Biosensors and Bioelectronics 28 (2011) 64–70

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Tri-enzyme functionalized ZnO-NPs/CHIT/c-MWCNT/PANI composite film for amperometric determination of creatinine

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ARTICLE INFO

Article history:
Received 15 April 2011
Received in revised form 22 June 2011
Accepted 28 June 2011
Available online 6 July 2011

Keywords:
Zinc oxide nanoparticles
c-MWCNT
Chitosan
Polyaniline
Creatinine biosensor

ABSTRACT

A new zinc oxide nanoparticles/chitosan/carboxylated multiwall carbonnanotube/polyaniline (ZnO-NPs/CHIT/c-MWCNT/PANI) composite film has been synthesized on platinum (Pt) electrode using electrochemical techniques. Three enzymes, creatinine amidohydrolase (CA), creatine amidinohydrolase (CI) and sarcosine oxidase (SO) were immobilized on ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode to construct the creatinine biosensor. The enzyme electrode was characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS). The enzyme electrode detects creatinine level as low as 0.5 μM at a signal to noise ratio of 3 within 10 s at pH 7.5 and 30 °C. The fabricated creatinine biosensor showed linear working range of 10–650 μM creatinine with a sensitivity of 0.030 μA μM^{-1} cm^{-2}. The biosensor shows only 15% loss of its initial response over a period of 120 days when stored at 4 °C. The fabricated biosensor was successfully employed for determination of creatinine in human blood serum.

1. Introduction

Determination of creatinine in various biological fluids is useful for evaluation of renal, muscular and thyroid dysfunctions. It is also useful for biomedical diagnosis of acute myocardial infarction as well as for quantitative hemodialysis therapy. In contrast with urea, the concentration of creatinine in these body fluids is not influenced by the protein intake and therefore it is a more reliable indicator of renal function (Kazmlerczak, 1991). Creatinine is mostly analyzed colorimetrically using the Jaffe's reaction (Jaffé, 1886) or enzymatically (Weber and Van Zenten, 1991). However, colorimetric methods are affected adversely by numerous metabolites and drugs found in biological samples (Lo and Tsai, 1994), while enzymatic assays are time consuming and expensive.

The goal of biosensing engineering has many advantages over other techniques used for creatinine analysis in clinical laboratory such as reducing time, complexity and cost of routine clinical analysis. Different types of biosensors have been reported for determination of creatinine (Lad et al., 2008; Tiwari and Shukla, 2009). Unfortunately, potentiometric biosensors suffer from interference by cations and endogenous ammonia those are present in blood and urine (Shih and Huang, 1999). However, amperometric biosensors have significant problems involving sensitivity, selectivity, elimination of interferences and sensor stability which are still to be improved. To overcome these problems, it is desired to design and prepare a functional film for the modification of the electrode and immobilize enzyme directly on the film coated electrode.

Carbon nanotubes (CNTs) discovered by Iijima (1991) have been used in biosensor, due to their high effective surface area, high surface/volume ratio, good electrical conductivity, strong adsorptive ability, excellent bioconsistency, high electrocatalytic effect and a fast electron transfer rate (Zhang et al., 2004a,b; Lin et al., 2005; Tsai et al., 2005). CNTs in a suspension individually can be cytotoxic but cytotoxicity is avoided by immobilizing CNTs on surface or within composite (Hussain et al., 2009). The addition of nanoparticles (NPs) to the CNTs films can generate new nanostructures with excellent behavior in the fields of optics, electronics, and electrocatalysis (Wang et al., 2007). Metal nanoparticle (NPs) modified electrodes present unusual advantages in electroanalysis such as improved catalysis, enhancement of electron transport, high effective surface area and control over electrode microenvironment (Luo et al., 2004; Welch and Compton, 2006; Singh et al., 2007). Among the metal oxide nanoparticles, ZnO-NPs have been exploited as a potential material for biosensing, because of their unusual properties, i.e. good biocompatibility, chemical stability, non toxicity, high electron communication, high surface area for strong adsorption and high isoelectric point (IEP) (~9.5) (Zhang et al., 2004a), making it possible to immobilize low IEP DNA or proteins by electrostatic adsorption in proper buffer solutions (Topoglidis et al., 2001) which is positively charged on the surface under acidic conditions.

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doi:10.1016/j.bios.2011.06.044

Chitosan (CHIT) is a polysaccharide aminosugar, with p*K*a values of 6.3 for $-NH_2$ group (Rinaudo et al., 1999). At pH below the p*K*a, most of the amino groups are protonated making CHIT a water-soluble, cationic polyelectrolyte. At pH above the p*K*a, CHIT amino groups are deprotonated, and thus become insoluble. CHIT pH-dependent solubility is therefore attractive, because it allows processing from aqueous solutions (Ligler et al., 2001), while a modest increase in pH to neutrality enables CHIT to be formed into various shapes. CHIT can be electrochemically deposited onto electrodes (Wu et al., 2002; Jiang et al., 2006). Chitosan 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. Thus, the excellent film-forming ability, good adhesion, biocompatibility and high mechanical strength of CHIT membrane have attracted scientists to use it to immobilize biomolecules in recent years (Chen and Gorski, 2001; Miao et al., 2001; Okuma and Watanabe, 2002).

In view of these advantages, a nanocomposite of ZnO-NPs/CHIT onto a MWCNT hybrid film is expected to be very promising for constructing novel biosensors. The aim of the present work was to synthesize electrochemically a novel ZnO-NPs/CHIT/c-MWCNT/PANI composite film on a Pt electrode and then immobilizing creatinine amidohydrolase (CA), creatine amidinohydrolase (CI) and sarcosine oxidase (SO) onto composite film for construction of an amperometric creatinine biosensor. The analytical performances of the newly designed electrochemical biosensor were evaluated.

2. Materials and methods

2.1. Materials and instruments

Creatinine amidohydrolase (CA, E.C. 3.5.2.10., from *Pseudomonas* sp.), creatine amidinohydrolase (CI, E.C. 3.5.3.3, from *Pseudomonas* sp.), sarcosine oxidase (SO, E.C. 1.5.3.1., from *Bacillus* sp.) and chitosan (CHIT) were from Sigma–Aldrich, USA. Carboxylated multiwalled carbon nanotubes (c-MWCNTs) were from Intelligent Materials Pvt. Ltd., Panchkula (Haryana), India. 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 throughout this work.

All electrochemical experiments were performed at room temperature ($25 \pm 1\,^{\circ}C$) using an Autolab Potentiostat/Galvanostat (Eco Chemie, The Netherlands). A conventional three electrode cell was used for electrochemical experiments in which enzymes/ZnO-NPs/c-MWCNT/PANI/Pt, Ag/AgCl and Pt electrode were used as the working, reference and auxillary electrode, respectively. UV–visible spectra from Shimazdu spectrophotometer and Fourier transform infrared (FTIR) spectra from Thermo FTIR spectrophotometer were carried out in our lab. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray diffraction (XRD) studies were carried out on commercial basis.

2.2. Construction of enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode

2.2.1. Preparation of c-MWCNT/PANI/Pt electrode

c-MWCNT (1 mg) was suspended into 1.0 ml mixture of concentrated H_2SO_4 and HNO_3 in 3:1 ratio and ultrasonicated for 2 h to obtain a homogeneous mixture. After sonication, c-MWCNTs were washed thoroughly with DW until the pH of the washing discard was 7.0. A solution for electrodeposition was prepared by adding aniline (50 μl) and finally dispersed c-MWCNT suspension (1 ml) into 1 N HCl (10 ml) in a glass cell. c-MWCNT/PANI film was electrodeposited onto Pt electrode through electropolymerization using a potentiostat. Prior to electrodeposition, the Pt wire ($1.85\,cm \times 1\,mm$) was ultrasonicated in 5.0 M HNO_3 and acetone for 15 min and then rinsed with distilled water. The three electrode-system was immersed into the solution and the potential scan was cycled for 20 times between −0.6 and 1.0 V at a scan rate of 50 mV/s (Yadav et al., 2011). During the electrochemical polymerization, the surface of Pt electrode became black gradually, indicating the deposition of c-MWCNT/PANI film on Pt wire. The c-MWCNT/PANI film coated Pt electrode was washed with deionized water and dried at room temperature.

2.2.2. Preparation of ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode

ZnO-NPs were prepared as described (Moghaddam et al., 2009) using a 0.45 M aqueous solution of zinc nitrate ($Zn(NO_3)_2{\cdot}4H_2O$) and 0.9 M aqueous solution of sodium hydroxide (NaOH). Zinc nitrate solution (50 ml) was added drop wise slowly for 40 min to the NaOH (100 ml) solution under high speed stirring. The beaker was sealed during this process for 2 h. The precipitated ZnO-NPs were cleaned with deionized water and ethanol and dried in air at 60 °C. The nanoparticles were recovered from the colloidal solution by successive precipitation with hexane. CHIT (0.1 g) flakes were dissolved in 5 ml of 1% acetic acid and kept overnight at room temperature. ZnO-NPs (5 mg) were dispersed into transparent CHIT solution and kept on magnetic stirring for about 30 min at room temperature followed by the sonication for about 1 h. The hybrid nanocomposite film of ZnO-NPs/CHIT solution was electrodeposited on c-MWCNT/PANI/Pt electrode by cyclic voltammetry using a potential range, −0.75 to 1.2 V at a scan rate of 50 mV/s and subsequently allowed to dry at room temperature. The ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode was washed with deionized water.

2.2.3. Preparation of enzyme electrode

The ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode was activated by spreading 2.5% glutaraldehyde solution on it and allowing it to stand at room temperature for 2 h. The excess of glutaraldehyde was removed by washing it repeatedly with 0.05 M phosphate buffer (PB), pH 7.5. The CA, CI and SO were placed onto glutaraldehyde activated surface of ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode and kept overnight for immobilization. The resulting enzyme electrode was dried and then stored in the refrigerator at 4 °C. The fabricated electrode was characterized by SEM, FTIR and EIS.

2.3. Cyclic voltammetric measurement and testing of creatinine biosensor

To evaluate the catalytic activity of enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode, the modified electrode was characterized by a cyclic voltammogram in the presence of creatinine at the potential range from −0.6 V to +1.2 V. Fig. 1a shows cyclic voltammogram of the enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode in 0.05 M phosphate buffer (PB), pH 7.5 with creatinine 1–300 μM. The maximum response was observed at 0.5 V and hence subsequent studies were carried out at this voltage. To test the functioning of biosensor, three electrodes system was immersed into 15 ml 0.05 M phosphate buffer (PB), pH 7.5. The reaction was started by adding creatinine solution to reach a final concentration of 100 μM and the current (mA) generated was recorded at 0.5 V.

2.4. Application and evaluation of creatinine biosensor

The present biosensor was employed for measuring creatinine in serum samples. Serum samples from of different age groups and sex were collected from PGIMS, Rohtak in tubes and stored at 4 °C

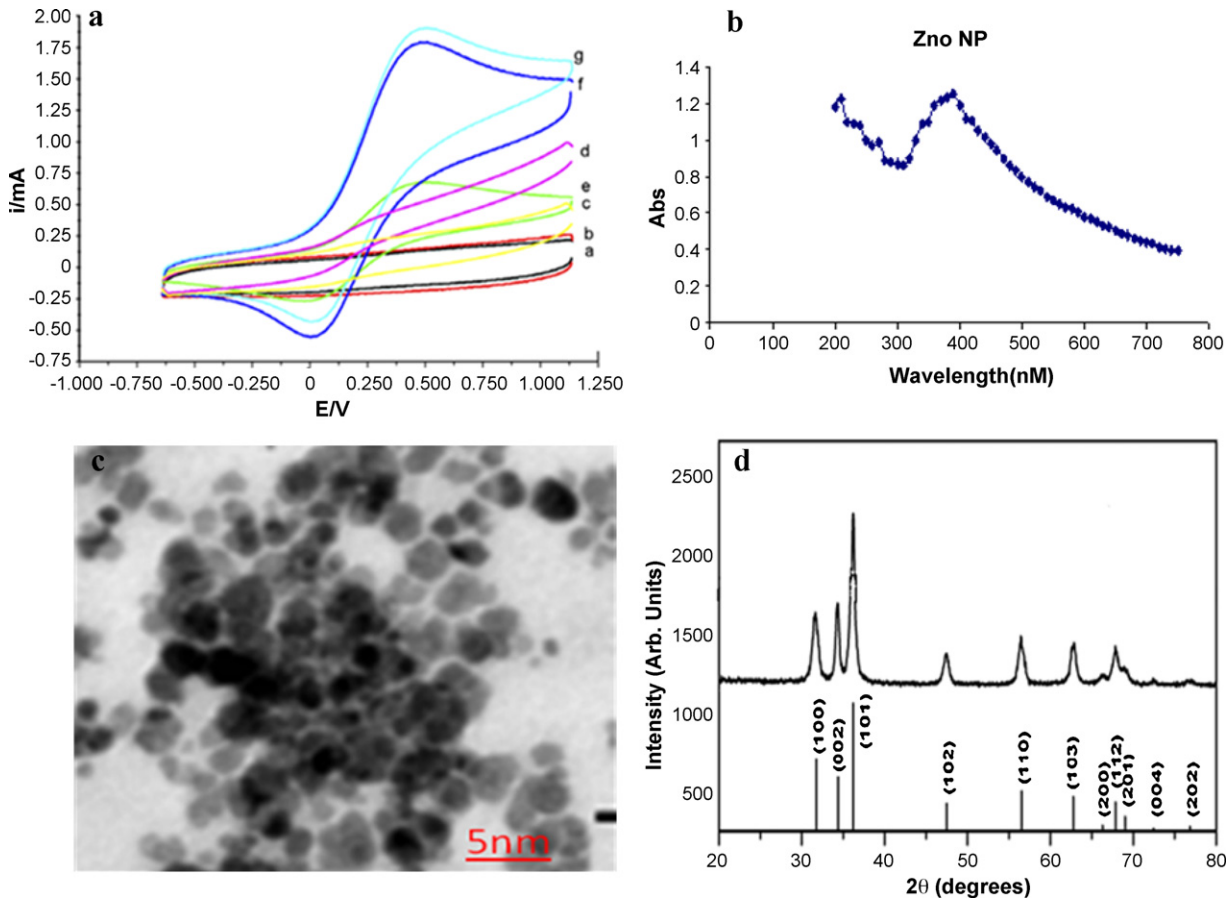

Fig. 1. (a) Cyclic voltammograms of amperometric response studies as a function of enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode in creatinine concentration from (a = 1 to g = 300 μM) using 0.05 M PB, pH 7.5. (b) UV spectrum of ZnO-NPs. (c) Transmission electron microscopic (TEM) images of ZnO-NPs. (d) X-ray diffraction (XRD) pattern ZnO-NPs.

until use. To determine serum creatinine, the same procedure was used, as described above for testing of biosensor under its optimal working conditions except that creatinine was replaced by serum. The following criteria were studied to evaluate the performance of the biosensor via linearity, analytical recovery, detection limit, sensitivity, precision and correlation with standard methods.

3. Results and discussion

3.1. Characterization of ZnO-NPs

The characterization of ZnO-NPs was carried out by UV spectra (Fig. 1b), TEM (Fig. 1c) and XRD (Fig. 1d). We found that the morphology of ZnO-NPs was crystallites rather than spherical with size 10–30 nm in diameter. In XRD, all peaks were consistent with the peaks of ZnO nanoparticles with high crystallinity. XRD studies confirmed that the synthesized materials were ZnO with wurtzite phase and all the diffraction peaks agreed with the reported joint committee on powder diffraction standard (JCPDS) data 15 and no characteristic peaks were observed other than ZnO. A definite line broadening of the diffraction peaks indicated that the synthesized materials were in nanometer range.

3.2. Construction of enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode

The fabrication of the creatinine biosensor based on enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt modified electrode is summarized in Fig. 2. Firstly, c-MWCNT/PANI composite film was deposited onto Pt electrode using cyclic voltammetry. Then, ZnO-NPs/CHIT biopolymer matrix was deposited electrochemically on the surface of PANI/c-MWCNT composite film. The electrodeposition method was selected to produce ZnO-NPs on electrode surfaces, because this method is easy to be carried out and thickness of the layer can be controlled. Secondly, CA, CI and SO enzymes were immobilized on ZnO-NPs/CHIT/c-MWCNT/PANI composite film through glutaraldehyde coupling as well as by electrostatic adsorption.

The cyclic voltammograms of c-MWCNT/PANI and ZnO-NPs/CHIT/c-MWCNT/PANI composite film are shown in Fig. 3. The ZnO-NPs/CHIT/c-MWCNT/PANI composite film exhibits higher currents than c-MWCNT/PANI composite film, which indicate that ZnO-NPs/CHIT/c-MWCNT/PANI composite film, have large effective surface area than c-MWCNT/PANI composite film and ZnO-NPs/CHIT/c-MWCNT/PANI composite film could provide a conducting path through the composite matrix for faster kinetics. Hence, the ZnO-NPs, acting as electron transfer mediator help in enhancing the sensor response of enzyme electrode and thus increase the sensitivity of the biosensor. These observations suggest the formation of ZnO-NPs/CHIT/c-MWCNT/PANI composite film which, provided large surface area for immobilization of the enzymes.

The electrochemical reactions involved in response measurement of creatinine biosensor are as follows:

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

$$\text{Creatine} + H_2O \xrightarrow{CI} \text{sarcosine} + \text{urea}$$

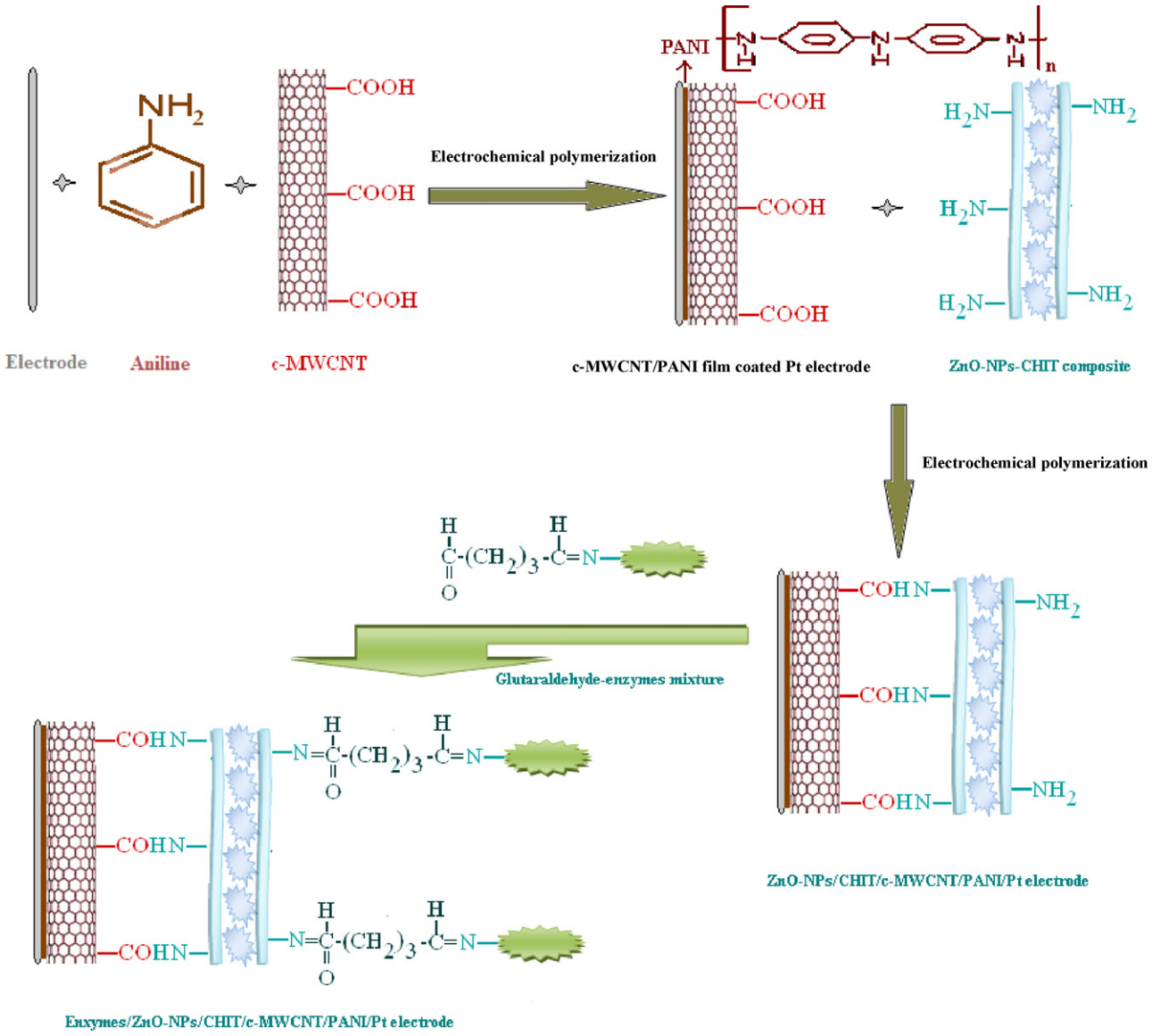

Fig. 2. Schematic representation of chemical reaction involved in the fabrication of enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt hybrid electrode.

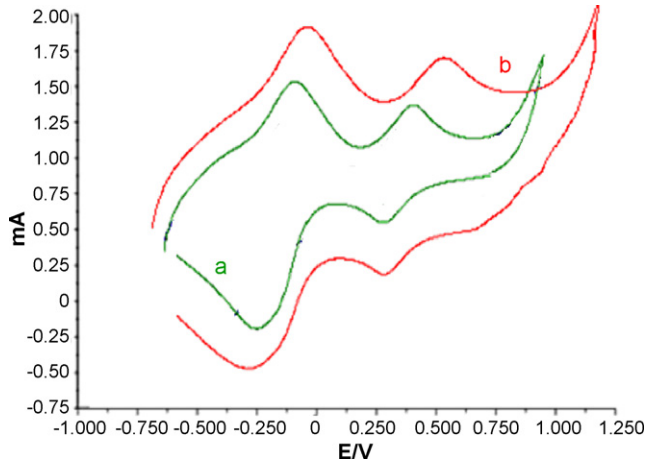

Fig. 3. Cyclic voltammogram for electrochemical deposition of (a) c-MWCNT/PANI/Pt electrode and (b) ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode.

$$\text{Sarcosine} + H_2O + O_2 \xrightarrow{SO} \text{formaldehyde} + \text{glycine} + H_2O_2$$

$$H_2O_2 \xrightarrow{0.5V} 2H^+ + O_2 + 2e^-$$

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

3.3. SEM studies

The morphology of c-MWCNT/PANI/Pt electrode, ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode and enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode was characterized by SEM studies. SEM images show c-MWCNTs/PANI/Pt electrode (Fig. 4a), presence of ZnO-NPs on c-MWCNT/PANI/Pt electrode (Fig. 4b) in a cluster forms and Fig. 4c shows the globular structure indicating the presence of immobilized CA, CI and SO on the nanocomposite hybrid electrode.

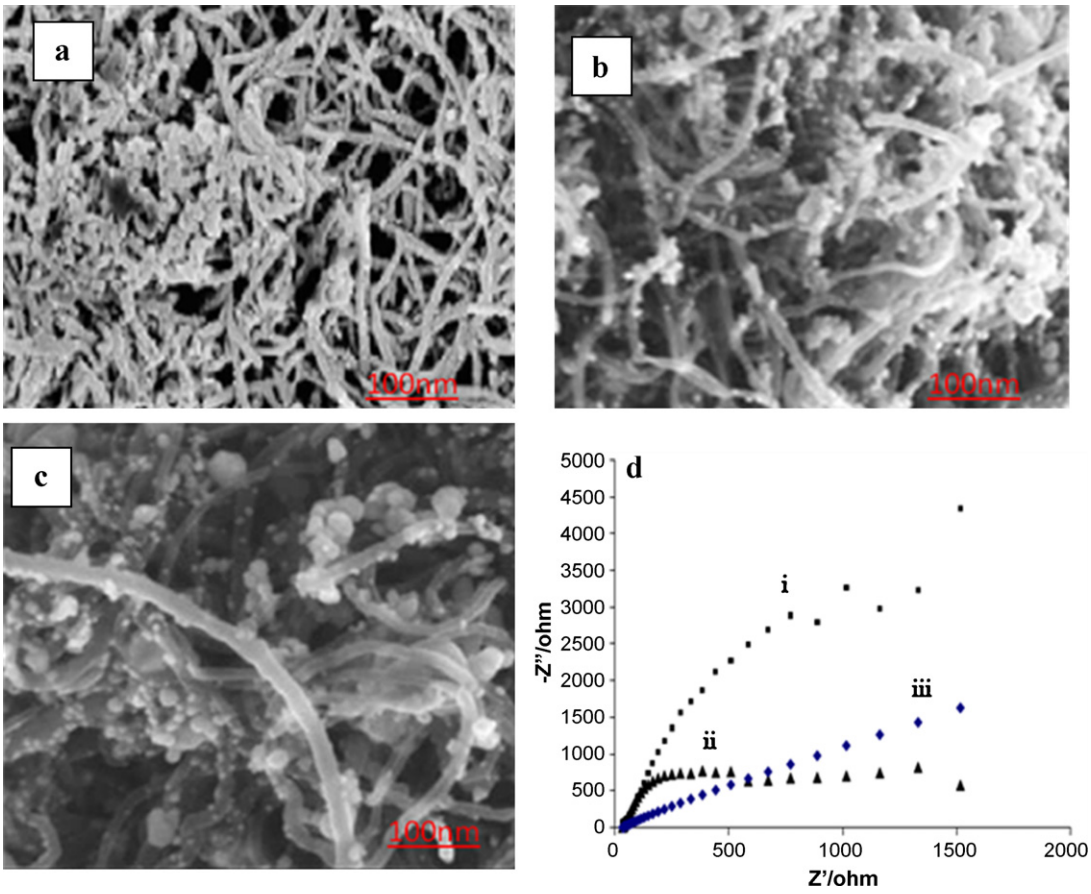

Fig. 4. (a) SEM images of c-MWCNT/PANI/Pt electrode. (b) SEM images of ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode. (c) SEM images of enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode. (d) Impedance spectra of (i) c-MWCNT/PANI/Pt electrode, (ii) ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode, and (iii) enzyme/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode 5 mM K3Fe(CN)6/K4Fe(CN)6 (1:1) containing 0.05 M PB, pH 7.5.

3.4. Electrochemical impedance spectroscopy (EIS)

EIS studies provides useful information on impedance changes of the electrode surface during the fabrication process and was carried out to investigate immobilization of enzymes onto ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode. The charge transfer process in enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode was studied by monitoring charge transfer resistance (R_{CT}) at the electrode and electrolyte interface. The diameter of semicircle portion at higher frequencies of Nyquist plot was equal to the charge transfer resistance (R_{CT}), which can be used to describe the interface properties of the electrode. Meanwhile, the linear part at lower frequencies corresponds to the diffusion process. Fig. 4d shows electrochemical impedance spectra (EIS) of (i) c-MWCNTs/PANI/Pt electrode, (ii) ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode and (iii) enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.05 M PB, pH 7.5. It was observed that the R_{CT} of ZnO-NPs/CHIT/c-MWCNT/PANI modified electrode (ii) is lower than c-MWCNT/PANI modified electrode (i). The results show that ZnO-NPs/CHIT/c-MWCNT/PANI modified electrode decreases the resistance of the electrode and hold high electron transfer efficiency, while the R_{CT} of enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI bioelectrode (iii) increases over to that of ZnO-NPs/CHIT/c-MWCNT/PANI modified electrode. This increase in R_{CT} 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 ZnO-NPs/CHIT/c-MWCNT/PANI composite.

3.5. FTIR spectra

The FTIR spectrum of electrochemically deposited c-MWCNT/PANI composite (Fig. 5a) shows benzenoid and quinoid ring stretching bands present at 1459.1 and 1624 cm^{-1}. The peaks obtained at 1043.3 and 3419.9 cm^{-1} attributed to B–N + = Q and –N–H stretching vibrations of PANI in the composite. In FTIR spectrum of ZnO-NPs/CHIT/c-MWCNT/PANI composite (Fig. 5b) showed addition peak at 492 cm^{-1} due to ZnO-NPs and a broad peak at 3421.3 cm^{-1} is a characteristic absorption bands of chitosan due to overlapping of –OH and $–NH_2$ stretching. The enzymes binding on ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode was indicated by the appearance of additional absorption bands at 1652.9 and 1559.6 cm^{-1} (Fig. 5c) which were assigned to carbonyl stretch (amide-1 band) and –N–H bonding (amide-11 band), respectively.

3.6. Optimization of experimental variables

The effect of pH on electrochemical response of bioelectrode was studied in the pH range 6.0–10.0. The optimum current response was obtained between pH 7.0 and 8.0. The optimum temperature of bioelectrode was studied by measuring the current response at different temperatures from 25 °C to 45 °C. The optimum current response was obtained at 35 °C. When creatinine was added into PB, pH 7.5 the biosensor responded rapidly to the substrate and achieved 95% of steady current within response time of 10 s which is much lower than those reported for creatinine biosensor based on PUR hydrogel matrix

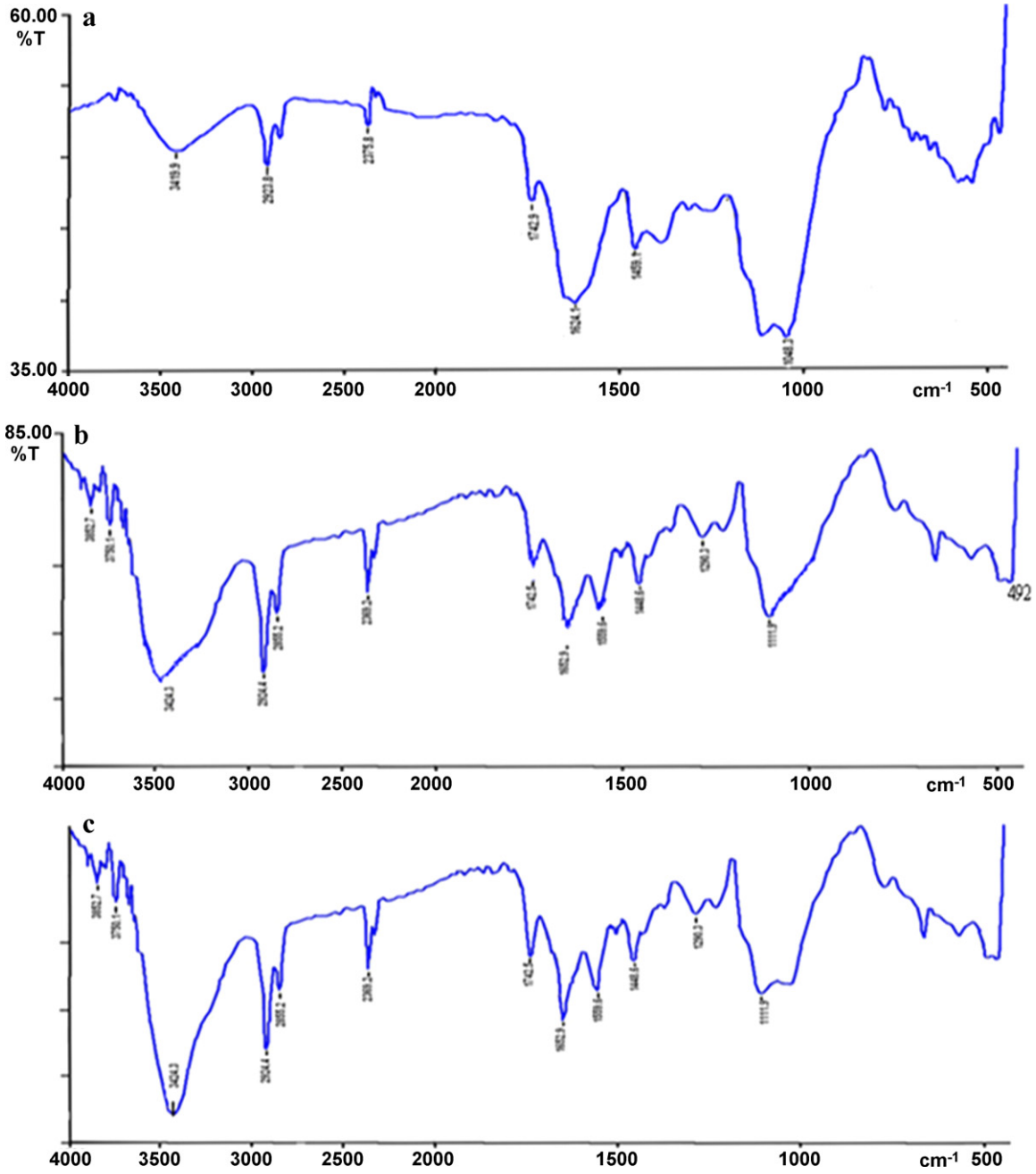

Fig. 5. FTIR spectra obtained for (a) PANI/c-MWCNT composite. (b) ZnO-NPs/CHIT/c-MWCNT/PANI composite. (c) Enzymes/ZnO-NPs/CHIT/c-MWCNT/PANI composite.

(5 min) (Marcel et al., 1996), carbon paste electrode (90 s) (Kim et al., 1999) and PbO_2 film (118 s) (Shin et al., 2001). This faster response is attributed to the synergetic influence of ZnO-NPs, CHIT and c-MWCNT. The steady state amperometric response of biosensor was investigated by increasing creatinine concentration from 0.5 μM to 1000 μM under optimal conditions. The resulted calibration plot for creatinine, over the concentration range 10–650 μM is shown in Fig. 6. The linear plot reveals that such electrode worked well in creatinine solution with a sensitivity of 0.030 $\mu A\,\mu M^{-1}\,cm^{-2}$. Lineweaver-Burk plot gave a K_m value of 0.35 mM for immobilized enzymes, which is lower than that of microfabricated creatinine biosensor (5.2 mM) (Marcel et al., 1996) and carbon paste electrode based creatinine biosensor (5.15 mM) (Kim et al., 1999). These results show that present biosensor possesses higher affinity to creatinine.

3.7. Application of creatinine biosensor

The present biosensor was used to investigate the level of creatinine in human serum and it was found in the range of 0.5 mg/dl to 1.9 mg/dl. When these results were compared with those obtained by chemical spectrophotometric method (Jaffé, 1886), there was a good correlation ($r = 0.989$). The results showed that the data obtained by present method and previously reported standard method are comparable. It proved that the present modified electrode ascertained the practical application of the biosensor in the clinical analysis.

3.8. Interference study and selectivity

The interference study of the present creatinine biosensor was carried out by comparing the amperometric response before and

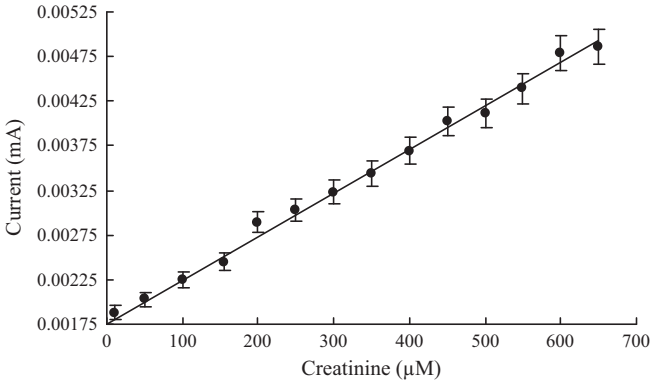

Fig. 6. Linear calibration plot corresponding to current responses for different creatinine concentration by creatinine biosensor based on ZnO-NPs/CHIT/c-MWCNT/PANI composite film.

after adding some interferents such as uric acid, ascorbic acid, glycine, acetaminophen and creatine at their physiological concentration into 100 μM creatinine in 0.05 M PB, pH 7.5. The results showed that uric acid, ascorbic acid, glycine and acetaminophen have no significant effect. However, an obvious interference was observed when creatine coexisting with creatinine, which is consistent with the results of previous mediator biosensors (Marcel et al., 1996). To avoid creatine interference, we determined total creatinine (creatinine + creatine) using the try-enzyme electrode (CA/CI/SO) and then the concentration of creatine in the same sample was determined by bienzyme electrode (CI/SO). The true creatinine was calculated by subtracting the creatine value from total creatinine.

3.9. Evaluation of creatinine biosensor

The detection limit of biosensor was 0.5 μM at a signal to noise ratio of 3, which is lower than those reported for creatinine biosensor based on PUR hydrogel matrix (Marcel et al., 1996), carbon paste electrode (Kim et al., 1999) and polymer film (polyanion polypyrrole) (Khan and Wernet, 1997) but comparable to that of microchip based creatinine biosensor employing an oxidizing layer (Shin et al., 2001). Analytical recovery of exogenously added creatinine in serum (0.5 mg/dl and 1 mg/dl) was 98.87% and 98.31%, respectively, showing the reliability of the method. The results of within and between batch coefficient of variation (CVs) for serum creatinine determination were <4.6% and <5.1%, respectively indicating the good reproducibility and consistency of the method.

3.10. Stability of enzyme electrode

The stability of the enzyme electrode was investigated every week under storage conditions at 4 °C. It was observed that the current response of the sensor maintained 85% of the initial current response even after 120 days of regular 100 uses which is better than PUR hydrogel matrix (90 days) (Marcel et al., 1996) and polymer film(polyanion polypyrrole) (<30 days) (Khan and Wernet, 1997). This suggests that ZnO-NPs/CHIT/c-MWCNT/PANI composite film ensure good stability.

4. Conclusion

A new amperometric creatinine biosensor was fabricated by immobilizing three enzymes (CA, CI and SO) on ZnO-NPs/CHIT/c-MWCNT/PANI/Pt electrode. The sensor exhibited high sensitivity, fast response time and good reproducibility. The study provides a feasible approach on developing new kind of ZnO nanoparticles based amperometric biosensors for constructing various hybrid type biosensors.

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