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Amperometric creatinine biosensor based on covalently coimmobilized enzymes onto carboxylated multiwalled carbon nanotubes/polyaniline composite film

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ABSTRACT

A mixture of commercial creatinine amidohydrolase (CA), creatine amidinohydrolase (CI), and sarcosine oxidase (SO) was coimmobilized covalently via *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy succinimide (NHS) chemistry onto carboxylated multiwalled carbon nanotube (c-MWCNT)/polyaniline (PANI) nanocomposite film electrodeposited over the surface of a platinum (Pt) electrode. A creatinine biosensor was fabricated using enzyme/c-MWCNT/PANI/Pt as working electrode, Ag/AgCl as reference electrode, and Pt wire as auxiliary electrode connected through potentiostat. The enzyme electrode was characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, and electrochemical impedance spectroscopy (EIS). The biosensor detected creatinine levels as low as 0.1 μM, estimated at a signal-to-noise ratio of 3, within 5 s at pH 7.5 and 35 °C. The optimized biosensor showed a linear response range of 10 to 750 μM creatinine with sensitivity of 40 μA/mM/cm^2. The fabricated biosensor was successfully employed for determination of creatinine in human serum. The biosensor showed only 15% loss in its initial response after 180 days when stored at 4 °C.

Creatinine (2-amino-1-methyl-5H-imidazol-4-one) is the end product of creatine catabolism. Creatinine is found together with creatine in muscle tissue and in blood. Determination of creatinine in blood and urine is essential for evaluation of renal, muscular, and thyroid dysfunctions. It is also useful for biomedical diagnosis of acute myocardial infarction as well as for quantitative description of hemodialysis therapy. In contrast to urea, the concentration of creatinine in these body fluids is not influenced by the protein intake, and therefore its level is a more reliable indicator of renal function [1]. Creatine and phosphocreatine are also excreted from the body. The amount of creatinine in the urine is proportional to the amount of creatine and creatine phosphate present in the body and also to muscle mass [2]. Creatinine is mostly analyzed colorimetrically using the Jaffe's reaction [3] or enzymic colorimetric method [4]. However, colorimetric methods are adversely affected by numerous metabolites and drugs found in biological samples [5], whereas enzymic assays are time-consuming and expensive. Highly selective and relatively fast creatinine determination in biofluids is possible with some chromatographic methods [6,7], but instruments required for these methods are costly and require sample preparation and skilled persons to operate, and therefore they are not suitable for routine analysis.

Nevertheless, amperometric determination of creatinine by biosensor is comparatively simple, rapid, and sensitive, and it requires no sample preparation. For routine creatinine analysis, various electrochemical methods have been proposed in the literature, including amperometric as well as potentiometric biosensors [8,9]. All of the amperometric creatinine biosensors are based on a multienzyme sequence proposed by Tsuchida and Yoda [10] consisting of creatinine amidohydrolase (CA,[1] EC 3.5.2.10), creatine amidinohydrolase (CI, EC 3.5.3.3), and sarcosine oxidase (SO, EC 1.5.3.1). The enzyme sequence catalyzes the conversion of creatinine via creatine and sarcosine to glycine, formaldehyde, and hydrogen peroxide as depicted in the following reaction sequence:

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

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

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

$$H_2O_2 \text{ Potential} \rightarrow 2H^+ + O_2 + 2e^-.$$

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[1] *Abbreviations used:* CA, creatinine amidohydrolase; CI, creatine amidinohydrolase; SO, sarcosine oxidase; CNT, carbon nanotube; PANI, polyaniline; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide; NHS, *N*-hydroxy succinimide; c-MWCNT, carboxylated multiwalled carbon nanotube; Pt, platinum; DW, double distilled water; FTIR, Fourier transform infrared; SEM, scanning electron microscopy; PB, phosphate buffer; EIS, electrochemical impedance spectroscopy; RCT, charge transfer resistance; CV, coefficient of variation.

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However, these amperometric biosensors have significant problems involving sensitivity, selectivity, elimination of interferences, and sensor stability that are still to be improved or solved. Unfortunately, potentiometric biosensors also suffer from interference by cations and endogenous ammonia that is present in blood and urine [11]. To overcome these problems, the enzymes need to be immobilized directly and covalently onto the film-coated electrode. The response times of previously reported biosensors were relatively high, ranging from 20 to 300 s [8]. In earlier reports, enzymatically generated H_2O_2 oxidized at relatively high positive potential (0.4–0.8 V) [12–17], leading to interferences from various electroactive species such as ascorbic acid, uric acid, and 4-acetamidophenol. Therefore, it is highly desirable to design and prepare such a functional material for modification of the electrode surface that efficiently lowers the H_2O_2 oxidation potential and maintains the enzyme activity. The emergence of nanotechnology offers great opportunities to improve the sensitivity, stability, and anti-interference ability of the biosensing systems.

Carbon nanotubes (CNTs) have attracted much attention as an electrode material for electrochemical sensors because of their excellent electrochemical properties, a large edge plane/basal plane ratio, rapid electron kinetics, semi- and superconducting electron transport, high tensile strength composites, and hollow core suitable for storing guest molecules [18,19]. The ability of CNTs to promote the electron transfer reactions of H_2O_2 suggests great promise for amperometric glucose biosensors because it can facilitate low-potential amperometric measurement H_2O_2 [20]. CNTs and conducting polymer with an expected synergistic effect have been explored for possible improvement in the electrical and mechanical properties of polymers [21], and hence CNT/polymer composite has been used to enhance the sensor performance. Among the various conducting polymers, polyaniline (PANI) is one of the most important polymers due to its electrical conductivity, stability, and facile synthesis [22,23]. Furthermore, PANI exhibits significant redox behavior.

In the current study, we report the construction and application of a novel, highly sensitive, and stable creatinine biosensor based on covalent immobilization of CA, CI, and SO using *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy succinimide (NHS) onto carboxylated multiwalled carbon nanotube (c-MWCNT)/PANI composite film, electropolymerized on platinum (Pt) electrode.

Materials and methods

Chemicals and nanomaterials

CA (from *Pseudomonas* sp.), CI (from *Pseudomonas* sp.) and SO (from *Bacillus* sp.) were obtained from Sigma–Aldrich (USA). c-MWCNTs were obtained from M/S Intelligent Materials (Panchkula, Haryana, India). EDC, NHS, and aniline (purified through vacuum distillation before use) were obtained from Sisco Research Laboratories (Mumbai, India). All other chemicals were of analytical reagent grade. Double distilled water (DW) was used in all experiments.

Instruments

All electrochemical experiments were performed at 25 ± 1 °C using an Autolab potentiostat/galvanostat (Eco Chemie, The Netherlands). A conventional three-electrode cell consisting of enzyme/c-MWCNT/PANI/Pt, Ag/AgCl, and Pt wire as the working, reference, and auxiliary electrodes, respectively, was used for electrochemical experiments. Fourier transform infrared (FTIR) spectroscopy was recorded in an FTIR spectrophotometer (Thermo Scientific, USA). The morphological studies were carried out by using scanning electron microscopy (SEM) on commercial basis.

Electrochemical deposition of c-MWCNT/PANI composite film on Pt electrode

c-MWCNT (1 mg) was suspended in a 1.0 ml mixture of concentrated H_2SO_4 and HNO_3 in a 3:1 ratio and ultrasonicated for 2 h to obtain a homogeneous mixture and then washed with DW. A solution for electrodeposition was prepared by adding aniline (50 μl) and finally dispersed c-MWCNT suspension (1 ml) in 1 N HCl (10 ml) in a glass cell. c-MWCNT/PANI film was electrodeposited onto Pt electrode through electropolymerization using a potentiostat/galvanostat. Prior to electrodeposition, the Pt wire (1.95 cm × 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 in the solution, and the potential scan was cycled 20 times between −0.1 and 0.9 V at a scan rate of 50 mV/s [24]. During the electrochemical polymerization, the surface of Pt wire gradually became black, indicating the deposition of c-MWCNT/PANI film on Pt wire. The c-MWCNT/PANI film coated Pt wire was washed with deionized water and dried at room temperature.

Immobilization of CA, CI, and SO on c-MWCNT/PANI composite film

The CA, CI, and SO were coimmobilized covalently onto electrochemically synthesized c-MWCNT/PANI film using EDC–NHS chemistry as described previously [20] with modification. First, free and unbound –COOH groups of c-MWCNT/PANI film were activated by immersing them into 0.1 M phosphate buffer (PB, pH 7.5) containing EDC and NHS of the same concentration (10 mM) for 6 h, and then excess of EDC and NHS was removed by washing with 0.1 M PB (pH 6.8). Finally, EDC–NHS-treated electrode was incubated in 0.05 M PB (pH 7.5) containing CA (44 U), CI (36 U), and SO (24 U) at 4 °C for 3 h and then washed with 0.05 M PB (pH 7.5). The fabricated working electrode was characterized by SEM, FTIR, and electrochemical impedance spectroscopy (EIS).

Testing of creatinine biosensor

The sensitivity of the creatinine biosensor was tested by measuring current using the three-electrode system. Fig. 1A shows the effect of working potential on the performance of the biosensor. The working potential was swept −0.1 to 0.9 V in 0.05 M PB (pH 7.5) containing 100 μM creatinine versus Ag/AgCl as reference electrode. The optimal current response was obtained at 0.2 V, and 0.2 V was selected as the working potential for amperometric detection of creatinine concentration.

The modified electrode was employed for measuring creatinine in serum samples. Serum samples of different age groups and genders were collected from PGIMS (Rohtak, Haryana, India) in tubes and stored at 4 °C until use. The following criteria were studied to evaluate the performance of this biosensor: linearity, analytical recovery, detection limit, sensitivity, precision, and correlation with standard method.

Results and discussion

Construction of modified enzyme electrode

The modification of the surface of working electrode was performed in two steps. First, c-MWCNT/PANI composite film was deposited onto Pt electrode using cyclic voltammetry. Second, CA, CI, and SO enzymes were covalently coimmobilized on

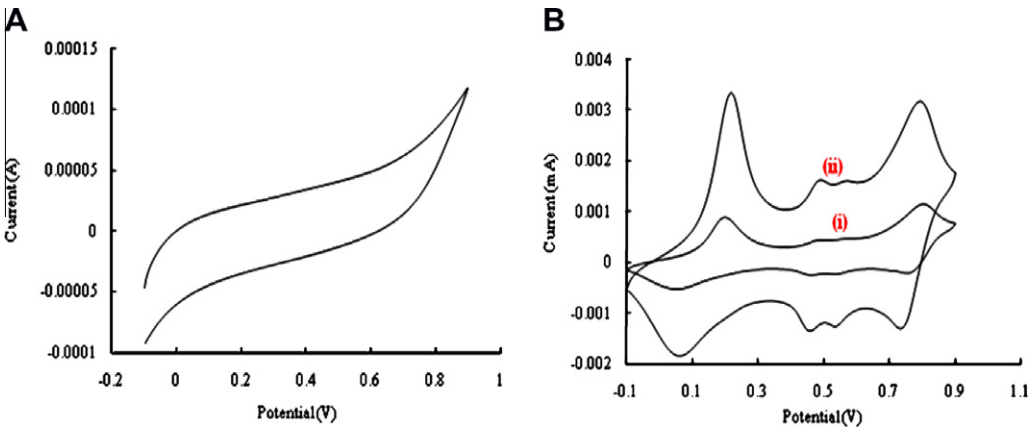


Fig.1. (A) Cyclic voltammogram for 100 μM creatinine solution in 0.05 M PB (pH 7.5). (B) Current response curves of pure conducting PANI (i) and c-MWCNT/PANI composite (ii) films at 100 μM creatinine.

c-MWCNT/PANI composite film using EDC–NHS chemistry. EDC–NHS was used to activate the free –COOH groups present on c-MWCNT/PANI composite film.

Fig. 1B displays the cyclic voltammograms of electrodeposition of pure conducting PANI (curve i) and c-MWCNT/PANI composite (curve ii) films. The composite film exhibits higher currents than its polymer counterpart, indicating that c-MWCNT/PANI composite film has a larger effective surface area than pure conducting PANI film and that PANI/c-MWCNTs could provide a conducting path through the composite matrix for faster kinetics. Hence, the c-MWCNTs, acting as electron transfer mediator, help to enhance the sensor response of enzyme electrode and to increase the sensitivity of the biosensor. These observations suggest the formation of c-MWCNT/PANI composite film to provide a large surface area for immobilization of the enzymes.

Scheme 1 summarizes the different chemical reactions involved in the fabrication of the creatinine biosensor based on covalent immobilization of CA, CI, and SO onto c-MWCNT/PANI film electrodeposited on Pt electrode surface through amide bond formation between the free and unbound –COOH groups of c-MWCNT/PANI composite film and the $-NH_2$ groups on the surface of enzymes.

Surface characterization by SEM

SEM was used for investigating the morphology of c-MWCNT/PANI/Pt electrode with and without immobilized enzymes. Fig. 2A shows SEM micrographs of c-MWCNT/PANI/Pt electrode that reveal the uniform and cable-like morphology of the nanostructure of c-MWCNT/PANI composite film. After immobilization of enzymes on c-MWCNT/PANI composite film, the hybrid bioelectrode shows deposition of globular structure on uniform structure (Fig. 2B), indicating that enzymes were successfully immobilized on the surface of c-MWCNT/PANI composite film.

FTIR spectra

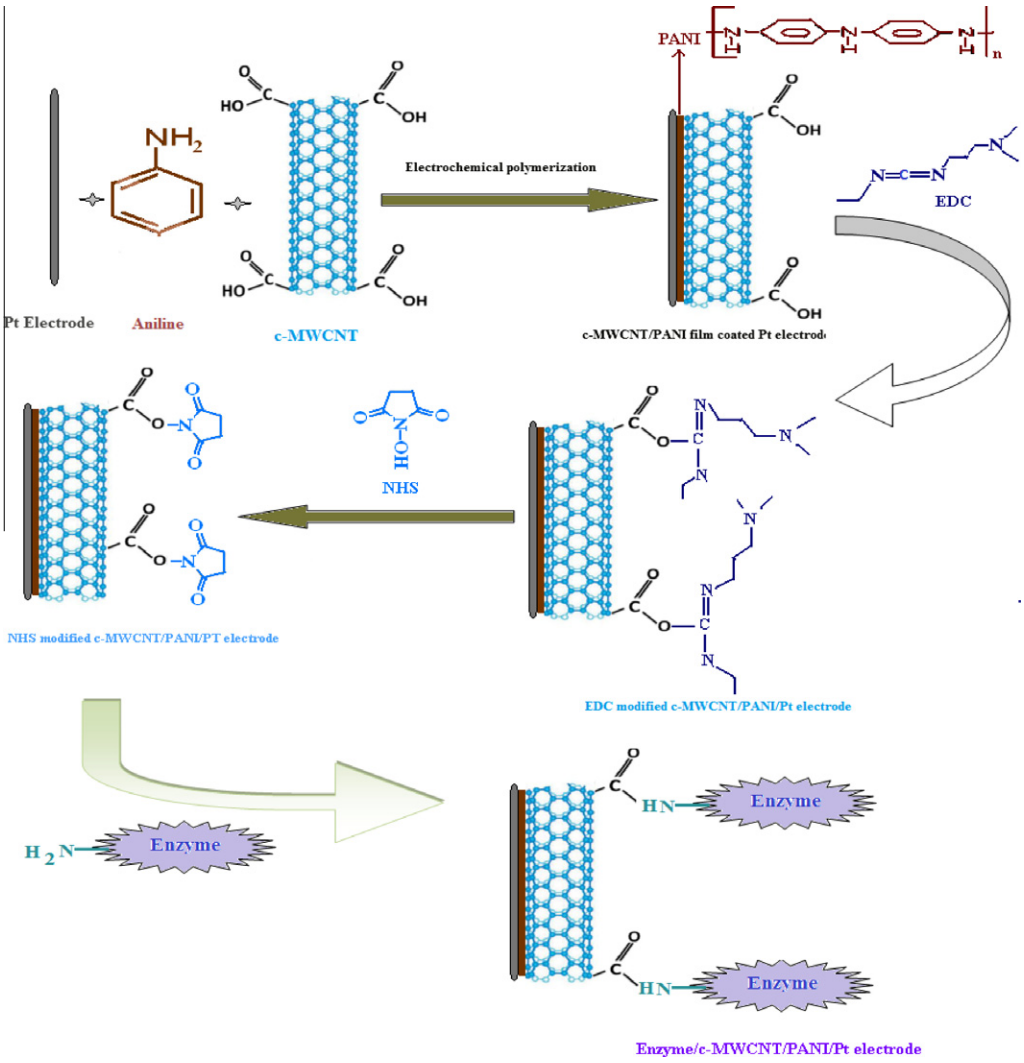
Fig. 3, shows FTIR spectra obtained for PANI, c-MWCNTs, c-MWCNT/PANI, and enzymes/c-MWCNT/PANI composites. When PANI and c-MWCNT composite form, no new absorption peaks result but peak shapes change to some extent due to interaction between c-MWCNTs and PANI (curve i and curve ii). The FTIR spectrum of electrochemically deposited c-MWCNT/PANI composite (curve iii) shows benzenoid and quinoid ring stretching bands present at 1404 and 1609.53 cm^{-1}. The peaks obtained at 1135.53 and 3399.32 cm^{-1} were attributed to B–N^+=Q and –N–H stretching vibrations of PANI in the composite. The –C=O stretching vibrations peak obtained at 1817.93 cm^{-1} indicated the presence of carboxyl group (–COOH) in the MWCNTs. The enzymes binding on c-MWCNT/PANI/Pt electrode are indicated by the appearance of additional absorption bands at 1589.10 and 1491.03 cm^{-1} (curve iv) that were assigned to carbonyl stretch (amide 1 band) and –N–H bonding (amide 11 band), respectively [25].

EIS studies

EIS studies provide useful information on impedance changes of the electrode surface during the fabrication process and were carried out to investigate immobilization of enzymes onto c-MWCNT/PANI/Pt electrode. The diameter of the semicircle portion at higher frequencies of the Nyquist plot was equal to the charge transfer resistance (R_{CT}), which controls the electron transfer kinetics of the redox probe at the electrode interface. Meanwhile, the linear part at lower frequencies corresponds to the diffusion process [26]. The Nyquist plot (Fig. 4) displays EIS studies of PANI/Pt (curve i), c-MWCNT/PANI/PT (curve ii), and enzyme/c-MWCNT/PANI/Pt (curve iii) in 0.05 M PB (pH 7.5) containing 5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1) as a redox probe. It was observed that the R_{CT} of c-MWCNT/PANI/Pt electrode (curve ii) was lower than PANI/Pt electrode (curve i), revealing its decreased resistance and high electron transfer efficiency. However, the R_{CT} of enzyme/c-MWCNT/PANI/Pt (curve iii) bioelectrode increased compared with that of c-MWCNT/PANI/Pt electrode. This increase in R_{CT} can be attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies and cause hindrance to electron transfer. These results also indicate the binding of enzymes onto c-MWCNT/PANI composite.

Optimization of experimental variables

The effect of pH on electrochemical response of bioelectrode was studied in the pH range 6.0 to 10.0 using the following buffers, each at a final concentration of 0.05 M: pH 6.0 to 8.0 PB, pH 8.5 to 9.0 Tris–HCl buffers, and 9.5 to 10.0 sodium carbonate/bicarbonate buffers. The optimal current response was obtained at pH 7.5 (Fig. 5), which is similar to previous amperometric biosensors based on nucleopore polycarbonate membrane [13], polymer film (polyanion polypyrrole) [14], and Nafion membrane [17] but slightly higher than polyurethane hydrogel matrix (pH 7.0) [12]. At below pH 7.0 and above pH 8.5, the response of the creatinine biosensor decreased sharply, probably due to decreased SO activity under these conditions [13]. The optimal temperature of bioelectrode was studied by measuring the current response at different



Scheme 1. Schematic representation of chemical reaction involved in the fabrication of enzyme/c-MWCNT/PANI/Pt hybrid electrode.

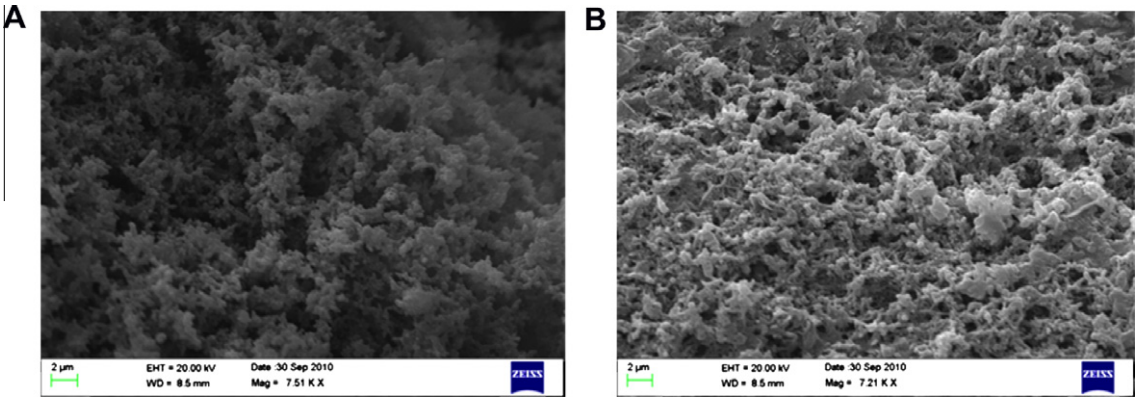


Fig.2. SEM images of c-MWCNT/PANI/Pt electrode without (A) and with (B) immobilized enzymes.

temperatures from 25 to 45 °C. The current response of the biosensor increased with increases in temperature up to 35 °C, after which it declined. This optimal temperature (35 °C) is comparable to that for an earlier biosensor based on nucleopore polycarbonate membrane (37 °C) [13]. The increase in current response of the biosensor up to 35 °C could be due to the activation energy of the reaction. After that, a decrease is often observed because of enzyme denaturation. The steady-state amperometric response of the biosensor was investigated by increasing the creatinine concentration from 0.1 to 1500 μM under optimal conditions. A Lineweaver–Burk plot gave the apparent K_m value of 0.26 mM for immobilized enzymes, and this value is lower than those of a microfabricated creatinine biosensor (5.2 mM) [12] and a carbon paste electrode-based creatinine biosensor (5.15 mM) [15]. These results show that the current biosensor possesses higher affinity to creatinine compared with earlier biosensors.

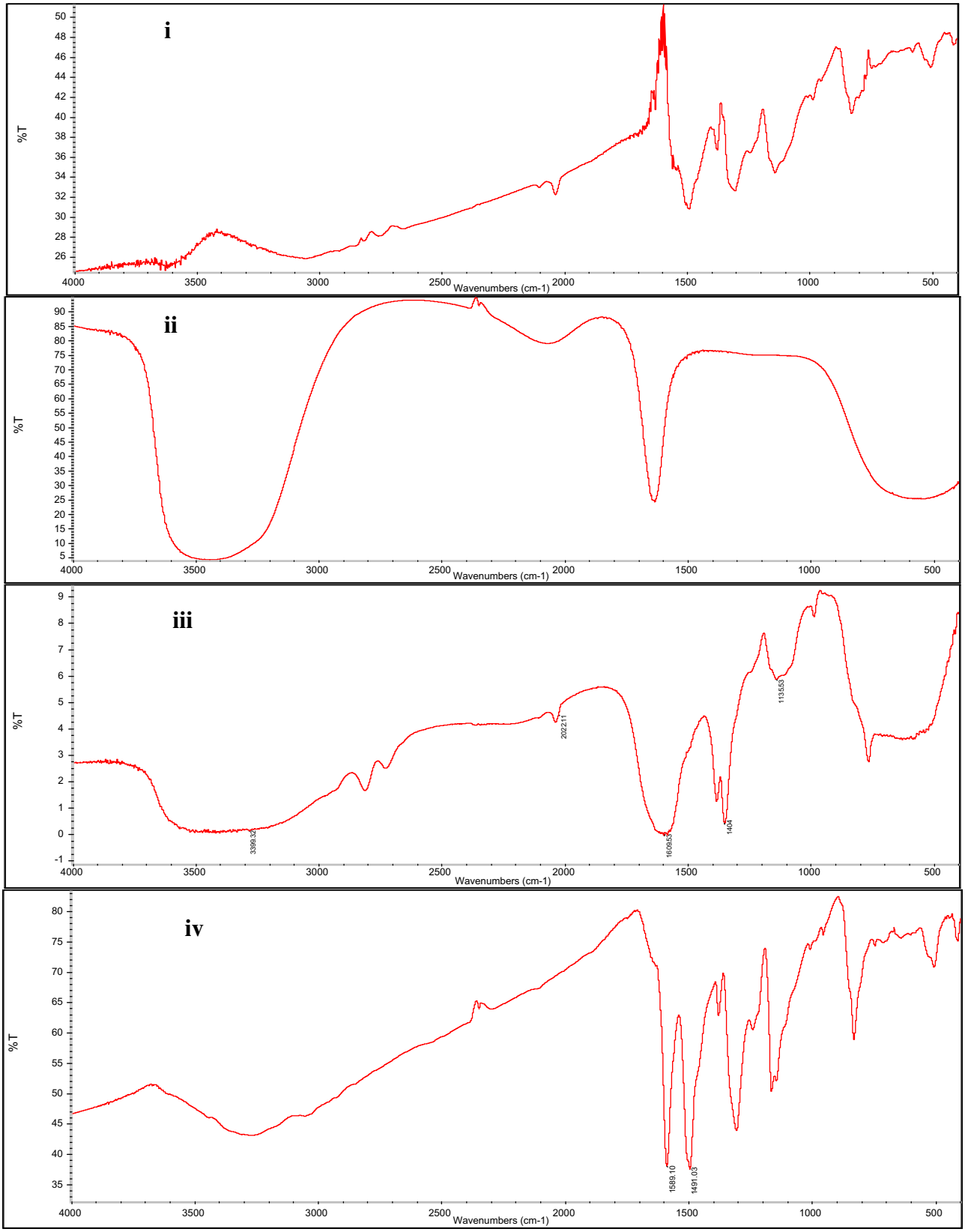


Fig.3. FTIR spectra obtained for PANI (i), c-MWCNT (ii), c-MWCNT/PANI (iii), and enzyme/c-MWCNT/PANI (iv) composites.

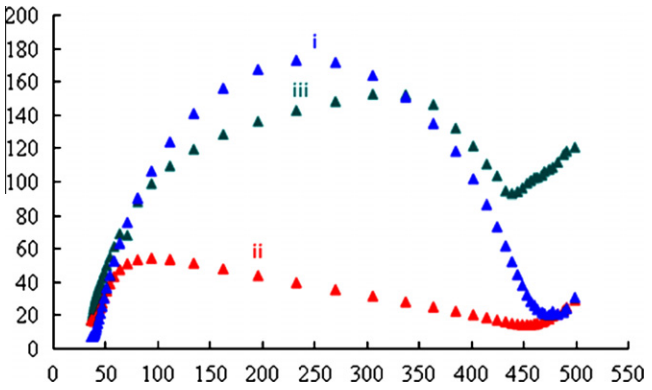


Fig.4. Nyquist plot of EIS of PANI/Pt (i), c-MWCNT/PANI/PT (ii), and enzyme/c-MWCNT/PANI/Pt (iii) in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.05 M PB (pH 7.5).

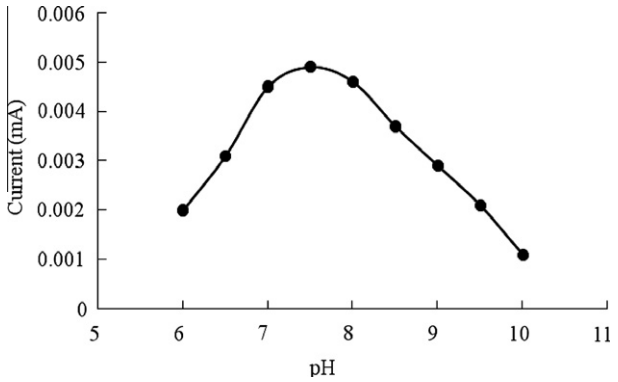


Fig.5. Effect of pH on c-MWCNT/PANI composite film-based creatinine biosensor response.

Interference study and selectivity

The interference study of the current creatinine biosensor was carried out by comparing the amperometric response before and after adding some interferents such as uric acid, ascorbic acid, glycine, acetaminophen, and creatine along with 100 μM creatinine in 0.05 M PB (pH 7.5) at their physiological concentrations. The results showed that uric acid, ascorbic acid, glycine, acetaminophen, and creatine have practically no interference (Table 1).

Evaluation of creatinine biosensor

When creatinine was added into PB (pH 7.5), the biosensor responded rapidly to the substrate and achieved 95% of steady current within 5 s. The resulting calibration plot for creatinine over the concentration range from 0.1 to 750 μM is shown in Fig. 6. The linear plot reveals that such electrode can work well in creatinine solution with a sensitivity of 40 μA/mM/cm^2. The detection limit of the biosensor was 0.1 μM at a signal-to-noise ratio of 3, and this value is much lower than those for earlier electrochemical biosensors [7,12,14,15] but is comparable to that of a microchip-based creatinine biosensor employing an oxidizing layer [16]. Analytical recoveries of exogenously added creatinine in serum (0.5 and 1 mg/dl) were 98.47% and 97.91%, respectively, showing the reliability of the method. The results of within- and between-batch coefficients of variation (CVs) for serum creatinine determination were less than 3.6% and 4.1%, respectively, which are very low. This shows the good reproducibility and consistency of the method.

Table 1
Effect of potential interferents on c-MWCNT/PANI composite film-based creatinine biosensor response.

Interferent	Physiological concentration (mM)	Relative activity (%)
No interferent		100
Uric acid	0.47	100
Ascorbic acid	0.11	105
Glycine	0.30	99
Acetaminophen	2.00	100
Creatine	0.09	103

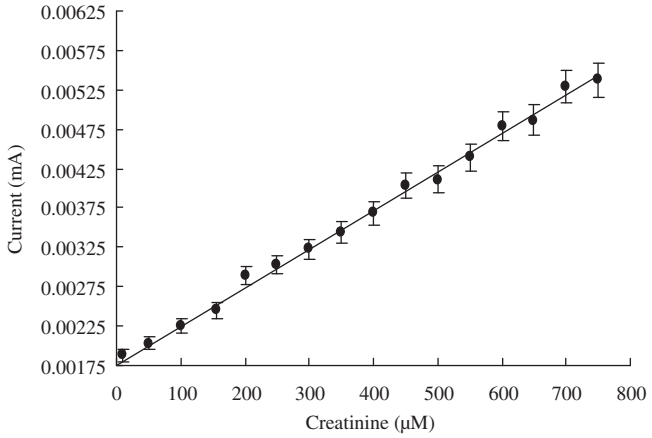


Fig.6. Linear calibration plot corresponding to current responses for different creatinine concentrations by the creatinine biosensor based on c-MWCNT/PANI composite film.

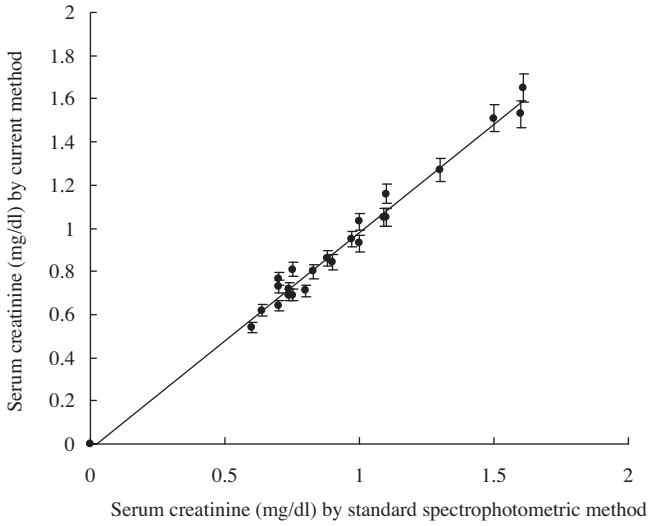


Fig.7. Correlation between serum creatinine values measured by chemical spectrophotometric method (*x* axis) and the current method (*y* axis) employing the creatinine biosensor based on c-MWCNT/PANI composite film.

Long-term stability of enzyme electrode

The long-term stability of the enzyme electrode was investigated by measuring current response of the biosensor every week under its storage at 4 °C. The biosensor maintained 85% of the initial current response even after 180 days of regular 150 uses. This suggests that use of c-MWCNT/PANI composite film ensures good stability of enzyme electrode.

Table 2
Comparison of various electrochemical amperometric creatinine biosensors with c-MWCNT/PANI composite film-based creatinine biosensor.

Property	Madaras et al. [12]	Schneider et al. [13]	Khan and Wernet [14]	Kim et al. [15]	Shin et al. [16]	Tombach et al. [17]	Current work
Support of immobilization	Polyurethane hydrogel matrix	Nucleopore polycarbonate	Polymer film (polyanion polypyrrole)	Carbon paste electrode	PbO_2 film	Nafion membrane	c-MWCNT/PANI composite film
Method of immobilization	Cross-linking	Gel entrapment	Cross-linking	Direct immobilization	Entrapment	–	Covalent
Response time	5 min	20 s	–	90 s	118 s	–	5 s
Linearity	Up to 2 mM	1–150 μM	Up to 5 mM	0.2–2 mM	1–1000 μM	0.06–1.7 mg/dl	10–750 μM
Detection limit	25 μM	0.3 μM	1–2 μM	–	0.8 μM	–	0.1 μM
Sensitivity	–	34 μA/mM/cm^2	23 μA/mM/cm^2	–		5.6 μA/mM/cm^2	40 μA/mM/cm^2
Correlation with standard method	>0.999	–	–	0.99	0.99997	–	0.989
Storage life	3 months	6 months	Reduced to 35% in 3 weeks	–	35 days	–	180 days
Number of uses	–	>100	–	–	–	–	150
K_m	5.2 mM	–	–	5.15 mM	–	–	0.26 mM
Interference	–	–	–	–	–	–	–
Potential	–	0.6 V	0.4 V	0.5 V	0.8 V	0.6 V	0.2 V

Application of creatinine biosensor

The creatinine level in human serum as measured by the current biosensor was in the range of 0.5 to 1.7 mg/dl. When these results were compared with those obtained by the chemical spectrophotometric method [3], there was a good correlation ($r = 0.989$) (Fig. 7).

The results obtained in the current study, along with those reported in the literature, are summarized in Table 2.

Conclusion

The use of c-MWCNT/PANI composite in the construction of a creatinine biosensor has led to its improved analytical performance in terms of low working potential (0.2 V), short response time (5 s), sensitivity (40 /μA/mM/cm^2), and high storage stability. Based on these observations, this composite could also be employed for the improvement of other biosensors.

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