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L-amino acid biosensor based on L-amino acid oxidase immobilized onto NiHCNFe/c-MWCNT/PPy/GC electrode

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ABSTRACT

A method is described for construction of a highly sensitive amperometric L-amino acid biosensor based on covalent immobilization of goat kidney L-amino acid oxidase (LAAO) onto carboxylated multiwalled carbon nanotubes/nickel hexacyanoferrate/polypyrrole hybrid film electrodeposited on surface of glassy carbon electrode. The biosensor showed optimum response within 5 s at pH 7.5 and 35 °C, when polarized at 0.15 V vs. Ag/AgCl. There was a linear relationship between biosensor response (μA) and L-phenylalanine concentration in the range, 0.5 μM to 100 mM. The sensitivity of the biosensor was 79.31 nA cm^{-2} μM^{-1} with a detection limit of 0.5 μM (S/N = 3). The enzyme electrode was used 160 times over a period of 140 days, when stored dry at 4 °C. The biosensor was evaluated and employed for determination of L-amino acid level in fruit juices and alcoholic beverages. The biosensor showed excellent analytical characteristics along with short response time, good shelf life, and negligible interference of commonly present interferents. The film could be used for improvement of other biosensors also.

1. Introduction

L-amino acids are essential compounds for life and ubiquitously found in nature. The determination of amino acids is important in the field of food biotechnology and wine industries [1,2]. L-amino acids are important for human nutrition and affect the quality of foods including taste, aroma, and color. Beers, fruit juices, and wines all contain free amino acids [3,4]. The L-amino acids in food samples such as fruit juices, wines and beers are important to determine the quality and the origin of the food product [3]. The chiral amino acids profile is also a useful tool for the monitoring of many fermentation processes, and detection of bacterial activity [2]. Amino acids are important precursors of flavor compounds and biogenic amines. Consequently, quantification of L-amino acids for assessing the quality and genuineness of food and drinks together with their nutritional and physiological relevance is of interest. Thus, there is an ongoing interest in the development of a reliable, rapid, and accurate method of L-amino acid analysis to determine the quality of foods for nutritional and regulatory purposes.

Various analytical methods for determination of L-amino acids are available such as colorimetric [5], enzymatic method [6], gas chromatography [7], capillary electrophoresis [8] and high-performance liquid chromatography (HPLC) [9]. However, these methods have certain drawbacks such as time-consuming sample preparation, labor-intensive, expert handling, and expensive equipment. The biosensors are useful tool for such an analysis, because of their simplicity, quick response time, ease of procedure, higher specificity and sensitivity. Few L-amino acid biosensors based on preactivated nylon membrane [10], rhodinised carbon [5], graphite–Teflon electrodes [11] and electrochemical detector anion-exchange chromatography [12] have been reported. Although these biosensors have the advantages of determining L-amino acids in lesser time with high sensitivity, these suffer from problems such as stability of electrode, complexity of immobilization of enzyme, low reproducibility and the requirement of low working potential range.

Transition metal hexacyanoferrate, a class of polynuclear inorganic compounds, are well known for their excellent electrocatalytic properties [13]. These compounds are able to form stable crystalline structure film, showing reversible redox processes without dissolution in solution [14]. Nickel hexacyanoferrate (II) is a Prussian blue analog, where Fe^{3+} ions are replaced by Ni^{2+}. NiHCNFe exhibits attractive redox properties in terms of charge compensation. NiHCNFe modified electrode present unusual advantages in electroanalysis such as catalysis, enhancement of electron transport and high effective surface area [15].

Recently, the biocompatible nanomaterials have opened a new field for development of third generation biosensors based on the direct electron transfer between the enzyme and the electrode [16–19]. Among these biocompatible nanomaterials, carbon nanotubes (CNT) are one of the popular materials for designing

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conducting thin films, including those for catalytic membranes actuation and mechanical thin film applications [20–22]. Carbon nanotubes have established themselves as a good material for immobilization of enzymes, as these tubes could retain enzyme activity, due to the desirable microenvironment and enhance the direct electron transfer between the enzyme's active sites and the electrode. It has wide applications in the fields of nano-electronics, nano-medicines, and nano-engineering due to its unique characteristics. The unusual high stiffness of CNT makes it suitable ingredients for composite materials [23]. Composite materials based on integration of carbon nanotubes (CNT) and some other materials possess properties of the individual components with a synergistic effect have gained growing interest. The conducting polymers have become the most attractive one in the formation of CNTs based composites [24]. Polypyrrole (PPy) is an inherently conducting polymer due to their facile preparation, high conductivity and good environmental stability, because of inter chain hopping of electrons [25].

The present study describes for the first time, the covalent immobilization of goat kidney LAAO onto NiHCNFe/c-MWCNT/PPy/GC electrode and its application in fabrication of a highly sensitive amperometric biosensor for determination of L-amino acid (LAA). The biosensor was evaluated and employed for measurement of LAA level in different fruit juices and alcoholic beverages.

2. Experimental design

2.1. Chemicals

Carboxylated multiwalled carbon nanotubes (c-MWCNTs) (functionalized MWCNT, 12 walls, 15–30 μm length, 90% purity, nil metal content) from M/S Intelligent Materials (Panchkula, Haryana, India). N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) (purity ≥98.0%) from Fluka, Sigma–Aldrich (USA), N-hydroxysuccinimide (NHS) (purity ≥97.0%) and pyrrole (98%, purified through vacuum distillation before use) from Sisco Research Laboratories (Mumbai, India) were used. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used in all experiments. Fruit juices and wine were purchased from local market. Glassy carbon electrode was purchased from M/S Metrohm, New Delhi, India.

2.2. Extraction and purification of L-amino acid oxidase

The extraction and purification of enzyme was carried out at 4 °C as described [26] with modifications. L-amino acid oxidase (LAAO: EC 1.4.3.3) is specific for L-amino acids (LAAs), but is not specific for amino acid side chains. The frozen goat kidney (50 g) was homogenized with cold 0.05 M sodium phosphate buffer (pH 6.5, 3:1 ratio, w/v) in a chilled pestle and mortar. The extract was filtered through a muslin cloth, and the filtrate was centrifuged at 10,000 × *g* for 30 min at 4 °C. The pellet was discarded and the supernatant was collected and treated as crude enzyme. It was tested for L-amino acid oxidase activity and protein concentration by the Lowry method. The crude enzyme was purified by 0–80% ammonium sulfate precipitation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephacel using linear gradient of KCl (0.1–0.6 M). The purity of enzyme was tested by polyacrylamide gel electrophoresis (PAGE) using coomassie brilliant blue stain, which showed a single band (results not shown) indicating its apparent homogeneity (~98.2 kDa). The molecular weight of LAAO was found corresponding to protein marker with Mr of 44.6 kDa (approximate) indicating homodimeric nature of enzyme. The enzyme was purified 31.4-fold with a yield of 63% after the final step. The purified enzyme had a specific activity of 43.2 unit/mg. The activity and protein content of various enzyme preparations were estimated as follows.

2.3. Enzyme assay

The assay of L-amino acid oxidase was carried out as described by [27] with slight modification. The reaction mixture contained 0.45 ml of Tris HCl buffer (0.05 M, pH 7.5), 0.1 ml of purified enzyme (30 U/ml, 40 mg/ml). The reaction was started by adding 0.1 ml of L-Phe (0.05 M) after incubation at 37 °C for 15 min. The reaction was stopped by adding 0.2 ml of trichloroacetic acid (TCA) (25%). The reaction mixture was centrifuged and 0.5 ml of supernatant was transferred to the test tube containing 2.5 ml of borate arsenate solution (1 M of sodium arsenate and 1 M of boric acid in final volume of 1 L, adjusted to pH 6.5 with concentrated HCl, filtered if necessary) and mixed thoroughly. The solution was allowed to stand for 30 min at room temperature. The change in A_{300} was read in a UV spectrophotometer (Shimagzu 1700, Japan). Enzyme was omitted from the blank solution.

One unit of enzyme is defined as amount of enzyme required to give an absorbance of 0.03 at 300 nm from oxidation of L-phe per min/ml under standard assay conditions.

Protein content in various enzyme preparations was determined by the Lowry method using bovine serum albumin (BSA) as standard protein.

2.4. Construction of LAAO/NiHCNFe/c-MWCNT/PPy modified GC electrode

2.4.1. Electrodeposition of c-MWCNT/NiHCNFe/PPy onto GC electrode

Prior to electrodeposition, the GC electrode (1.5 cm^2) was polished with 0.05 mm alumina slurry followed by ultrasonic cleaning with DW. One milligram c-MWCNT was suspended in 4 ml mixture of concentrated H_2SO_4 and HNO_3 in 3:1 ratio (v/v) and ultrasonicated for 2 h to get a finally dispersed black colored solution of c-MWCNTs. The c-MWCNT solution was washed with DW thoroughly to remove acid. The dispersed c-MWCNT solution (1.0 ml) was mixed into a mixture of 0.5 ml 0.2 M EDC and 0.5 ml 0.2 M NHS (pH adjusted to 6.0) and kept at room temperature for 1 h. The electrodeposition of c-MWCNT/NiHCNFe/PPy onto the cleaned GC electrode was carried out by immersing the electrode into a mixture of 22 ml of 0.1 M KCl, 10 μl of 5 mM pyrrole, 200 μl of EDC & NHS activated c-MWCNT, 1.0 ml of 5 mM of $K_3Fe(CN)_6$, 200 μl of 5 mM $NiCl_2$ and then applying a triangular potential sweep in the range from −0.3 to +0.9 V (vs. Ag/AgCl) for 10 cycles at a scan rate of 50 mV s^{-1}. The resulting c-MWCNT/NiHCNFe/PPy modified GC electrode was rinsed with DW to remove unbound matter and kept in a dry Petri-plate at 4 °C.

2.4.2. Immobilization of LAAO onto c-MWCNT/NiHCNFe/PPy modified GC electrode

NiHCNFe/c-MWCNT/PPy/GC electrode was dipped into 1.5 ml of LAAO solution (30 U/ml, 40 mg/ml protein) and kept overnight at 4 °C for covalent interaction. The resulting electrode with immobilized LAAO was washed 3–4 times with 0.1 M sodium phosphate buffer, pH 7.5 to remove residual unbound protein. The resulting LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode was used as working electrode and stored at 4 °C, when not in use.

2.4.3. Characterization of enzyme electrode (LAAO/NiHCNFe/c-MWCNT/PPy/GC)

The fabricated electrode was characterized by scanning electron microscope (SEM, Zeiss EV040) and Fourier transform infrared

(FTIR) spectroscopy techniques at different stages of its construction. To record FTIR spectra of hybrid material deposited onto GC electrode, the hybrid material was scrapped off the GC electrode, mixed with dried KBr and its pellet was formed by hydraulic pellet press, The pellet was kept into the socket of the FTIR spectrometer and its spectra was recorded.

2.4.4. Cyclic voltammetry measurement and testing of L-amino acid biosensor

All electrochemical characterizations and measurements were carried out using a conventional three-electrode system consisting of a LAAO/cMWCNTs/NiHCNFe/PPy-modified GC electrode as the working electrode, an Ag/AgCl electrode as the reference electrode and Pt wire as the auxiliary electrode connected through potentiostat (Echochemie, Autolab, the Netherlands). Cyclic voltammetric measurements were carried out in a three-electrode cell containing 0.1 ml of L-Phe (0.5 mM) and 25 ml Tris–HCl buffer (0.05 M, pH 7.5). Current was measured using cyclic voltammetry (CV) in the potential range between 0 and +0.5 V. All electroanalytical measurements were performed at room temperature.

The following electrochemical reactions occurred during the current response measurements of the L-amino acid biosensor:

$$\text{L-amino acid} + O_2 + H_2O_2 \xrightarrow{\text{LAAO}} \alpha\text{-Keto acid} + NH_3 + H_2O_2$$

$$H_2O_2 \xrightarrow{0.15\ \text{V}} 2H^+ + O_2 + 2e^-$$

$$2e^- \xrightarrow{\text{LAAO/NiHCNFe/c-MWCNT/PPy}} \text{GC electrode}$$

2.5. Optimization of L-amino acid biosensor

To optimize working conditions of the biosensor, effects of pH, incubation temperature, time and substrate (L-phenylalanine) concentration on biosensor response were studied. To determine optimum pH, the pH was varied between pH 4.0–9.5 at an interval of pH 0.5 using the following buffer, each at a final concentration of 0.05 M: pH 4.0–5.5 sodium succinate buffer, 6.0–7.0 sodium phosphate buffer and pH 7.5–9.5 Tris HCl buffer. Similarly to determine optimum temperature the reaction mixture was incubated at different temperature (15–50 °C). To determine the optimal response time, the current response was measured from 2 to 12 s at intervals of 2 s. The effect of L-Phe concentration on biosensor response was determined by varying the concentration of L-Phe in the range 0.001–100 mM. Apparent K_m and I_{max} for L-Phe were calculated from a Lineweaver–Burk plot based on the following equation:

$$\frac{1}{I} = \frac{K_m}{I_{max}}\frac{1}{[S]} + \frac{1}{I_{max}}$$

where K_m is the Michaelis–Menten constant, I_{max} is the maximum current, and [S] is the L-Phe concentration (Fig. 1a and b).

2.6. Evaluation of L-amino acid biosensor

The following criteria were studied to evaluate the analytic performance of the biosensor, e.g. linearity, analytical recovery, detection limit, precision and correlation. Analytical recovery was studied using mixed juice with two different standard concentrations (5 mM and 10 mM) of L-Phe. To test the reproducibility and reliability of the present biosensor, L-amino acid in six fruit juice samples was measured five times on single day (within batch) and five times again after their storage at −20 °C for 1 week (between batch). Both within and between batches

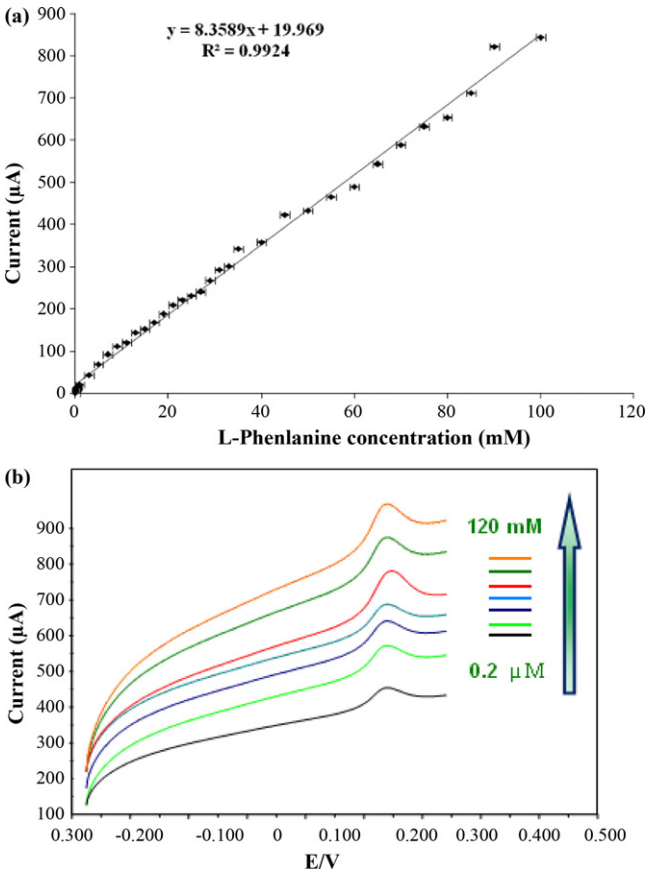


Fig. 1. (a) Effect of L-phenylalanine concentration on response of L-amino acid biosensor based on LAAO/NiHCNFe/c-MWCNT/PPy based glassy carbon (GC) electrode. (b) Linear square voltammetry net current responses of biosensor in 0.05 M Tris HCl buffer (pH 7.5) containing different concentrations (0.2 μM to 120 mM) of L-phenylalanine at a scan rate of 50 mV s^{-1}.

coefficients of variation (CV) in fruit juices 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)

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

To study the correlation, the L-Phe level was measured in 15 brands of commercially available fruit juices by the standard colorimetric method and the present method and their correlation was studied using regression equation. To examine the long-term storage stability, the activity of enzyme electrode was tested up to 4 months at an interval of 1 week, when stored at 4 °C. To reuse the enzyme electrode, it was washed 3–4 times with reaction buffer, before its use in next assay.

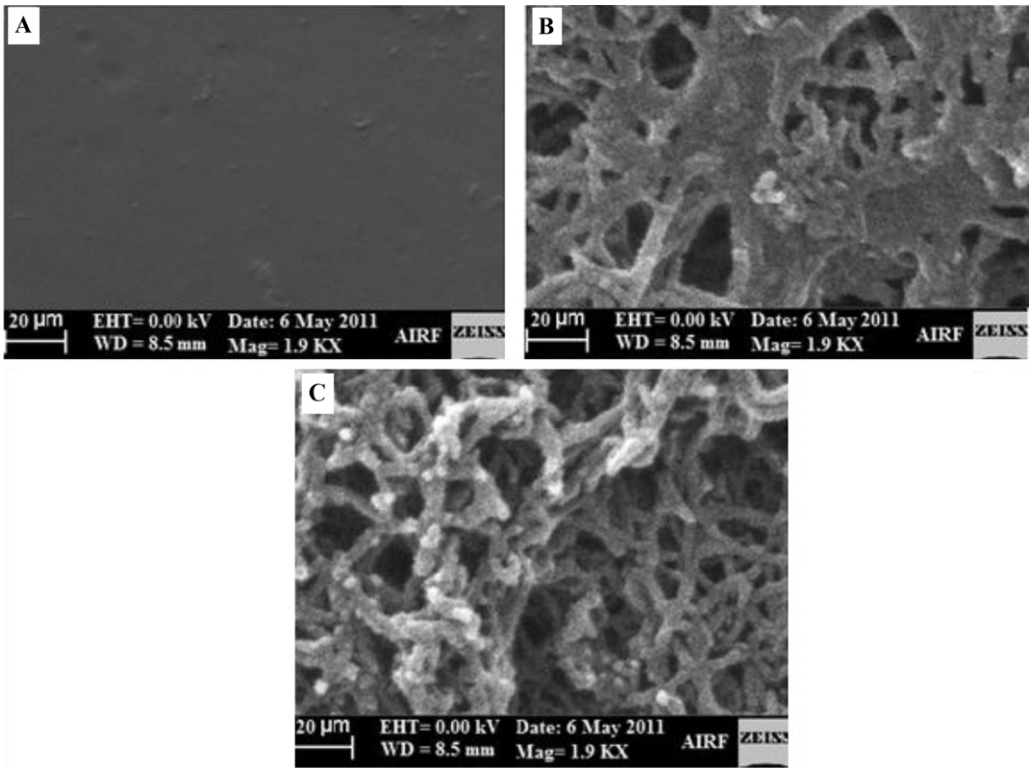


Fig. 2. Scanning electron micrograh of (a) Bare GC electrode, (b) NiHCNFe/c-MWCNT/PPy electrodeposited onto GC electrode and (c) scanning electron micrographs of LAAO/NiHCNFe/cMWCNT/PPy electodeposited onto GC electrode.

2.7. Application of biosensor in measurement of L-amino acid in fruit juices and alcoholic beverages

The commercially available fruit juices and alcoholic beverages of different brands were collected from the local market and the samples were diluted by 10 times with Tris HCl buffer (0.05 M, pH 7.5). Total LAA content in fruit juices and alcoholic beverages was determined by the biosensor in the same manner as described above for its response measurement under its optimal working conditions except that L-Phe was replaced by these samples. The current (μA) was recorded, and the amount of total LAA content was extrapolated from standard curve between L-Phe concentrations and current (μA) prepared under optimal working conditions.

3. Results and discussion

3.1. Electrode surface characterization by SEM and FTIR

The SEM images of the surface of bare GC electrode, NiHCNFe/c-MWCNT/PPy/GC electrode and LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode are shown in Fig. 2(a)–(c) respectively. The stepwise modification of electrode could be seen clearly from these SEM images. The SEM image of the bare GC electrode showed a smooth and featureless morphology (Fig. 2a). The NiHCNFe/c-MWCNT/PPy/GC micrograph showed homogenous coating, which indicate that c-MWCNT, NiHCNFe and polypyrrole were well dispersed over the GC electrode. The composite film exhibited a interwoven fibrous, more uniform and porous structure (Fig. 2b), hence effective surface area was larger, which was favorable for the interaction as well oxidation of analytes. On immobilization of LAAO more globular structural morphology appears due to the covalent interaction between NiHCNFe/c-MWCNT/PPy/GC electrode with LAAO (Fig. 2c).

Fig. 3 displays the FTIR spectra for NiHCNFe/c-MWCNT/PPy/GC (curve a) and LAAO/NiHCNFe/c-MWCNT/PPy/GC (curve b). The FTIR spectrum of electrodeposited NiHCNFe/c-MWCNT/PPy/GC composite (curve a) shows absorption band of with characteristic IR peak being observed at 1710 cm^{-1} indicating the C=O stretching, C—O stretching at 1225 cm^{-1}, 2050 cm^{-1} appears which reveals the CN groups or incorporation of NiHCNFe film. The intensity of bands at 1045 cm^{-1}, 1125 cm^{-1} and 1150 cm^{-1} display background of pyrrole bands. The presence of these peaks reveals the existence of polypyrrole, NiHCNFe and cMWCNT on the GC electrode. The FTIR spectrum of LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode (curve b) showed characteristic peaks at 1550 cm^{-1} and 1600 cm^{-1} attributed to the primary and secondary amide linkages between —NH_2 on the surface group on the surface of enzyme molecules and free —COOH groups of c-MWCNT. The fabrication of the LAA biosensor based on NiHCNFe/c-MWCNT/PPy/GC electrode is summarized in Scheme 1A and B. Firstly, pyrrole was electropolymerised on the surface of GC electrode, as this method is easy and

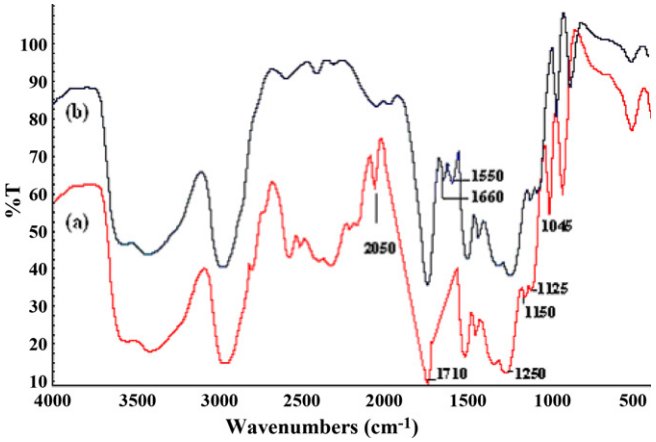
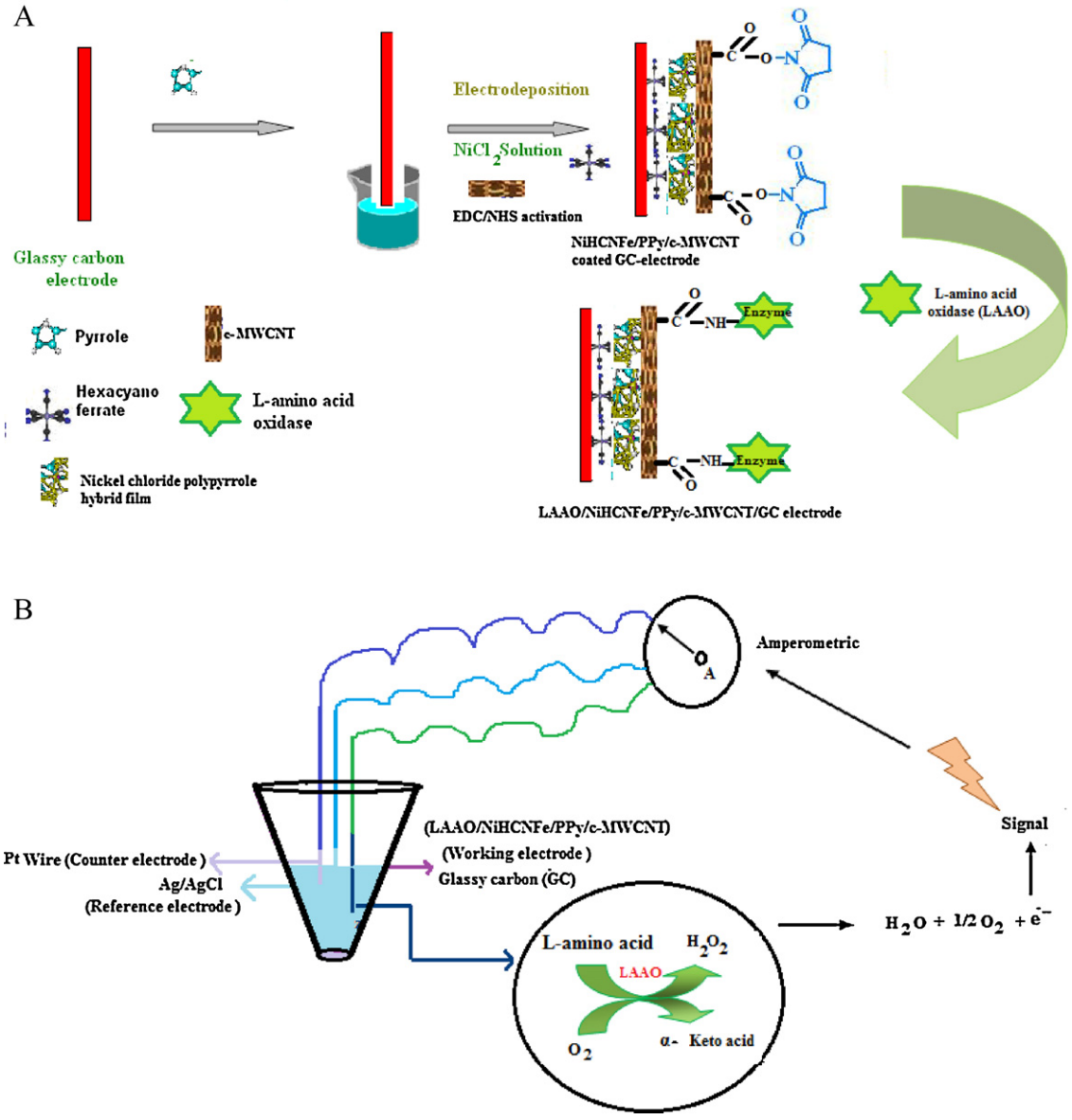


Fig. 3. FTIR spectra of (a) NiHCNFe/c-MWCT/PPy and (b) LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode.



Scheme 1. (A) Schematic diagram of the L-amino acid biosensor. (B) Schematic representation of chemical reaction involved in the fabrication of LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode.

the layer thickness could be controlled. After that cMWCNT and NiHCNFe were co-electrodeposited on the polypyrrole coated GC electrode. To construct the working electrode, LAAO was immobilized onto NiHCNFe/c-MWCNT/PPy/GC electrode through covalent coupling. Incubation of LAAO solution with activated NiHCNFe/c-MWCNT/PPy/GC electrode leads to effective collision between —COOH group of cMWCNT and —NH_2 groups on the surface of the LAAO to form an amide bond (—CO NH—) through EDC and NHS chemistry.

3.2. Electrochemical characterization

To characterize the electronic and transport properties of NiHCNFe/c-MWCNT/PPy hybrid film, electrochemical impedance experiments were performed. Fig. 4 showed Nyquist plots of the imaginary vs. the real part of the impedance for bare GC electrode Fig. 4(a) and the hybrid material (R_{CT} 625 Ω) Fig. 4(b). For both systems, the intersection at high frequencies on the real axis corresponds to the electrolyte resistance. The impedance values attained for the hybrid material are smaller than for bare GC electrode (R_{CT} 410 Ω) and LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode (R_{CT} 580 Ω) Fig. 4(c). The increased R_{CT} value of LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode was due to the immobilization of enzyme onto NiHCNFe/c-MWCNT/PPy/GC surface. This increase in R_{CT} is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 kHz) and cause hindrance to the electron transfer [28]. The presence of conducting polymer improve both electronic and ionic conductivity of the hybrid film: they act as a “wire” connecting the redox sites of NiHCNFe, thus increasing the charge transfer rate.

3.3. Cyclic voltammetry and response studies

Fig. 5 showed cyclic voltammogrammes for bare GC electrode (curve a), NiHCNFe/c-MWCNT/PPy/GC (curve b) and LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode (curve c), respectively. Fig. 5 (curve a) shows cyclic voltammograms (CV) of GC

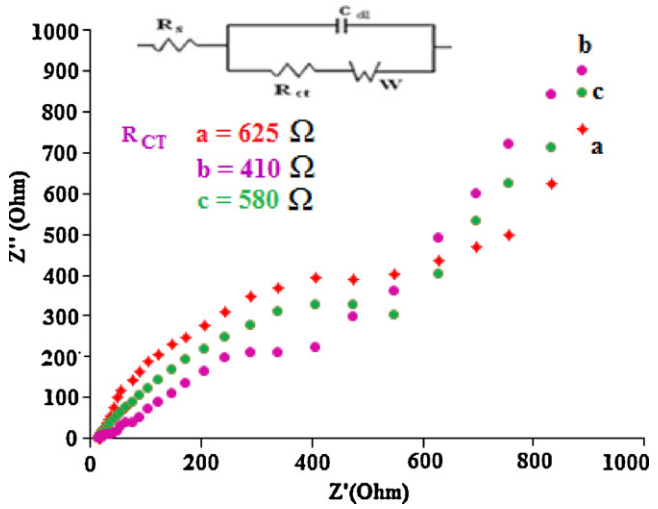


Fig. 4. Impedance spectra of (a) bare GC electrode, (b) NiHCNFe/c-MWCNT/PPy and (c) LAAO/NiHCNFe/c-MWCNT/PPy electrode in 0.05 M Tris HCl buffer, pH 7.5 containing $[K_3Fe(CN)_6]^{3-}/[K_3Fe(CN)_6]^{4-}$ (0.05 M).

electrode in a solution containing 0.1 M KCl solution along with 0.5 mM L-Phe. The bare GC electrode (curve a) shows no redox peak, while the c-MWCNTs/NiHCNFe/PPy/GC electrode (curve b) shows redox peak current which leads to an increased electroactive area. After immobilization of enzyme onto the modified electrode, the current response get enhanced, which revealed that the NiHCNFe/c-MWCNT/PPy hybrid film provides a biocompatible environment to the enzyme, and resulting in a fast electron transfer between enzyme and electrode and thus increases its sensitivity (79.31 nA cm^{-2} μM^{-1}). When 0.5 mM L-Phe was added, well-defined oxidation (0.15 V) and reduction peaks (0.08 V) (curve b) were observed, which clearly indicate the catalytic properties of the modified electrode. It can be seen that the sensor responded very rapidly, producing 95% of the steady-state current within 5 s (Fig. 5).

3.4. Optimization of L-amino acid biosensor

The biosensor showed maximum response at pH 7.5 (Fig. 6a), which is lower than that of earlier reported LAA biosensors, bound to cellophane and "O" ring on Pt electrode (pH 7.8) [29], ferrocene mono carboxylic acid (pH 8.3) [30], plexi cell (pH 8.8) [6], poly carbamoyl sulfonate hydrogel (pH 9.5) [12], but comparable to nylon membrane based electrode (pH 7.3) [10] rhodinised carbon electrode (pH 7.5) [5]. Therefore, a pH of 7.5 was used in

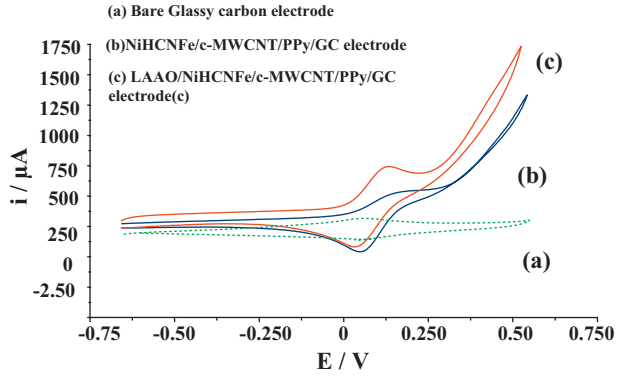


Fig. 5. Cyclic voltammograms (CVs) for electrochemical deposition of (a) bare GC electrode, (b) NiHCNFe/c-MWCNT/PPy/GC electrode and (c) LAAO/NiHCNFe/c-MWCT/PPy/GC electrode.

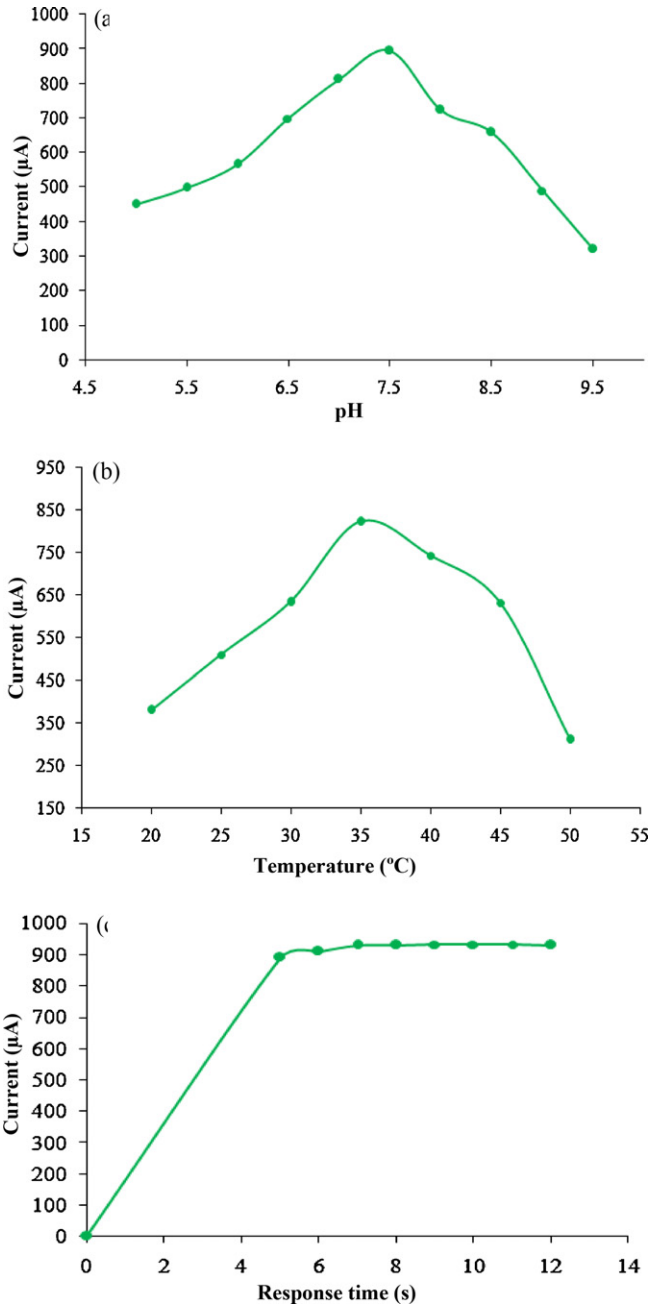


Fig. 6. Effect of pH (a) temperature, (b) on the current response and (c) effect of response time (s) on modified LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode.

subsequent experiments. The optimum working temperature of biosensor was reported at 35 °C (Fig. 6b), which is comparable to that of cellophane and "O" ring on Pt electrode (35 °C) [29], ferrocene mono carboxylic acid (35 °C) [30], but lower than plexi cell (40 °C) [6], poly carbamoyl sulfonate hydrogel (40 °C) [12], which favors the use of modified enzyme electrode. The effect of applied potential was maximum at 0.15 V, hence in subsequent electrochemical studies, the enzyme electrode was polarized at 0.15 V. The biosensor showed optimum response observed within 5 s (Fig. 6c). A fast response of present biosensor might be due to improved electronic and ionic transport capacity of NiHCNFe/c-MWCNT/PPy hybrid film, as c-MWCNT provided a 3D electro-conductive network, which extended throughout the ion conductive matrix of NiHCNFe [25] and PPy facilitate the fast electron transport. Also,

Table 1
Analytical recovery of added L-phenylalanine in fruit juice, as measured by LAA biosensor based on LAAO bound to c-MWCNT/NiHCNFe/PPy/GC electrode.

L-amino acid added (mM)	L-amino acid (mM)	% Recovery
–	5.25	100
5	10.19	98.4
10	14.89	96.4

Reported values were averaged from three independent analyses of sample ($n = 3$).

there might be a synergistic catalytic effect between NiHCNFe and c-MWCNT toward the oxidation of H_2O_2.

3.5. Amperometric determination of LAA

An amperometric method for determination of LAA was developed using the present biosensor. The method is based on measurement of current i.e. e^- generated from electro-oxidation of H_2O_2, a product of oxidation of LAA by immobilized LAAO.

3.6. Evaluation of biosensor

3.6.1. Linearity

There was a linear relationship between the current (in μA) and the L-Phe concentration in the range 0.5 μM–100 mM, which is better than biosensor based on LAAO immobilized onto matrix of cellophane and "O" ring on Pt electrode (0.1–0.5 mM) [29], plexi cell (0.2–3 mM) [6] and poly carbamoyl sulfonate hydrogel (0.2–1.0 mM) [12].

3.6.2. Detection limit

The detection limit of the present sensor was 0.5 μM (S/N = 3), which is better than that of enzyme electrode based on cellophane and "O" ring on Pt electrode (1.0 mM) [29], plexi cell (0.005 mM) [6] and poly carbamoyl sulfonate hydrogel (2.75 μM) [12].

3.6.3. Analytical recovery

The average recoveries of L-Phe added to fruit juice (at levels of 5 mM and 10 mM) was 98.4% and 96.4% demonstrating the satisfactory reliability of the present biosensor (Table 1).

3.6.4. Precision

Within-batch and between-batch coefficients of variation for the determination of LAA in mixed fruit juice on the same day and after 1 week of storage were 1.64% and 2.6%, respectively (Table 2). These high precisions highlight the good reproducibility and consistency of the present method, which can be attributed to the covalent immobilization of LAAO onto the NiHCNFe/c-MWCNT/PPy/GC electrode.

Table 2
Within and between assay coefficients of variation for determination of LAA in fruit juices, as measured by LAA biosensor based on LAAO bound to NiHCNFe/c-MWCNT/PPy/GC electrode.

n[a]	Mean (mM)	CV (%)
Within assay (6)		
5.24		
5.26		
5.21	5.283	1.64
5.25		
5.27		
5.25		
Between assay (6)		
5.29		
5.42		
5.43	5.38	2.6
5.21		
5.62		
5.31		

[a] $n = 6$.

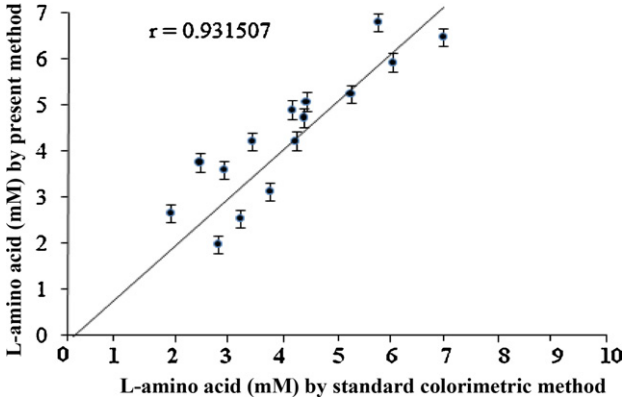


Fig. 7. Correlation between fruit juices L-amino acid values measured by colorimetric method (x axis) and the current method (y axis) employing the L-amino acid biosensor based on LAAO/NiHCNFe/c-MWCNT/PPy/GC composite film.

3.6.5. Accuracy

To determine the accuracy of the method, the level of LAA in alcoholic beverages was determined by standard colorimetric method (x) and the present method (y). The LAA values obtained by both the methods were in agreement with each other with a good correlation ($r = 0.931$) (Fig. 7) indicating the high accuracy of the method. These evaluation studies showed that the method was fairly reliable with high recovery and in agreement with the standard method.

3.7. Application of LAA biosensor

The LAA level in commercially available fruit juices and alcoholic beverages as measured by the current biosensor (Table 3), which ranged from 1.85 mM ± 0.3 to 8.92 mM ± 0.4 (mean ± SD) in fruit juices, 1.15 mM ± 0.2 to 1.88 mM ± 0.1 (mean ± SD) in different brands of alcoholic beverages and 1.96 mM ± 0.1 to 6.89 mM ± 0.3 (mean ± SD) in beer samples. These values are comparable to those stated in earlier reports [1,31].

3.8. Interference study and selectivity

The interference study of the current LAA biosensor was carried out by comparing the amperometric response before and after

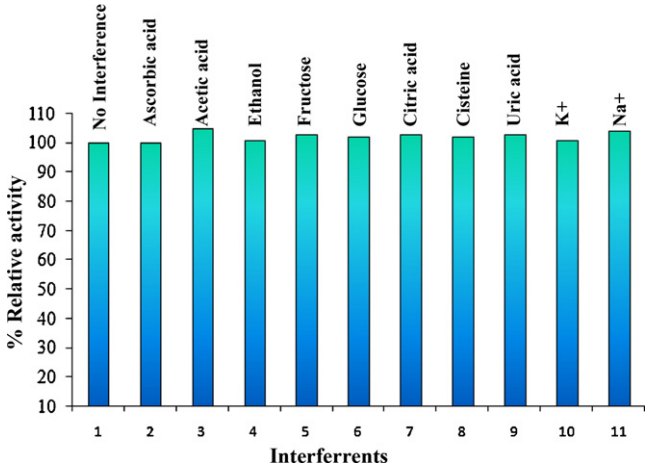


Fig. 8. Effect of potential interferents on biosensor response.

Table 3
L-amino acid levels in different brands of fruit juices and alcoholic beverages as measured by the LAAO/NiHCNFe/c-MWCNT/PPy/GC electrode.

Samples[a]	Total L-amino acid content (mM) by present method (means ± SD, $n = 3$)
Fruit juices	
Pineapple	5.44 ± 0.2
Orange	3.95 ± 0.3
Guava	5.78 ± 0.2
Mosambi	5.23 ± 0.1
Strawberries	3.62 ± 0.2
Black grapes	8.83 ± 0.5
Green grapes	8.92 ± 0.4
Litchi	1.85 ± 0.3
Appy fizz	4.41 ± 0.2
Lemon	2.38 ± 0.1
Mango	3.98 ± 0.3
Mixed	5.82 ± 0.2
Range = 1.85 mM ± 0.3 to 8.92 mM ± 0.4	
Alcoholic beverages	
Brand 1	1.82 ± 0.1
Brand 2	1.15 ± 0.2
Brand 3	1.22 ± 0.5
Brand 4	1.55 ± 0.3
Brand 5	1.87 ± 0.2
Brand 6	1.35 ± 0.3
Brand 7	1.88 ± 0.1
Brand 8	1.83 ± 0.5
Range = 1.15 mM ± 0.2 to 1.88 mM ± 0.1	
Beer samples	
Brand 1	3.85 ± 0.1
Brand 2	4.11 ± 0.2
Brand 3	3.32 ± 0.2
Brand 4	5.95 ± 0.3
Brand 5	5.68 ± 0.5
Brand 6	2.85 ± 0.2
Brand 7	2.23 ± 0.4
Local brand 8	1.96 ± 0.1
Fruit beer 9	6.89 ± 0.3
Range =1.96 mM ± 0.1 to 6.89 mM ± 0.3	

Reported values were averaged from three independent analyses of samples ($n = 3$). SD = standard deviation.
[a] Samples were diluted 1:10 times (sample:buffer).

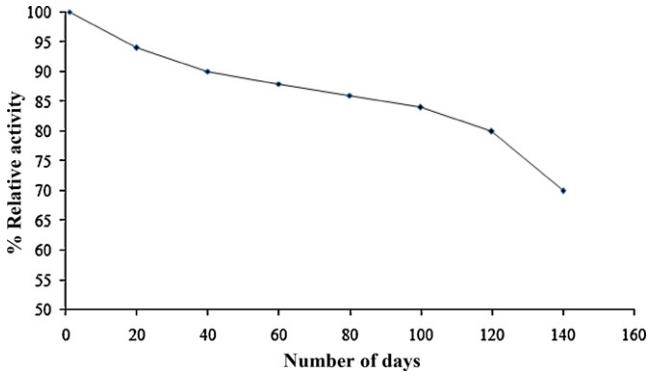


Fig. 9. Effect of storage at 4 °C on the response of LAAO/NiHCNFe/c-MWCNT/PPy electrodes.

adding some possible interferents such as ascorbic acid, calcium citrate, glucose, fructose, Na^+ and K^+ along with 100 μM L-Phe in 0.05 M Tris HCl buffer (pH 7.5). The results showed that ascorbic acid, calcium citrate, glucose, fructose, Na^+ and K^+ have practically negligible interference (Fig. 8).

3.9. Long-term stability of enzyme electrode

The shelf life and reproducibility of the enzyme electrode was investigated by measuring the response signal seven times in continuous manner which exhibited a better reproducibility, storage at 4 °C. In our study the biosensor was quite stable which has proven its stability for more than three months with a slight loss of sensitivity. The enzyme electrode maintained 70% of its initial activity even after 140 days of regular 160 uses (Fig. 9). These observations indicates the better shelf life of enzyme electrode than earlier enzyme electrodes [5,6,12].

4. Conclusion

The use of a new organic/inorganic hybrid material consisting of polypyrrole, nickel hexacyanoferrate and carboxylated multi-walled carbon naotubes, as a support for immobilization of LAAO has resulted into the better analytic performance of LAA biosensor than that of earlier reported L-amino acid biosensors. The film could be used for improvement of other biosensors also.

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