Enzyme and Microbial Technology 50 (2012) 247–254

Contents lists available at SciVerse ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Immobilization of creatininase, creatinase and sarcosine oxidase on iron oxide nanoparticles/chitosan-g-polyaniline modified Pt electrode for detection of creatinine

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ARTICLE INFO

Article history:
Received 3 October 2011
Received in revised form 20 January 2012
Accepted 25 January 2012

Keywords:
Creatinine
Creatinine biosensor
Iron oxide nanoparticles
Chitosan
Polyaniline

ABSTRACT

Commercial enzymes, creatininase (CA) from Pseudomonas sp, creatinase (CI) from Pseudomonas sp, sarcosine oxidase (SO) from Bacillus sp were co-immobilized onto iron oxide nanoparticles/chitosan-graft-polyaniline (Fe_3O_4-NPs/CHIT-g-PANI) composite film electrodeposited on surface of Pt electrode through glutaraldehyde coupling. Transmission electron microscopy (TEM) was used for characterization of Fe_3O_4-NPs. A creatinine biosensor was fabricated using Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode as working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode. The enzyme electrode was characterized by cyclic voltammetry (CV), scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopic and electrochemical impedance spectroscopy (EIS). The biosensor exhibited an optimum response within 2 s at pH 7.5 and 30 °C, when polarized at 0.4 V vs Ag/AgCl. The electrocatalytic response showed a linear dependence on creatinine concentration ranging from 1 to 800 μM. The sensitivity of the biosensor was 3.9 μA μM^{-1} cm^{-2}, with a detection limit of 1 μM (S/N = 3). Apparent Michaelis–Menton (K_m) value for creatinine was 0.17 mM. The biosensor showed only 10% loss in its initial response after 120 uses over 200 days, when stored at 4 °C. The biosensor measured creatinine in the serum of apparently healthy persons which correlated well with a standard colorimetric method (r = 0.99).

1. Introduction

Creatinine, a dehydrogenated form of creatine is a metabolic byproduct of amino acid that provides energy to muscles tissue. It is an important clinical analyte for the diagnosis and medical management of renal and muscular dysfunction [1]. Normally, the level of serum creatinine in apparently healthy person is in the range, 44–106 μM. However, it can exceed up to 1000 μM during nephrons malfunction [2]. Therefore, precise monitoring of creatinine in the blood is compulsory for monitoring renal function. Creatinine is mostly analyzed either by the colorimetric method using the Jaffe's reaction [3] or enzymatic colorimetric method [4]. However, the colorimetric method is time consuming and sensitive to numerous metabolites and drugs found in biological samples [5], while the enzymatic colorimetric method is expensive and also prone to metabolites. The biosensing method has many advantages over these routine techniques in terms of reducing time, simplicity, sensitivity and cost of analysis. Various electrochemical methods have been proposed for routine creatinine analysis, including potentiometric biosensors [6–9]. However, these electrochemical biosensors had problems such as lack of stability, sensitivity and low reproducibility, which are still to be improved. To overcome these problems, the enzyme is required to be immobilized directly and covalently onto the film coated electrode.

The emergence of nanotechnology offered great opportunities to improve the sensitivity, stability and anti-interference ability of biosensing systems. Applications of nanomaterials to biosensors have recently aroused much interest as these materials exhibit large surface-to-volume ratio, high surface reaction activity, high catalytic efficiency and strong adsorption ability that are helpful for immobilization of biosensing molecules. Moreover, nanoparticles have a unique ability to promote fast electron transfer between the electrode and the active site of the enzyme [10]. In this context, different types of nanoparticles such as gold (AuNPs) [11], zinc-oxide (ZnO-NPs) [12,13], iron-oxide (Fe_3O_4-NPs) [14,15], have been suggested as promising matrices for enzyme immobilization to improve the stability and sensitivity of the biosensor. Among various metal oxide nanoparticles, Fe_3O_4-NPs have been considered as interesting for the immobilization of desired biomolecules due to biocompatibility, strong superparamagnetic behavior which provide better contact and low toxicity [16–18]. Immobilization of bioactive molecules onto a surface charged with

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doi:10.1016/j.enzmictec.2012.01.008

superparamagnetic nanoparticles is of special interest, since the magnetic behavior of these bioconjugates may result in improved delivery and recovery of biomolecules for desired biosensing applications [19–21]. A very important property of this type of nanoparticle for electrochemical biosensors is their ability to provide a favorable microenvironment for biomolecules to exchange electrons directly with an electrode, thus improving the sensitivity of electrochemical biosensors. Besides this, the existing problem of aggregation and rapid biodegradation of Fe_3O_4-NPs onto a given matrix containing biomolecules can perhaps be overcome by modifying these nanoparticles using chitosan (CHIT) [22–24]. CHIT is an abundant natural biopolymer with excellent film forming abilities, biocompatibility, nontoxicity, good water permeability, high mechanical strength [15] and susceptible to chemical modification due to the presence of reactive hydroxyl and amino functional groups. CHIT can accumulate metal ions through various mechanisms, such as chelation, electrostatic attraction and ion exchange, depending on the nature of the metal ion and pH of the solution. CHIT along with polyaniline (PANI) a conducting polymer provides a suitable matrix for covalent immobilization and stabilization condition for the biomolecules [7]. PANI as a conducting polymer has become very attractive, because of its facile synthesis [25], stable chemical and environmental properties [26] and inherent reversible doping/dedoping states [27]. Therefore, it is a suitable matrix for achieving electromagnetic shielding or absorption materials by doping with magnetic Fe_3O_4-NPs.

The present reports describe herein the use of unique properties of Fe_3O_4-NPs, CHIT and PANI for fabrication of an improved creatinine biosensor.

2. Experimental

2.1. Reagents

Creatininase (CA, E.C. 3.5.2.10., from Pseudomonas sp), creatinase (CI, E.C. 3.5.3.3., from Pseudomonas sp), sarcosine oxidase (SO, E.C. 1.5.3.1., from Bacillus sp), glutaraldehyde (25%) and CHIT were from Sigma–Aldrich, USA. Aniline (purified through vacuum distillation before use) and zinc nitrate were from SISCO Research Lab., Mumbai, India. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used in all experiments.

2.2. Instruments

All electrochemical experiments and electrochemical impedance spectroscopic (EIS) measurements were performed at 25 ± 1 °C using an potentiostat/galvanostat equipped with an Autolab PGSTAT-302N, GPES and FRA software (Eco-Chemie, Utrecht, The Netherlands) with a three electrode system consisting Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode used as working, Ag/AgCl as reference and Pt wire as auxillary electrode. Fourier transform infrared (FTIR) spectroscopy was recorded in FTIR spectrophotometer (Thermo Scientific, USA). Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray diffraction (XRD) studies were carried out on commercial basis.

2.3. Construction of Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode

The Fe_3O_4-NPs were prepared by the co-precipitation of Fe(II) and Fe(III) under a base condition as described by Kim et al. [28], with slight modification. Five milliliters of iron ion solution containing 0.25 M Fe^{2+} and Fe^{3+} (1:1 ratio) was added drop wise into 50 mL NaOH (2 mol/L) solution under vigorous mechanical stirring for 35 min at 80 °C. The precipitates were washed firstly with DW and then ethanol 2–3 times and collected by applying an external magnetic field until the supernatant solution turned neutral and finally, these were dried in oven at 70 °C. The morphology, particle size and structure of the Fe_3O_4-NPs were determined by a TEM.

Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode was prepared by cyclic voltammetry method. CHIT solution was prepared by dissolving 2.0 g of CHIT flakes into 100 mL of 1.0% acetic acid and stirred for 3 h at room temperature until completely dissolved. The CHIT solution was stored in a refrigerator when not in use. Fe_3O_4-NPs (400 μL) were dispersed into transparent CHIT solution and kept on magnetic stirring for about 30 min at room temperature followed by the sonication for 4 h. Finally a highly viscous solution of CHIT with uniformly dispersed Fe_3O_4-NPs was obtained. A solution for electrodeposition was prepared by adding aniline (180 μL) + Fe_3O_4-NPs/CHIT (75 μL) + 0.5 M HCl (10 mL) in a glass cell. A nanocomposite film of Fe_3O_4-NPs/CHIT-g-PANI was electrodeposited on Pt electrode by cyclic voltammetry using three electrode-systems. A Pt electrode (1.9 cm × 1 mm) (length × diameter) was ultrasonicated in 5.0 M HNO_3 and acetone for 15 min and then rinsed with D.W., and then immersed into a electrodepositing solution, the potential scan was cycled for 30 times between −0.1 and 1.0 V vs Ag/AgCl at a scan rate of 50 mV/s and subsequently allowed to dry at room temperature. The resulting Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode was washed with D.W.

2.4. Immobilization of enzymes on Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode

The CA, CI and SO were co-immobilized onto the Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode surface through glutaraldehyde coupling. First 10 μL of 2.5% glutaraldehyde solution in 0.05 M phosphate buffer (PB) pH 7.5 was spread over the Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode and kept for 5 h at room temperature, washed in 0.05 M PB, pH 7.5 and then 100 μL mixture of CA (44 unit), CI (36 unit) and SO (24 unit) was mounted on surface of the electrode and dried. The resulting Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode was washed thoroughly with 0.05 M PB of pH 7.5 to rinse off any loosely bound enzymes from the electrode. The resulting enzyme electrode was dried at room temperature and then stored in a refrigerator at 4 °C, when not in use. The fabricated electrode was characterized by cyclic voltammetric, SEM, FTIR and EIS studies.

2.5. Cyclic voltammetric measurement and optimization of creatinine biosensor

The sensitivity of creatinine biosensor was tested by measuring current using three electrode system. The working (Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt) electrode along with Ag/AgCl as reference electrode and Pt wire as auxillary electrode were connected through potentiostat/galvanostat to construct creatinine biosensor. Fig. 1A showed the effect of working potential on the performance of biosensor. The electrode system was dipped into a reaction mixture containing 10 mL 0.05 M PB, pH 7.5 and 0.5 mL creatinine solution (50–250 μM). The electrode current response was measured applying a potential range of −0.1 V to 1.0 V vs Ag/AgCl. The steady state current response increased with increase in working potential and optimum current response was obtained at 0.4 V vs Ag/AgCl. Therefore, 0.4 V vs Ag/AgCl was selected as the working potential for amperometric detection of creatinine concentration.

To optimize the working condition of electrode, the pH of reaction buffer was varied from 6.0 to 10.0 at an interval of pH 0.5 using 0.05 M PB in the pH range 6–8 and 0.05 M sodium carbonate/bicarbonate buffer in the pH range 8.5–10. The optimum temperature was studied by incubating the reaction mixture at different temperature (25–45 °C at an interval of 5 °C). Similarly the current response was measured at 2–20 s at an interval of 2 s and creatinine conc. was varied from 0.1 to 1200 μM in reaction buffer under optimal conditions.

2.6. Amperometric determination of creatinine in serum

The modified electrode was employed for measuring creatinine in serum samples. Serum samples from apparently healthy persons of different age groups and sex were collected from hospital of PGIMS, Rohtak in tubes and stored at 4 °C until use. The content of creatinine in sera was determined by the present biosensor as described for its testing under optimal working conditions except that creatinine solution was replaced by serum. The content of creatinine was determined from standard curve between creatinine conc. vs current (mA) prepared under optimal conditions.

The following criteria were studied to evaluate the performance of this biosensor viz. linearity, analytical recovery, detection limit, sensitivity, precision and correlation with standard method.

3. Results and discussion

3.1. Characterization of Fe_3O_4-NPs

A TEM image of synthesized Fe_3O_4-NPs dispersed in CHIT (Fig. 1B) indicates that Fe_3O_4-NPs were nanocrystalline and composed predominantly of a large number of well dispersed spherical nanoparticles with some hexagonal shaped nanoparticles. The average size of the spherical nanoparticles was ∼20 nm. Moreover, Fe_3O_4-NPs were agglomerates due to their high surface area and magnetic dipole–dipole interactions between the particles [29] which could be overcome after modifying these NPs with CHIT. The XRD pattern (Fig. 1C) of synthesized Fe_3O_4-NPs shows that the Fe_3O_4-NPs nanoparticles are polycrystalline in nature. The peak positions are well in agreement with joint committee on powder diffraction standard (JCPDS) and are indexed to (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0), respectively. The results obtained agree with standard magnetite (Fe_3O_4) XRD patterns and identified that the Fe_3O_4-NPs are in a cubic spinel structure [17].

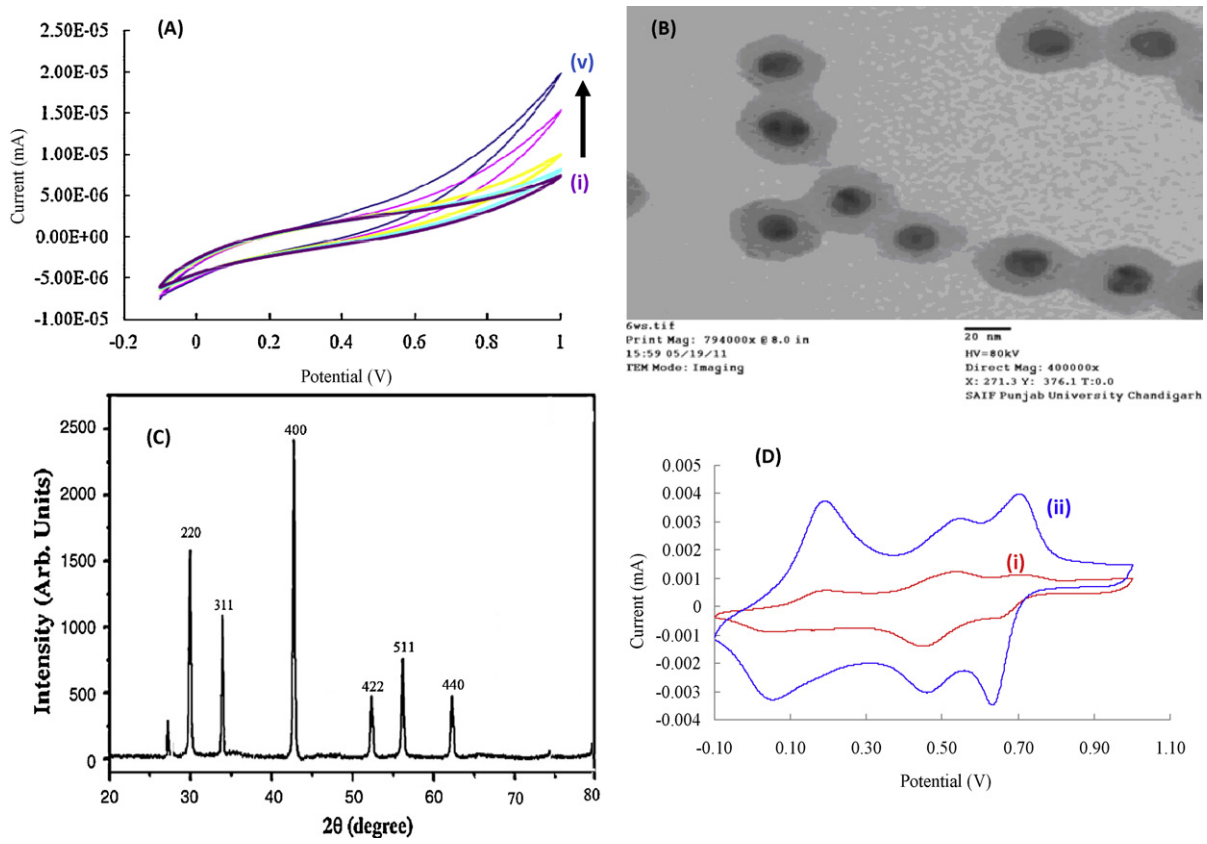


Fig. 1. (A) Cyclic voltammograms of amperometric response studies as a function of Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode in creatinine concentration from (i = 50 to v = 250 μM) using 0.05 M PB, pH 7.5. (B) Transmission electron microscopic (TEM) images of Fe_3O_4-NPs dispersed in CHIT. (C) X-ray diffraction (XRD) pattern of Fe_3O_4-NPs. (D) Current responses curves of CHIT-g-PANI (curve i) and Fe_3O_4-NPs/CHIT-g-PANI composite (curve ii) films at 100 μM creatinine.

3.2. Construction of Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode

Fig. 1D displays the cyclic voltammograms of CHIT-g-PANI (curve i) and Fe_3O_4-NPs/CHIT-g-PANI (curve ii) composite film. It can be seen that the surface charged Fe_3O_4-NPs interact with the cationic biopolymer matrix of CHIT via electrostatic interactions and hydrogen bonding with $-NH_2$/OH groups to form a hybrid nanobiocomposite [22]. The Fe_3O_4-NPs/CHIT-g-PANI composite film exhibits higher currents than the CHIT-g-PANI composite film, which indicate that the Fe_3O_4-NPs/CHIT-g-PANI composite film, has a larger effective surface area than the CHIT-g-PANI composite film. The Fe_3O_4-NPs/CHIT-g-PANI composite film appears to provide a conducting path through the composite matrix for faster kinetics. Hence, the Fe_3O_4-NPs, acting as an electron transfer mediator and may help in enhancing the sensor response of enzyme electrode and thus increase the sensitivity of the biosensor. These observations suggest the formation of Fe_3O_4-NPs/CHIT-g-PANI composite film provides a large surface area for the immobilization of enzymes.

A method is described for construction of an amperometric biosensor using Fe_3O_4-NPs/CHIT-g-PANI film electrodeposited onto Pt electrode. The fabrication of the creatinine biosensor based on Enzymes/Fe_3O_4-NPs/CHIT-g-PANI modified electrode is summarized in Scheme 1. To achieve it, firstly a Fe_3O_4-NPs/CHIT-g-PANI composite film was electrodeposited onto Pt electrode using cyclic voltammetry. The electrodeposition method was selected to produce Fe_3O_4-NPs on electrode surfaces, for its simplicity and so that the layer thickness can be controlled. Secondly, commercially available CA, CI and SO enzymes were co-immobilized on Fe_3O_4-NPs/CHIT-g-PANI composite film by through glutaraldehyde coupling. One end of the glutaraldehyde was attached to the $-NH_2$ group of the Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode through a reaction between the —CHO end group of glutaraldehyde and the $-NH_2$ groups of terminal PANI and CHIT. The other end of the glutaraldehyde is attached to enzymes through a reaction between the —CHO group of glutaraldehyde and $-NH_2$ group of enzymes, which resulted in Enzymes/Fe_3O_4-NPs/CHIT-g-PANI electrode.

The following electrochemical reactions occur during response measurements of present biosensor:

$$\text{Creatinine} + H_2O \xrightarrow{CA} \text{Creatine}$$

$$\text{Creatine} + H_2O \xrightarrow{CI} \text{Sarcosine} + \text{Urea}$$

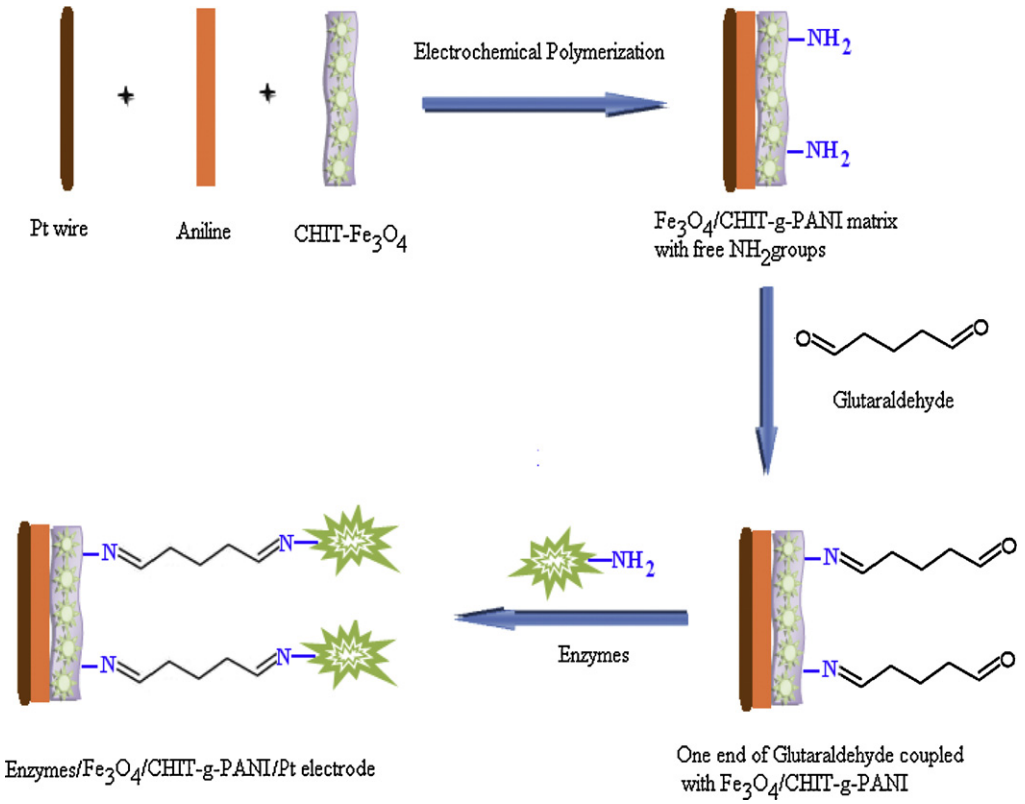
$$\text{Sarcosine} + H_2O + O_2 \xrightarrow{SO} \text{Formaldehyde} + \text{Glycine} + H_2O_2$$

$$H_2O_2 \xrightarrow{0.4\,V} 2H^+ + O_2 + 2e^-$$

$$2e^- \rightarrow \text{Pt electrode}$$

3.3. Surface characterization by SEM

The surface morphologies of CHIT-g-PANI/Pt, Fe_3O_4-NPs/CHIT-g-PANI/Pt and Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt were investigated by SEM (Fig. 2). Globular porous morphology of Fe_3O_4-NPs/CHIT-g-PANI (Fig. 2B) biocomposite film reveals incorporation of the Fe_3O_4-NPs in CHIT-g-PANI (Fig. 2A), indicating the formation of Fe_3O_4-NPs/CHIT-g-PANI biocomposite film. However, after the



Scheme 1. Schematic representation of chemical reaction involved in the fabrication of Enzymes/Fe_3O_4/CHIT-g-PANI/Pt electrode.

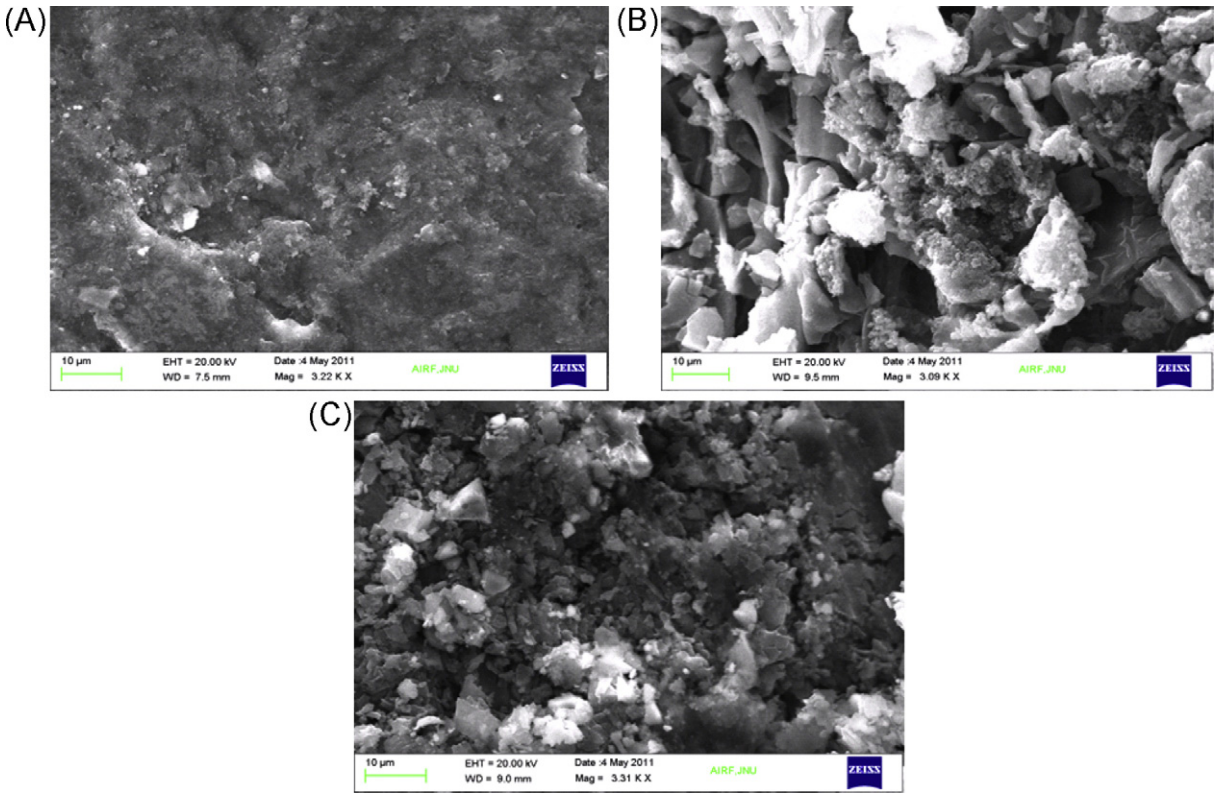


Fig. 2. (A) SEM images of CHIT-g-PANI/Pt electrode. (B) SEM images of Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode. (C) SEM images of Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode.

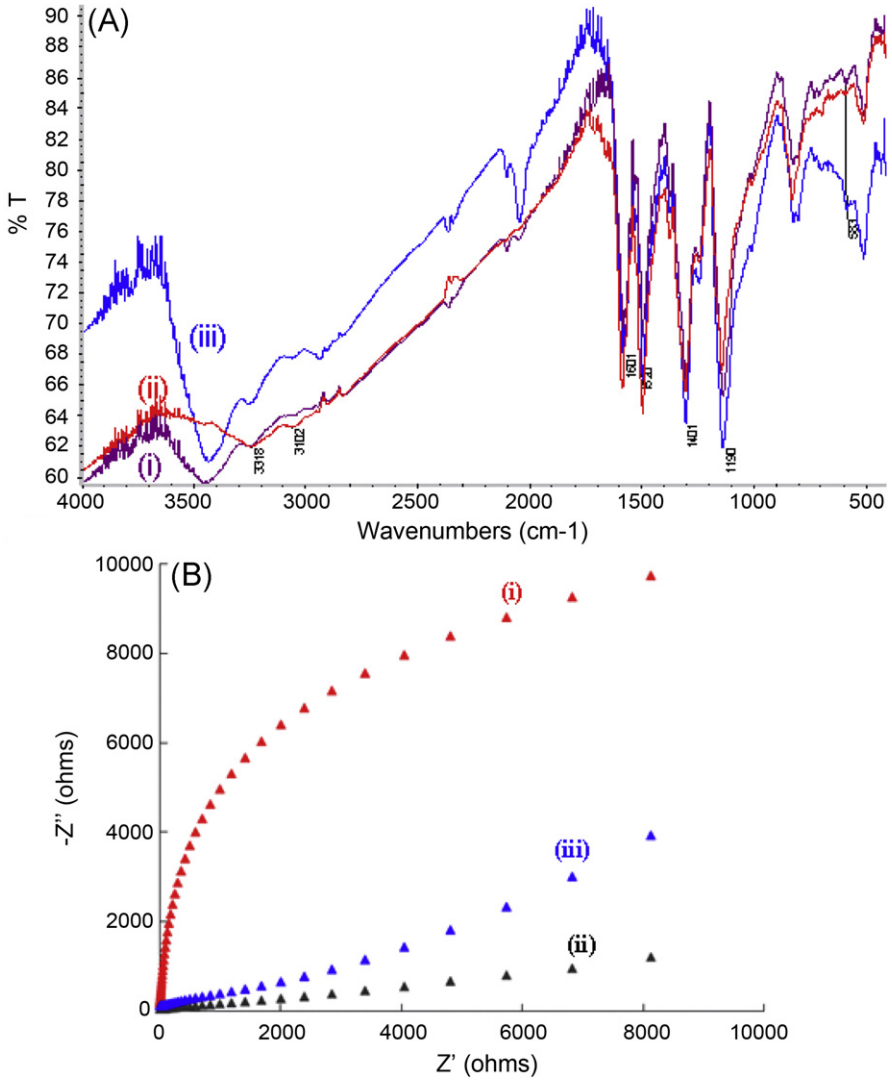


Fig. 3. (A) FTIR spectra obtained for CHIT-g-PANI (curve i), Fe_3O_4-NPs/CHIT-g-PANI (curve ii), Enzymes/Fe_3O_4-NPs/CHIT-g-PANI (curve iii). (B) The Nyquist plot of the EIS of CHIT-g-PANI/Pt (curve i), Fe_3O_4-NPs/CHIT-g-PANI/Pt (curve ii), Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt (curve iii) in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.05 M PB (pH 7.5).

immobilization of enzymes onto Fe_3O_4-NPs/CHIT-g-PANI composite film, the globular morphology changed to regular form (Fig. 2C). This suggests that Fe_3O_4 nanoparticles provide a favorable environment for high loading of enzymes.

3.4. FTIR spectra

Fig. 3A showed the FTIR spectra obtained for CHIT-g-PANI, Fe_3O_4-NPs/CHIT-g-PANI and Enzymes/Fe_3O_4-NPs/CHIT-g-PANI composites. The FTIR spectrum of the CHIT-g-PANI composite (curve i) illustrated the characteristic peaks of PANI, as well as CHIT [30]. The stretching bands of benzenoid and quinoid ring were observed at 1520 and 1601 cm^{-1}. The absorption band of the N=Q=N bending vibration of protonated PANI was observed at 1190 cm^{-1} in the CHIT-g-PANI copolymer. The FTIR spectrum of Fe_3O_4-NPs/CHIT-g-PANI composite (curve ii) exhibits characteristic IR bands of the functional group corresponding to CHIT-g-PANI and the Fe_3O_4-NPs. The absorption band of Fe_3O_4-NPs appears at 583 cm^{-1} belonging to the stretching vibration mode and the torsional vibration mode of Fe—O bonds in the tetrahedral sites and in the octahedral sites. The FTIR spectrum of the Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/ITO electrode (curve iii) showed peaks broadening at 3102–3318 cm^{-1} (addition of N—H stretching vibration) due to the attachment of enzymes with the Fe_3O_4-NPs/CHIT-g-PANI composite. Hence, FTIR spectra confirmed the immobilization of enzymes onto the Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode.

3.5. Electrochemical impedance spectroscopy (EIS) studies

EIS is an effective method for probing the features of surface modified electrodes. The Nyquist plot of impedance spectra includes a semicircle portion and a linear portion, with the former at higher frequencies corresponding to the electron transfer limited process and the latter at lower frequencies corresponding to the diffusion process. The electron transfer resistance (R_{CT}) at electrode surface is equal to the semicircle diameter, which can be used to describe the interface properties of the electrode. Fig. 3B presents the Nyquist plot of the impedance spectroscopy of CHIT-g-PANI/Pt, Fe_3O_4-NPs/CHIT-g-PANI/Pt and Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.05 M PB, pH 7.5. The diameter of semicircle for Fe_3O_4-NPs/CHIT-g-PANI composite film (curve ii) is smaller than that of CHIT-g-PANI composite film (curve i) which suggests

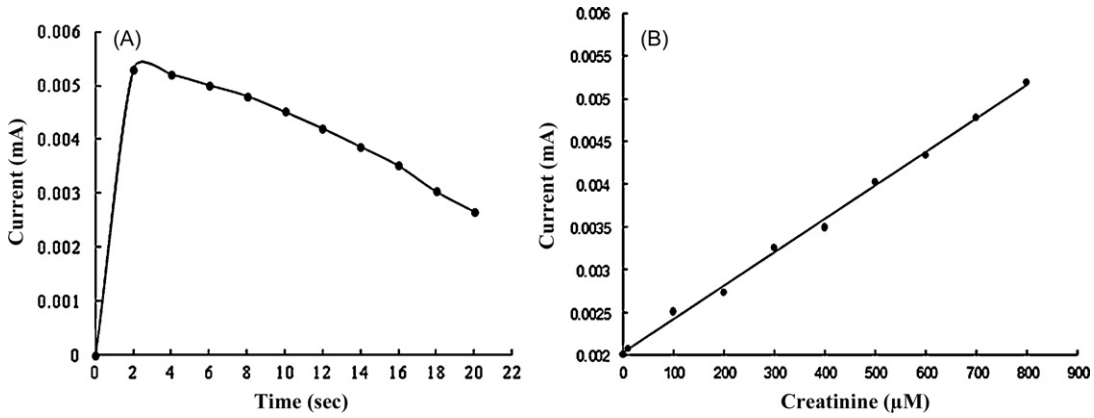


Fig. 4. (A) Effect of incubation time on response of creatinine biosensor based on Fe_3O_4-NPs/CHIT-g-PANI composite film. (B) Linear calibration plot corresponding to current responses for different creatinine concentration by creatinine biosensor based on Fe_3O_4-NPs/CHIT-g-PANI composite film.

that electron transfer in Fe_3O_4-NPs/CHIT-g-PANI composite film is easier between the solution and electrode i.e. Fe_3O_4-NPs not only provide the hydrophilic surface but also act as a nanoscale electrode and promote electron transfer due to permeable structure of CHIT-g-PANI/Pt. However the diameter of the semicircle obtained for Enzymes/Fe_3O_4-NPs/CHIT-g-PANI/Pt electrode (curve iii) further increases. This increase in diameter is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductor at low frequencies and cause hindrance to electron transfer. These results also indicate binding of enzymes onto Fe_3O_4-NPs/CHIT-g-PANI composite film.

3.6. Optimization of experimental variables

The optimum current response was obtained at pH 7.5 which is similar to earlier amperometric biosensors based on ZnO-NPs/CHIT/c-MWCNT/PANI composite film [8], c-MWCNT/PANI composite [9], nucleolore polycarbonate membrane [31], Polymer film (polyanion polypyrrole) [32], nafion membrane [33] but slightly higher than polyurethane hydrogel matrix (pH 7.0) [34]. The optimum temperature of biosensor was 30 °C, which is lower than earlier biosensor based on c-MWCNT/PANI composite [9], nucleolore polycarbonate membrane (37 °C) [31]. When creatinine was added into PB, pH 7.5 the biosensor responded rapidly to the substrate and achieved 95% of steady current within 2 s (Fig. 4A), which is lower than those earlier reported creatinine biosensor based on ZnO-NPs/CHIT/c-MWCNT/PANI composite film (10 s) [8], c-MWCNT/PANI composite (5 s) [9], PUR hydrogel matrix (5 min) [34], carbon paste electrode (90 s) [35] and PbO_2 film (118 s) [36]. This faster response can be attributed to the synergetic influence of Fe_3O_4-NPs and CHIT-g-PANI composite film. The resulted calibration plot for creatinine, over the concentration range from 1 to 800 μM is shown in Fig. 4B. The linear plot reveals that such electrode can work well in creatinine solution with a sensitivity of 3.9 $\mu A\,\mu M^{-1}\,cm^{-2}$, which is better than those earlier reported creatinine biosensor based on ZnO-NPs/CHIT/c-MWCNT/PANI composite film (0.030 $\mu A\,\mu M^{-1}\,cm^{-2}$) [8], c-MWCNT/PANI composite (0.040 $\mu A\,\mu M^{-1}\,cm^{-2}$) [9]. Lineweaver–Burk plot gave a apparent K_m value of 0.17 mM for immobilized enzymes, which is lower than that of earlier reported creatinine biosensor based on ZnO-NPs/CHIT/c-MWCNT/PANI composite film (0.35 mM) [8], c-MWCNT/PANI composite (0.26 mM) [9], carbon paste electrode based creatinine biosensor (5.15 mM) [35] and microfabricated creatinine biosensor (5.2 mM) [34] indicate that our biosensor possesses higher affinity for creatinine than that for earlier reported biosensors.

3.7. Interference study and selectivity

The effect of several possible interfering substances such as uric acid, ascorbic acid, bilirubin, creatine, sarcosine, pyruvic acid and urea on the present creatinine biosensor was investigated, at their physiological concentration using 100 μM creatinine in 0.05 M PB, pH 7.5. The results showed that all these compounds had practically no interference, except ascorbic acid and creatine which showed a slight increase in biosensor response (Table 1).

3.8. Application of creatinine biosensor

The biosensor was employed to determine the creatinine level in blood serum samples. The serum creatinine level was found in the range of 0.63 mg/dL to 1.1 mg/dL with a mean of 1.02 mg/dL in males and 0.77 mg/dL in females.

3.9. Evaluation of creatinine biosensor

The detection limit of biosensor was 1 μM (S/N = 3), which is much lower than that of earlier electrochemical creatinine biosensors [32,33,35] but comparable to that of ZnO-NPs/CHIT/c-MWCNT/PANI composite film and microchip based creatinine biosensor employing an oxidizing layer [8,36]. Analytical recovery of exogenously added creatinine in serum (0.5 mg/dL and 1 mg/dL) was 98.97% and 98.91% respectively showing the reliability of the method. The results of within and between batch analysis matched with each other and the coefficient of variation for serum creatinine determination were <4.15% and <5.58% respectively showing the good reproducibility and consistency of the method. The creatinine values of serum (n = 21) measured by the present biosensor were in good agreement with those by standard colorimetric method [3], with regression coefficient (r = 0.99) (Fig. 5).

Table 1
Effect of potential interferents on Fe_3O_4-NPs/CHIT-g-PANI composite film based creatinine biosensor response.

Interferents	% Relative activity
No interferent	100
Uric acid	100
Ascorbic acid	120
Bilirubin	100
Sarcosine	103
Urea	99
Pyruvic acid	101
Creatine	115

Table 2
A comparison of various analytical properties of present creatinine biosensor based on Fe_3O_4-NPs/CHIT-g-PANI composite film with earlier biosensors.

Electrode material	Detection limit (μM)	Linear range	Response time (s)	Sensitivity ($\mu A\,\mu M^{-1}\,cm^{-2}$)	Applied potential (V)	Ref.
PUR hydrogel matrix	25	Up to 2 mM	300	–	–	[34]
Polymer film (polyanion polypyrrole)	1–2	Up to 5 mM	–	0.023	0.4	[32]
Carbon paste electrode	–	0.2–2 mM	90	–	0.5	[35]
PbO_2 film	0.8	1–1000 μM	118	–	0.8	[36]
ZnO-NPs/CHIT/c-MWCNT/PANI composite film	0.5	10–650 μM	10	0.030	0.5	[8]
c-MWCNT/PANI	0.1	10–750 μM	5	0.040	0.2	[9]
Fe_3O_4-NPs/CHIT-g-PANI	1	1–800 μM	2	3.9	0.4	Present work

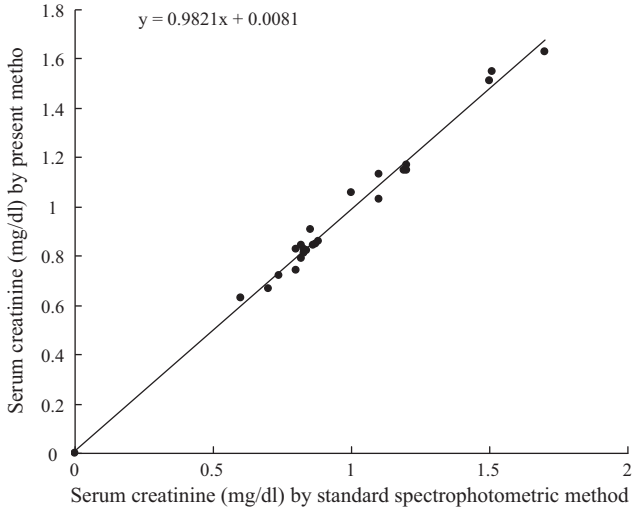


Fig. 5. Correlation between serum creatinine values measured by chemical spectrophotometric method (x) and present method (y) employing creatinine biosensor based on Fe_3O_4-NPs/CHIT-g-PANI composite film.

This shows the accuracy of the method for practical application of the biosensor in the clinical analysis.

3.10. Long-term stability of enzyme electrode

The long-term stability of the enzyme electrode was investigated every week under its storage at 4 °C. The biosensor showed only 10% loss in its initial current response after 120 uses over 200 days, which is better than ZnO-NPs/CHIT/c-MWCNT/PANI composite film (120 days) [8], polymer film (polyanion polypyrrole) (<30days) [32] and PUR hydrogel matrix (90 days) [34] based creatinine biosensors. The Fe_3O_4-NPs/CHIT-g-PANI composite film also helps in keeping the bioactivity of modified Pt electrode.

A comparison of analytical properties of this biosensor with earlier creatinine biosensors is summarized in Table 2.

4. Conclusion

The use of Fe_3O_4-NPs/CHIT-g-PANI composite film for construction of amperometric creatinine biosensor has resulted in improved analytical performance in terms of relatively rapid response (2 s), higher sensitivity ($3.9\,\mu A\,\mu M^{-1}\,cm^{-2}$), broad linear range (1–800 μM), good reproducibility and long term stability (200 days) of the biosensor. The study could provide a feasible approach on developing new kinds of oxidase-based amperometric biosensors, using Fe_3O_4-NPs/CHIT-g-PANI nanocomposite film.

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