The aim of the present work was to develop an improved creatinine biosensor in terms of its analytical performance using nanoparticles (c-MWCNT) and conducting polymer (PANI) composite film. To achieve this aim c-MWCNT/PANI composite film was electrodeposited on the surface of a platinum (Pt) electrode and then mixture of commercially available CA, CI and SO enzymes co-immobilized covalently onto this c-MWCNT/PANI composite film using EDC and NHS chemistry. The enzyme electrode was optimized and employed for amperometric determination of creatinine in serum and urine. The creatinine values obtained by this enzyme electrode were evaluated and compared with those by standard chemical spectrophotometric method. The following results were obtained from the present study. The activity and specific activity of free CA were calculated and found to be 2 µmol/ml/min and 132 U/mg respectively. The activity and specific activity of free CI were found to be 8 µmol/ml/min and 12.8 U/mg respectively while activity and specific activity of free SO were found to be 1.4 µmoles/ml/min and 27.1 U/mg respectively. The activity of combined free CA, CI & SO was also observed and found to be 1.3 µmol/ml/min.

4.1 CONSTRUCTION OF ENZYME (Enzymes/c-MWCNT/PANI/Pt) ELECTRODE

The construction of enzyme/working electrode was carried out in two steps. Firstly, c-MWCNT/PANI composite film was electrodeposited onto Pt electrode using cyclic voltammetry. Secondly, CA, CI, and SO enzymes were co-immobilized covalently onto this c-MWCNT/PANI composite film using EDC–NHS chemistry.

4.1.1 Fabrication of PANI and c-MWCNT/PANI composite film on platinum (Pt) electrode

The PANI and c-MWCNT/PANI composite films were electrodeposited onto Pt electrode through electropolymerization using a potentiostat/galvanostat. The numerous methods were employed to synthesize PANI, which have produced several products and which differ in their nature & properties. These products must represent the results of a multitude of polymerization mechanisms of aniline. In the present study, PANI was prepared by direct oxidation of aniline by electrochemical oxidation on surface Pt electrode. Both the polymerization of aniline and the subsequent transformations of polyaniline are regarded as typical redox processes, where the direction and establishment of equilibrium are dependent on the oxidation potentials and concentrations of the

reactants (and also on pH of the medium, affecting the values of oxidation potential of the reactants). This method was used, as the electrochemical methods offer some advantages over classical chemical methods. Particularly there was no need for added oxidants and electrodeposited conducting polymers were naturally integrated as a continuous uniform layer/film on the electrode. The resulting product was clean and did not necessarily need to be extracted from the initial monomer/oxidant/solvent mixture. In the electrochemical method, potential was cycled, with the value of the applied potential being in the order of -0.1 to 0.9 V vs Ag/AgCl at a scan rate of 50 mV/s. The potential cycling leads to a more homogenous product.

The mechanism of aniline polymerization is based on kinetic studies of the electrochemical polymerization of aniline **(Fig 16).** In general, polymerization proceeds via the radical cation of the monomer, which then reacts with a second radical cation of the monomer to give a dimmer by eliminating two protons. The slowest step in the polymerization of aniline was the oxidation of aniline monomer to form dimeric species (i.e. p-aminodiphenylamine, PADPA, N-N'-diphenylhydrazine and benzidine), because the oxidation potential of aniline is higher than those of dimers, subsequently formed oligomers and polymer. At the potential required to oxidize the monomer, the dimer or higher oligomer would also be oxidized, and thus could react further with the radical cation of the monomer via an electrophilic aromatic substitution to build up the aniline chain. Polyaniline deposited on an inert electrode was conducting in both anodic and cathodic regions. The observations on the electrochemical behaviour of PANI suggest a redox mechanism. Notably, the cyclic voltammograms showed well defined electro-active regions with at least two rapidly reversible and clearly defined electrochemical systems.

PANI exists in a variety of forms that differ in chemical and physical properties. PANI exists in three well defined oxidation states: leucoemeraldine, emeraldine and pernigraniline **(Fig 17).** Cyclic voltammetry actually revealed two redox processes (or the so-called oxidative doping of polyaniline). They correspond to the transitions from leucoemeraldine to emeraldine and from emeraldine to pernigraniline, although the existence of a limited potential window of conducting state (emeraldine) and the corresponding three-state switching, have been regarded as a surprising behavior, as compared to the conventional semiconductors. Leucoemeraldine and pernigraniline are the fully reduced (all the nitrogen atoms are amine) and the fully oxidized (all the nitrogen atoms are imine) forms, respectively, and in emeraldine the ratio is 0.5. Starting

from the electrically insulating leucoemeraldine, electrically conducting emeraldine can be obtained by electrochemical oxidation, as with other conducting polymers. But, upon further oxidation a sond redox process occurs, which yields a new insulating material, pernigraniline. The electrochemical activity of PANI (evaluated by the positions of the oxidation and reduction peaks, the shape of the transitions, anodic and cathodic charge) depends mainly on the pH of the medium; in neutral and alkaline media PANI loses its electrochemical activity.

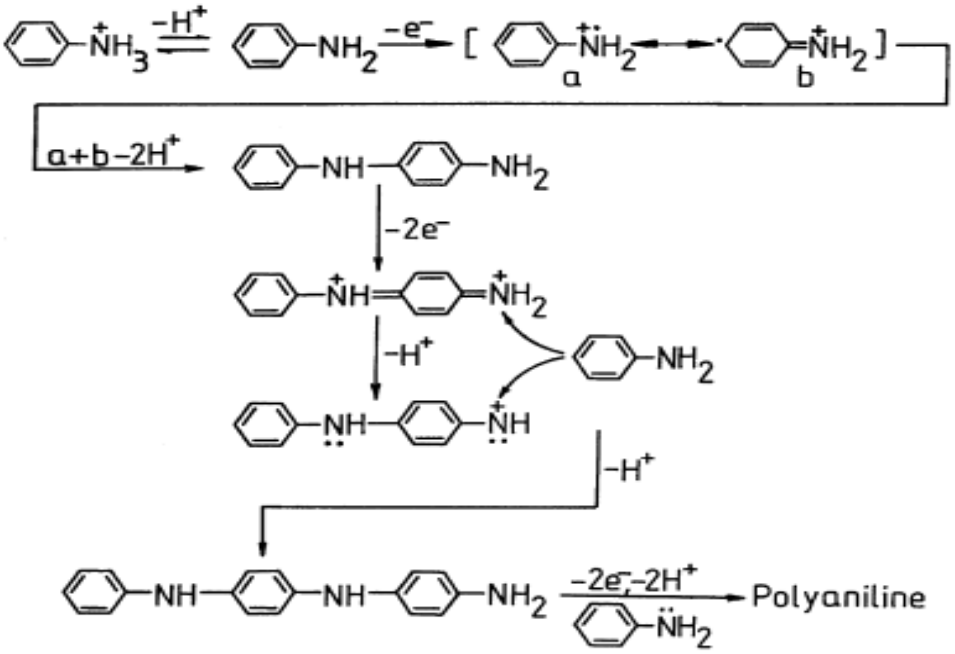


Fig. 16. Mechanism of the polymerization of aniline (Source: Wei *et al.*, 1989; 1990a,b)

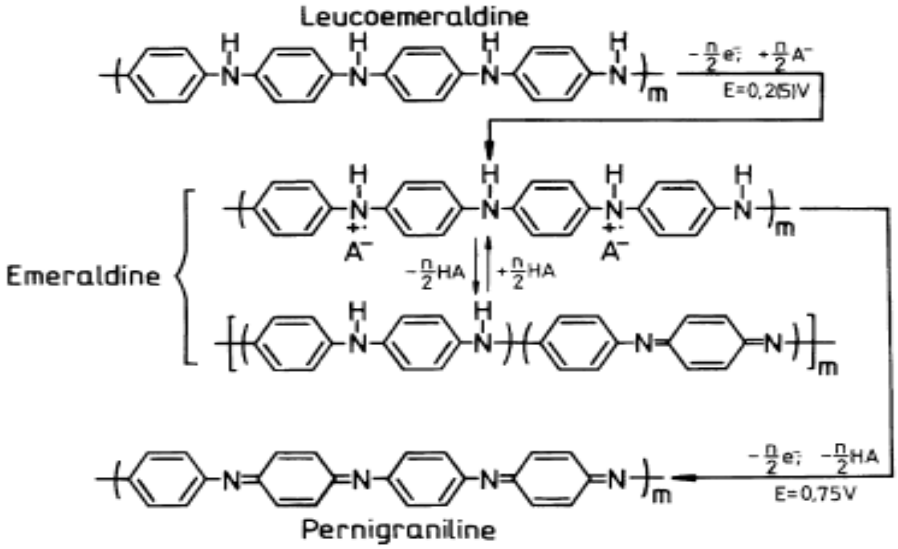


Fig. 17. Generalized scheme of the oxidative and non-oxidative (protonic acid) doping of PANI: n, number of aniline units, m = 4n (Source: Gospodinova and Terlemezyan, 1998)

Fig 18A displays the cyclic voltammograms of electrodeposition of pure conducting PANI onto Pt electrode surface during the polymerization of aniline into PANI. It was believed that the anodic oxidative polymerization of aniline in acid medium produces soluble benzidines as well as the products deposited on the electrode. The cyclic voltammogram exhibited three pairs of redox current peaks centred roughly at 0.2 V (a, a'), 0.5 V (b, b') & 0.8 V (c, c') and their peak heights increases with increase in number of potential cycles, suggested that the film was conductive and electroactive. The first redox current peak (a, a') at 0.2 V was commonly corresponds to the electron transfer from/to the PANI film (oxidation of PANI in state of leucoemeraldine: leucoemeraldine/emeraldine). In order to compensate the charge on PANI film, anion doping/dedoping of the PANI film will occur. The third redox current peak (c, c') at 0.8 V correspond to the deprotonation and protonation process (further oxidation of emeraldine; emeraldine/pernigraniline). The second redox current peak (b, b') at 0.5 V was due to side reaction in the PANI film (Benzo-hydroquinone or BQ/HQ couple). At both negative and positive ends of potential scan, the pure PANI film turned into its non conductive form.

To elucidate the effect of c-MWCNT on the property of PANI film, electrochemical performance of c-MWCNT/PANI composite film was evaluated by carrying out cyclic voltammogram as shown in **Fig 18B.** The cyclic voltammogram of c-MWCNT/PANI composite film showed strong and broad redox current peaks. There was no obvious difference in redox current peak potentials between two modified electrodes. This shows that change of the PANI film from Pt electrode to c-MWCNT has no significant effect on the electrochemical behaviour of PANI film, however, the c-MWCNT/PANI composite film not only revealed large background current in the potential sweep than the pure PANI film, particularly at the peak potentials and demonstrated sharply defined peaks but also there exist faradaic current, which is believed to arise from the contribution of loaded c-MWCNT. 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 & c-MWCNT/PANI composite have higher electroactivity over pure PANI and that c-MWCNT/PANI could provide a conducting path through the composite matrix for faster kinetics, which can be attributed to the higher electronic conductivity of the MWCNT networks. 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. The electrochemical activities of PANI film coincide with the degree of protonation; hence a promotion in the protonation with a doping effect of c–MWCNTs on the PANI was expected. A possible explanation may be that c-MWCNTs have large Π-bonded surface which might interact strongly with the conjugated structure of PANI via Π-stacking and that the resulting highly conjugated Π-system would promote the degree of electron delocalization and then lead to the preferential protonation of the amine nitrogen atoms.

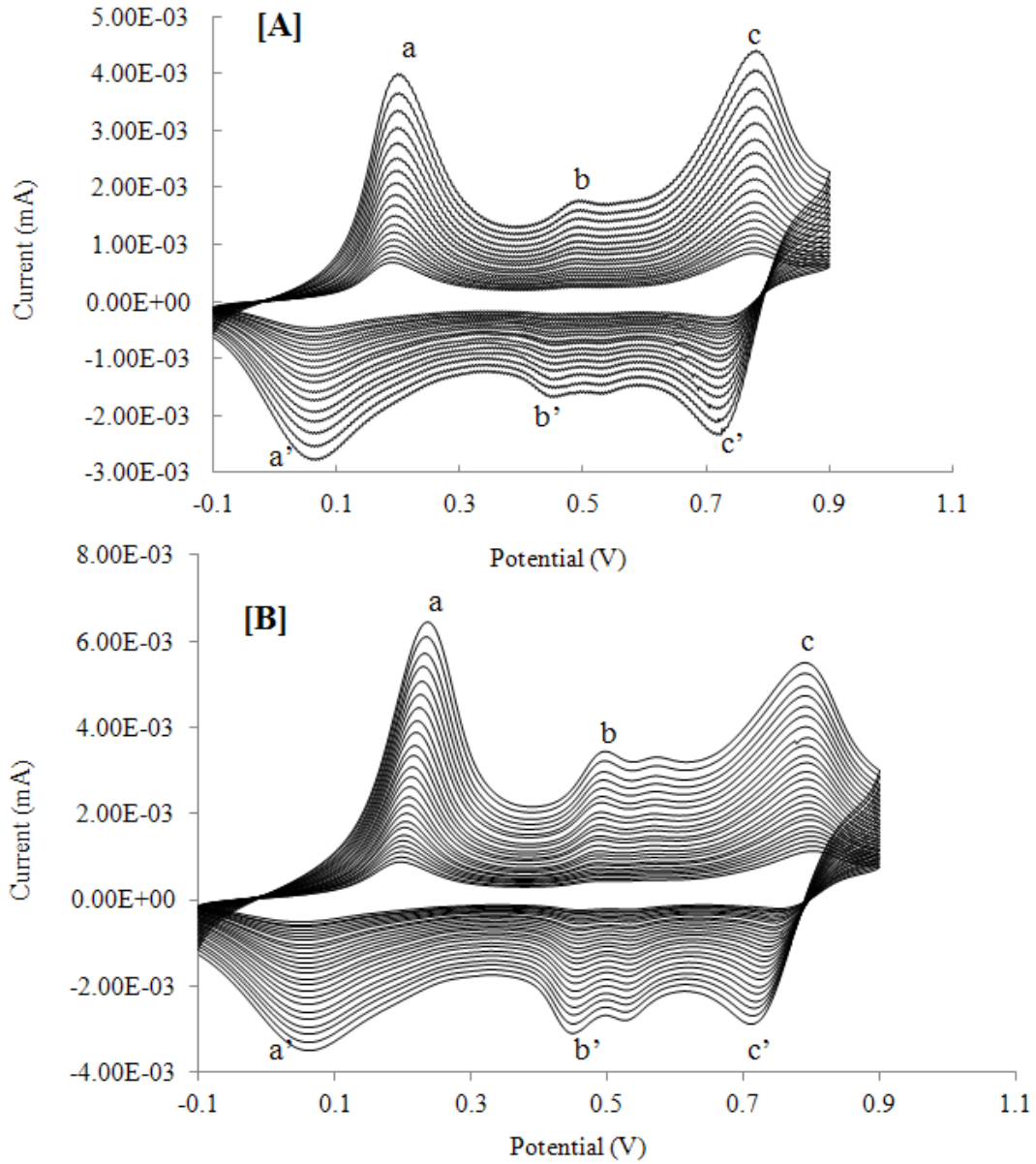


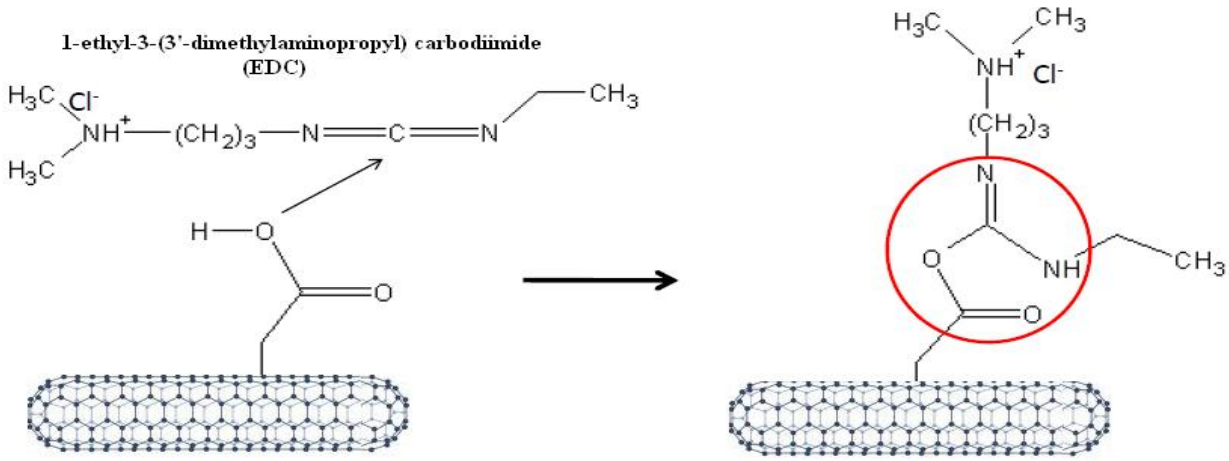
Fig. 18. Cyclic voltammograms for electrochemical deposition of PANI film [A] and c-MWCNT/PANI composite film [B] onto surface Pt electrode. Supporting electrolyte: 1N HCl solution; scan rate: 50 mV/s.

4.1.2 Co-immobilization of CA, CI and SO enzymes onto c-MWCNT/PANI composite film coated Pt electrode

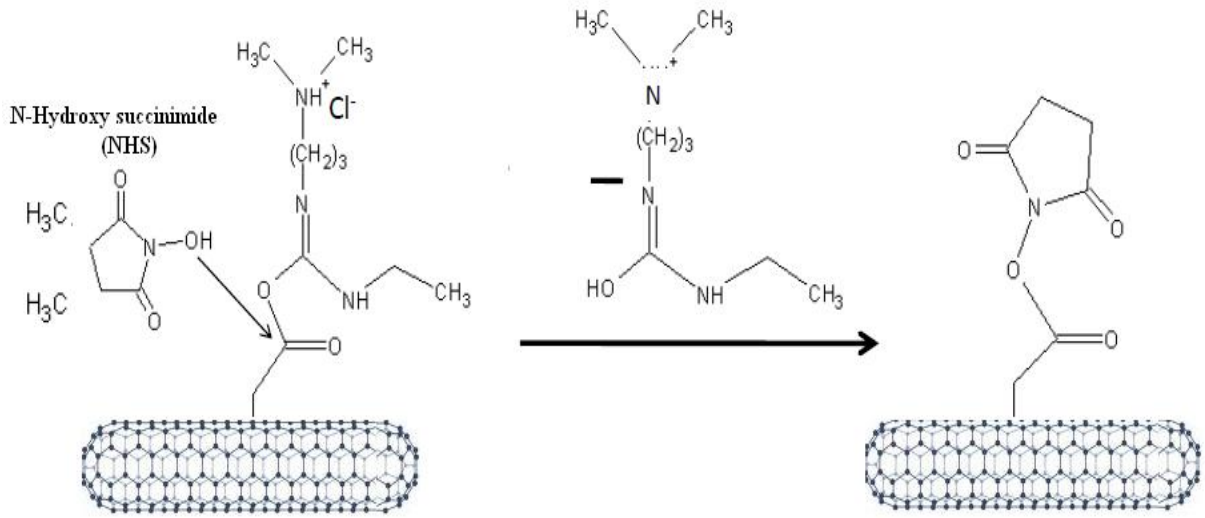
The enzymes CA, CI and SO were co-immobilized covalently onto c-MWCNT/PANI composite film electrodeposited on surface of a Pt electrode using EDC-NHS chemistry through amide bond formation between the free and unbound –COOH groups of c-MWCNT/PANI composite film and the $–NH_2$ groups on the surface of enzymes. EDC and NHS were used to activate the free –COOH groups of c-MWCNT/PANI composite layer. EDC was used to conjugate the free carboxyl (-COOH) groups of c-MWCNT/PANI composite film to amine ($-NH_2$) groups of the enzymes, using NHS as a catalyst. The immobilization of enzymes onto c-MWCNT/PANI/Pt is supposed to involve three chemical processes. In the first step reaction EDC converts free –COOH groups of c-MWCNT/PANI composite film into a reactive intermediate, which is susceptible to amine attacks. EDC catalyzes the formation of amide bonds between –COOH groups and $–NH_2$ groups by activating carboxyl to form an O-urea derivative. This intermediate was unstable and random reactions were results in undesired products. In the second step reaction NHS was often used to assist the carbodiimide coupling in the presence of EDC. The reaction includes the formation of an intermediate ester (the product of condensation of the free -COOH groups of c-MWCNT/PANI composite film and NHS). In the third step reaction the active ester intermediate further reacts with the $–NH_2$ groups on the surface of enzymes to yield the final amide bond confirming the covalent co-immobilization of enzymes on the surface of c-MWCNT/PANI composite film through amide bond formation **(Fig 19)**.

This covalent coupling of the enzymes onto c-MWCNT/PANI composite film does not allow the leaching of the enzymes during the repeated washing of enzyme electrode for its reuse. **Fig 20** summarizes the different chemical reactions involved in the fabrication of the creatinine biosensor.

Step 1:



Step 2:



Step 3:

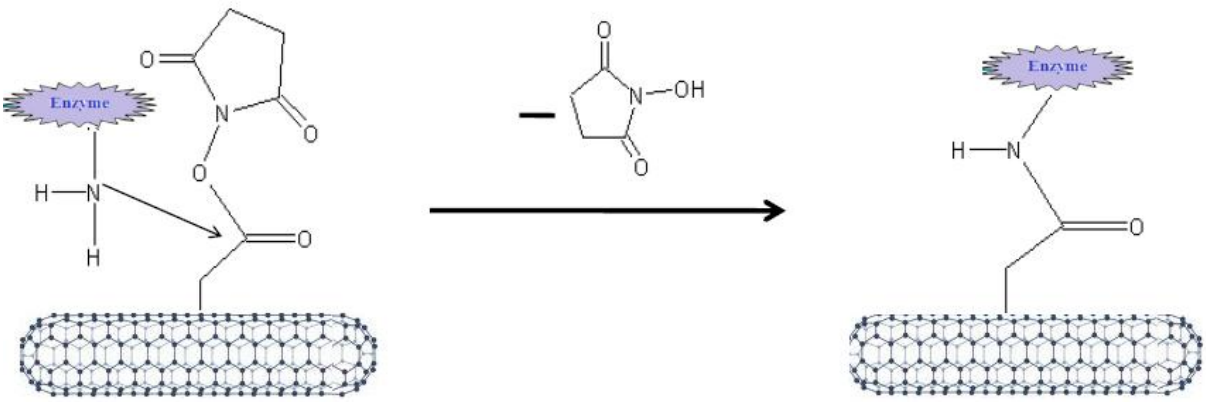


Fig. 19. Different steps involved in the immobilization of enzymes on to c-MWCNT/PANI composite film

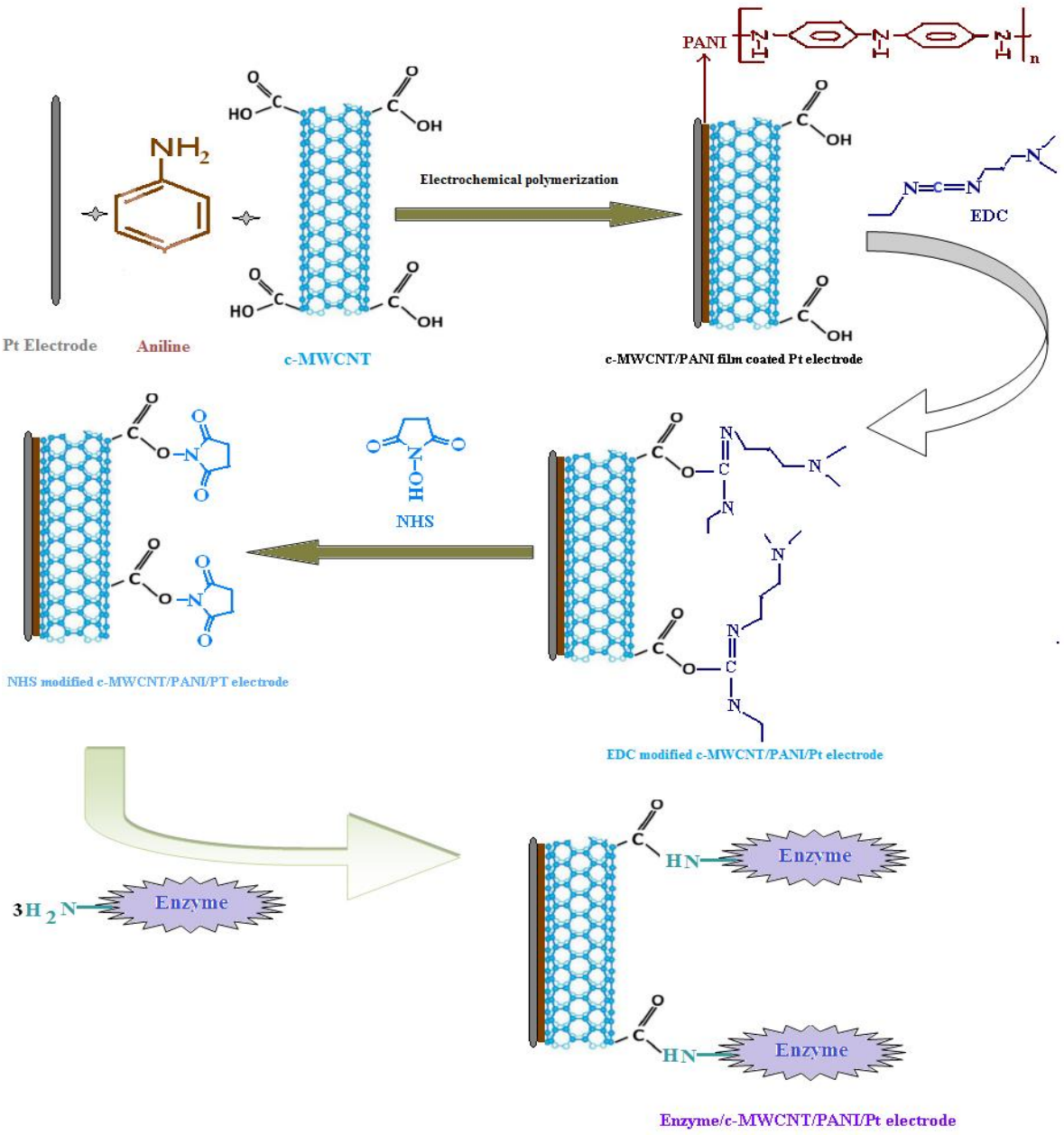


Fig. 18. Schematic representation of chemical reaction involved in the fabrication of Enzymes/c-MWCNT/PANI/Pt hybrid electrode

4.2 CHARACTERIZATION OF ENZYME ELECTRODE (Enzymes/c-MWCNT/ PANI/Pt)

The fabricated enzyme electrode was characterized using scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS) studies to confirm the immobilization of enzymes onto c-MWCNT/PANI composite film coated Pt electrode.

4.2.1 Surface characterization of enzyme electrode by SEM

Morphological features are the key features that provide the surface information of the modified electrode at different stages. SEM was employed to investigate the deposition of PANI, c-MWCNT/PANI and immobilization of enzymes onto c-MWCNT/PANI composite film electrodeposited onto Pt electrode. The SEM images of bare Pt, PANI/Pt, c-MWCNT/PANI/Pt and Enzymes/c-MWCNT/PANI/Pt electrodes were shown in **Fig 21**. **Fig 21A** shows the uniform structure of Pt electrode. The pure PANI film shows a loose and fibrillar structure **(Fig 21B)** on the uniform structure of Pt electrode. The PANI represents the net structure with spherical grains, and it's not very uniform. **Fig. 21C** shows SEM micrographs of c-MWCNT/PANI/Pt electrode that reveal the uniform, homogenous and cable-like morphology of the nanostructure of c-MWCNT/PANI composite film, which indicate that c-MWCNTs were well dispersed in the composite film. Both pure PANI and c-MWCNT/PANI composite films were composed of nanofibrils but for the pure PANI they are slight thicker than those of in the c-MWCNT/PANI composite and showed net structure, but the pore is smaller and denser in c-MWCNT/PANI composite due to presence of c-MWCNTs. After immobilization of enzymes on c-MWCNT/PANI composite film, the hybrid bioelectrode shows the sporadic appearance of globular/beaded structure on uniform structure of c-MWCNT/PANI composite film **(Fig 21D)**, indicating the successful immobilization of enzymes onto the surface of c-MWCNT/PANI composite film. It was observed that the enzymes were attached at sidewalls and the ends of c-MWCNTs. These results confirm the presence of c-MWCNT in the form of network on PANI surface along with immobilized enzyme.

4.2.2 Fourier transforms infrared (FTIR) spectroscopy

Fig 22, shows FTIR spectra obtained for PANI, c-MWCNTs, c-MWCNT/PANI and enzymes/c-MWCNT/PANI composites. The spectrum for PANI (Fig 22A) exhibits the clear presence of benzoid at 1491.03 cm^{-1} and the quinoid ring vibration at 1548.24 cm^{-1}, indicating the oxidation state of emaraldine salt of PANI. The strong band around 1139.61 cm^{-1} was attributed to B-N^{+}=Q (characteristic peak of PANI conductivity) and a measure of the degree of the delocalisation of electrons (Quillard *et al.,* 1994). The very weak and broad band around 3435 cm^{-1} was assigned to the N-H stretching mode of PANI. In the FTIR spectra of c-MWCNT **(Fig 22B)**, a very broad peak at 3399.3 cm^{-1} corresponds to the -OH group present on c-MWCNTs surface, due to presence of

moisture. The –C=O stretching vibrations peak obtained at 1634.05 cm^{-1} indicated the presence of carboxyl group (–COOH) in the MWCNTs. A peak observed at 1569.5 cm^{-1} due to carboxylate ion. Therefore, it was considered that carboxylic groups (-COOH) have been present onto the surface of the MWCNTs. The two weak peaks at 2345.06 and 2385.93 cm^{-1} corresponds to the –CH stretching mode. 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 **(Fig 22C).** The N-H stretching region near 3420 cm^{-1} showed strong and broad peaks, but weak and broad peaks were present in the pure PANI spectrum. The interaction between the c-MWCNTs and PANI due to charge transfer may result an increase in the N-H stretching intensity. There was shift in peak from 3435 to 3419 cm^{-1} and 1491.03 to 1593.19 cm^{-1}. The enzymes binding on c-MWCNT/PANI/Pt electrode were indicated by the appearance of additional absorption bands at 1589.10 and 1491.03 cm^{-1} **(Fig 22D)** that were assigned to carbonyl stretch (amide 1 band) and -N-H bonding (amide 11 band), respectively. Also change in peak position was observed (1636 to 1647.5 cm^{-1} and 1570 to 1540 cm^{-1}), this change indicates that the enzymes were attached to c-MWCNT/PANI composite film. The successful covalent co-immobilization of enzymes onto c-MWCNT/PANI composite film was indicated by the appearance of the IR absorption of the amide I and amide II (Matharu *et al.,* 2007).

4.2.3 Electrochemical impedance spectroscopic (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 Waberg diffusion process (Feng *et al.,* 2005). The Nyquist plot **(Fig 23)** displays EIS studies of PANI/Pt (curve i), c-MWCNT/PANI/Pt (curve ii), and Enzymes/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. The R_{CT} values for the PANI/Pt, c-MWCNT/PANI/Pt and enzyme/c-MWCNT/PANI/Pt electrodes have been obtained as 475 Ω, 210 Ω and 450 Ω respectively. 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. This means that

the c-MWCNTs inside the PANI matrix may lead to a faster electron transport in the bulk film and charge transfer in the parallel PANI film interface and c-MWCNT interface, compared to that in the originally pure PANI interface. In c-MWCNT/PANI composites, it has been suggested that either the polymer functionalizes the c-MWCNTs or the PANI are doped with c-MWCNTs. This fact may suggest that the c-MWCNT has an obvious improvement effect, which makes the composite have more active sites for faradaic reactions and a larger capacitance that pure PANI film. This results in enhanced conductivity, lowers the resistance and facilitates the charge-transfer of the composite. However, the R_{CT} of Enzymes/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.

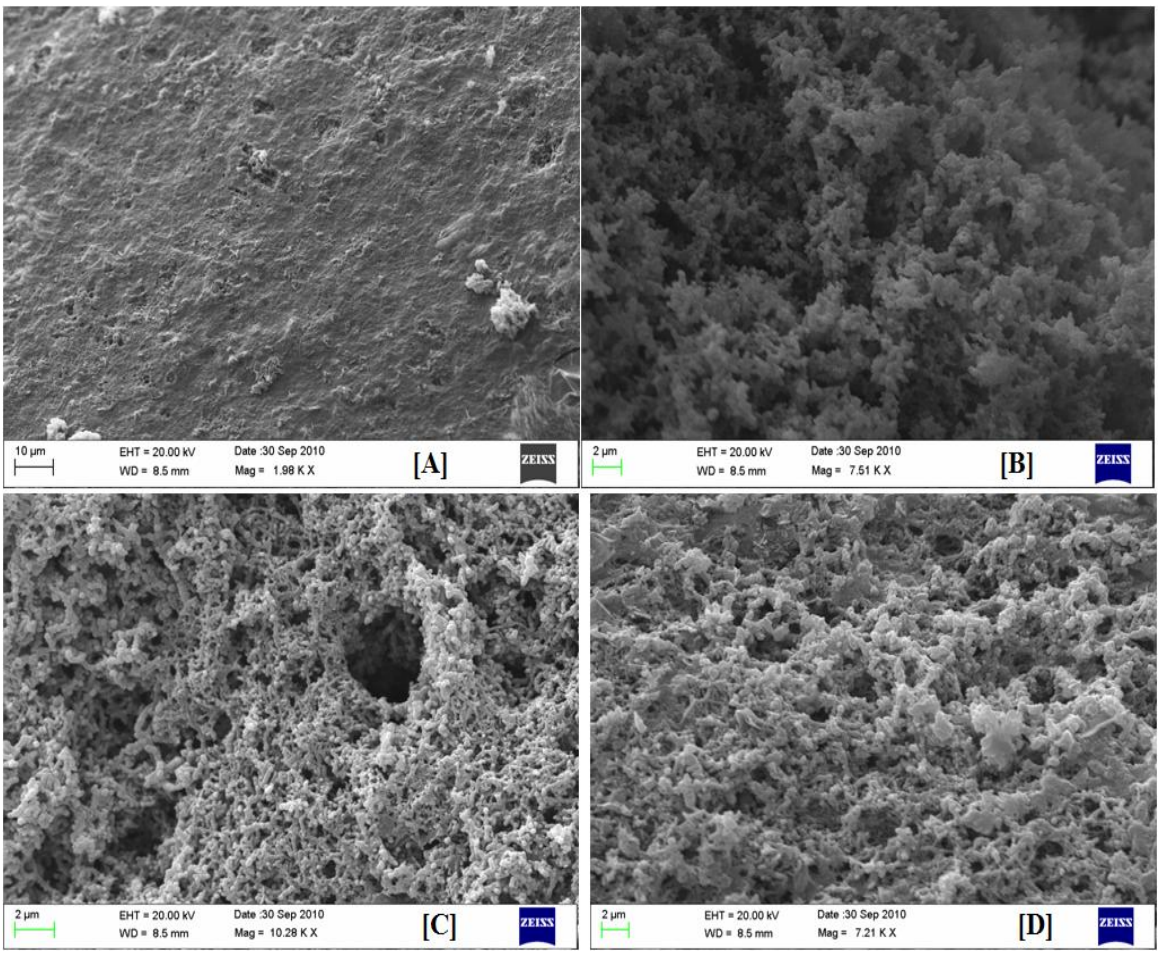


Fig. 21. Scanning electron microscopy of bare Pt electrode [A] PANI film [B] c-MWCNT/PANI composite film [C] immobilized enzymes onto c-MWCNT/PANI composite film deposited on Pt electrode

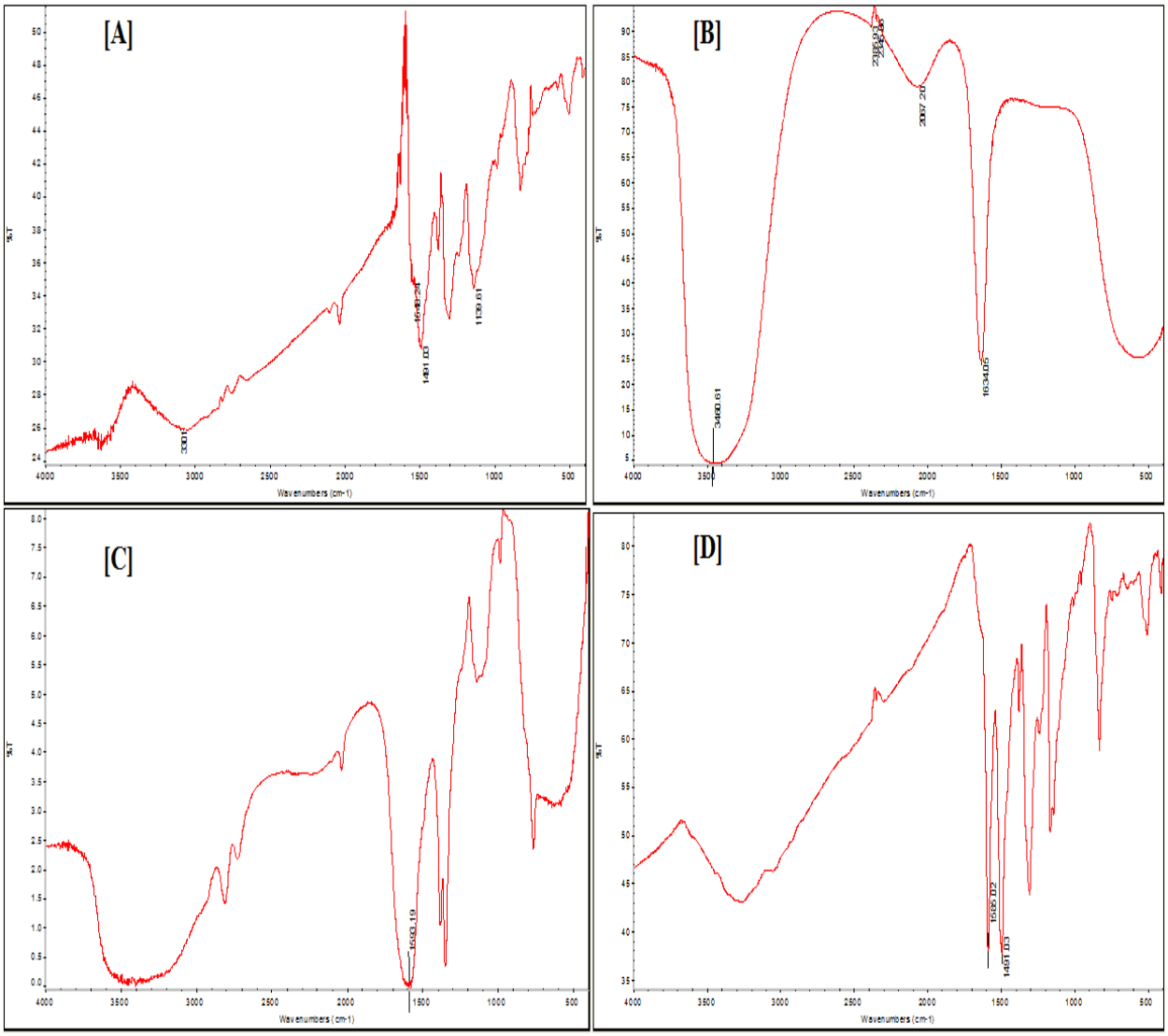


Fig. 22. FTIR spectra for pure PANI film [A] c-MWCNTs [B] c-MWCNT/PANI composite [C] Enzymes/c-MWCNT/PANI composite

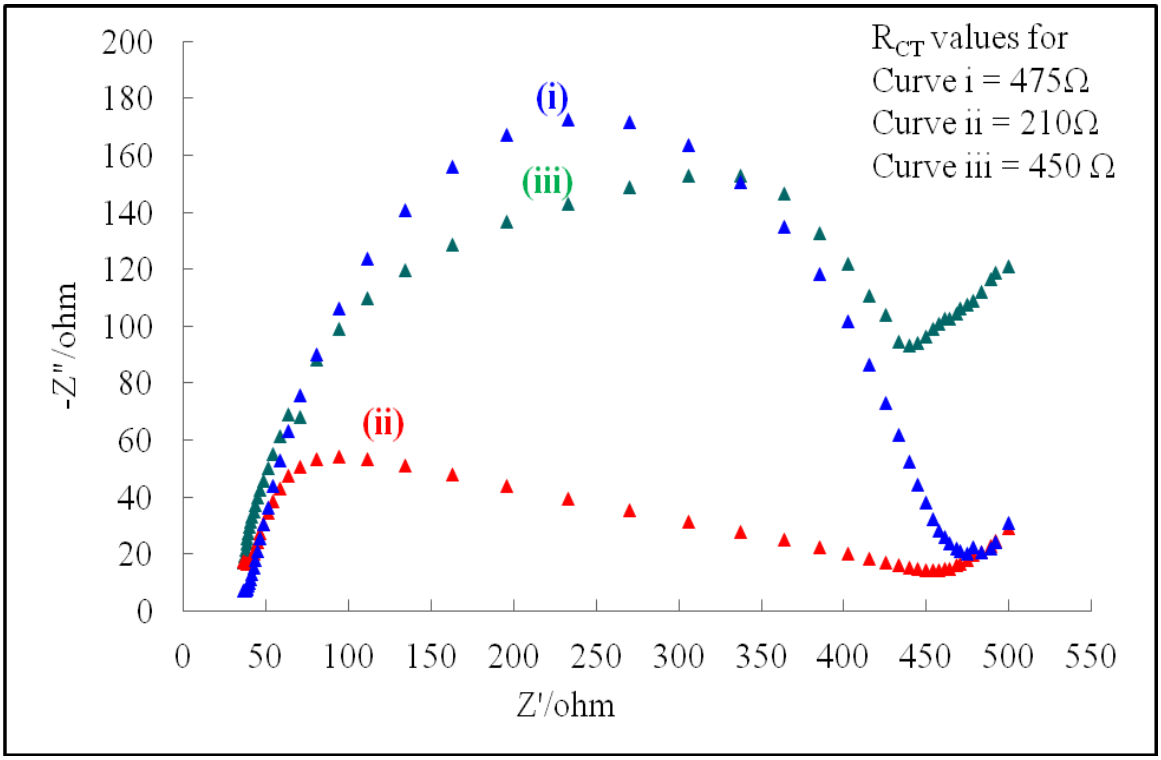


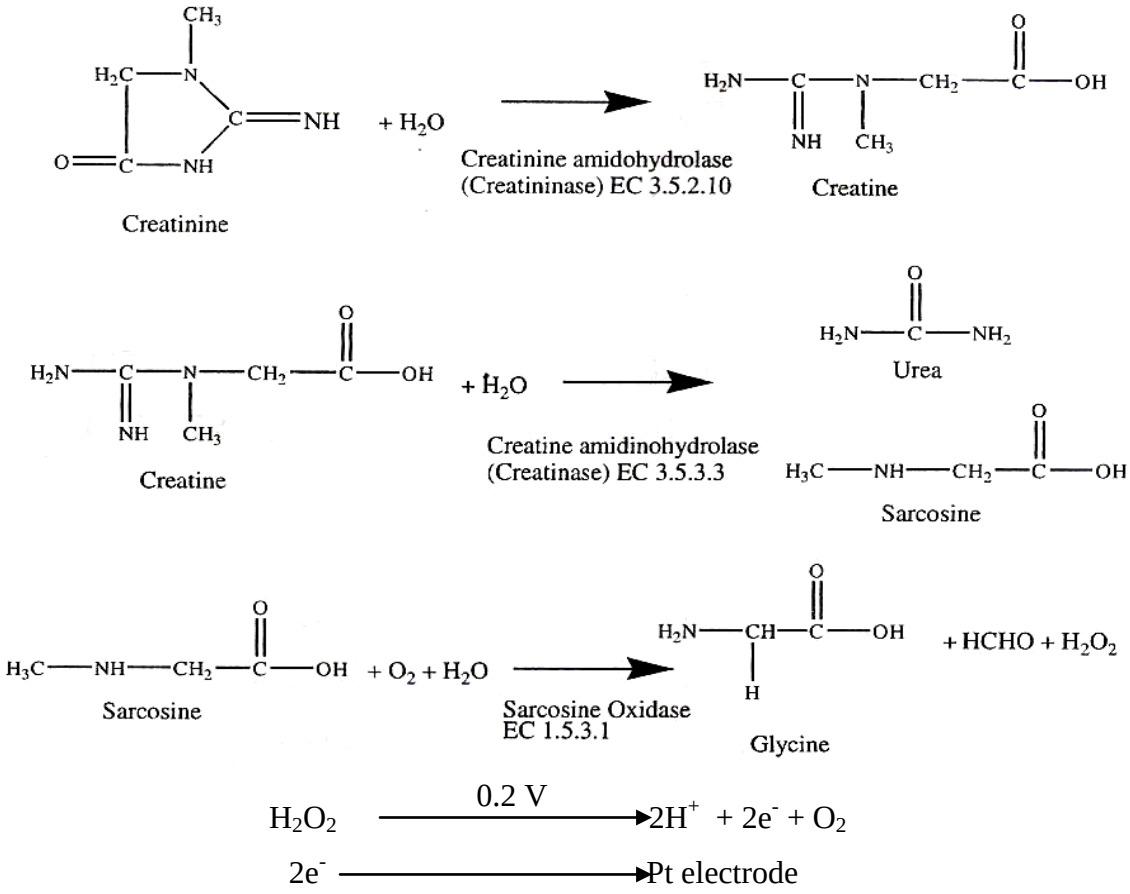
Fig. 23. Nyquist plot of electrochemical impedance spectra for PANI/Pt electrode (curve i), c-MWCNT/PANI/Pt electrode (curve ii) and Enzymes/c-MWCNT/PANI/Pt electrode (curve iii) in 0.05 M PB (pH 7.5) containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) as a redox probe

4.3 CONSTRUCTION OF AN AMPEROMETRIC CREATININE BIOSENSOR

An amperometric creatinine biosensor was constructed using a three-electrode electrochemical cell system, consisting of a working electrode (Enzymes/c-MWCNT/PANI/Pt), a silver/silver chloride (Ag/AgCl) as reference electrode and Pt wire as auxillary electrode. These electrodes were connected through Autolab Potentiostat/Galvanostat **(Fig 14).** The electrochemical response of Enzymes/c-MWCNT/PANI/Pt electrode was studied by using cyclic voltammetry. This study was carried out by using a three electrode cell in 0.05 M PB, pH 7.5. The electrode system was dipped into a reaction mixture containing 0.05 M PB, pH 7.5 and 100 µM creatinine solution. The electrode response was measured in terms of ampere (A) applying a potential range of -0.1 to 0.9 V vs Ag/AgCl. The optimal current response was obtained at 0.2 V **(Fig 24)** and 0.2 V was selected as the optimum working potential for amperometric detection of creatinine concentration in this work to assure high sensitivity and fast sensor operation. The optimum working potential of present creatinine biosensor

was lower than earlier reported amperometric creatinine biosensors based on cellulose acetate membrane (0.65V) (Tsuchida and Yoda, 1983), controlled pore glass (0.65 V) (Sakslund and Hammerrch, 1992), polypyrrole doped with sulfonated phenoxy resin (0.4 V) (Yomato *et al.,* 1995), gas permeable membrane (0.525 V) (Osborne and Girault, 1995), poly (carbamoyl) sulfonate hydrogel matrix (0.6 V) (Schneider *et al.,* 1996), poly-2-hydroxy ethyl methyacrylate (0.6 V) (Madaras *et al.,* 1996), poly (1,3-diaminobenzene (0.65 V) (Madaras and Buck, 1996), platinized-S (shapable electroconductive) film (0.4 V) (Khan and Wernet, 1997), carbon paste electrode containing 10% Pt powder (0.5 V) (Kim *et al.,* 1999), PbO_2 oxidizing layer over HPU (0.8 V) (Shin *et al.,* 2001), poly (carbamoyl) sulfonate-hydrogel with nafion membrane (0.6 V) (Tombach *et al.,* 2001), polished Pt electrode with alumina and diamond suspension (0.7 V) (Walsh and Dempsy, 2002), polyvinyl alcohol (0.8 V) (Choi *et al.,* 2002), nafion/poly (1,2-diaminobenzene) (0.5 V) (Yao and Kotegawa, 2002), poly (carbamoyl) sulfonate hydrogel (0.6 V) (Erlenkotter *et al.,* 2002), carbon paste electrode (0.65 V) (Stefan *et al.,* 2003; Steden *et al.,* 2006), ZnO-NPs/CHIT/c-MWCNT/PANI composite film (0.5 V) (Yadav *et al.,* 2011) and Fe_3O_4/CHIT-g-PANI composite film (0.4 V) (Yadav *et al.,* 2012). The lowering of the working potential in the present biosensor might be due to the presence of c-MWCNT in the matrix of PANI, which provides an environment for the enhanced electrocatalytic effect and a fast electron-transfer rate. The c-MWCNT and PANI had a synergistic electrocatalytic effect toward the oxidation of H_2O_2. The synergistic influence of c-MWCNT and PANI contributes to the excellent performance for the sensor. The existence of c-MWCNT and PANI provides a favorable potential window and electrocatalytic behavior for the H_2O_2 electron transfer to the electrode. The ability of c-MWCNTs to promote the electron transfer of H_2O_2, suggests that c-MWCNTs have great promise as oxidase based biosensors.

In the present working Enzymes/c-MWCNT/PANI/Pt electrode, the electronic signal was generated as a result of biocatalytic redox process. Hence, the amperometric detection was based on CA, CI and SO enzymes generated product i.e. H_2O_2 of biocatalytic reactions. The reaction involved degradation of creatinine and then H_2O_2 which was broken by applied potential between working electrode and counter electrode. The electrons released by H_2O_2 breakage were transferred to the working electrode to be relayed to potentiometer in which it was read as current in mA. The electrochemical reactions involved in response measurement of creatinine biosensor are given below:



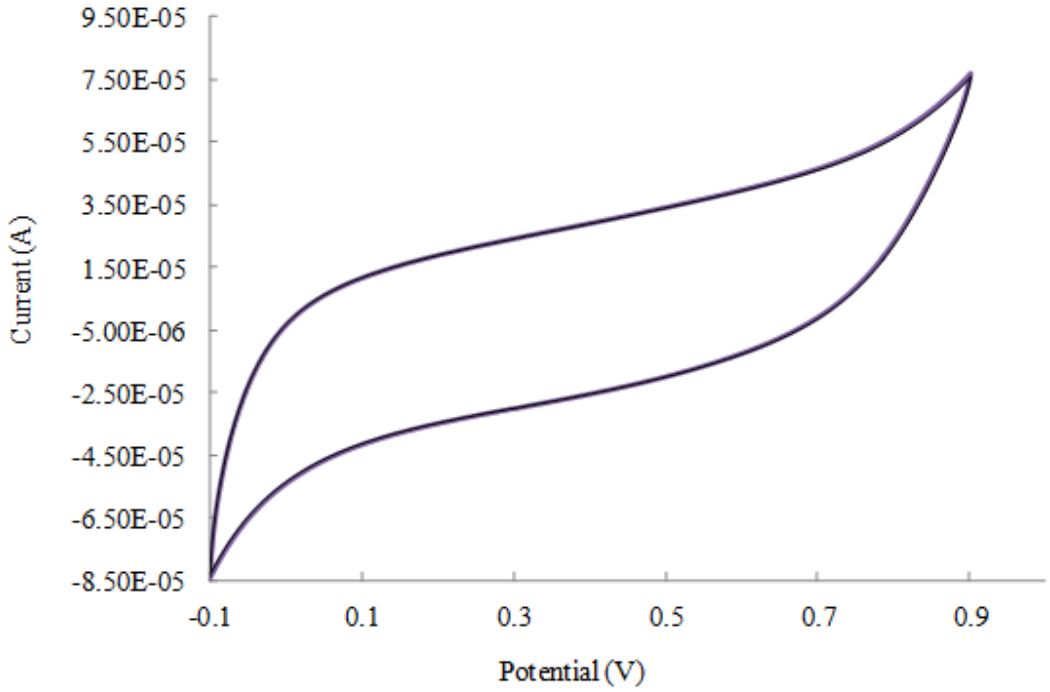


Fig. 24. Cyclic voltammogram of Enzymes/c-MWCNT/PANI/Pt electrode in 0.05 M PB (pH 7.5) at 100 µM creatinine solution

The resulting current reflects the turnover rate of the electronic exchange between substrate and biocatalyst and is directly related to the substrate concentration, provided electron transfer between electrode and redox enzyme is sufficiently fast. The acceleration to the flow of electron is being provided by c-MWCNT electropolymerized with PANI on Pt electrode. In fact, the redox centers are often buried inside the cores of the proteins and therefore they are so electronically insulated that it is not easy to observe a direct electronic transfer across the enzyme electrode. That is the case where the separation distance is very large and the redox enzyme lack electrical contact with the support. In order to assess a fast electronic transfer in amperometric biosensors, preferential pathways between the enzyme redox site and the electrode surface are designed, where the biofunctions of the protein are driven and stimulated by electron transfer or the perturbation originated from the electrical contact is directly used as a transduction signal of biochemical event (Hirst *et al.,* 1998, Bourdillon 2006). In the present study, both the parameters were achieved by immobilizing CA, CI and SO enzymes covalently, on to c-MWCNT/PANI/Pt coated surface. The biosensor is designed in such a way that there is a proper communication between the support and redox protein. The electron flow in the present electrode was shown in **Fig 25.**

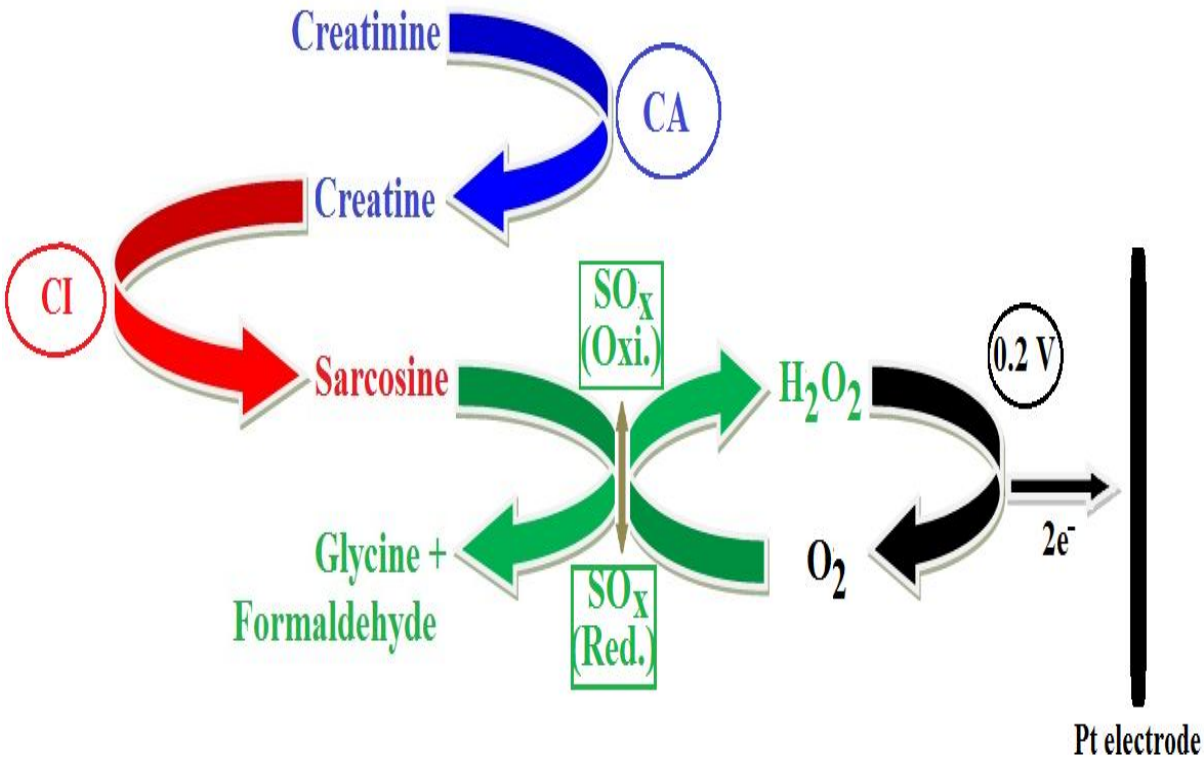


Fig. 25. Flow of electrons in present creatinine biosensor based on of Enzymes/c-MWCNT/PANI/Pt electrode

4.4 OPTIMIZATION OF WORKING CONDITIONS OF CREATININE BIOSENSOR

Various kinetic properties of co-immobilized enzymes onto c-MWCNT/PANI composite film coated Pt electrode were studied such as effect of pH, incubation temperature, time of incubation and effect of substrate (creatinine) concentration to optimize the working conditions of enzyme electrode. The measurements were carried out with electrode through potentiostat/Galvanostat at a constant potential of 0.2 V vs Ag/AgCl.

4.4.1 Effect of pH

Enzymatic reactions are pH dependent, as every enzyme has a characteristic optimum pH at which it shows maximum activity. Optimum pH of the enzyme indicates the types of chemical groups involved at the active centre of enzyme. The optimum pH may or may not be altered after immobilization of enzyme onto some support. The effect of pH on electrochemical response of enzyme electrode was studied in the pH range 6.0 to 10.0. Highest current responses were obtained between pH 7.0 and 8.0 **(Fig 26).** 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 (Schneider *et al.,* 1996). Therefore, 0.05 M PB at pH 7.5 was selected as the optimum carrier buffer, which was found in the same pH region as for free enzymes (Yoshimoto *et al.,* 1976; Rikitake *et al.,* 1979; Hayashi *et al.,* 1983). The pH optima for the free enzymes were pH 6.5–7.5 for CA, pH 6.5–7.5 for CI and pH 7.5–8.5 for SO. The described immobilization procedure had, therefore, no effect on the optimal pH for enzymatic activity. The optimum pH of the present biosensor was similar to that of previously reported amperometric creatinine biosensors based on cellulose acetate membrane (Tsuchida and Yoda, 1983), controlled pore glass (Sakslund and Hammerrch, 1992), poly (carbamoyl) sulfonate hydrogel matrix (Schneider *et al.,* 1996), polyurethane hydrogel matrix (Madaras *et al.,* 1996), platinized-S (shapable electroconductive) film (Khan and Wernet, 1997), carbon paste electrode containing 10% Pt powder (Kim *et al.,* 1999), poly (carbamoyl) sulfonate-hydrogel with nafion membrane (Tombach *et al.,* 2001), polished Pt electrode with alumina and diamond suspension (Walsh and Dempsy, 2002), polyvinyl alcohol (Choi *et al.,* 2002), nafion/poly (1,2-diaminobenzene) (Yao and Kotegawa, 2002), poly (carbamoyl) sulfonate hydrogel matrix (Erlenkotter *et al.,* 2002), carbon paste electrode (Stefan *et al.,* 2003; Steden *et al.,* 2006), ZnO-NPs/CHIT/c-MWCNT/PANI composite film (Yadav *et al.,*

2011) and Fe_3O_4/CHIT-g-PANI composite film (Yadav *et al.,* 2012), but slight higher than polypropylene (pH 7.0) (Hsiue *et al.,* 2004) and lower than gas permeable membrane (pH 8.5-9.5) (Osborne and Girault, 1995), ferrocene embedded carbon paste (pH 8.0) (Kinoshita *et al.,* 1997), polypropylene (pH 8.0) (Nguyen *et al.,* 1999), silicone gas permeable membrane (pH 9.0) (Suzuki *et al.,* 2001).

4.4.2 Effect of incubation temperature

Each enzyme exhibits its maximum activity at a particular incubation temperature. The rate of enzyme reaction increases as the incubation temperature increases upto an optimum temperature, after which it declines rapidly, because of the denaturation of enzyme protein. The temperature for maximum activity of enzyme is expected to be altered after its immobilization on some support. In the present study, the effect of incubation temperature was studied on the activity of CA, CI and SO enzymes immobilized onto c-MWCNT/PANI/Pt electrode. The optimal temperature of enzyme electrode was studied by measuring the current response at different temperatures from 15 to 50 °C. It showed that the current response of the biosensor increased with increasing temperature and reached a maximum at approximately 35 ºC, and then went down as the temperature turned higher, hence 35 ºC was selected as optimum temperature **(Fig 27).** The increase in current response of the biosensor upto 35 ºC could be due to the increase in activation energy of the reaction. After that, a decrease was often observed because of enzyme denaturation. The optimal temperature (35 °C) of present biosensor was comparable to that for an earlier amperometric creatinine biosensors based on cellulose acetate membrane (37 ºC) (Tsuchida and Yoda, 1983), poly (carbamoyl) sulfonate hydrogel matrix (37 ºC) (Schneider *et al.,* 1996), silicone gas permeable membrane (37 ºC) (Suzuki *et al.,* 2001), nafion/poly (1,2-diaminobenzene) (35 ºC) (Yao and Kotegawa, 2002) but higher than controlled pore glass (25 ºC) (Sakslund and Hammerrch, 1992), polypropylene (31 ºC) (Nguyen *et al.,* 1999), ZnO-NPs/CHIT/c-MWCNT/PANI composite film (30 °C) (Yadav *et al.,* 2011), Fe_3O_4/CHIT-g-PANI composite film (30 °C) (Yadav *et al.,* 2012) and lower than poly (carbamoyl) sulfonate hydrogel matrix (current response increases with temperature from 30-50 ºC) (Schneider *et al.,* 1996), poly (carbamoyl) sulfonate hydrogel matrix (40 ºC) (Erlenkotter *et al.,* 2002). Schneider et al. (1996) assumed that the increase in current at higher temperatures arose from overlapping effects consisting of higher enzyme activities on the one hand and the decrease of the diffusion barrier of the gel matrix on the other hand. Although the enzyme electrode

current response was higher at higher temperatures, but we performed the creatinine measurement in blood serum at 25 °C. This avoids an eventual loss of enzymatic activity following long-term use at higher temperatures.

4.4.3 Effect of incubation time

Effect of time of incubation on the current response of enzyme electrode employing covalently co-immobilized CA, CI and SO enzymes onto c-MWCNT/PANI composite film was determined to know the suitable incubation time. For this purpose, the enzyme electrode was dipped into reaction mixture and current response was measured at different time intervals upto 20s at an interval of 2s. When creatinine was added into PB, pH 7.5 the biosensor responded rapidly to the substrate and achieved 95 % of steady current within 5s **(Fig. 28)**, hence enzyme electrode showed optimum current response at 5s. The response time of present creatinine biosensor was quite low than that of earlier reported amperometric creatinine biosensors based on cellulose acetate membrane (20s) (Tsuchida and Yoda, 1983), polypyrrole doped with sulfonated phenoxy resin (100s) (Yomato *et al.,* 1995), poly (carbamoyl) sulfonate hydrogel matrix (20s) (Schneider *et al.,* 1996), polyurethane hydrogel matrix (300s) (Madaras *et al.,* 1996), poly (1,3-diaminobenzene) (60s) (Madaras and Buck, 1996), ferrocene embedded carbon paste (120s) (Kinoshita *et al.,* 1997), carbon paste electrode containing 10% Pt powder (90s) (Kim *et al.,* 1999), polyaniline-nafion (60s) (Shih and Huang, 1999), PbO_2 oxidizing layer over HPU (98s) (Shin *et al.,* 2001), polished Pt electrode with alumina & diamond suspension (60s) (Walsh and Dempsy, 2002), polyvinyl alcohol (104s) (Choi *et al.,* 2002), poly (carbamoyl) sulfonate hydrogel matrix (25-80s) (Erlenkotter *et al.,* 2002), carbon paste electrode (30s) (Stefan *et al.,* 2003) and ZnO-NPs/CHIT/c-MWCNT/PANI composite film (10s) (Yadav *et al.,* 2011) but higher than Fe_3O_4/CHIT-g-PANI composite film based creatinine biosensor (2s) (Yadav *et al.,* 2012). This faster response can be attributed to the synergetic influence of c-MWCNT and PANI composite film, which provides an environment for the enhanced electrocatalytic effect and a fast electron-transfer rate. The synergistic influence of c-MWCNT and PANI contributes to the excellent performance for the sensor. The existence of c-MWCNT and PANI provides a favorable potential window and electrocatalytic behavior for the H_2O_2 electron transfer to the electrode.

4.4.4 Effect of substrate (creatinine) concentration

The effect of substrate (creatinine) concentration on the activity of enzyme electrode employing covalently co-immobilized CA, CI and SO enzymes onto c-MWCNT/PANI composite film was determined by varying the creatinine concentration from 0.1-1500 µM in the reaction mixture. A hyperbolic relationship was observed between immobilized enzymes activity and creatinine concentration. The current response was linear in the range of 0.1-750 µM for the enzyme electrode, after which it became constant **(Fig 29).** The linear plot reveals that such electrode can work well in creatinine solution with a sensitivity of 0.040 µA/µM/cm^2. The sensitivity of the present creatinine was higher/better than that of earlier reported amperometric creatinine biosensor based on controlled pore glass (0.0000208 µA/µM/cm^2) (Sakslund and Hammerrch, 1992), gas permeable membrane (0.000001 µA/µM/cm^2) (Osborne and Girault, 1995), polyurethane hydrogel matrix (0.0000139 µA/µM/cm^2) (Madaras *et al.*, 1996), poly (carbamoyl) sulfonate hydrogel matrix (0.034 µA/µM/cm^2) (Schneider *et al.,* 1996), platinized-S (shapable electroconductive) film (0.023 µA/µM/cm^2) (Khan and Wernet, 1997), poly (carbamoyl) sulfonate-hydrogel with nafion membrane (0.005 µA/µM/cm^2) (Tombach *et al.,* 2001), polyvinyl alcohol (0.0001256 µA/µM/cm^2) (Choi *et al.,* 2002), poly (carbamoyl) sulfonate hydrogel matrix (0.00024-0.00046 µA/µM/cm^2) (Erlenkotter *et al.,* 2002) and ZnO-NPs/CHIT/c-MWCNT/PANI composite film (0.030 µA/µM/cm^2) (Yadav *et al.,* 2011) but lower than Fe_3O_4/CHIT-g-PANI composite film based creatinine biosensor (3.9 µA/µM/cm^2) (Yadav *et al.,* 2012). The higher sensitivity for Enzymes/c-MWCNT/PANI/Pt electrode documents the importance of the modification of electrode by c-MWCNT. This higher sensitivity can be attributed to the synergetic influence of c-MWCNT and PANI composite film, which provides an environment for the enhanced electrocatalytic effect and a fast electron-transfer rate. The synergistic influence of c-MWCNT and PANI contributes to the excellent performance for the sensor.

The apparent Michaelis-Menton constant (App K_m), which gives an indication of the enzyme-substrate kinetics for the enzyme electrode was calculated from the linear part of the calibration graph, using the electrochemical version of Lineweaver-Burk equation

$$\frac{1}{I} = \frac{K_m}{I_{max}}\left(\frac{1}{S}\right) + \frac{1}{I_{max}}$$

$$\text{Where slope} = \frac{K_m}{I_{max}} \; ; \quad \text{intercept} = \frac{1}{I_{max}}$$

Where K_m = Michaelis-Menten constant

I_{max} = Maximum current response of enzyme electrode

I = Steady state current after addition of substrate (creatinine)

S = Concentration of substrate (creatinine)

Analysis of the slope and intercept of the plot for the steady state current vs reciprocal of substrate (creatinine) concentration allows the determination of app K_m and I_{max}. The App K_m and I_{max} values for the immobilized enzymes were found to be 0.26 mM and 0.0055mA respectively **(Fig 30).** The app K_m value for the present enzyme electrode was found to be lower than those of earlier reported amperometric creatinine biosensors based on cellulose acetate membrane (2.4 mM) (Tsuchida and Yoda, 1983), polyurethane hydrogel matrix (5.2 mM) (Madaras *et al.,* 1996), carbon paste electrode containing 10% Pt powder (5.15 mM) (Kim *et al.,* 1999) and ZnO-NPs/CHIT/c-MWCNT/PANI composite film (0.35 mM) (Yadav *et al.,* 2011) but lower than Fe_3O_4/CHIT-g-PANI composite film based creatinine biosensor (0.17 mM) (Yadav *et al.,* 2012). The small app K_m value indicates that diffusion of substrate (creatinine) and product through the composite film is much easier in the Enzymes/c-MWCNT/PANI/Pt electrode and the biosensor possesses higher affinity to creatinine as compared with earlier amperometric creatinine biosensors. When an enzyme is coupled to an solid support, kinetic pattern of enzyme reaction changes considerably leading to change in K_m & I_{max}. The kinetic of immobilized enzyme is affected by many factors such as conformational change, chemical modification of enzyme, steric effect, which occurs because, often a proportion of enzyme molecule immobilized on a position relative to support surface such that active site is relatively inaccessible to substrate molecule, partitioning effect which may arise from electrostatic or hydrophobic interaction between the matrix and low molecular weight species present in solution. It leads to a modified microenvironment, mass transfer or diffusional effect, arising from diffusional resistance to transport of substrate from bulk solution to catalytic site and from the diffusion of the product of reaction back to bulk solution (Kennedy, 1985).

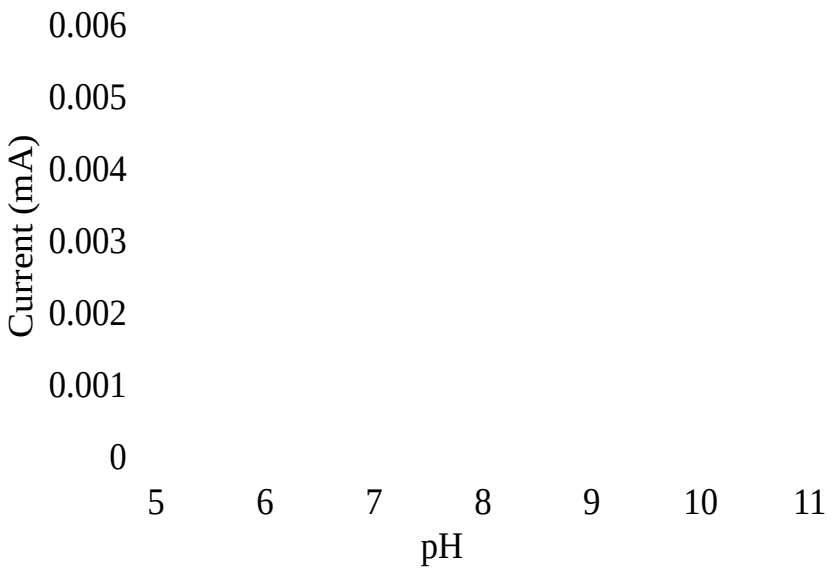


Fig. 26. Effect of pH on current response of c-MWCNT/PANI composite film based creatinine biosensor

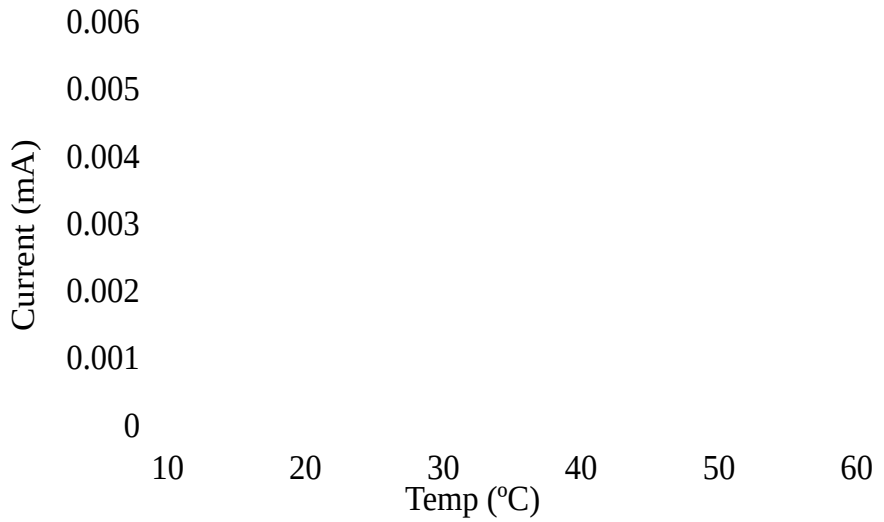


Fig. 27. Effect of incubation temperature on current response of c-MWCNT/PANI composite film based creatinine biosensor

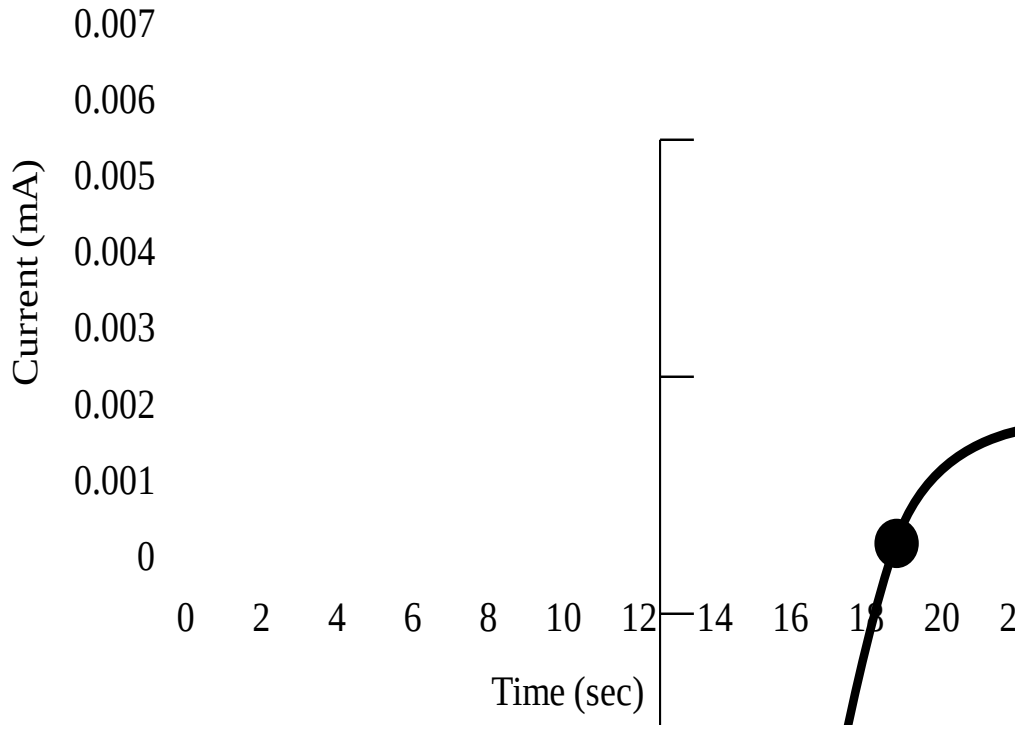


Fig. 28. Effect of incubation time on current response of c-MWCNT/PANI composite film based creatinine biosensor

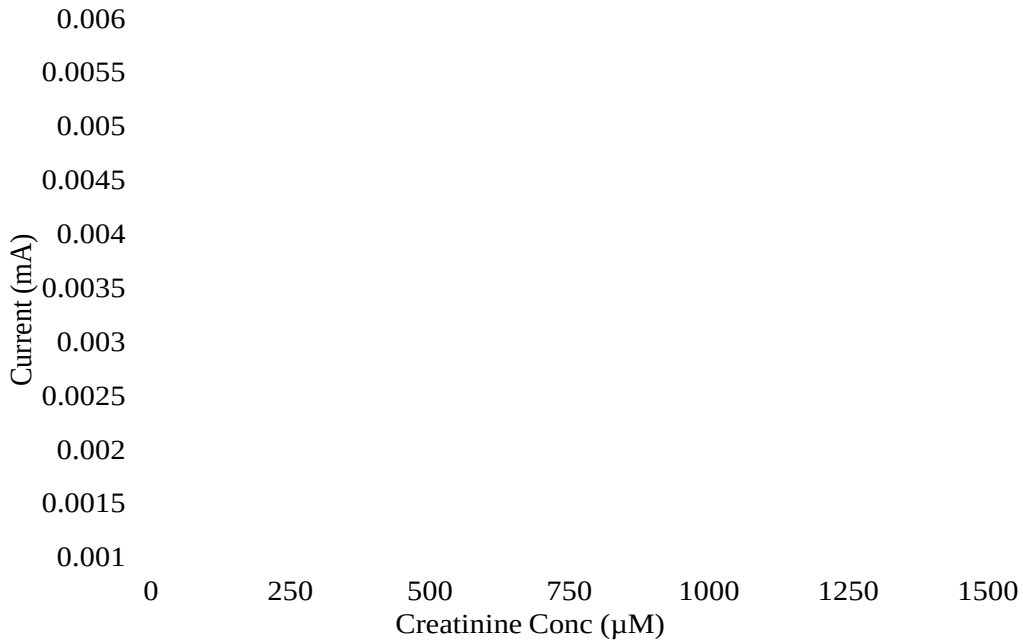


Fig. 29. Effect of substrate (creatinine) concentration on current response of c-MWCNT/PANI composite film based creatinine biosensor. Standard assay conditions were used except varying the creatinine concentration from 0.1-1500 µM

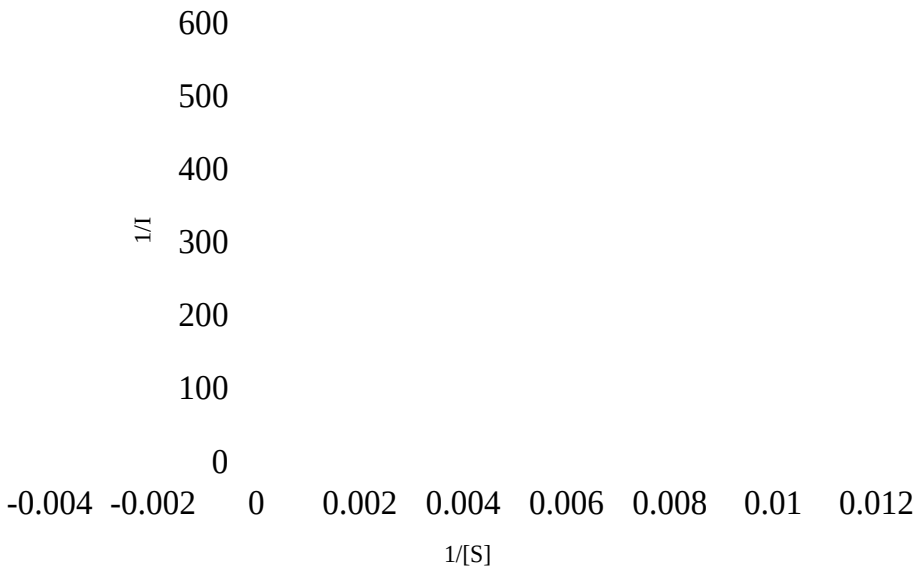


Fig. 30. Lineweaver-Burk plot for effect of substrate (creatinine) concentration on current response of c-MWCNT/PANI composite film based creatinine biosensor

4.5 DETERMINATION OF SERUM AND URINE CREATININE EMPLOYING ENZYME ELECTRODE (Enzymes/c-MWCNT/PANI/Pt)

The creatinine in serum and urine samples of apparently healthy and diseased individuals suffering from various kinds of kidney and muscular diseases, both male and female of different age groups was determined by present biosensor employing CA, CI and SO enzymes bound electrode.

4.5.1 Serum creatinine value in apparently healthy and diseased persons

The serum creatinine content in apparently healthy persons was ranged from 0.752 to 1.363 mg/dl with a mean of 1.013±0.143 mg/dl for males and 0.571 to 0.991 mg/dl with a mean of 0.777±0.150 mg/dl for females, where as in diseased persons suffering from various kind of kidney and muscular diseases was ranged from 2.823 to 5.603 mg/dl with a mean of 4.145±0.187 mg/dl for males and 2.542 to 4.990 mg/dl with a mean of 3.693±0.178 for females **(Table 7).** The increased levels of creatinine in diseased individuals might be due to irregularities in the function of kidneys or any condition that impair the function of the kidneys can cause improper filtering of the urine and alterations in metabolism of creatinine. The increased creatinine levels are associated with a number

of physiological conditions such as glomerulonephritis, acute tubular necrosis, nephritis, rhabdomyolysis, pre-eclampsia, diabetic nephropathy, haemolytic-uraemic syndrome (HUS), bilateral nephrectomy, cardio vascular disease, hypoxic-ischemic encephalopathy (HIE), acute lymphoblastic leukemia (ALL) and acute pancreatitis. The creatinine content determined by present biosensor are comparable to those determined by earlier reported creatinine biosensors based on poly (carbamoyl) sulfonate-hydrogel with nafion membrane (0.23-11.25 mg/dl) (Tombach *et al.,* 2001).

4.5.2 Urine creatinine value in apparently healthy and diseased persons

Because urinary creatinine concentrations are so widely used to adjust or correct urinary concentrations of environmental and workplace chemicals or their metabolites, the formation of urinary creatinine and the ways in which various factors may affect its concentration are important to review. The urine creatinine content in apparently healthy persons was ranged from 30.123 to 56.952 mg/dl with a mean of 43.881±1.196 mg/dl for males and 25.125 to 48.457 mg/dl with a mean of 38.132±1.186 mg/dl for females, where as in diseased persons suffering from various kind of kidney diseases was ranged from 15.325 to 28.975 mg/dl with a mean of 21.825±1.145 mg/dl for males and 13.247 to 24.761 mg/dl with a mean of 19.022±1.151 for females **(Table 8).** There was a decrease in urine creatinine level in kidney dysfunction persons as compared to normal persons which is consistent with earlier results (Barr *et al.,* 2005).

4.6 EVALUATION OF ANALYTICAL PERFORMANCE OF CREATININE BIOSENSOR EMPLOYING ENZYME ELECTRODE (Enzymes/c-MWCNT/PANI/ Pt)

A new method was developed for amperometric determination of creatinine in serum samples employing working enzyme (Enzymes/c-MWCNT/PANI/Pt) electrode. The method was based on oxidation of H_2O_2 produced from oxidation of creatinine by immobilized CA, CI and SO enzymes. The amperometric biosensor was based on the measurement of current response through potentiostat/galvanostat, which was generated from oxidation of H_2O_2. The current measured was directly proportional to the concentration of creatinine in the given sample. The present method has the advantage that it is simple, sensitive, specific and rapid. The electrodes employed in the method are fairly reusable and stable. The following criteria were studied to evaluate the analytical

performance of the present creatinine biosensor employing the enzyme electrode (Enzymes/c-MWCNT/PANI/Pt):

4.6.1 Linearity

There was a linear relationship between current (mA) and creatinine concentration ranging from 0.1 to 750 µM in 0.05 M PB, pH 7.5 for CA, CI and SO bound electrode **(Fig 15)**. The linear relationship of present method was better than earlier reported amperometric creatinine biosensor based on controlled pore glass (25-750 µM) (Sakslund and Hammerrch, 1992), poly (carbamoyl) sulfonate hydrogel matrix (1-150 µM) (Schneider *et al.,* 1996), ferrocen embedded carbon paste (upto 15 µM) (Kinoshita *et al.,* 1997), polyaniline-nafion (0.5-500) (Shih and Huang, 1999), poly (carbamoyl) sulfonate-hydrogel with nafion membrane (5-100 µM) (Tombach *et al.,* 2001), polished Pt electrode with alumina & diamond suspension (4.5-500 µM) (Walsh and Dempsy, 2002), nafion/poly (1,2-diaminobenzene) (1-100 µM) (Yao and Kotegawa, 2002), carbon paste electrode (0.004-0.1 µM and 0.004-0.4 µM) (Stefan *et al.,* 2003, Steden *et al.,* 2006) and polypropylene (3.2-320 µM) (Hsiue *et al.,* 2004) but comparable to that of cellulose acetate membrane (upto 880 µM) (Tsuchida and Yoda, 1983), PbO_2 oxidizing layer over HPU (1-1000 µM) (Shin *et al.,* 2001), polyvinyl alcohol (10-1000 µM) (Choi *et al.,* 2002), poly (carbamoyl) sulfonate hydrogel (5-1000 µM) (Erlenkotter *et al.,* 2002), ZnO-NPs/CHIT/c-MWCNT/PANI composite film (10-650 µM) (Yadav *et al.,* 2011) and Fe_3O_4/CHIT-g-PANI composite film (1-800 µM) (Yadav *et al.,* 2012). The linearity of present biosensor was lower than earlier reported amperometric creatinine biosensors based on polypyrrole doped with sulfonated phenoxy resin (200-5000 µM) (Yomato *et al.,* 1995), gas permeable membrane (20-1000 µM) (Osborne and Girault, 1995), poly-2-hydroxy ethyl methyacrylate (upto 2000 µM) (Madaras *et al.,* 1996), poly (1,3-diaminobenzene (900-1200 µM) (Madaras and Buck, 1996), polypyrrole (upto 2000 µM) (Trojanowicz *et al.,* 1996), platinized-S (shapable electroconductive) film (10-5000 µM) (Khan and Wernet, 1997) and carbon paste electrode containing 10% Pt powder (200-2000 µM) (Kim *et al.,* 1999) towards the higher limit of linearity range.

Table 7. Serum creatinine level of apparently healthy and diseased persons as measured by c-MWCNT/PANI composite film based creatinine biosensor

Age Group (n=10) (Years)	Sex	Serum creatinine (mg/dl) of healthy persons (Mean±SD)	Serum creatinine (mg/dl) of diseased persons (Mean±SD)
<10	M	0.752±0.132	2.823±0.123
	F	0.571±0.141	2.542±0.131
11-20	M	0.893±0.153	2.952±0.145
	F	0.687±0.159	2.692±0.138
21-30	M	0.982±0.159	3.921±0.163
	F	0.841±0.157	3.541±0.171
31-40	M	1.284±0.163	4.615±0.184
	F	0.942±0.161	3.701±0.178
41-50	M	1.363±0.148	5.603±0.179
	F	0.812±0.141	4.990±0.168
51-60	M	0.991±0.175	4.791±0.165
	F	0.765±0.168	4.403±0.161
61 & above	M	0.825±0.164	4.312±0.142
	F	0.645±0.173	3.984±0.151
Mean	M	1.013±0.143	4.145±0.187
	F	0.777±0.150	3.693±0.178

Table 8. Urine creatinine level of apparently healthy and diseased persons as measured by c-MWCNT/PANI composite film based creatinine biosensor

Age Group (n=10) (Years)	Sex	Urine creatinine (mg/dl) of healthy persons (Mean±SD)	Urine creatinine (mg/dl) of diseased persons (Mean±SD)
11-20	M	30.123±1.198	15.325±1.141
	F	25.125±1.181	13.247±1.135
21-30	M	45.103±1.121	22.465±1.124
	F	39.145±1.129	19.486±1.129
31-40	M	56.952±1.146	28.975±1.145
	F	48.457±1.151	24.761±1.156
41-50	M	48.789±1.192	26.694±1.114
	F	45.695±1.186	22.679±1.109
51-60	M	42.954±1.145	19.946±1.121
	F	37.649±1.141	17.479±1.116
61 & above	M	39.365±1.112	17.547±1.165
	F	32.824±1.122	16.478±1.159
Mean	M	43.881±1.196	21.825±1.145
	F	38.132±1.186	19.022±1.151

4.6.2 Detection limit

The detection limit of the present method was 0.1 µM at a signal to noise ratio of 3 (S/N=3), which was much lower than that of earlier reported amperometric creatinine biosensors based on cellulose acetate membrane (8.8 µM) (Tsuchida and Yoda, 1983), controlled pore glass (25 µM) (Sakslund and Hammerrch, 1992), sulfonated phenoxy resin (200 µM) (Yomato *et al.,* 1995), poly-2-hydroxy ethyl methyacrylate (30 µM) (Madaras *et al.,* 1996), poly (1,3-diaminobenzene (20 µM) (Madaras and Buck, 1996), polypyrrole (88 µM) (Trojanowicz *et al.,* 1996), platinized-S (shapable electroconductive) film (1-2 µM) (Khan and Wernet, 1997), carbon paste electrode containing 10% Pt powder (200 µM) (Kim *et al.,* 1999), PbO_2 oxidizing layer over HPU (0.8 µM) (Shin *et al.,* 2001), poly (carbamoyl) sulfonate-hydrogel with nafion membrane (5 µM) (Tombach *et al.,* 2001), polished Pt electrode with alumina & diamond suspension (4.5 µM) (Walsh and Dempsy, 2002), polyvinyl alcohol (10 µM) (Choi *et al.,* 2002), Fe_3O_4/CHIT-g-PANI composite film (1 µM) (Yadav *et al.,* 2012) but comparable to that of poly (carbamoyl) sulfonate hydrogel matrix (0.3 µM) (Schneider *et al.,* 1996), ZnO-NPs/CHIT/c-MWCNT/PANI composite film (0.5 µM) (Yadav *et al.,* 2011) and higher than that of ferrocene embedded carbon paste (0.01 µM) (Kinoshita *et al.,* 1997), carbon paste electrode (0.002 and 0.004-0.006 µM) (Stefan *et al.,* 2003; Steden *et al.,* 2006).

4.6.3 Analytical recovery

In order to check the accuracy of the method, the analytical recovery of added creatinine in the serum samples was determined. The mean analytic recoveries of exogenously added 0.5 mg/dL and 1.0 mg/dL creatinine (final conc. in reaction mixture) were 98.47±1.1 and 97.91±1.8 % respectively **(Table 9)**, which were better than earlier reported amperometric creatinine biosensor based on polished Pt electrode with alumina & diamond suspension (87.4 %) (Walsh and Dempsy, 2002) but lower than that of cellulose acetate membrane based creatinine biosensor (109.6 %) (Tsuchida and Yoda, 1983) and comparable to that of nafion/poly (1,2-diaminobenzene) (94-98 %) (Yao and Kotegawa, 2002), ZnO-NPs/CHIT/c-MWCNT/PANI composite film (98.87 % and 98.31 %) (Yadav *et al.,* 2011), Fe_3O_4/CHIT-g-PANI composite film (98.97 % and 98.91 %) (Yadav *et al.,* 2012).

4.6.4 Precision

To check the reproducibility and reliability of the present creatinine biosensor, the creatinine content of the sample in one run (Within batch) and after storage at -20 ^{o}C for one week (Between batch) were determined. The results showed that the creatinine value of these determination agreed with each other and within batch and between batch coefficient of variation (CVs) were 3.6 % and 4.1 % **(Table 10)** showing the good reproducibility and reliability of the method, which is better than earlier amperometric creatinine biosensors based on cellulose acetate membrane (8.4 % & 11.5 %) (Tsuchida and Yoda, 1983), triamine & acetyl cellulose membrane (6.7 % & 8.8 %) (Kubo *et al.*, 1983), propylamine & succinate CPG (5 %) (Rui *et al.*, 1992), poly-2-hydroxy ethyl methyacrylate (6.2 %) (Madaras *et al.,* 1996), poly (carbamoyl) sulfonate-hydrogel with nafion membrane (5.1-11.4 %) (Tombach *et al.*, 2001) and comparable to that of ZnO-NPs/CHIT/c-MWCNT/PANI composite film (<4.6 % and <5.1 %) (Yadav *et al.*, 2011), Fe_3O_4/CHIT-g-PANI composite film (<4.15 % and <5.58 %) (Yadav *et al.*, 2012) but higher than polypropylene (2.5 %) (Nguyen *et al.*, 1991), propylamine & succinate CPG (2.34 % & 3.94 %) (Rui *et al.*, 1993a), polished Pt electrode with alumina & diamond suspension (1.14 %) (Walsh and Dempsy, 2002).

4.6.5 Accuracy

To study the accuracy of the present method, creatinine level in 25 serum samples from as determined by the present method (y) were compared with those obtained by standard chemical spectrophotometric method (x) (Jaffe, 1986). When the serum creatinine values obtained by our method were compared with those obtained by chemical spectrophotometric method, there was a good correlation (r=0.9833) with the following regression equation: y = 1.0031x + 0.00212 **(Fig 31).** These results showed that the data obtained by present method and previously reported standard method are comparable. It proved that the modified electrode ascertained the practical application of the biosensor in the routine quantitative analysis of creatinine. The correlation coefficient obtained by present biosensor was comparable to that of earlier reported amperometric creatinine biosensors based on cellulose acetate membrane (0.985) (Tsuchida and Yoda, 1983), polypropylene (0.99) (Nguyen *et al.*, 1991, Hsiue *et al.*, 2004), poly-2-hydroxy ethyl methyacrylate (0.991) (Madaras *et al.*, 1996), poly (1,3-diaminobenzene (0.957) (Madaras and Buck, 1996), poly (carbamoyl) sulfonate hydrogel matrix (0.99) (Schneider *et al.,* 1996), carbon paste electrode containing 10% Pt powder (0.994) (Kim *et al.,* 1999),

PbO_2 oxidizing layer over HPU (0.99) (Shin *et al.,* 2001), poly (carbamoyl) sulfonate-hydrogel with nafion membrane (0.978-0.994) (Tombach *et al.,* 2001), nafion/poly (1,2-diaminobenzene) (0.99) (Yao and Kotegawa, 2002), poly (carbamoyl) sulfonate hydrogel (0.98-0.99) (Erlenkotter *et al.,* 2002), carbon paste electrode (0.99) (Stefan *et al.,* 2003; Steden *et al.,* 2006), ZnO-NPs/CHIT/c-MWCNT/PANI composite film (0.989) (Yadav *et al.,* 2011) and Fe_3O_4/CHIT-g-PANI composite film (0.99) (Yadav *et al.,* 2012).

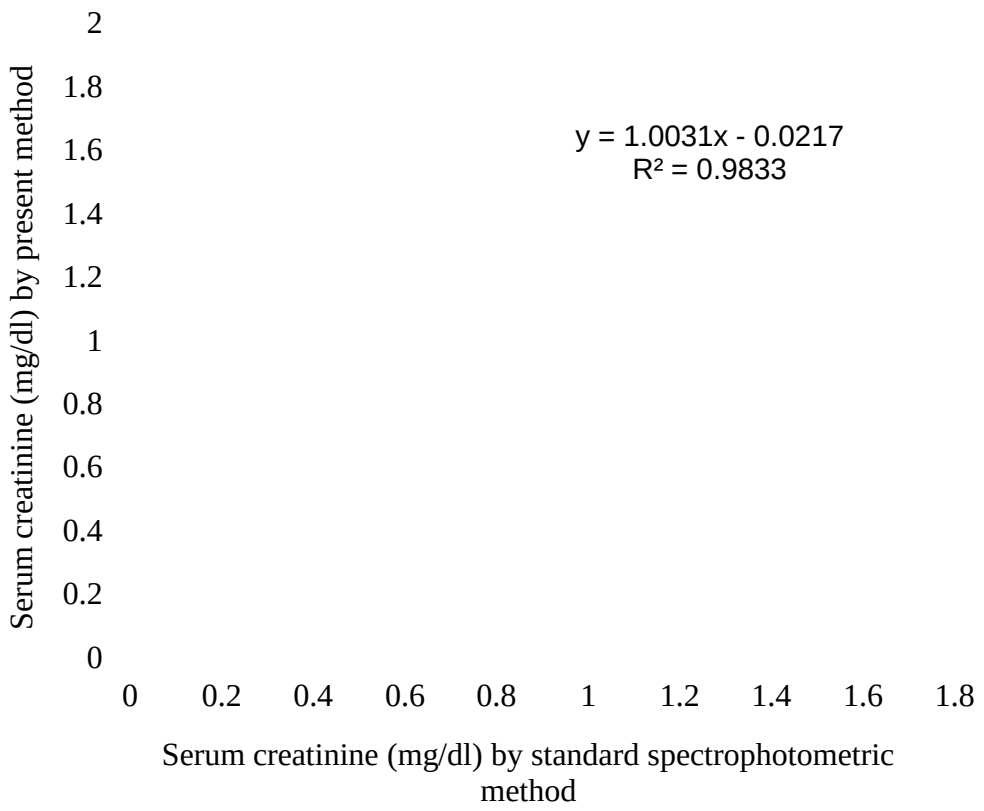


Fig. 31. Correlation between serum creatinine values as determined by standard chemical spectrophotometric method (x-axis) and present creatinine biosensor (y-axis) based on c-MWCNT/PANI composite film

Table 9. Analytical recovery of added creatinine in the serum samples, as measured by c-MWCNT/PANI composite film based creatinine biosensor

Creatinine added (mg/dl)	Creatinine found (mg/dl)	% Recovery
-	0.60	-
0.5	1.092	98.47±1.1
1.0	1.579	97.91±1.8

Table 10. Within and between assay coefficients of variation for determination of creatinine in serum samples, as measured by c-MWCNT/PANI composite film based creatinine biosensor

N	Creatinine (mg/dl)	CV (%)
Within assay (5)		
0.8		
0.83		
0.87	0.83	3.6
0.81		
0.86		
Between assay (5)*		
0.99		
1.0		
0.94	0.99	4.1
0.97		
1.05		

* Samples were assayed after storage at -20 °C for one week.

4.7 EFFECT OF INTERFERING SUBSTANCES

To check the interference the amperometric current response of enzyme electrode was measured in presence of various observed potential interfering metabolites and metal ions at their physiological concentration.

4.7.1 Effect of various serum metabolites

The effect of various metabolites found in the serum such as creatine, sarcosine, ascorbic acid, uric acid, urea, bilirubin, glucose, sodium pyruvate, triglycerides & cholesterol was studied at their physiological concentration on the response of present creatinine biosensor employing the Enzymes/c-MWCNT/PANI/Pt electrode **(Table 11).** Results given in the **Table 11** showed that serum metabolites had no effect on the response of biosensor, which were similar to that of earlier reported amperometric creatinine biosensors based on triamine & acetyl cellulose membrane (Kubo *et al.,* 1983), poly(γ-methyl-L-glutamate) (Kubo and Karube, 1986), ferrocene embedded carbon paste (Kinoshita *et al.,* 1997), nafion/poly (1,2-diaminobenzene) (Yao and Kotegawa, 2002). However some earlier reported amperometric creatinine biosensors showed interference by various metabolites based on polypropylene (creatine and sarcosine) (Nguyen *et al.,* 1991), propylamine & succinate CPG (ascorbic acid) (Rui *et al.,* 1993a), poly-2-hydroxy ethyl methyacrylate (creatine and sarcosine) (Madaras *et al.,* 1996), poly (1,3-diaminobenzene (creatine and sarcosine) (Madaras and Buck, 1996), platinized-S (shapable electroconductive) film (creatine and sarcosine) (Khan and Wernet, 1997), ZnO-NPs/CHIT/c-MWCNT/PANI composite film (creatine) (Yadav *et al.,* 2011) and Fe_3O_4/CHIT-g-PANI composite film (creatine and sarcosine) (Yadav *et al.,* 2012).

4.7.2 Effect of various metal ions

The effect of various metal ions found in the serum such as $CaCl_2$, $CdCl_2$, $MgCl_2$, $AgNO_3$, $HgCl_2$, $MnSO_4$, $NiCl_2$, $BaCl_2$, $FeSO_4$ & $FeCl_3$ at their physiological concentration was also studied on the response of present creatinine biosensor employing the Enzymes/c-MWCNT/PANI/Pt electrode **(Table 12).** Results given in the **Table 12** showed that current response of present biosensor was stimulated only by Cd^{+2} and Ni^{+2} metals ions but rest metal ions had practically no effect on current response.

4.8 STORAGE STABILITY AND REUSABILITY OF ENZYME ELECTRODE (Enzyme/c-MWCNT/PANI/Pt)

The long term storage stability and reusability of Enzymes/c-MWCNT/PANI/Pt electrode was investigated by measuring current response of the biosensor every week under its storage in 0.05 M PB, pH 7.5 at 4 °C over a period of 6 months. It was revealed that current response of the biosensor maintained 85% of the initial current response even after regular 150 uses over a period 180 days **(Fig 32)**, which is better than earlier reported amperometric creatinine biosensors based on propylamine & succinate CPG (30 days) (Rui *et al.,* 1993a), poly-2-hydroxy ethyl methyacrylate (creatine and sarcosine) (90 days) (Madaras *et al.,* 1996), platinized-S (shapable electroconductive) film (≤ 30 days) (Khan and Wernet, 1997), nafion/poly (1,2-diaminobenzene) (90 days) (Yao and Kotegawa, 2002), polypropylene (90 days and 21 days) (Nguyen *et al.,* 1991, Hsiue *et al.,* 2004), poly (carbamoyl) sulfonate hydrogel (70 days) (Erlenkotter *et al.,* 2002) and ZnO-NPs/CHIT/c-MWCNT/PANI composite film (120 days) (Yadav *et al.,* 2011), but comparable to that of controlled pore glass (180 days) (Sakslund and Hammerrch, 1992), poly (carbamoyl) sulfonate hydrogel matrix (180 days) (Schneider *et al.,* 1996) and Fe_3O_4/CHIT-g-PANI composite film (200 days) (Yadav *et al.,* 2012) based amperometric creatinine biosensors. This suggests that use of c-MWCNT/PANI composite film ensures good stability and keeping the bioactivity of enzyme electrode.

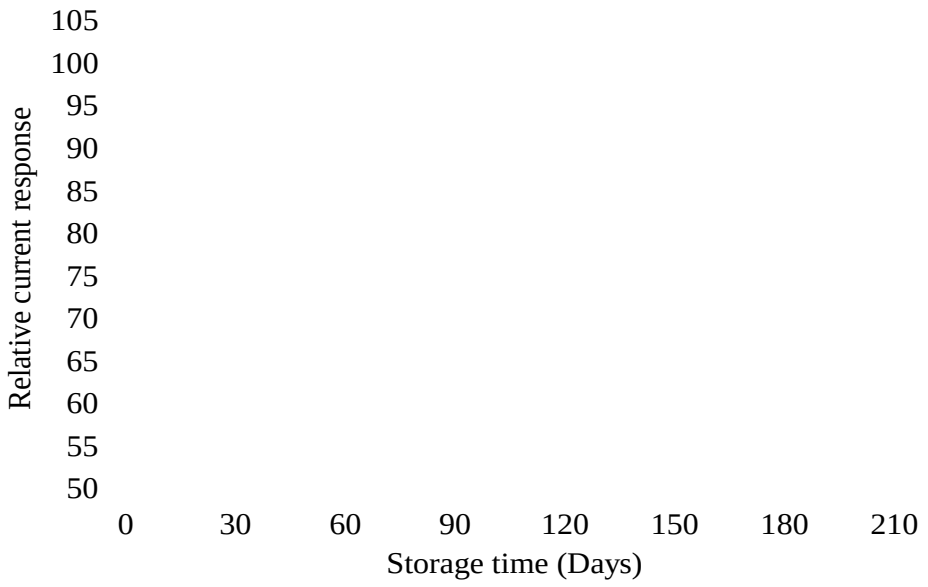


Fig. 32. Effect of storage at 4 °C on the response of creatinine biosensor based on c-MWCNT/PANI composite film

Table 11. Effect of various serum metabolites on response of c-MWCNT/PANI composite film based creatinine biosensor

Compound added	**Physiological conc. (mg/dl)**	**% Relative response of biosensor**
None	-	100
Creatine	0.65	103
Sarcosine	14.16	101
Ascorbic acid	2.0	99
Uric acid	8.0	100
Urea	30.0	100
Bilirubin	1.0	99
Glucose	100.0	101
Sodium pyruvate	1.0	100
Triglyceride	100.0	98
Cholesterol	225.0	100

Table 12. Effect of various serum metal ions on response of c-MWCNT/PANI composite film based creatinine biosensor

Compound added	**Physiological conc. (µg/dl)**	**% Relative response of biosensor**
None	-	100
$CaCl_2$	10.0×10^3	101
$CdCl_2$	0.1	93
$MgSO_4$	2×10^3	99
$AgNO_3$	2.5	102
$HgCl_2$	2.0	89
$MnSO_4$	25.0	99
$NiCl_2$	75.0	102
$BaCl_2$	0.06	100
$FeSO_4$	160.0	98
$FeCl_3$	160.0	101