3.1 SOURCES OF CHEMICALS AND BIOCHEMICALS

Creatininase/creatinine amidohydrolase (CA, EC 3.5.2.10, 140 units/mg, from *Pseudomonas sp*), creatinase/creatine amidinohydrolase (CI, EC 3.5.3.3, 13.2 units/mg, from *Pseudomonas sp*), sarcosine oxidase (SO, EC 1.5.3.1, 39 units/mg from *Bacillus sp*), 4-aminophenazone, creatinine, creatine and sarcosine were purchased from Sigma–Aldrich (USA). Carboxylated multiwalled carbon nanotubes (c-MWCNT) (functionalized MWCNT, 12 walls, 15–30 μm length, 90% purity, nil metal content) were purchased from M/S Intelligent Materials (Panchkula, Haryana, India). N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), aniline (purified through vacuum distillation before use), potassium ferrocyanide, potassium ferricyanide, dimethylaminobenzaldehyde (DAB), picric acid, potassium bromide (Spectronic grade), bilirubin, cholesterol, ascorbic acid, pyruvic acid, glucose, uric acid, glycine, acetaminophen, acetone, nitric acid, sulphuric acid, hydrochloric acid, sodium carbonate, sodium bicarbonate, tris buffer, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate were purchased from Sisco Research Laboratories (Mumbai, India). All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used in all experiments.

3.2 INSTRUMENTS AND EQUIPMENTS USED

The following instruments and laboratory items were used in the present study: Autolab Potentiostat/Galvanostat equipped with an Autolab PGSTAT-302N, general purpose electrochemical system (GPES) and frequency response analysis (FRA) software (Eco-Chemie, Utrecht, The Netherlands, Model: AUT83785), Fourier transform infrared (FTIR) spectrophotometer (Nicolet iS10, Thermo Scientific USA) along with hydraulic press (cap 15T), Misonix ultrasonic liquid processor (XL-2000 series, Newtown City, USA) along with sonobox, refrigerated centrifuge (Sigma 3-30K), biomedical freezer (-40°C, Sanyo), refrigerator (GL-325TMG4/2007, LG), all quartz double distillator apparatus (881B, Labco, India), electronic balance (Sartorious, BT224S), Temperature controlled digital water bath shaker & oven (NSW, New Delhi), digital pH meter (335D, systronics, Ahmadabad), Spectronic-20D^{+} (Thermo Scientific, USA), ice flaking machine (AICIL, Chandigarh) and magnetic stirrer with hot plate. All glasswares used were from M/S Borosil Glass Works Ltd. Mumbai.

3.3 ASSAY OF FREE ENZYMES

Assays of different enzymes CA, CI and SO separately as well as their combined assay were carried out to check their activity.

3.3.1 Assay of creatininase (CA)

The measurement of CA activity was based on the Jaffe reaction and carried out according to modified method of Tsuchida and Yoda, (1983).

Principle:

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

$$\text{Creatinine} + \text{Picric acid} \xrightarrow{OH^-} \text{Orange dye}$$

The orange dye (molar absorptivity = 4.65×10^3 cm^2/mmol) has an absorption maximum at 520 nm.

To determine the activity of the CA, 0.9 ml of 0.1 M creatine in 0.05 M potassium phosphate buffer (PB, pH 7.5) was preincubated for 5 min at 37 °C and then 0.1 mL of CA solution (1.8-2.4 U/ml enzyme solution) was added to the same buffer. After 10 min of incubation at 37 °C, 0.1 ml of reaction mixture was transferred to 0.9 ml of ice-cold water and then added 1.0 ml of 1 M sodium hydroxide solution & 1.0 ml of 10 g/L picric acid solution. The mixture was incubated for 20 min at 25 °C and absorbance of the resulting solution was measured at 520 nm vs the blank in a Spectronic-20.

One unit (U) activity of CA was defined as the amount of enzyme which causes the formation of 1 µmol of creatinine/min/ml under above standard conditions.

3.3.2 Assay of creatinase (CI)

CI activity was assayed according to modified method of Tsuchida and Yoda, (1983) and based on the following reactions:

Principle:

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

$$\text{Urea} + 2\ \text{p-dimethylaminobenzaldehyde} \longrightarrow \text{Yellow dye} + 2H_2O$$

The yellow dye (molar absorptivity = 3.21×10^2 cm^2/mmol) formed from urea and p -dimethylaminobenzaldehyde has an absorption maximum at 435 nm.

To determine the activity of CI, 0.9 ml of 0.1 M creatine solution in 0.05 M PB (pH 7.5) was preincubated for 5 min at 37 °C, then 0.1 ml of CI solution (9-10 U/ml enzyme

solution) was added to the same buffer. After 10 min of incubation at 37 °C, 2.0 ml solution of 2.0 g of p-dimethylaminobenzaldehyde in 100 ml of dimethyl sulfoxide followed by 15 ml of concentrated HCI were added. After keeping it at room temperature (25 °C) for 20 min, the absorbance at 435 nm was recorded vs blank in the Spectronic-20.

One unit (U) of activity of CI was defined as the amount of CI required to generate 1 µmol of yellow dye per min/ml under the standard conditions.

3.3.3 Assay of sarcosine oxidase (SO)

SO activity was assayed according to modified method of Tsuchida and Yoda, (1983) and based on the following reactions:

Principle:

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

$$\text{Formaldehyde} + \text{4-amino-3-hydrazino-5-mercepto-1,2,4-trizole} \xrightarrow[+\,OH^-]{+NaIO_4} \text{Tetrazine dye}$$

The tetrazine dye (molar absorptivty = 3.72×10^4 cm^2/mmol) has an absorption maximum at 550 nm.

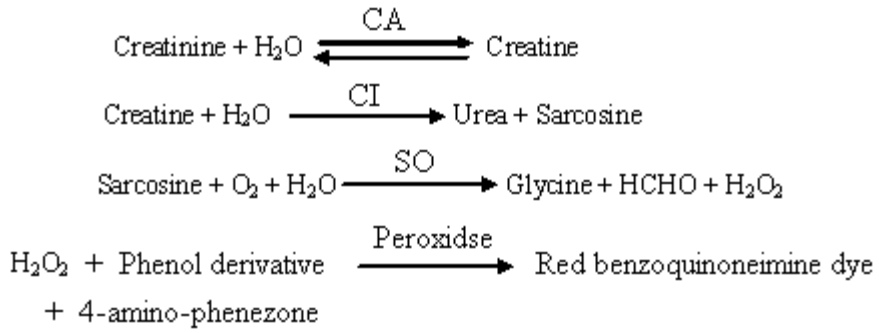
To measure SO activity, 0.3 ml of 0.1 M sarcosine aqueous solution and 0.1 ml of 0.05 M PB (pH 7.7) were pre-incubated at 37 °C for 3 min and then 0.1 ml of SO solution (1.3-1.5 U/ml enzyme solution) was added to the mixture. After incubation at 37 °C for 10 min, 1.0 ml of 0.05 M potassium hydroxide and 1.0 ml of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (6 g/L) in 0.5M aqueous HCl were added. After keeping it at room temperature (25 °C) for 10 min, 1.0 ml of sodium periodate solution (7.5 g/L) was added under stirring, and the absorbance of dye was measured at 550 nm vs the blank in the Spectronic-20.

One unit (U) of SO activity was defined as amount of SO, which causes the formation of 1 µmol of formaldehyde per min/ml under the standard conditions.

3.3.4 Assay of mixture of free CA, CI and SO

The assay of mixture of free enzymes was carried out as described by Fossati *et al.*, (1983) with modification and based on the following reactions:

***Principle*:**



To measure the combined activity of free enzymes, 1 ml of the chromogen reagent was added to 50 µl of creatinine solution (0.175 mM), then added 10 µL of CA solution (9-10 units). After keeping at room temperature (25 °C) for 30 min, the absorbance at 510 nm was measured vs the blank in the Spectronic-20.

Preparation of chromogen reagent: The chromogen reagent consisted of 0.1 M sodium phosphate buffer (pH 7.5) containing, per liter, CI 8 kU, SO 1.3 kU, peroxidase 1.5 kU, 0.2 mM 4-aminophenazone and 3 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid. The chromogen reagent was stored in amber colored bottle, when not in use and stable for one month at 2-8 °C; slight pink discoloration was unimportant but prepared fresh after one week.

3.4 CONSTRUTION OF ENZYME ELECTRODE (Enzymes/c-MWCNT/PANI/ Pt)

3.4.1 Dispersion of c-MWCNTs

c-MWCNT powder (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 hr to obtain a homogeneous black colored solution **(Fig 11)** and then washed with DW until the pH of the washing discard was 7.0.

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

The PANI and c-MWCNT/PANI composite film coated Pt electrodes were prepared according to method of Du *et al.,* (2009) with modification. The PANI and c-MWCNT/PANI composite films were electrodeposited onto Pt electrode through electropolymerization using a potentiostat/galvanostat. Prior to electrodeposition, the Pt electrodes (1.95 cm × 1 mm) (length × diameter) were ultrasonicated in 5.0 M HNO_3 and acetone for 15 min and then rinsed with DW. For electrodeposition of PANI (purified through vacuum distillation before use) and c-MWCNT/PANI composite films, solutions for electrodeposition were prepared by adding 50 µl aniline in 10 ml of 1N HCl and 50 µl

aniline along with finally dispersed 1 ml c-MWCNT suspension in 10 ml of 1 N HCl in a glass cell respectively. The three-electrode system was immersed in the electrodepositing solutions, and the potential scan was cycled 20 times between -0.1 and 0.9 V v/s Ag/AgCl at a scan rate of 50 mV/s. During the electrochemical polymerization, the surface of Pt electrode gradually became black, indicating the deposition of c-MWCNT/PANI composite film on Pt electrode **(Fig 12).** The PANI and c-MWCNT/PANI composite film coated Pt electrodes were washed with DW and subsequently kept in a desiccator for 24 h at room temperature.

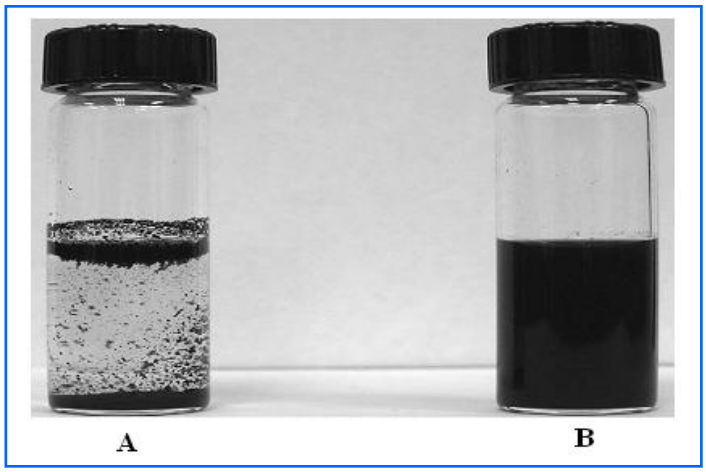


Fig. 11. Partially dispersed c-MWCNT [A] Completely dispersed c-MWCNT [B]

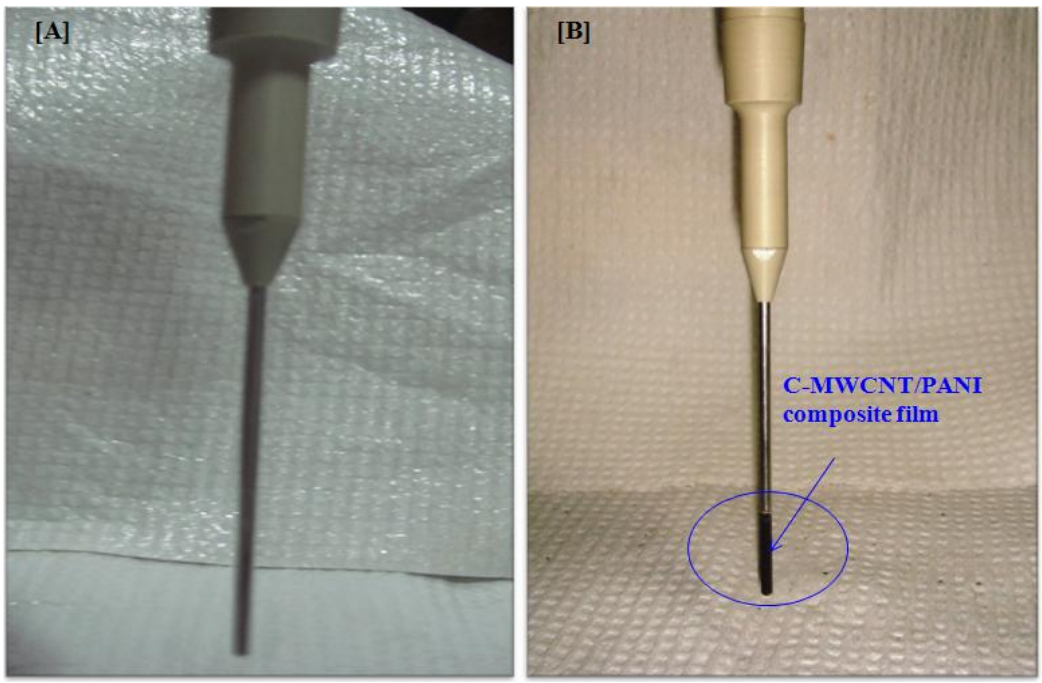


Fig. 12. Pt electrode with out (A) and with (B) electrodeposited c-MWCNT/PANI composite film

3.4.3 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 coated Pt electrode using N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC)-N-hydroxysuccinimide (NHS) chemistry, as described by Rahman *et al.,* (2009b) with modifications. First, free and unbound –COOH groups of c-MWCNT/PANI composite film were activated by immersing them into 0.1 M 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 5ml 0.05 M PB (pH 7.5) containing CA (44 U), CI (36 U) and SO (24 U) at 4 °C for 3 hr and then washed with 0.05 M PB (pH 7.5). The resulting enzyme electrode was dried and stored in a refrigerator at 4 °C

3.4.4 Characterization of enzyme electrode (Enzymes/c-MWCNT/PANI/Pt)

The fabricated enzyme electrodes were characterized using scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS).

3.4.4.1 Scanning electron microscopy

SEM studies were used for investigating the morphology of c-MWCNT/PANI/Pt electrode with and without immobilized enzymes. SEM studies were carried out on commercial basis at Advanced Instrumentation Research Facility (AIRF) of Jawaharlal Nehru University, New Delhi. SEM used a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derived from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, crystalline structure and orientation of materials making up the sample. The electrodes were cut into small pieces (1 cm) and placed on a specimen chamber of 2 cm diameter using a spray gun, generally mounted rigidly on a specimen holder called a specimen stub and micrographs were taken with a scanning electron microscope. The samples were coated with an ultrathin coating of electrically conducting material gold. In SEM, data were collected over a selected area of the surface of the sample and 2-dimensional images were generated that displayed spatial variations in these properties.

3.4.4.2 Fourier transform infrared spectroscopy

Infrared spectroscopy is one of the most powerful analytical techniques, which offers the possibility of chemical identification. One of the most important advantages of infrared spectroscopy over the other usual methods of structural analysis is that it provides useful information about the structure of molecules and bonding quickly, without tire-some evaluation methods. The technique is based upon the simple fact that a chemical substance shows marked selective absorption in the infrared region giving rise to close-packed absorption bands, called an IR absorption spectrum, which may extend over a wide wavelength range. Various bands present in an IR spectrum correspond to the characteristic functional groups and bonds present in a chemical substance. The FTIR technique works almost exclusively on samples with covalent bonds. To record FTIR spectra of enzyme electrode at different stages of its construction, the deposited material was scrapped off the Pt electrodes, ground with dry potassium bromide (KBr) and this powder mixture was then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrophotometer could pass **(Fig 13).** Then this pellet was kept in the socket of FTIR spectrophotometer and its spectrum was recorded. The infrared spectrum of a sample was recorded by passing a beam of infrared light through the sample. Band intensities in IR spectrum were expressed as transmittance (T). Transmittance is defined as the ratio of the radiant power transmitted by a sample to the radiant power incident on the sample. In most of the spectrum transmittance (T) versus wave number (cm^{-1}) has been plotted. The infrared portion of the electromagnetic spectrum is usually divided into three regions; the near-, mid- and far- infrared, named for their relation to the visible spectrum. In the present study the mid-infrared, approximately 4000–400 cm^{-1} (2.5–25 μm) was used to study the fundamental vibrations and associated rotational-vibrational structure. Unlike a dispersive instrument, i.e. grating monochromator or spectrograph, FTIR spectrophotometers collected all wavelengths simultaneously. Analysis of the position, shape and intensity of peaks in this spectrum reveals details about the molecular structure of the sample.

3.4.4.3 Electrochemical impedance spectroscopy

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 EIS studies were conducted on Autolab Potentiostat/Galvanostat equipped with FRA in the frequency range of 0.01 to

10^5 Hz, amplitude 5 mV after dipping the three electrodes system 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. During an impedance measurement, a FRA was used to impose a small amplitude AC signal to the electrode via the load. The AC voltage and current response of the electrode was analyzed by the FRA to determine the resistive, capacitive and inductive impedance behavior of the electrode at that particular frequency. When conducted over a broad range of frequencies, impedance spectroscopy is used to identify and quantify the impedance associated with these various processes. EIS data for electrochemical cells such as enzyme electrode are most often represented in Nyquist plot. A complex plane or Nyquist plot depicts the imaginary impedance, which is indicative of the capacitive and inductive character of the cell, versus the real impedance of the cell. Nyquist plots have the advantage that activation-controlled processes with distinct time-constants show up as unique impedance arcs and the shape of the curve provides insight into possible mechanism or governing phenomena.

3.5 CONSTRUCTION AND TESTING OF AMPEROMETRIC CREATININE BIOSENSOR

An amperometric creatinine biosensor was constructed using a three-electrode electrochemical cell system, consisting of Enzymes/c-MWCNT/PANI/Pt as a working electrode, 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 electrode system was dipped into a reaction mixture containing 10 ml 0.05 M PB, pH 7.5 and 0.5 ml creatinine solution (100 µM). The electrode response was measured in terms of milliampere (mA) applying a potential range of -0.1 to 0.9 V vs Ag/AgCl. The reaction involved degradation of creatinine and ultimately generation H_2O_2, which is 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.

Fig. 13. FTIR spectrophotometer [A] Hydraulic press [B] Dye set for pellet preparation along with pellet holding socket [C]

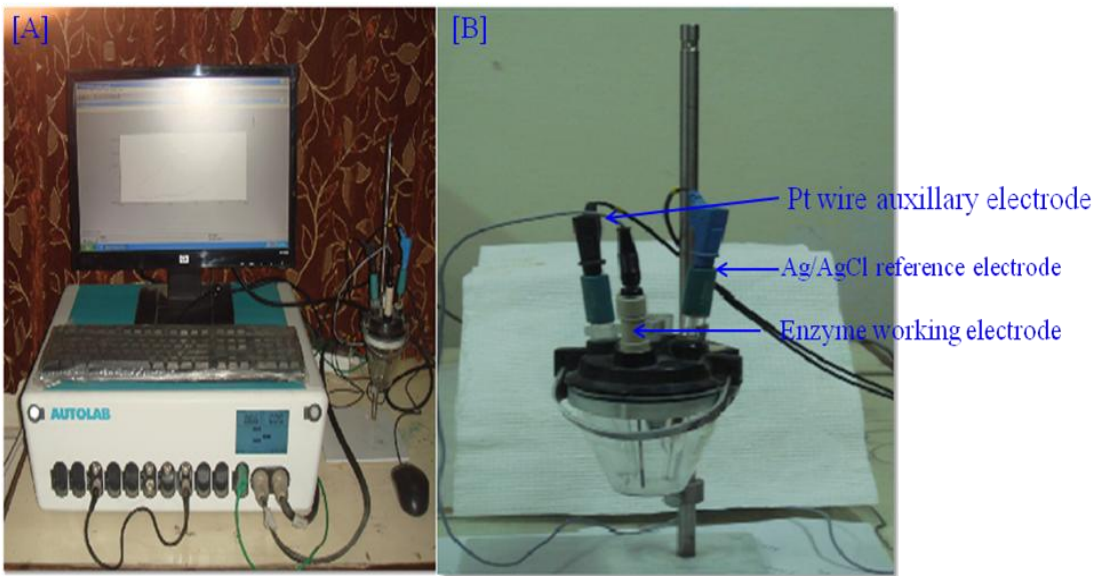


Fig. 14. Autolab Potentiostat/Galvanostat equipped with an Autolab PGSTAT-302N, general purpose electrochemical system (GPES) and frequency response analysis (FRA) software [A] and three-electrode electrochemical cell system, consisting of a working electrode (Enzymes/c-MWCNT/PANI/Pt), a silver/silver chloride (Ag/AgCl) as reference electrode and Pt wire as auxillary electrode [B]

3.6 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 optimum 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.

3.6.1 Effect of pH

To determine the optimum pH of the enzyme electrode, the pH of the reaction buffer was varied from pH 6.0 to 10.0 using the following buffer, each at a final conc. of 0.05M; pH 6.0 to 8.0 potassium phosphate buffer, pH 8.5 to 9.0 Tris-HCl buffer and 9.5 to 10.0 sodium carbonate/bicarbonate buffer. The response current in terms of mA was measured.

3.6.2 Effect of incubation temperature

To determine the optimum incubation temperature for maximum response of the enzyme electrode, the reaction mixture was incubated at different temperatures ranging from 15 to 50 ºC at 5 ºC interval. The current response in terms of mA was measured.

3.6.3 Effect of incubation time

To study the effect of incubation time for maximum response of the enzyme electrode, the reaction mixture was incubated at different time intervals upto 20s. The current in terms of mA was measured at an interval of 2s.

3.6.4 Effect of substrate (creatinine) concentration

The effect of substrate concentration on the initial velocity of co-immobilized enzymes reaction was studied varying the final concentration of creatinine from 0.1-1500 µM. The current responses were measured in terms of mA by enzyme electrode.

3.6.5 Determination of K_m and I_{max}

A Lineweaver Burk plot was made between reciprocal of creatinine concentration (I/[S]) vs enzyme electrode current response (1/I) of the enzyme reaction. Apparent K_m and I_{max} were calculated from the plot using the following Michaelis-Menten equation:

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

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

K_m = Michaelis-Menten constant

I_{max} = Maximum current response of enzyme electrode

3.7 AMPEROMETRIC DETERMINATION OF CREATININE IN SERUM AND URINE

3.7.1 Collection of blood samples

Fresh blood samples from apparently healthy individuals and diseased persons of different sex and age groups were obtained from hospital of local Pt BDS P.G. Institute of Medical Science, Rohtak. One ml of fresh blood was transferred to a vial and allowed to coagulate at room temperature (25 °C) for 30 min. It was centrifuged at 5000 rpm for 10 min at room temperature. The supernatant (serum) was collected and stored at -20 °C until use.

3.7.2 Collection of urine samples

Urine creatinine is also widely used matrix for biomonitoring the functioning of kidney. One of the major advantages of using urine creatinine in biomonitoring is its ease of collection for spot or grab (untimed) urine samples but not for 24-hr urine voids, because 24-hr collection can be cumbersome, often resulting in improper or incomplete collection. Therefore, spot urine samples, whether first-morning voids or "convenience" samples, are generally used for biomonitoring. The major disadvantages of spot urine samples include the variability in the volume of urine and the concentrations of endogenous and exogenous chemicals from void to void. Urine sample from apparently healthy persons of different sex and age groups were collected in plastic bottle containing conc. HCl enough for maintaining pH 2.0 from hospital of local Pt BDS P.G. Institute of Medical Science, Rohtak. The pH of acidified urine sample was then adjusted nearly pH 6.0 by adding NaOH (0.5 M) dropwise with constant shaking and stored at 4 °C until use.

3.7.3 Measurement of serum and urine creatinine level with enzyme electrode (Enzymes/c-MWCNT/PANI/Pt)

Creatinine level in serum and urine samples was determined by the present biosensor in the similar manner as described above for its testing/response measurement, under its optimal working conditions except that creatinine was replaced by serum and urine sample. The content of creatinine was interpolated from calibration curve **(Fig 15).**

3.7.4 Preparation of calibration curve for creatinine with enzyme electrode (Enzymes/ c-MWCNT/PANI/Pt)

The calibration curves using enzyme, the working electrode was prepared with different concentrations of creatinine in the range 0.1-750 µM under optimal working conditions, as described above. A curve was plotted between creatinine concentrations vs current in mA**.**

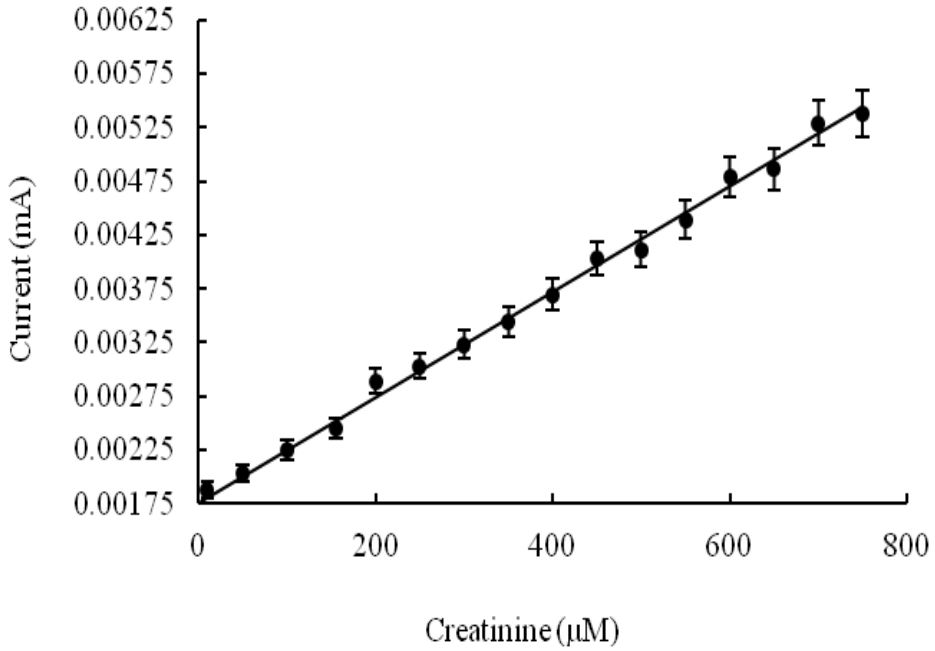


Fig. 15. Calibration curve of creatinine by c-MWCNT/PANI composite film based creatinine biosensor

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

The following parameters were studied in order to evaluate creatinine biosensor employing enzyme electrode for creatinine determination.

3.8.1 Detection limit

In order to check limit of detection of the method, amperometric responses for enzyme electrode were studied at varying creatinine concentration ranging from 0.1-1500 µM and current response was measured through potentiostat/galvanostat.

3.8.2 Percent recovery

To determine the reliability of the methods, two concentration of creatinine (0.5 mg/dl and 1.0 mg/dl) were added to the serum samples and creatinine content was determined before and after addition of creatinine into the serum samples by the present method. The % recovery of added creatinine was calculated.

3.8.3 Precision

To study reproductively of the method, the creatinine content was determined in six serum sample repeatedly six times on the same day (within batch) and in the same samples after their storage at -20 °C for one week (between batch) by the present enzyme electrode. Both within and between batches coefficients of variation (CV) in serum creatinine were calculated as follows:

Coefficient of variation (CV)

$$CV = \frac{\sigma \times 100}{\alpha}$$

Where σ = SD

α = means of series

Standard deviation (SD)

$$(\sigma) = \sqrt{\frac{\Sigma x^2}{n}}$$

Where x = deviation from mean

n = number of samples

Standard error (SE)

$$SE = \frac{\sigma}{\sqrt{n}}$$

3.8.4 Accuracy

In order to determine accuracy of present method, the creatinine values in 25 serum samples were determined by the present enzyme electrode method (y) and compared with

those obtained by chemical spectrophotometric method (x), the values obtained by both the methods were co-related using regression equation. The regression plot between the two methods were drawn and the correlation coefficient was determined by using following formula-

Correlation coefficient (r)

$$r = \frac{n\Sigma xy - \Sigma x\Sigma y}{\{n\Sigma x^2 - (\Sigma x^2)\}\{n\Sigma x^2 - (\Sigma y^2)\}}$$

Where x = value obtained by reference chemical spectrophotometric method.

y = values obtained by present enzyme electrode method

3.8.5 Determination of serum creatinine by chemical spectrophotometric (Jaffe) method

The standard chemical spectrophotometric method for creatinine determination was performed as follows:

Principle: The chemical spectrophotometric method (Jaffe reaction) was involved the alkaline sodium picrate for creatinine determination. Creatinine reacts with picric acid in alkaline conditions to form a color complex which absorbs at 510 nm. The rate of formation of color was proportional to the creatinine concentration in the sample. In the endpoint method the difference in absorbance measurements after color formation yields a creatinine value corrected for interfering substances. The Jaffe reaction method involves the following reaction:

$$\text{Creatinine + Sodium Picrate} \xrightarrow{\text{Alkali}} \text{Creatinine-Picrate complex (yellow-orange)}$$

Assay of serum creatinine: In a 15 ml test tube, 1 ml of an appropriate dilution of serum sample or of standard creatinine solution was mixed with 1 ml of 0.92 % picric acid and 4.0 ml of 0.1 N sodium hydroxide. After 45 min of incubation at room temperature, the optical density at 510 µM was read in comparison with a creatinine-free blank prepared with 1 ml of water. A standard solution of creatinine was prepared by dissolving 1 g of creatinine in 1 L of 0.1 N HCl and a fresh standard solution was prepared each day to eliminate the possibility of decomposition.

3.9 EFFECT OF INTERFERING SUBSTANCES

The amperometric current response of enzyme electrode was measured in presence of observed potential interfering metabolites such as creatine, sarcosine, ascorbic acid, uric acid, urea, bilirubin, glucose, sodium pyruvate, triglycerides & cholesterol and metal ions

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. The current in terms of mA was measured using autolab potentiostat/galvanostat.

3.10 REUSABILITY AND STORAGE STABILITY OF ENZYME ELECTORDE (Enzymes/c-MWCNT/PANI/Pt)

To reuse the working electrode, it was washed by dipping it in 0.05 M, pH 7.5 reaction buffer. The long-term storage and stability of the biosensor was investigated over a 6 months period, when enzyme electrode was stored in a refrigerator at 4 °C in 0.05 M PB, pH 7.5.