS10, Thermoelectron, USA). Scanning electron microscopy (SEM) measurements were carried out at Department of Chemistry, M. D. University, Rohtak. Transmission electron microscopy (TEM) was performed at Punjab University, Chandigarh. Ultrasonication was performed on Misonix Ultrasonic Liquid Processors (mode XL-2000 series). X-ray diffraction (XRD) experiments were conducted on a X-ray diffractometer (Make: Rigaku, D/Max2550,

Tokyo, Japan) at Department of Physics, G.J. University, Hisar, collecting data in steps of 2-theta from 20 to 70 (2- theta). The database of the Joint Committee on Powder Diffraction Standards (JCPDS) was used for phase identification.

*2.3. Synthesis of $Fe_3O_4$NP*

$Fe_3O_4$NP were prepared according to the method of Predoi [28]. The 0.5 M ferrous chloride tetrahydrate ($FeCl_2.4H_2O$) in 2M HCl and 0.5 M ferric chloride hexahydratate ($FeCl_3.6H_2O$) in DW were mixed 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 by centrifugation at 8000×g and dried at 40$^o$C.

*2.4. Preparation of ZrO @ Fe3O4NP*

ZrO @ $Fe_3O_4$NP were prepared by hydrolysis and condensation method of Chaubey [29]. Fe nanoparticles (3 g) and zirconium (IV) tert-butoxide (10 ml) were taken in a 50 ml beaker in inert atmosphere. To this mixture, 50 ml of ethanol was added and stirred mechanically for 2 h. The mixture was centrifuged at 10000 rpm for 5 min. The encapsulated particles were removed and washed several times with ethanol. This zirconia coated iron oxide nanoparticles (ZrO @ $Fe_3O_4$NP) were kept at 40°C for drying. The characterization of zirconia coated iron oxide nanoparticles was carried out by recording its UV & visible spectra in a UV-visible spectrophotometer, X-ray diffraction pattern in an X-ray diffractometer (XRD) and Transmission electron micrograph in Transmission electron microscopy.

*2.5 Construction of ZrO@ $Fe_3O_4$NP/ CHIT hybrid film onto Au electrode*

The surface of Au electrode was polished with alumina slurry and was dipped into sodium phosphate buffer (0.1 M, pH 7.0) containing chitosan (500µl) and ZrO @ $Fe_3O_4$NP suspension (500 µl) and subjected to 10 successive deposition cycles at - 0.1 to 0.1 V using a Potentiosatat-Galvanostat (Fig 1). The modified Au electrode was washed thoroughly with DW to remove unbound matter.

*2.6 Preparation of enzyme electrode for electrochemical sensing of analgesic drug*

The purified HRP enzyme was immobilized onto the surface of ZrO@ $Fe_3O_4$NP/ CHIT hybrid film. First, Au electrode was dipped into glutaraldehyde (2.5%) at room temperature and washed thoroughly with DW. This modified electrode was dipped into 10 µl of enzyme solution (40 mg $ml^{-1}$ protein) and kept undisturbed for approximately 12 h at $4^{o}$ C. The electrode was finally washed with 0.1M Tris HCl buffer (pH 8.5) to remove unbound enzyme. The resulting HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode was used as working electrode and stored at $4^{o}$C, when not in use. This working electrode was characterized by SEM at different stages of its construction (Scheme 1.).

*2.7. Electrochemical characterization of HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode*

Cyclic voltammetry studies were carried out using a three electrode system composed of HRP/ZrO@$Fe_3O_4$NP/ CHIT /Au electrode as working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode. Cyclic voltammograms of bare Au electrode, ZrO@$Fe_3O_4$NP/ CHIT /Au electrode, and HRP/ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode were recorded in sodium phosphate buffer (0.1 M, pH 7.0, containing 0.1 mM $H_2O_2$) in potential ranging between -0.1 to +1 V $s^{-1}$ at a scan rate of 50 mV $s^{-1}$.

*2.8.Preparation of analgesic drug solution*

Paracetamol (acetaminophen) was prepared in phosphate buffer solution (pH 7.0). Solutions of different concentrations of Paracetamol (acetaminophen) ranging from 0.01 to 10000 µM were prepared in 0.1 M sodium phosphate buffer (pH 7.0) and stored at 4°C until use.

*2.9. Optimization of analgesic drug biosensor*

To optimize working conditions of the biosensor, effects of pH, incubation temperature, time and substrate concentration on biosensor response were studied. To determine optimum pH, the pH of reaction buffer sodium phosphate buffers was varied from 5.0 to 8.0, each at a final concentration of

0.1M. To determine optimum temperature, the reaction mixture was incubated at different temperature (20–50ºC) at intervals of 5ºC. The effect of substrate concentration on biosensor response was determined by varying the concentration of acetaminophen in the range 0.01 to 10000 μM. To optimize the applied potential for the acetaminophen determination, the effect of applied potential on the response current was investigated in the range -0.1 to +0.1 V vs Ag/AgCl. The optimal current was measured at -0.75 V vs. Ag/AgCl. Hence subsequent electrochemical studies were carried at -0.07 V vs Ag/AgCl.

*2.10. Amperometric determination of analgesic drug*

Each pharmaceutical product (1.0 ml) was stirred until complete dissolution and then diluted to 10, 20 and 50 ml with phosphate buffer solution (0.1 M; pH 7.0). Finally, each pharmaceutical product (400 μl) was added to the cell containing 10 ml of phosphate buffer solution (0.1 M; pH 7.0). The measurements were performed after successive additions of pharmaceutical product. After each addition, cyclic voltammograms was recorded by cycling the potential between -0.1 and +0.1 V at a scan rate of 100 mV $s^{-1}$. Acetaminophen content in pharmaceutical product was determined by the present biosensor and recording the current (mA) under its optimal working conditions (Fig.1).

*2.11. Storage stability of HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au 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 1 month at 4ºC.

## 3. Results and Discussions

*3.1. Characterization of ZrO@ $Fe_3O_4$NP*

Fig. 2A shows XRD of ZrO@ $Fe_3O_4$NP hybrid film, peaks observed were matched up to only bcc Fe structure and shows no peak for Fe oxide or crystalline $ZrO_2$. TEM image of ZrO@ $Fe_3O_4$NP (Fig. 2B)

shows the occurrence of spherical particles tending to form chains, indicating ferromagnetic interaction. Iron nanoparticles aggregation led to the trapping of zirconia shell during coating process (30 nm). These observations confirm formation of ZrO@ $Fe_3O_4$NP.

*3.2. Scanning electron microscopy (SEM), electrochemical impedance studies (EIS) and Fourier transform infrared (FTIR) spectroscopy of modified Au electrode*

To confirm enzyme immobilization on the ZrO@$Fe_3O_4$NP film, the surface morphologies of bare electrode (a), ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode (b) and HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au bioelectrode (c) were investigated by SEM (Fig. 3). The bare electrode showed smooth surface (top image). The granular morphology with roughness of ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode showed the coating of nanoparticles on chitosan (middle image). Some flakes like structure observed after immobilization of enzyme on the electrode which confirms the immobilization of enzyme (bottom image).

Fig.3B shows electrochemical impedance spectra (EIS) of CHIT/Au electrode (a), ZrO@ $Fe_3O_4$NP/ CHIT/Au electrode (b) and HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode (c). The Rct values for the CHIT/Au electrode, ZrO@ $Fe_3O_4$NP/CHIT/Au electrode and HRP/ZrO@ $Fe_3O_4$ NP/CHIT/Au electrode were obtained as 800, 380 and 600 Ω, respectively. Upon immobilization of enzyme, Rct value of HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode gets increased. It is due to the fact that most biological molecules, including enzymes, are poor electrical conductors which cause hindrance to the electron transfer.

` Fig. 4C shows FTIR spectra of ZrO@ $Fe_3O_4$NP/ CHIT/Au electrode (upper curve) and HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode (lower curve). The CHIT exhibited characteristic absorption bands of amino saccharide at 3421 $cm^{-1}$ (due to overlapping of OH and $NH_2$ stretching), 2811 $cm^{-1}$ (due to–$CH_2$ stretching) and 1647 $cm^{-1}$ (due to C–O stretching), while the 634 $cm^{-1}$ in ZrO@ $Fe_3O_4$NP are characteristic bands for Zr–O film (curve i). The enzyme mixture was immobilized onto chitosan through covalent binding with glutaraldehyde. One CHO group of glutaraldehyde was linked to $NH_2$ group on surface of enzymes, while other CHO group was bound to $NH_2$ group of chitosan on CHIT/ ZrO@ $Fe_3O_4$NP composite film, which provided physically more stable complex. FTIR spectra of HRP/CHIT/ ZrO@ $Fe_3O_4$NP /Au electrode showed broadening of the peak at 3264 $cm^{-1}$ and 1653 $cm^{-1}$

due to the addition of carbonyl and amino groups confirming the binding of enzyme with the CHIT/ ZrO@ $Fe_3O_4$NP matrix (curve ii). This change indicates that the enzyme was attached to ZrO@ $Fe_3O_4$NP/ CHIT/Au composite film.

**Approximate position for Fig.2.**

### 3.3 *Construction of HRP/ZrO@ $Fe_3O_4$NP/ CHIT modified Au electrode and cyclic voltammetric measurement*

To confirm electron transfer regime, CV technique was employed at the electrode surface. Unmodified electrode is not able to take redox reactions at an electrode surface. Modification of electrode with ZrO@ $Fe_3O_4$NP/ CHIT resulted into fast electron transfer reactivity. As is shown in Fig. 5, a pair of well defined, quasi-reversible redox peaks can be obtained with a ZrO@ $Fe_3O_4$NP/ CHIT modified electrode for 0.1M pH 7.0 PBS. $Fe_3O_4$ NPs also act as electron-transfer mediators, but also play an important role in the preparation of immobilized enzymes due to their desirable characteristics: large pore size and volume, and good electron conductivity (CV curve i) [18]. $Fe_3O_4$ NPs also create suitable microenvironment which benefit the exposition of the active center, and increase the activity of enzyme [19]. In contrast, decrease in peaks observed at the enzyme modified electrode as protein might cause hindrance in transfer of electrons (CV curve ii). No peak exists on the voltammogram at unmodified electrode (CV curve iii).Results were matched with the EIS study.

**Approximate position for Fig.3**

### *3.4. The principle of ZrO@ $Fe_3O_4$NP/ CHIT hybrid film modified Au electrode for electrochemical sensing of analgesic drug*

It is likely that an electrocatalytic mechanism initiated by HRP catalyzes the oxidation of paracetamol to *N*-acetyl-*p*-benzoquinoneimine (Scheme 2.). The resulting current is proportional to the concentration of phenolic compounds in solution. It is expected that because of the participation of proton(s) in the oxidation reaction of acetaminophen to N-acetyl-*p*-benzoquinone-imine, and vice-versa within a quasi-reversible two-electron process [30]. So, significantly increased redox peak currents, greatly increased electron transfer rate of APAP at the ZrO@ $Fe_3O_4$NP/ CHIT/AuE. As can be seen in Fig 6, oxidation

peak signal significantly increases to 750 μA. Fig 7 shows the CV curve showing the electrode modified with HRP only and with HRP/ ZrO@ $Fe_3O_4$NP/ CHIT/AuE, oxidation peak signal significantly increases to 1000 μA when nanocomposite get decorated on the Au electrode . These results demonstrated that the electrochemical reactivity of APAP is remarkably improved on the ZrO@ $Fe_3O_4$NP/ CHIT/AuE

**Approximate position for Fig.5.**

*3.5. Optimization of the biosensor*

To improve the performance of the biosensor, the effect of the determination conditions such as the working potential, pH value, response time and temperature on the response of the ZrO@ $Fe_3O_4$NP/ CHIT/Au electrode was investigated in detail.

The effect of the working potential on the response current of the ZrO@ $Fe_3O_4$NP/ CHIT/Au electrode is studied. When the applied potential was changed from -0.1 to +0.1 V, the response current increased obviously. The maximum response current was achieved at around −0.07 V. When the applied potential became more negative, there may be interfering reactions from other electroactive species in the solution. Therefore, an applied potential of −0.07V was selected to give a high detection sensitivity and good signal/noise ratio. The effect of the pH value on the response current of the ZrO@ $Fe_3O_4$NP/ CHIT/Au electrode was studied between 5.0 and 8.0 in 0.05 M PBS. As shown in Fig. 2B, the response current increased from 5.0 to 7.0 and decreased from 7.0 to 8.0, and so the maximum current response was at pH 7.0.(Fig.8a). Therefore, the pH 7.0 was suitable for the maximum activity of immobilized HRP, and was in agreement with that reported for soluble HRP. The response time was less than 4 s, which shows a quick response and the immobilized HRP could well catalyze the reduction of $H_2O_2$ (Fig.8c). The faster response was mainly ascribed to the fact that ZrO@ $Fe_3O_4$NP are providing favorable orientation and conductive pathway to transfer electrons. Additionally, through ZrO@ $Fe_3O_4$NP exposed surface $H_2O_2$ molecule can freely diffuse to the HRP molecules. Effect of temperature on biosensor was also studied in order to ensure the optimization. The current response reaches a maximum at approximately 50°C, and then goes down as the temperature turn higher. In contrast, the modified electrode without ZrO@ $Fe_3O_4$NP shows that the response declines when temperature is higher

than 40°C. The result indicates that enzyme bioconjugated with ZrO @ $Fe_3O_4$NP has good thermodynamic stability and life span. In order to keep consistent with the temperature of human body, 35°C was selected for this work (Fig.8b).

*3.6. Voltammetric determination of analgesic drug*

Cyclic voltammetry (CV) was used to determine acetaminophen in array to obtain higher sensitivity. CV curves of different concentration of acetaminophen at ZrO@ $Fe_3O_4$NP/ CHIT/AuE modified electrode were obtained (Fig 5). The peak current increased linearly with increase in acetaminophen concentration. The limit of detection was 0.01 μM.

*3.7. Reproducibility*

The repeatability of the biosensor was valued at a acetaminophen concentration of 0.1 mM in PBS (0.1 M) with the same enzyme electrode. The relative standard deviation (R.S.D.) was 1.2% for ten successive assays.

*3.8. Selectivity and real sample analysis*

The effect of substances that might interfere with the response of the biosensor was studied. The selectivity of the biosensor was examined in the presence of acetaminophen (0.2 mM). The addition of the same concentration citric acid, sodium benzoate, stearic acid, sodium metabisulphite and saccharin did not cause observable interference. Only stearic acid decreased the response 10% and has a significant interference. The proposed procedure was applied to determine paracetamol in pharmaceutical formulations. Table 1 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 pharmacopoeia method (x) and the present method (y). The values obtained by both the methods matched with each other with a good correlation (r = 0.95).

### *3.9. Stability of the enzyme electrode*

The stability of the biosensor is investigated and the current response of biosensor is retained about 90 % of its original response after 40 times uninterrupted detection. In addition, the long-term stability is also tested after a month. It is revealed that the current response of the sensor maintains 84% of the initial current response. This means that ZrO@ $Fe_3O_4$NP ensure well stability of the biosensor.

A comparison of present biosensor with other biosensing methods is given in Table.2.

## 4. Conclusions

A novel strategy for developing a composite electrode consisting HRP/ZrO@$Fe_3O_4$NP/ CHIT/AuE, which showed relatively rapid response, high sensitivity, broad linear range, low detection limit, good reproducibility, and long term stability. Moreover, this biosensor almost eliminated the interference. Therefore, this novel biosensor could be readily extended to the detection of other clinically important antigens by using ZrO@ $Fe_3O_4$NP to develop other simple and practical biosensors.

## ACKNOWLDGMENTS

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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## Captions to Figures

**Scheme . 1.** Graphical representations of the stepwise amperometric sensor fabrication process.

**Fig. 1** Linear response of concentrations of acetaminophen (Substrate concentration/μM) vs. current (I/mA).

**Scheme. 2.** Electrochemical reaction at HRP/ZrO@$Fe_3O_4$NP/ CHIT hybrid film modified Au electrode.

**Fig. 2** **(A)** Transmission electron microscope (TEM) image of ZrO@ $Fe_3O_4$NP and **(B)** X-ray diffraction (XRD) pattern of ZrO@ $Fe_3O_4$NPs.

**Fig. 3** **(A)** SEM of bare Au electrode (*top*), ZrO@$Fe_3O_4$NP/ CHIT hybrid film modified Au electrode (*middle*) and HRP/ZrO@ $Fe_3O_4$NP/ CHIT hybrid film modified Au electrode (*bottom*). **(B)** EIS of ZrO@ $Fe_3O_4$NP/ CHIT hybrid film (a), HRP/ZrO@ $Fe_3O_4$NP/CHIT hybrid film modified Au electrode (b) and bare Au electrode (c) in a solution containing 1 mM $Fe(CN)_6^{3-/4-}$ with 0.1 M KCl at 0.20 mV $s^{-1}$ (frequency range of 0.01 Hz –10 kHz). **(C)** FTIR spectra of ZrO@ $Fe_3O_4$NP/ CHIT/Au electrode (upper curve) and HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode (lower curve).

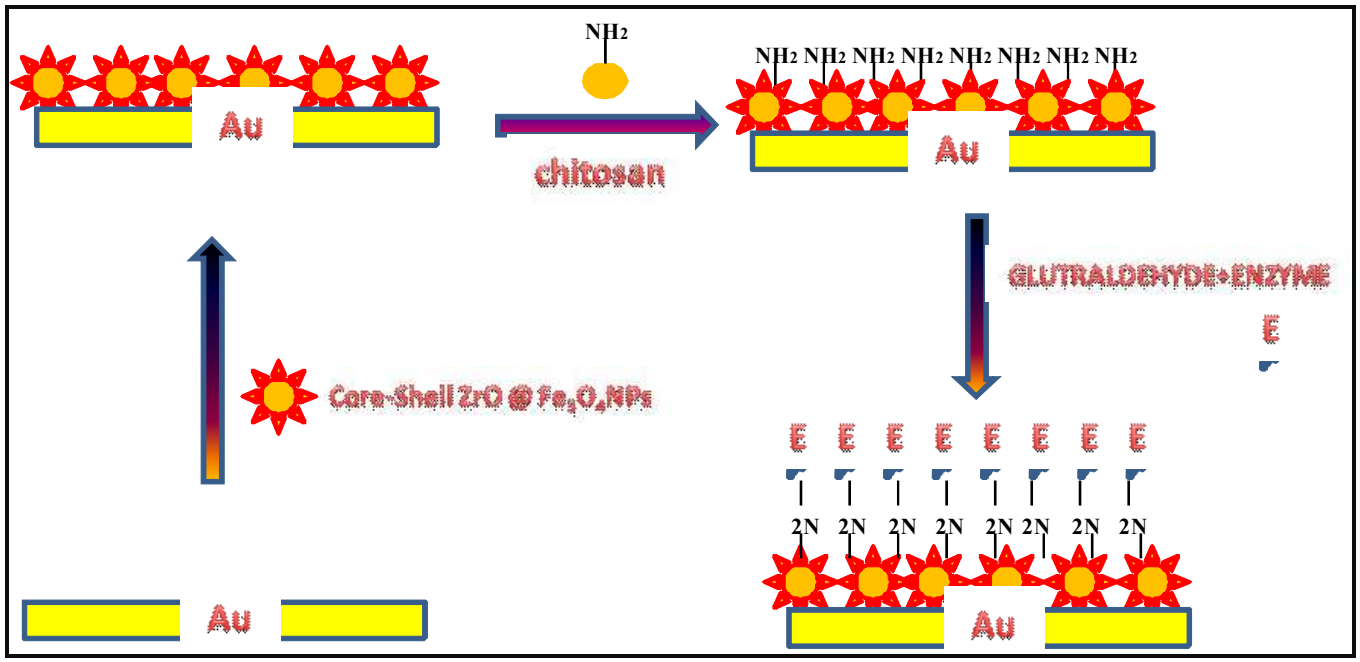
**Fig. 4** Cyclic voltammograms of (i) ZrO@ $Fe_3O_4$NP/ CHIT hybrid film, (ii) HRP/ZrO@ $Fe_3O_4$NP/ CHIT hybrid film and (iii) bare Au electrode modified Au electrode in a 2.5 mM $K_3Fe(CN)_6$ /$K_4Fe(CN)_6$ solution and sodium phosphate buffer 0.05M (pH 7.2) at a scan rate of 50 $mVs^{-1}$.

**Fig. 5** Cyclic voltammograms for HRP/ZrO@ $Fe_3O_4$NP/ CHIT /Au electrode in PBS (pH 7.0) in presence of substrate (i) and in absence of substrate (ii), at scan rate of 5 mV $s^{-1}$.

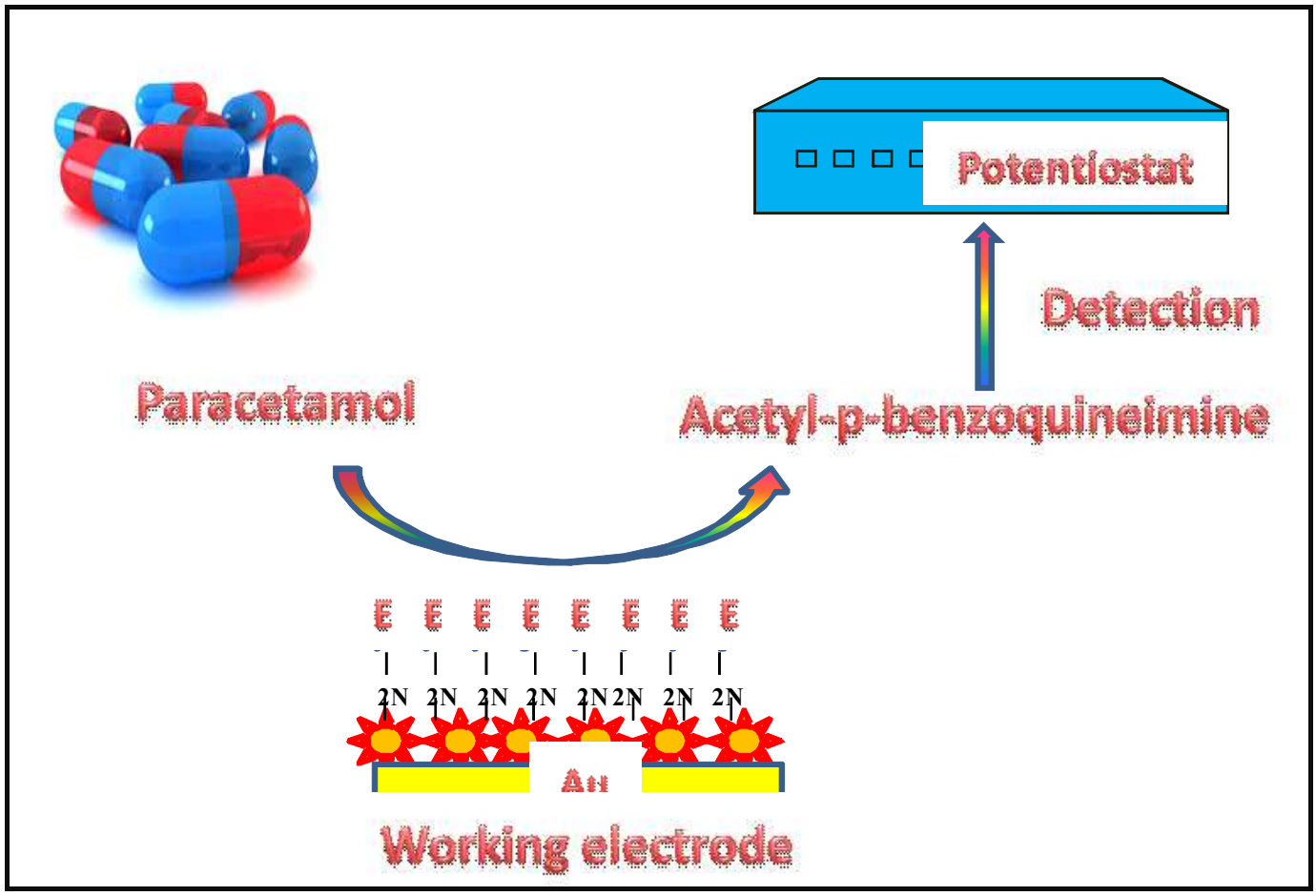
**Fig. 6** Cyclic voltammograms of HRP/ZrO@ $Fe_3O_4$NP/ CHIT at various concentrations of acetaminophen.

**Fig. 7** Cyclic voltammograms of (i) HRP/Au (ii) HRP/ZrO@ $Fe_3O_4$NP/ CHIT hybrid film modified Au electrode in a 2.5 mM $K_3Fe(CN)_6$ /$K_4Fe(CN)_6$ solution and sodium phosphate buffer 0.05M (pH 7.2) at a scan rate of 50 $mVs^{-1}$.

**Fig. 8** Effects of pH (a) and temperature (b) on the electrochemical response of fabricated acetaminophen biosensor based on HRP/ZrO@ $Fe_3O_4$NP/ CHIT in 0.1 M sodium phosphate buffer.




**Scheme 1.**




**Scheme 2.**

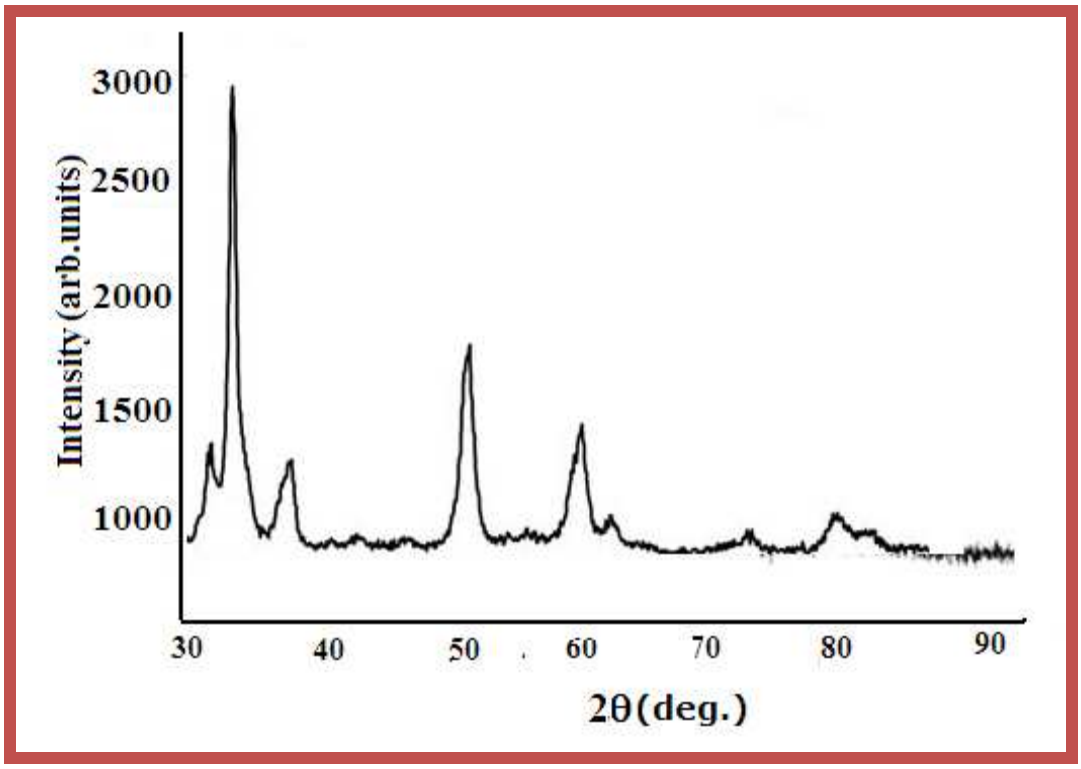



**Fig.1 A**

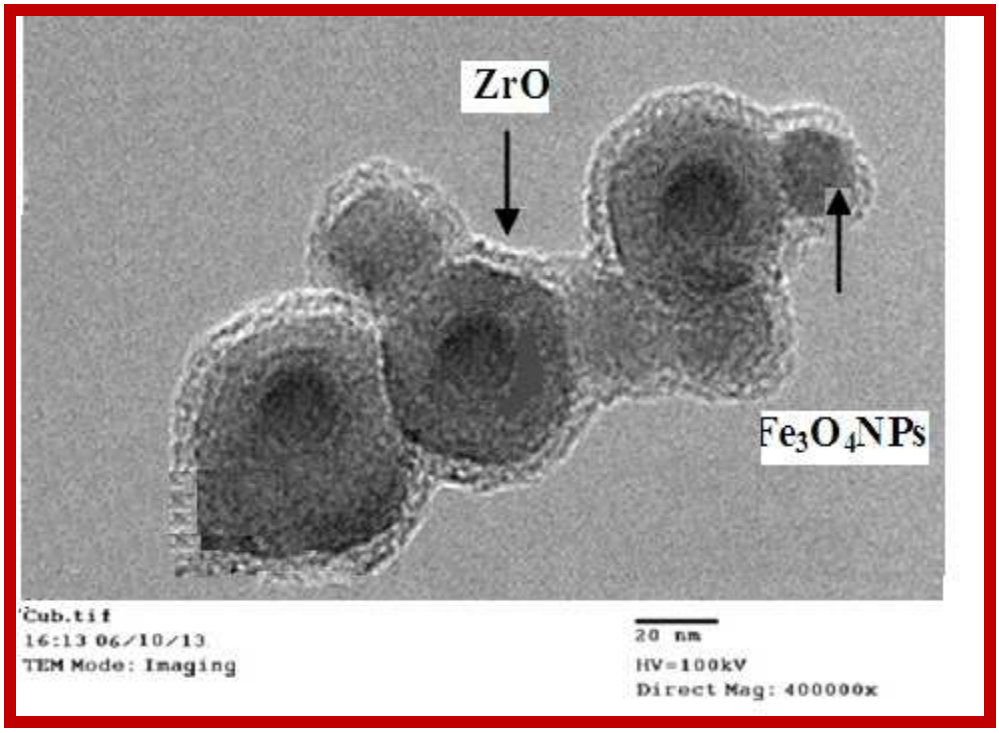



**Fig.1 B**

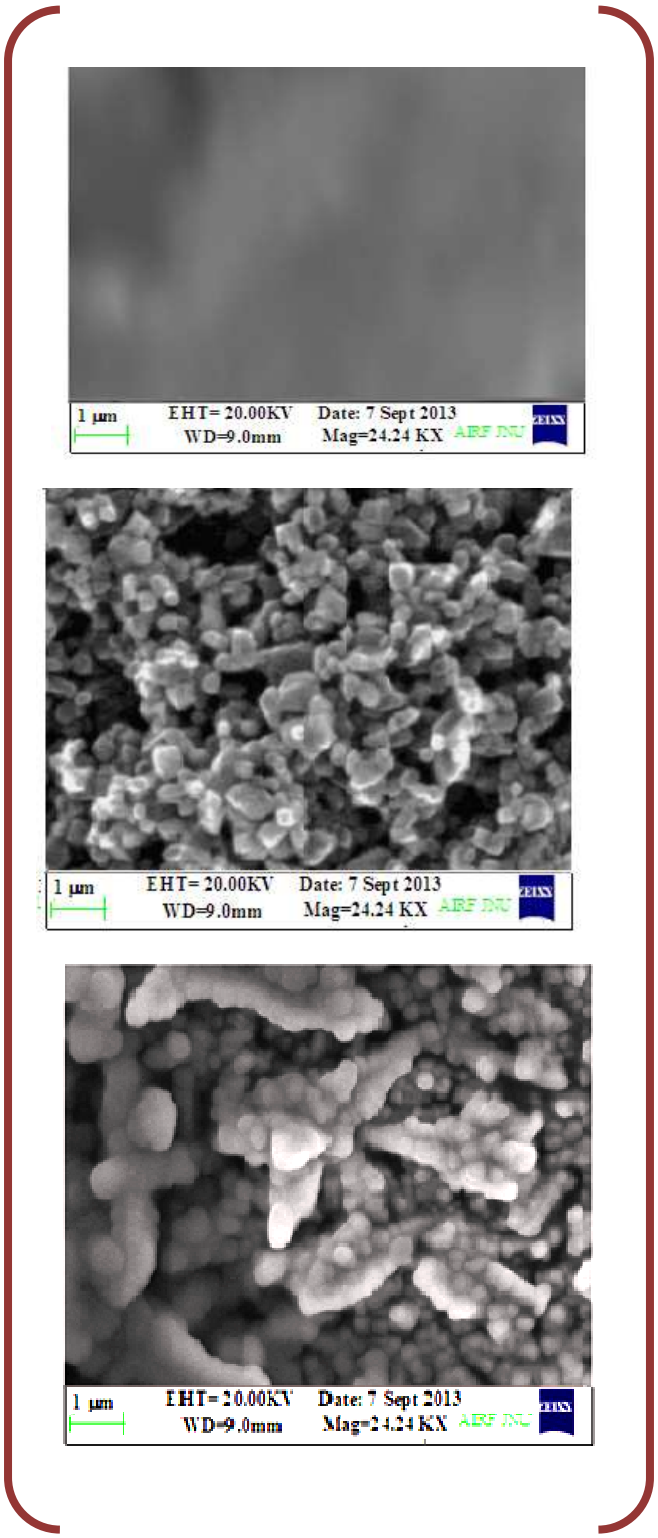



**Fig.2A**

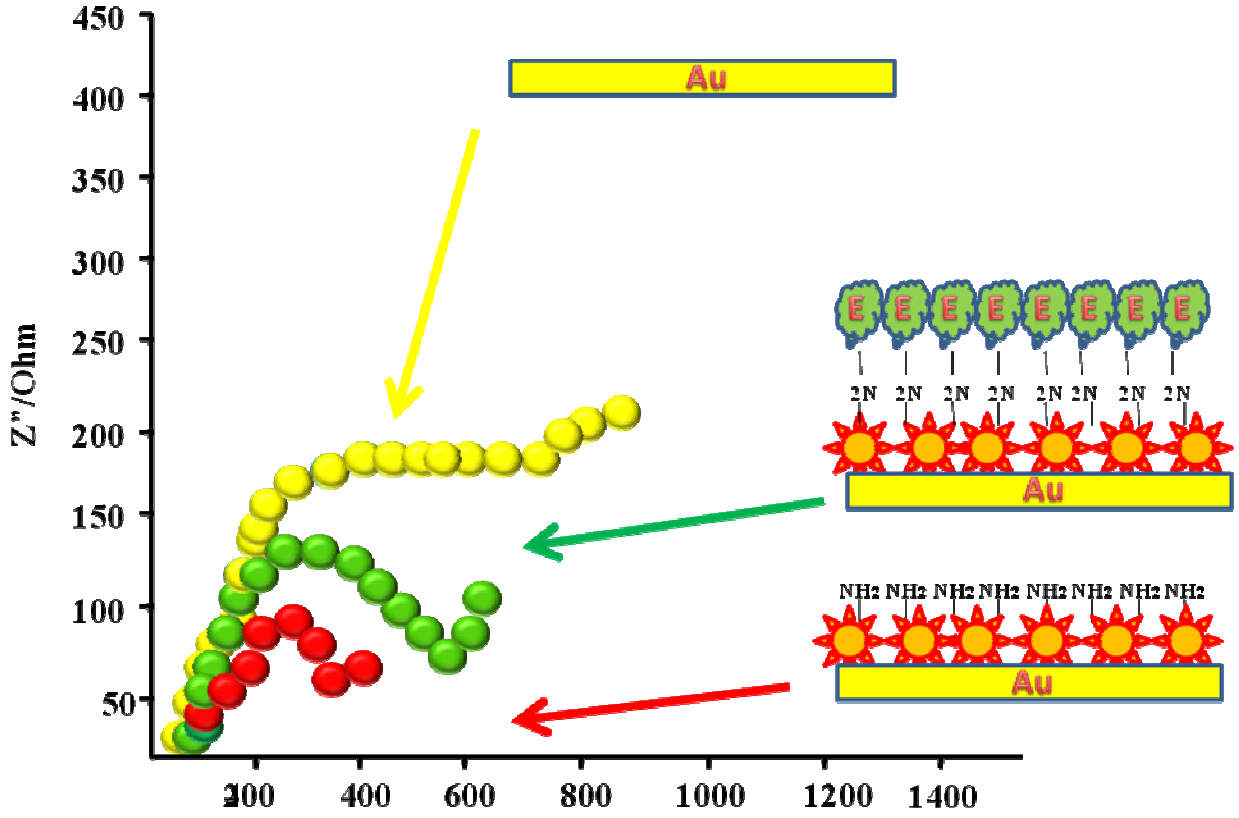



**Fig.2B**

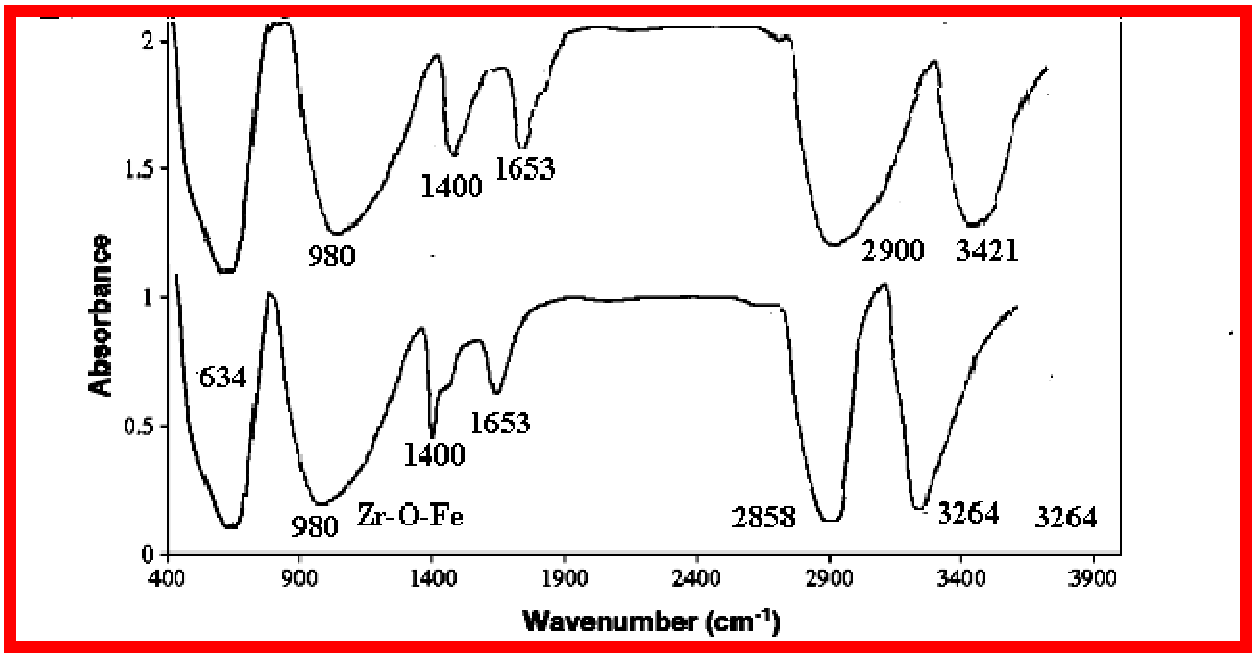



**Fig.2C**

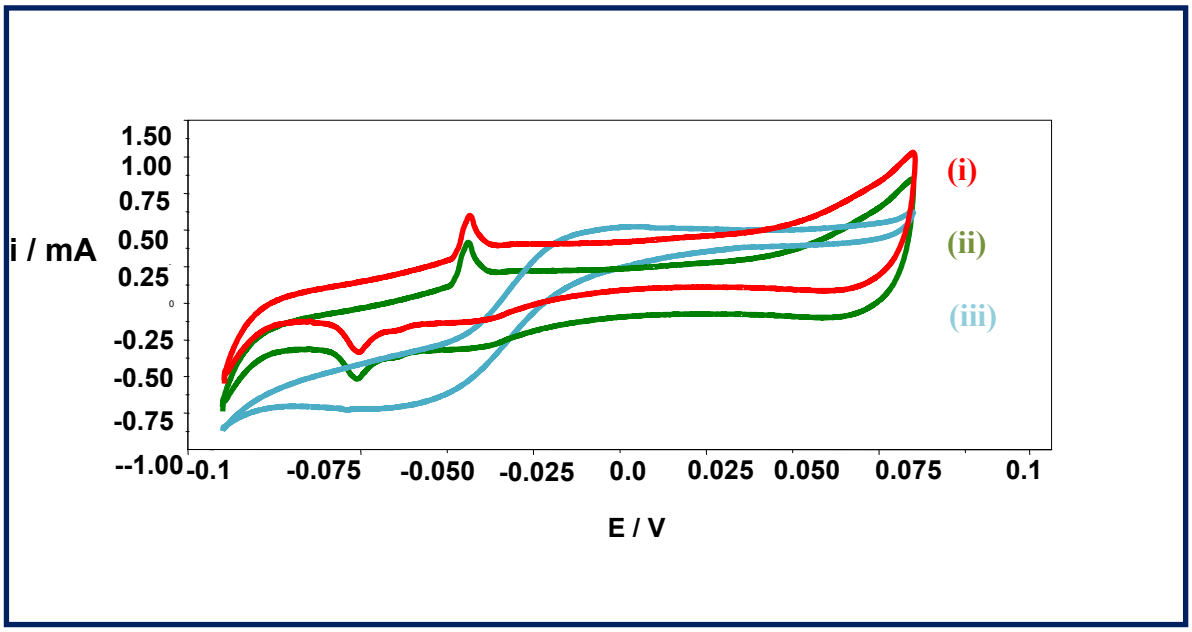



**Fig.3.**

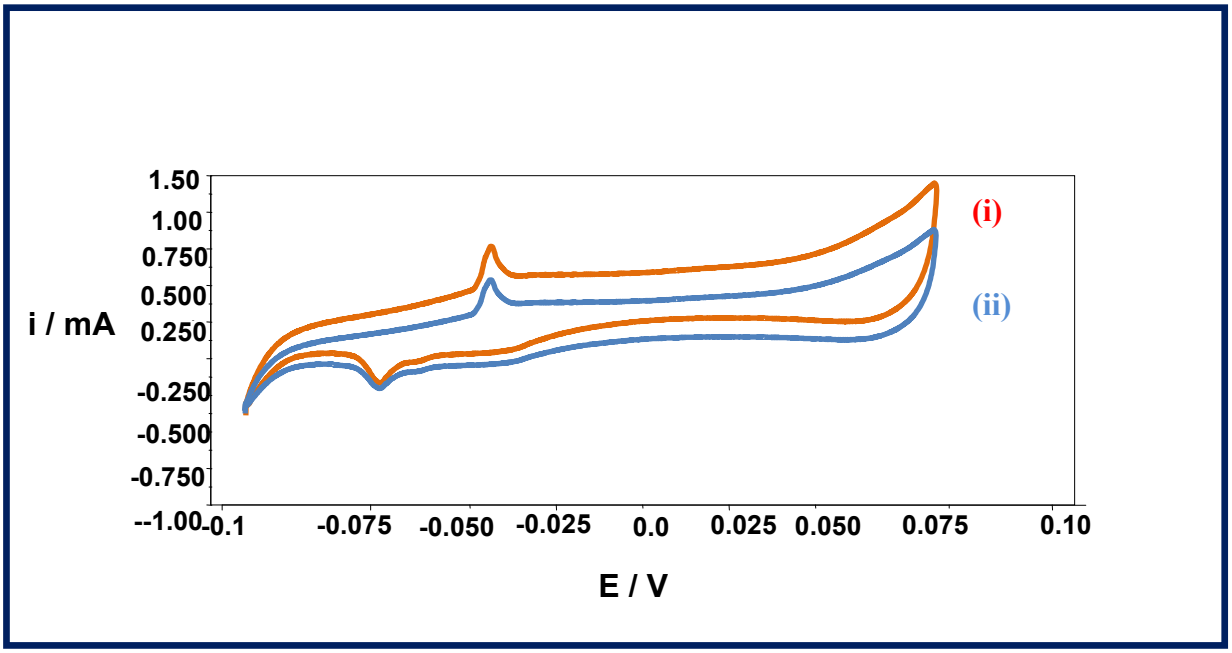



**Fig.4.**

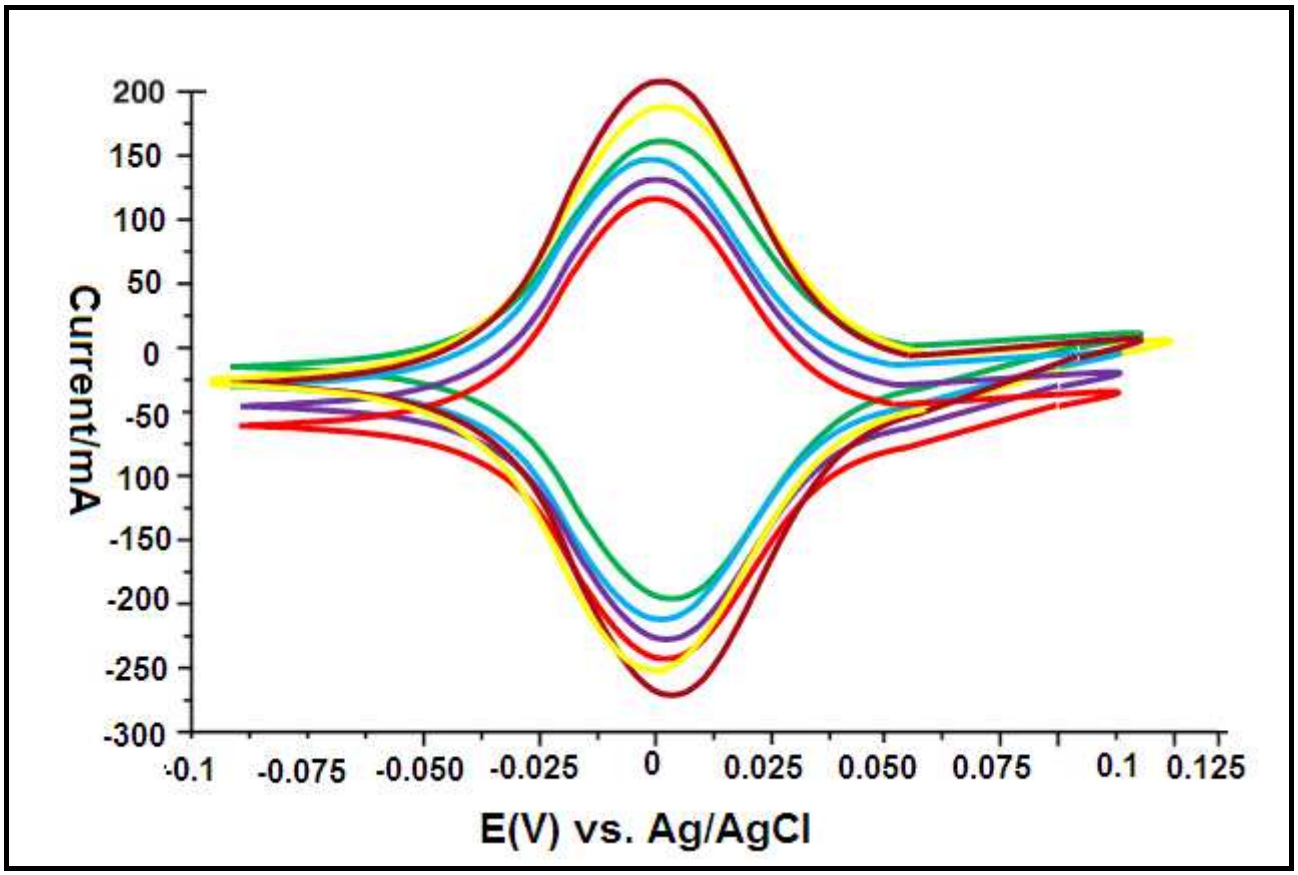
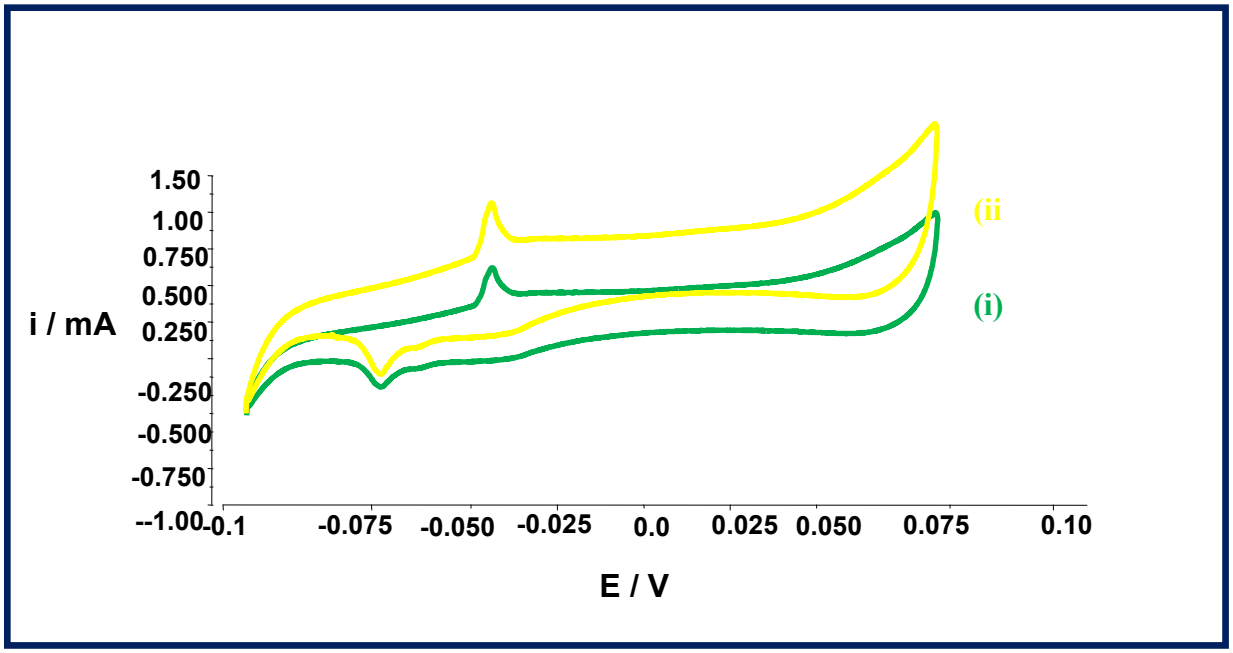



**Fig.5.**

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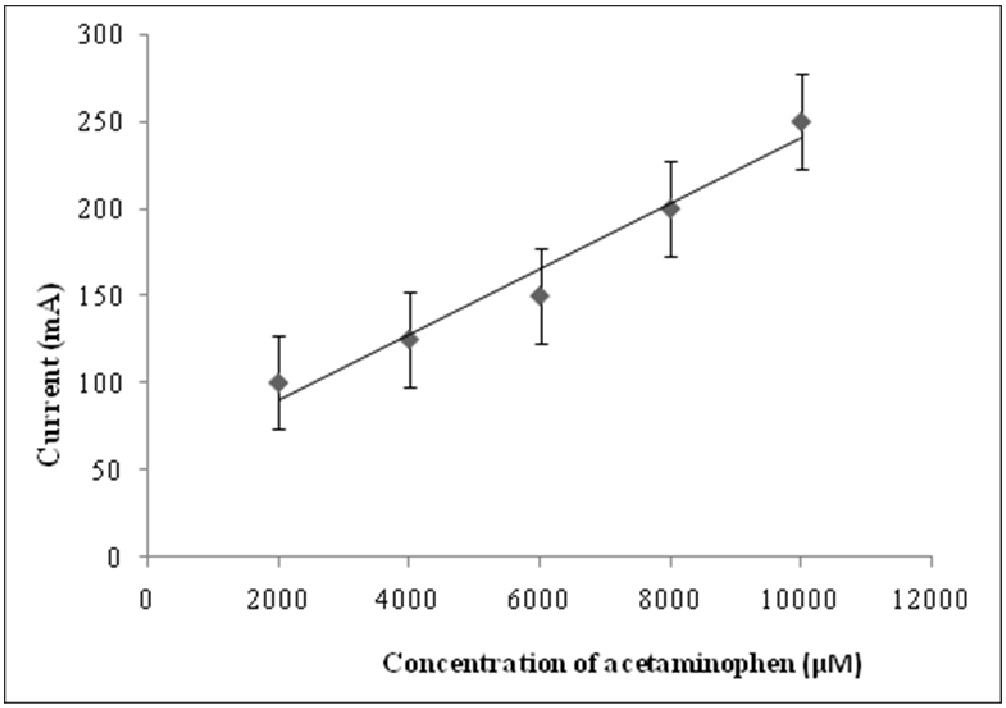


**Fig.7.**

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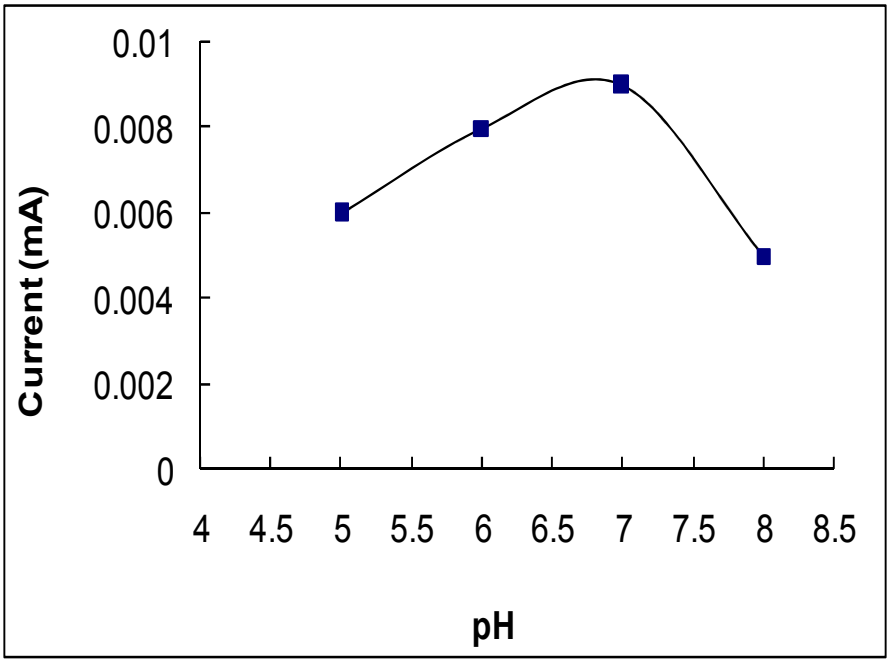


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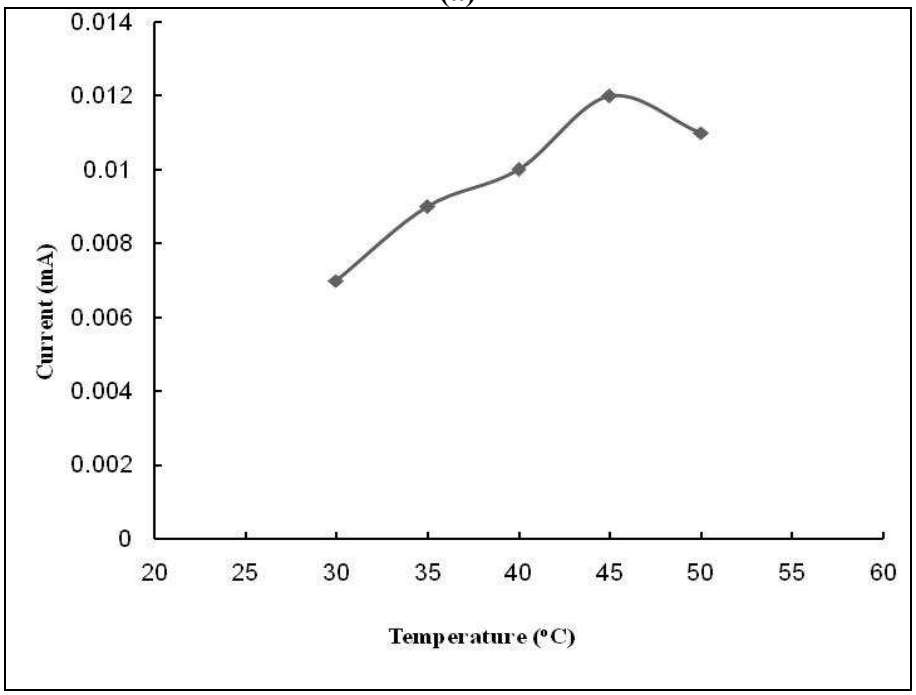
**(a)**

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**(b)**

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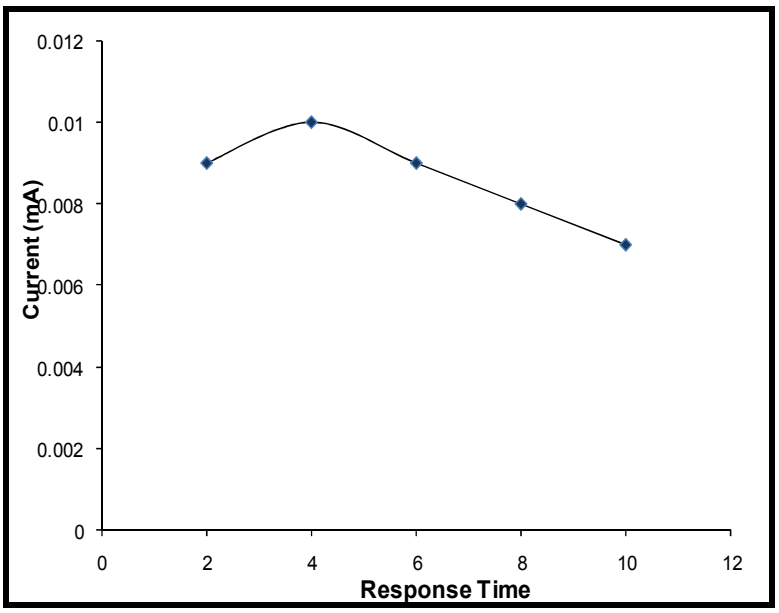


**(c)**

**Fig.8.**

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## Captions to tables

**Table.1.** Determination of acetaminophen by present sensor based on HRP/ZrO@ $Fe_3O_4$NP/ CHIT/Au electrode and standard enzymatic method.

**Table.2.** Comparison of present method with other biosensing methods.

**Table.1.**

| S.No. | Sample | Label value (mg) | Pharmacopoeia Method (mg) | Present Method (mg) |
|---|---|---|---|---|
| 1. | A | 500 | 500 | 499 |
| 2. | B | 250 | 250 | 255 |
| 3. | C | 100 | 100 | 98 |
| 4. | D | 50 | 50 | 49 |
| 5. | E | 100 | 100 | 102 |
| 6. | F | 450 | 440 | 450 |
| 7. | G | 350 | 360 | 350 |
| 8. | H | 550 | 540 | 552 |
| 9. | I | 150 | 145 | 152 |
| 10. | J | 500 | 490 | 500 |

**Table.2.**

| Matrix/method | Enzyme | Response time | Detection limit (µM) | Linearity (µM) | Stability | Reference |
|---|---|---|---|---|---|---|
| C-Ni/GCE /DPV Chronoamperometry | - | - | - | 7.8-110 | - | [20] |
| MWCNT-film coated electrode | - | - | 0.04 | 0.1–20 | - | [21] |
| Carbon nanoparticles (CNPs)/GCE /Voltammetry | | | 0.05 | 0.1–100 | - | [22] |
| Cobalt hydroxyl nanoparticles/Cyclic voltammetery | - | - | 10 | 2.5–1000 | - | [23] |
| Nanogold/ITOE /Cyclic voltammetery | - | - | 0.18 | 0.2-1500 | | [24] |
| HRP /ZrO@ $Fe_3O_4$NP/ CHIT /Au | HRP | 1s | 0.01 | 0.01 to 10000 | | Present |