

The aim of the present work was to use c-MWCNT/PANI composite film to improve the analytic performance of creatinine biosensor for the determination of creatinine in serum and urine required in the diagnosis and medical management of kidney failure, muscular dystrophy, glomerulonephritis, acute tubular necrosis, nephritis, rhabdomyolysis, pre-eclampsia, diabetic nephropathy, haemolytic-uraemic syndrome (HUS), bilateral nephrectomy, cardiovascular disease, hypoxic-ischemic encephalopathy (HIE), acute lymphoblastic leukemia (ALL) and acute pancreatitis. To achieve this aim c-MWCNT/PANI composite film was electrodeposited on the surface of platinum (Pt) electrode (1.95 cm × 1 mm) (length × diameter). The cyclic voltammograms were obtained for electrodeposition of pure conducting PANI and c-MWCNT/PANI composite films. The composite film exhibited higher current response 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. A method is described for the construction of an amperometric creatinine biosensor based on covalent co-immobilization of creatinine amidohydrolase (CA), creatine amidinohydrolase (CI) and sarcosine oxidase (SO) onto c-MWCNT/PANI composite film electrodeposited on the surface of Pt electrode using N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) chemistry. EDC was used to conjugate the free carboxyl (-COOH) groups of c-MWCNT/PANI composite film to amine (-NH₂) groups on the surface of enzymes, using NHS as a catalyst. First, free and unbound -COOH groups of c-MWCNT/PANI film were activated by immersing them into 0.1M phosphate buffer (PB, pH 7.5) containing EDC and NHS of the same concentration (10 mM) for 6h, 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 covalent co-immobilization of enzymes and then washed with 0.05M PB (pH 7.5).

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 -NH₂ 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 assisted 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. This working electrode along with Ag/AgCl as reference and Pt wire as auxiliary electrode were dipped into in 15 ml 0.05 M PB (pH 7.5) containing 100 μ M creatinine and connected through potentiostat/galvanostat (Autolab, Eco Chemie, The Netherland. Model: AUT83785). The electrodes were polarized at different potential (volts) and current (mA) generated was measured. The optimal current response was obtained at 0.2 V, and hence 0.2 V was selected as the optimum working potential for all amperometric determination of creatinine.

The fabricated working electrode was characterized by SEM, FTIR and electrochemical impedance spectroscopic (EIS) studies. SEM micrographs for both pure PANI and c-MWCNT/PANI composite films showed that they were composed of nanofibrils but for the pure PANI they were slight thicker than those of in the c-MWCNT/PANI composite and showed net structure, but the pore was smaller and denser in c-MWCNT/PANI composite, due to presence of c-MWCNTs. SEM micrographs of c-MWCNT/PANI/Pt electrode revealed 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 the sporadic appearance of globular/beaded structure on uniform structure of c-MWCNT/PANI composite film, indicating that enzymes were successfully immobilized on the surface of c-MWCNT/PANI composite film. It was observed that the enzymes were attached at sidewalls as well as at the ends of c-MWCNTs. FTIR spectra were 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 shape changes to some extent due to interaction between c-MWCNTs and PANI. The FTIR spectrum of electrochemically deposited c-MWCNT/PANI composite shows benzenoid and quinoid ring stretching bands present at 1491.03 and 1548.24 cm^{-1} . The peaks obtained at 1139.53 and 3435 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 1634.05 cm^{-1} indicated the

presence of carboxyl group ($-\text{COOH}$) in the MWCNTs. The enzymes binding on c-MWCNT/PANI/Pt electrode was revealed by the appearance of additional absorption bands at 1589.10 and 1491.03 cm^{-1} , which are assigned to carbonyl stretch (amide I band) and $-\text{N}-\text{H}$ bonding (amide II band), respectively. 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. 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 R_{CT} value of c-MWCNT/PANI/Pt electrode ($210\ \Omega$) was lower than PANI/Pt electrode ($475\ \Omega$), showing its decreased resistance and high electron transfer efficiency. However, the R_{CT} of Enzyme/c-MWCNT/PANI/Pt bioelectrode ($450\ \Omega$) increased compared with that of c-MWCNT/PANI/Pt electrode, indicating the co-immobilization of enzymes on c-MWCNT/PANI/Pt electrode.

The biosensor detected creatinine levels as low as $0.1\ \mu\text{M}$, estimated at a signal-to-noise ratio of 3, within 5s at pH 7.5 and $35\ ^\circ\text{C}$. The optimized biosensor showed a linear response range, 0.1 to $750\ \mu\text{M}$ for creatinine with a sensitivity of $40\ \mu\text{A}/\text{mM}/\text{cm}^2$. A Lineweaver–Burk plot gave the apparent K_{m} value of $0.26\ \text{mM}$ for immobilized enzymes, which is lower, than for free enzymes indicating that the affinity of enzymes was increased for substrate after immobilization. The method showed a good reproducibility as the analytical recoveries of added triolein ($0.5\ \text{mg}/\text{dl}$ and $1.0\ \text{mg}/\text{dl}$) in serum sample were $98.47\ \%$ and $97.91\ \%$. The results of within- and between-batch coefficients of variation (CVs) for serum creatinine determination were less than $3.6\ \%$ and $4.1\ \%$, respectively, showing a good repeatability of the method. A good correlation ($r = 0.989$) was obtained between present biosensor and standard chemical spectrophotometric method for measurement of creatinine. Among the various serum metabolites tested such as creatine, sarcosine, ascorbic acid, uric acid, urea, bilirubin, glucose, sodium pyruvate, triglycerides & cholesterol and metal ion such as CaCl_2 , CdCl_2 , MgCl_2 , AgNO_3 , HgCl_2 , MnSO_4 , NiCl_2 , BaCl_2 , FeSO_4 & FeCl_3 along with $100\ \mu\text{M}$ creatinine in $0.05\ \text{M}$ PB (pH 7.5) at their physiological concentrations, none had practically any significant interference except Cd^{+2} and Ni^{+2} ions, which caused a slight stimulation in biosensor response. The biosensor was employed for determination of creatinine in human serum and urine samples of healthy and diseased persons (suffering from kidney and muscular diseases). The creatinine level in human serum, as measured by the present biosensor was in the range of 0.571 to $1.363\ \text{mg}/\text{dl}$ in case of apparently healthy persons and in range of

2.542 to 5.603 mg/dl in case of diseased persons, while in urine samples ranged from 25.125 to 56.952 mg/dl and 13.247 to 28.975 mg/dl in apparently healthy and kidney diseased persons respectively. The biosensor maintained 85 % of the initial activity after 180 days of regular 150 uses, when stored dry at 4 °C.

Conclusion

The use of c-MWCNT/PANI composite film in the preparation of an amperometric creatinine biosensor has improved its performance in terms of low working potential (0.2 V), short response time (5 sec), high sensitivity ($40 \mu\text{A}/\text{mM}/\text{cm}^{-2}$) and high storage stability compared to earlier biosensors. Based on these observations, this composite film could also be employed for improvement of other biosensors.