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ORIGINAL PAPER

Fabrication of an amperometric D-amino acid biosensor based on nickel hexacyanoferrate polypyrrole hybrid film deposited on glassy carbon electrode

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Abstract A nickel hexacyanoferrate polypyrrole film was synthesized through an electrochemical two-step methodology leading to a very stable and homogenous robust hybrid film. A highly sensitive, specific and rapid amperometric D-amino acid biosensor was constructed by immobilizing D-amino acid oxidase on this film deposited over the surface of a glassy carbon electrode. The modified electrode was characterized by scanning electron microscopy, electrochemical impedance spectroscopy and Fourier transform infrared spectrophotometry. The biosensor showed optimum response within 1 s, when operated at 50 mV s^{-1} in 0.01 M Tris HCl buffer, pH 7.0 at 30 °C. The biosensor exhibited excellent sensitivity with a detection limit of 1.5 μM ($S/N = 3$) for D-amino acids and wider linear range, 20–500 μM. Analytical recovery of added D-alanine (5 and 10 mM) in serum samples was 98.00 and 98.80 %, respectively. Within-batch and between-batch coefficients of variation in serum samples were 1.36 and 2.77 %, respectively. The enzyme electrode was used more than 50 times over 2 months, when stored at 4 °C. The proposed modified electrode exhibited sufficient mechanical and electrochemical stability and high sensitivity compared to earlier electrochemical D-amino acid biosensors. Interference by ascorbic acid and uric acid, the main interfering species in the biological samples, was negligible.

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Keywords D-Amino acid · D-Amino acid biosensor · Nickel hexacyanoferrate polypyrrole hybrid film · GC electrode

Introduction

Monitoring of D-amino acid content in biological materials has become increasingly important for different purposes. The white and grey matter of Alzheimer brains contain D-amino acid one to four times higher than the respective region of normal brains [1]. The individuals with renal failure have high levels of D-amino acids in urine and serum [2]. Therefore, the determination of D-amino acids is very important in clinical chemistry [3]. The occurrence of D-amino acids in food is normally associated with a decrease in protein digestibility, thus affecting the bioavailability of amino acids and ultimately impairing the nutritional value of food [4]. The content of D-alanine >1 ppm is indicative of bacterial contamination of the juice [5]. The content of free D-Ala in milk samples increases significantly, when milk is stored at 4 °C for long time. Several D-amino acids have also been detected in fermented products, where they arise from "natural" processes (D-Ala, D-Gln, and D-Asp are abundant in cheese, yoghurt, wine and vinegar).

Various techniques, such as spectrophotometric [6], HPLC [7], electrophoresis [8] etc., have been employed for determination of amino acids in food, beverages, and other biological samples. Besides being highly sensitive, these methods have some limitations, such as labor-intensive, time-consuming sample pretreatment, expensive equipment and require a skilled person to operate. Nevertheless, biosensing methods are simple, sensitive, rapid and do not require pretreatment of sample.

DAO is an FAD-containing flavoenzyme known to oxidize the D-isomers of amino acids into α-ketoacid, H_2O_2 and NH_3 with absolute stereospecificity

$$\text{D-Aminoacid} + O_2 + H_2O \xrightarrow{\text{DAO}} \alpha\text{-ketoacid} + H_2O_2 + NH_3 \quad (1)$$

DAO have been immobilized on different transducers by employing various methods of immobilization such as cross-linking on eggshell membrane [9], adsorption on prussian blue (PB) and Nafion layer [10] and covalent coupling [11] for determining D-amino acid with high sensitivity in less time [12]. However, these DAO-based biosensors have some common drawbacks such as instability due to leaching of enzyme from membrane/support, low conductivity, high response time, low reproducibility, requirement of high working electrode potential and low stability in electrolytes containing Na^+, which is present in most biological fluids such as blood, urine as well as biological tissues. Therefore, there is still, a demand for the development of a biosensor, which is stable, reproducible and sensitive for D-amino acid determination in various real samples. The use of metal hexacyanoferrate (NiHCNFe) film in modified electrodes has been extensively studied, as this material has been found to be an excellent catalyst for H_2O_2 oxidation at low potential, minimizing the interference caused by reductant species. NiHCNFe also showed a well-defined, reversible and reproducible electrochemical response in supporting electrolytes containing not only K^+ but also Na^+, Rb^+ and Cs^+ contrary to PB that only shows good electroactivity in electrolytes containing K^+. The novelty of the present work is the incorporation of NiHCNFe into the structure of a polypyrrole (PPy) network to construct a D-amino acid biosensor. The resulting composite showed a robust film with better stability, good conductivity as well as capability of catalyzing the electro-oxidation of H_2O_2 efficiently in the presence of Na^+ and K^+.

Materials and methods

Reagents

$NiCl_2$, $K_3Fe(CN)_6$, and KCl of AR grade were from Merck, Mumbai, India, pyrrole was from SRL Mumbai, glassy carbon electrode (GC) was from Metrohm, Delhi. D-Amino acid oxidase was purified from goat kidney and its homogeneity was checked in poly-acrylamide gel electrophoresis. All other chemicals were of analytical reagent (AR) grade. Double distilled (DW) water was used throughout the study.

Instrumentation

Cyclic voltammetry (CV) and amperometric measurements were performed on a potentiostat/galvanostat (Autolab, Eco Chemie, The Netherland. Model: AUT83785) with a three electrode system composed of a Pt wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode and DAAO/NiHCNFe/PPy/GC as a working electrode. Scanning electron microscopy (SEM) studies were carried out on a commercial basis at J.N. University, New Delhi. Fourier transform Infra-red (FTIR) spectroscopy was recorded in FTIR spectrometer (Thermo Scientific, USA).

Extraction, purification and assay of D-amino acid oxidase from goat kidney

Extraction and purification of DAO from fresh goat kidney cortex region was done as described [13]. The activity and protein content of enzyme were measured as previously described [14] with modifications. One activity unit was defined as the production of 1 μmol of hydrogen peroxide per min/ml at 25 °C. All assays were performed in triplicate.

Pretreatment of glassy carbon electrode

Before modification, glassy carbon electrode (3 mm) surface was polished with successively fine grade aqueous alumina slurry (grain size, 0.5 μm) and then sonicated for 5 min in DW. Working of the electrode was checked with the potentiostat/galvanostat (Autolab, model PGSTAT30) by applying potential between −0.75 and 0.75 V at a scan rate of 50 mV/s in 0.1 M KCl electrolyte.

Construction of DAAO/NiHCNFe/PPy/GC electrodes

Nickel hexacyanoferrate/polypyrrole hybrid deposition

NiHCNFe/PPy film was formed in a two-step procedure. In the first stage, a hexacyanoferrate (III)-doped polypyrrole film was deposited onto the electrode by applying triangular potential sweep (at scan rate 50 mV s^{-1}) in the potential range of −0.75 to 0.75 V versus Ag/AgCl reference electrode in solution containing 2×10^{-2} M $K_3Fe(CN)_6$ + 0.1 M KCl + 1.5×10^{-2} M pyrrole. The second step consisted of placing the $Fe(CN)_6^{3-}$/PPy-modified electrode in a solution containing 2×10^{-2} M $NiCl_2$ + 0.1 M KCl during 2 h. After that, the electrode was cycled 20 times in the same electrolyte, transferred to a solution containing 0.1 M KCl and cycled until stabilization of the peaks corresponding to nickel hexacyanoferrate oxidation/reduction.

Fabrication of D-amino acid biosensor

D-Amino acid oxidase (DAO) was immobilized onto the electrode surface after its bovine serum albumin (BSA)–glutaraldehyde (GA) cross-linking as described [15]. To prepare 30 μL of this mixture, 10 μL of glutaraldehyde (2.5 % v/v diluted in water) and 40 mg of BSA were added to 25 μL of the enzyme solution in 1 mL of 0.01 M Tris HCl buffer pH 7.0. 10 μL of this mixture was placed onto the surface of the working electrode and allowed to dry at room temperature. The electrode was washed in 0.01 M Tris HCl buffer pH 7.0 to remove unbound protein. The fabricated electrode was characterized by SEM, EIS and FTIR spectroscopy. Protein concentration in wash out solution was determined by Lowry method. Enzyme electrode was stored at 4 °C when not in use.

Cyclic voltammetric measurement and testing of D-amino acid biosensor

Cyclic voltammogram (CV) of DAO/NiHCNFe/PPy/GC electrode was recorded in potentiostat–galvanostat from −0.75 to 0.75 V versus Ag/AgCl as reference and Pt wire as counter electrode in a 0.01 M Tris HCl (pH 7.0) containing 100 μM D-alanine. The maximum response was observed at 0.25 V and hence subsequent studies were carried out at this voltage. The reaction was started by adding D-alanine solution to reach a final concentration of 10 mM and the current (mA) generated was recorded at 0.25 V.

Optimization of D-amino acid biosensor

The experimental conditions affecting the biosensor response were studied in terms of effect of pH, incubation temperature, time and substrate (D-alanine) concentrations. To determine optimum pH, the pH was varied from pH 4.5 to 8.5 at an interval of 0.5. Similarly to determine optimum temperature, the reaction mixture was incubated at different temperatures (20–50 °C) and times (1–20 s). The effect of D-alanine concentration on biosensor response was determined by varying the concentration of D-alanine from 20 to 500 μM. The amperometric response was also measured in the presence of potential interfering metabolites present in biological fluids, such as glucose, urea, ascorbic acid, pyruvate, and cholesterol at a final concentration of 200 μM in 0.01 M Tris HCl buffer, pH 7.0.

Determination of D-amino acid in real samples

Fresh serum and urine samples from apparently healthy individuals and persons suffering from kidney disorder of different sex/age groups were collected from the hospital of the local Pt B.D.S P.G. Institute of Medical Science, Rohtak (India). The serum, urine and commercial fruit juice samples were diluted by fivefold in buffer and analyzed for D-amino acid content using the present biosensor. To determine D-amino acids content in different samples, same procedure was used as described for sensor's response measurement under the optimal working conditions except that D-amino acids was replaced by the sample. The D-amino acids content was interpolated from standard curve between D-amino acids concentrations versus current (mA) prepared under optimal working conditions (Fig. 1).

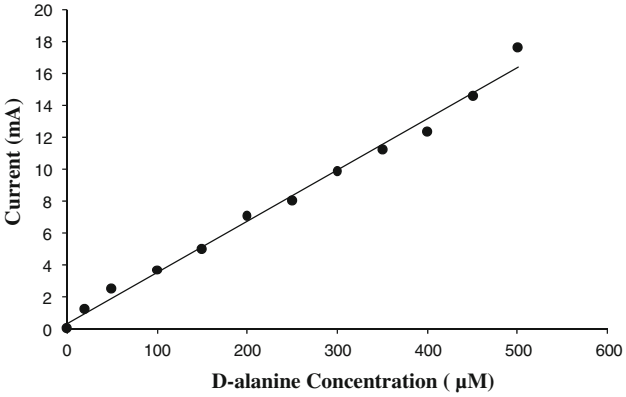


Fig. 1 Effect of D-alanine concentration on response of D-amino acid biosensor based on DAO/NiHCNFe/PPy Gc electrode

Evaluation

The performance of this sensor was evaluated by studying its analytical recovery, detection limit, precision and correlation.

Storage stability of DAO/NiHCNFe/PPy/GC electrode

The storage stability of the enzyme electrode was studied at 4 °C for more than 6 months by performing the assay on a weekly basis.

Results and discussion

SEM studies of GC electrode during modification

The SEM images of the surfaces of the bare GC electrode, NiHCNFe/PPy/GC electrode and DAO/NiHCNFe/PPy/GC electrode are shown in Fig. 2a, b and 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/PPy composite film shows net

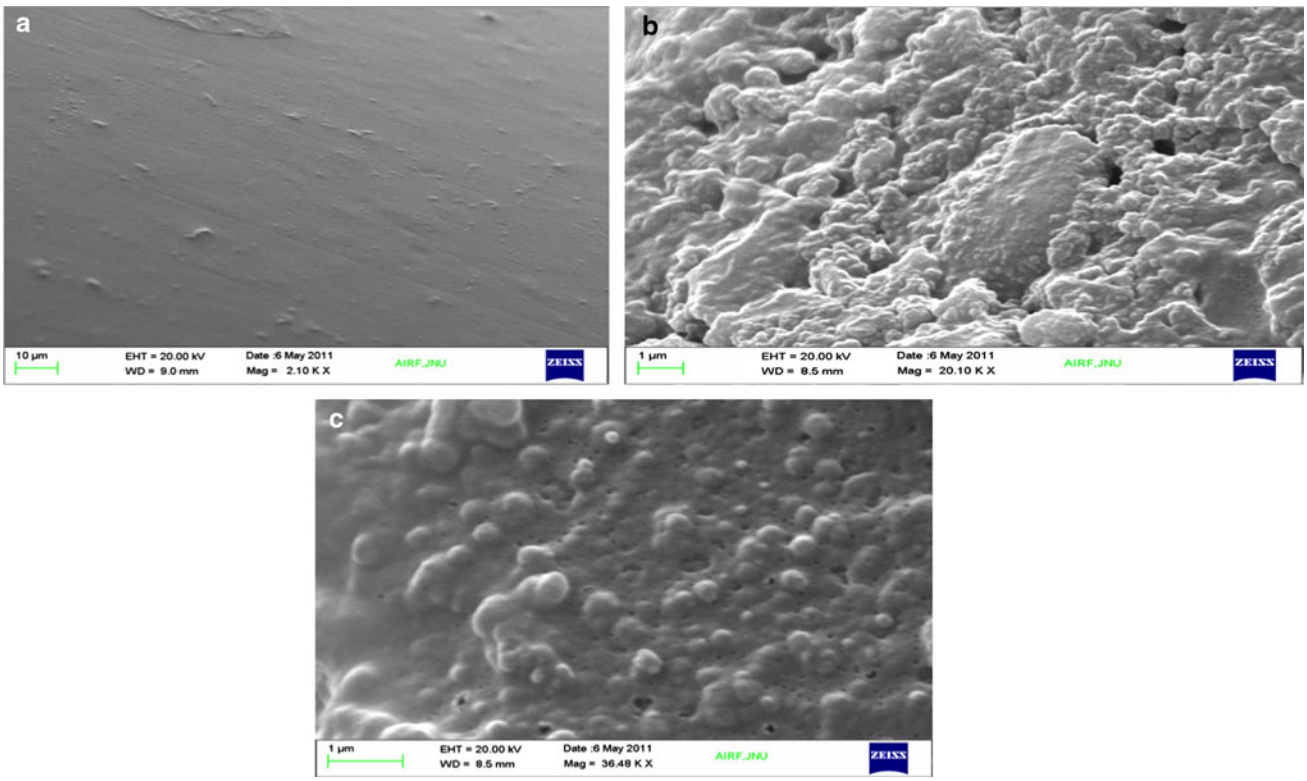
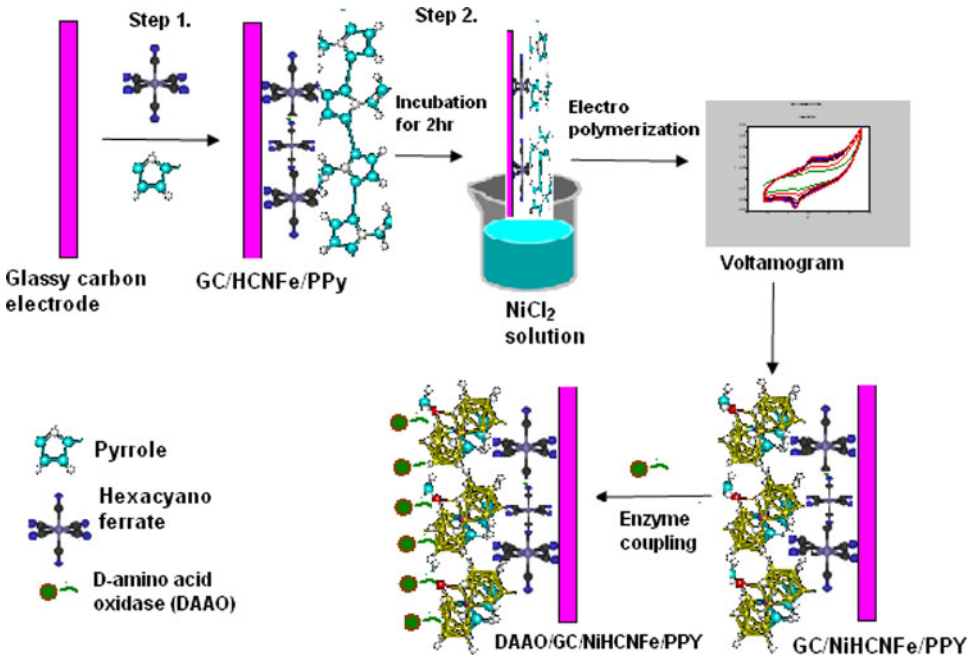


Fig. 2 SEM images of **a** Bare GC electrode, **b** NiHCNFe/PPy/GCelectrode and **c** DAO/NiHCNFe/PPy/GC electrode

Scheme 1 Schematic representation of chemical reaction involved in the fabrication of DAO/NiHCNFe/PPy/GC electrode



structure. Film is more uniform and porous and hence effective surface area is larger (Fig. 2b). On immobilization of DAO, the globular structures of DAO on the surface of NiHCNFe/PPy/GC film were observed (Fig. 2c).

Construction of D-amino acid biosensor

The potentiodynamic profile was recorded during the electrochemical synthesis of the hexacyanoferrate/poly-

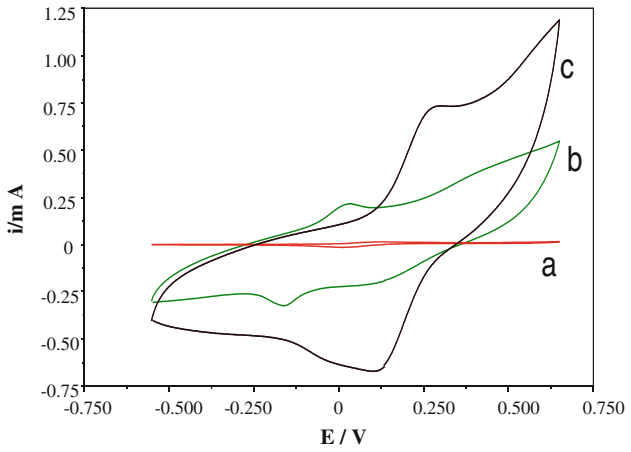


Fig. 3 **a** Cyclic voltammograms (CVs) of **a** bare GC electrode, **b** electrodeposition of $Fe(CN)_6^{-4}$-doped polypyrrole. Electrolyte: $K_3Fe(CN)_6$ 2×10^{-2} mol L^{-1} + pyrrole 1.5×10^{-2} mol L^{-1} + KCl 0.1 mol L^{-1}; 10 cycles. **c** Electrodeposition of $Fe(CN)_6^{-4}$-doped polypyrrole in $NiCl_2$ 2×10^{-3} mol L^{-1} + KCl 0.1 mol L^{-1}. 10 cycles

pyrrole matrix by applying potential −0.75 to 0.75 V. The electrode was cycled 10 times in the potential range −0.75 to 0.75 V. The positive potential limit chosen was lower than 1.0 V in order to avoid over oxidation of the polymer, which leads to loss of its conductivity. After the deposition process, a homogenous blue film was observed on the electrode, with good mechanical stability. Next, the $Fe(CN)_6^{3-}$/PPy-modified electrode was placed in a solution containing 2×10^{-2} M $NiCl_2$ + 0.1 M KCl during 2 h, in order to allow the insertion of Ni^{2+} ions into the polymer network. After this process, it is supposed that both $Fe(CN)_6^{3-}$ and Ni^{2+} ions are present inside the polymer matrix (Scheme 1). The electrode was then cycled 10 times in the same electrolyte. The appearance and growth of a new oxidation peak at 0.25 V was seen. This redox couple corresponds to the oxidation/reduction of nickel hexacyanoferrate [16] and the increase of current with the number of cycles indicates growth of the nickel hexacyanoferrate inside the polymeric matrix. When the electrode is transferred to an aqueous electrolyte containing only 0.1 mol L^{-1} KCl and cycled between −0.75 and 0.75 V, the peaks corresponding to oxidation/reduction of the nickel hexacyanoferrate still increased. Due to this reason, the electrode was cycled until complete stabilization of these peaks. These results indicate the incorporation of nickel hexacyanoferrate into the polymer network (Fig. 3).

Response measurements of D-amino acid biosensor

Amperometric response of enzyme electrode was recorded toward oxidation of D-alanine. There was a good linear relationship between current and D-alanine concentration in the range 20–500 μM. The detection limit calculated as a concentration that gave a signal equal to three times the standard deviation of the blank signal was 1.5 μM. An oxidation wave was noticed around 0.25 V at a modified electrode in comparison to the negligible at the bare GC electrode. CV curves show that as the concentrations of the D-alanine increased, the oxidation current also increased (Fig. 4).

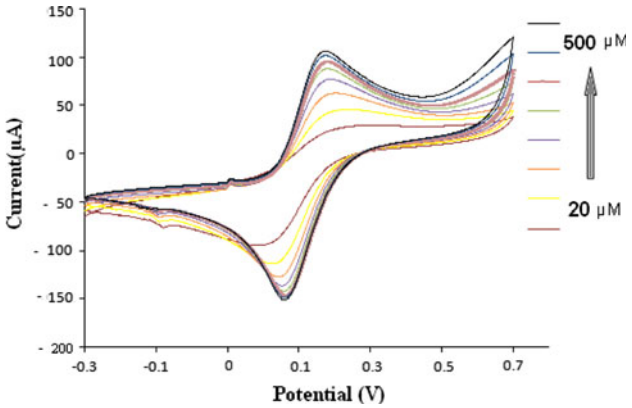


Fig. 4 Cyclic voltammetry in 0.01 M Tris HCl buffer, pH 7.0, D-alanine (20–500 μM)

Electrochemical impedance spectroscopy (EIS) study

To characterize the electronic and transport properties of NiHCNFe/PPy hybrid films, electrochemical impedance experiments were performed at 0.25 V. Figure 5 shows complex plane spectra for bare glassy carbon, NiHCNFe/PPy and DAO/NiHCNFe/PPy films. As can be seen, the presence of the conducting polymer dramatically modifies the impedance response of the system. In case of hybrid material, a semicircle is clearly observed at high frequencies, the diameter of this semicircle being related to the resistance of the charge transfer (R_{CT}) reaction and the electronic resistance of the film. By analyzing the low frequency region, it is possible

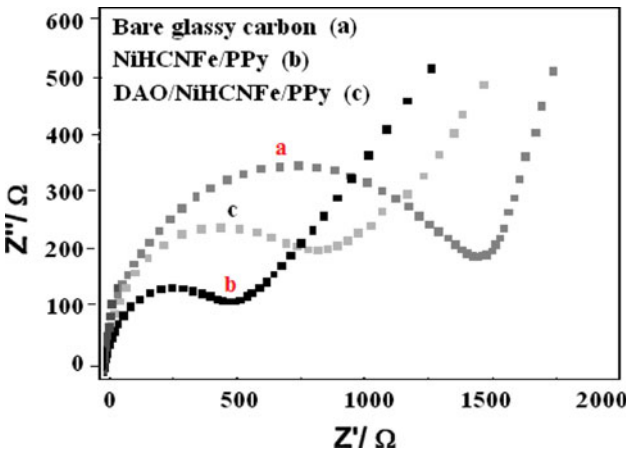


Fig. 5 Impedance spectra of **a** bare GC electrode, **b** NiHCNFe/PPy and **c** DAO/NiHCNFe/PPy electrode in 0.01 M Tris HCl buffer, pH 7.0 containing $[K_3Fe(CN)_6]$ (5 mM)

to observe a transition from semi-infinite diffusion. This impedance diagram shows the typical shape expected for a thin conducting polymer film. On the other hand, the impedance diagram of the bare GC electrode shows a higher value of the film resistance in the high frequency region whereas, from the low frequency region, we could conclude that the ionic movement inside the bare electrode and NiHCNFe was slower than that inside the hybrid one. This improvement being related to the increased electronic conductivity in the hybrid is due to the polypyrrole network. The conducting polymer could act as a "wire", connecting the redox sites of NiHCNFe, thus increasing the charge transfer rate.

FTIR spectra

Figure 6 shows FTIR spectra obtained for NiHCNFe/PPy electrode (curve A) and DAO/NiHCNFe/PPy films bioelectrode (curve B). FTIR spectra of electrodeposited NiHCNFe/PPy composite showed peaks at 1,547 and 1,343.93 cm^{-1} for typical pyrrole ring vibration and that of C–H in plane vibration peaks at 1,294, 1,045 cm^{-1} shows a broad peak at 1,164 cm^{-1} which is assigned as N–C stretching band and bands observed at 763, 500 cm^{-1} for the presence of polymerized pyrrole and that of metal oxide band at 1,625.88 cm^{-1} due to the addition of carbonyl and amino groups confirming the binding of D-amino acid oxidase with the NiHCNFe/PPy composite [17].

Optimization of experimental conditions

Considering that the electrochemical reactions are influenced by the total hydrogen ion concentration of buffer solutions, this effect had to be optimized. The catalytic current observed for DAO at different pH was studied. The current of the NiHCNFe/PPy-modified electrode increased as the pH was increased up to pH 7.0 after which it declined (Fig. 7). Thus the highest current response was achieved at pH 7.0. Figure 8 shows that the biosensor response increased with temperature up to 35 °C, the response gradually decreased. Therefore, 35 °C was chosen as the optimum temperature for the biosensor. The optimum current was obtained within 1 s. There was a hyperbolic relationship between biosensor response and D-alanine concentration up to a final concentration of 700 μM, after which it was constant.

Evaluation of D-amino acid biosensor

The following parameters were studied to evaluate the performance of this biosensor.

Linearity

There was a linear relationship between D-alanine concentration ranging from 20 to 500 μM and current (μA), which is better than the earlier biosensor based on prussian blue and Nafion layer (50–200 μM) [10] (0.25–5.0 mM) [14].

Detection limit

The detection limit of the biosensor was 1.5 μM (S/E = 3), which is lower than that of activated controlled pore glass-modified D-amino acid biosensor (30 μM) [18] and

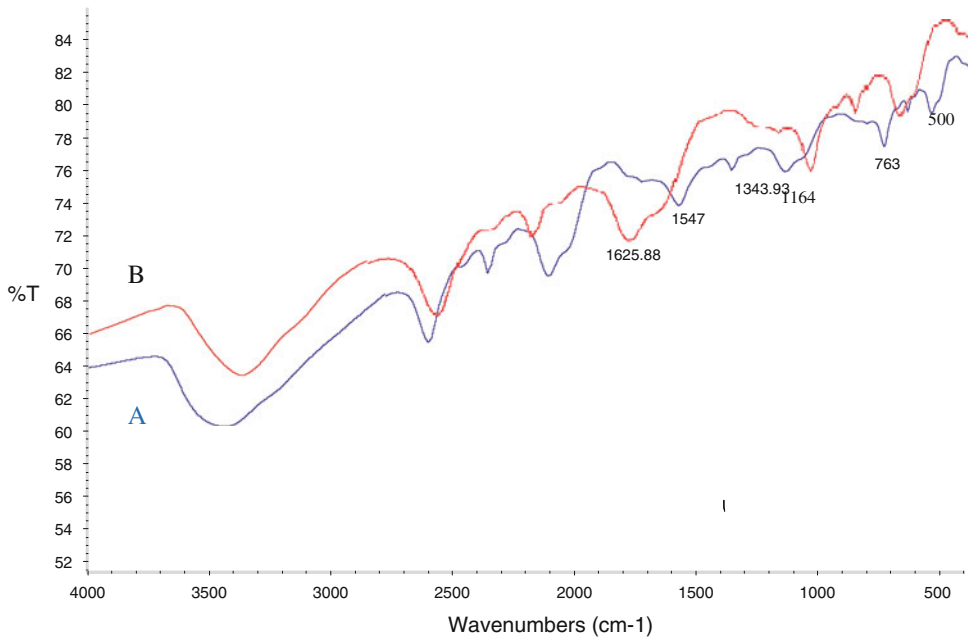


Fig. 6 FTIR spectra of **a** NiHCNFe/PPy and **b** DAO/NiHCNFe/PPy/GC electrode

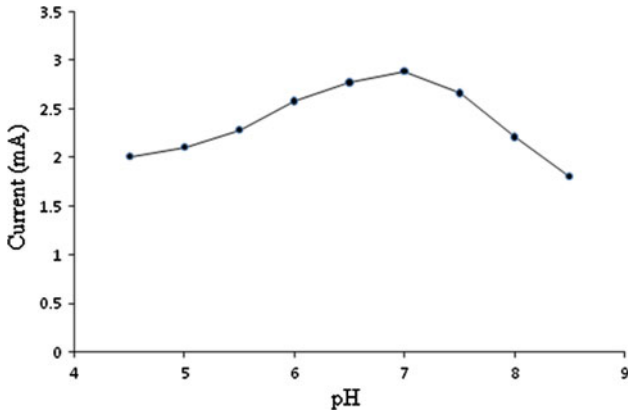


Fig. 7 Effect of pH on the current response of modified DAO/NiHCNFe/PPy/GC electrode

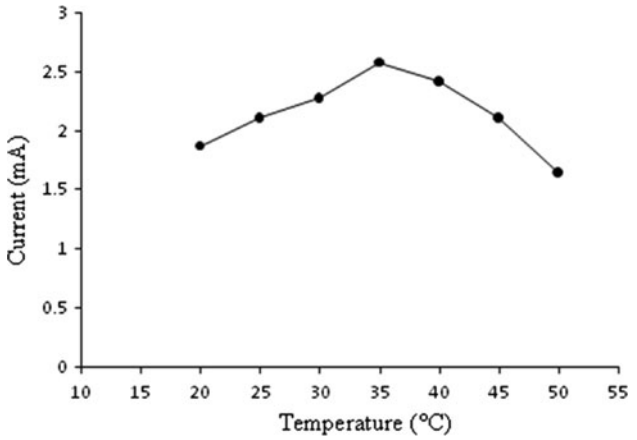


Fig. 8 Effect of temperature on the current response of modified DAO/NiHCNFe/PPy/GC electrode

self-assembled Amberzyme oxirane support (50 μM) [14], but comparable to prussian blue and Nafion layer-modified D-amino acid biosensor (1 μM for lowest noise and 30 μM in worst case) [10].

Analytical recovery

Analytical recoveries of exogenously added D-alanine (5, 10 mM) in serum samples by present method were 98.00 and 98.80 %, respectively, showing the reliability of the method (Table 1).

Table 1 Analytical recovery of added D-alanine in serum samples determined by D-amino acid biosensor based on NiHCNFe/PPy/GC electrode

D-Alanine (mM)	D-Alanine found (mM)	% Recovery
–	2.41	–
5.0	7.31	98.00
10.0	12.29	98.80

Table 2 Within- and between-batch assay coefficient of variation (CV) for determination of serum D-amino acid by D-amino acid biosensor based on DAO/NiHCNFe/PPy/GC electrode

(*n*)	Mean D-amino acid (mM)	CV (%)
Within batch (6)		
2.42	2.423	1.36
2.43		
2.39		
2.41		
2.48		
2.39		
Between batch (6)		
2.52	2.49	2.77
2.5		
2.51		
2.58		
2.48		
2.37		

Precision study

In order to check the repeatability and reproducibility of the method, the D-alanine content in six serum samples was determined six times on a single day (within batch) and again after storage at −20 °C for 1 week (between batch). The results showed that determinations were almost consistent. Within- and between-batch coefficients of variation for serum samples determination were 1.36 and 2.77 %, showing the good reproducibility and reliability of the method (Table 2).

Determination of D-amino acid in fruit juices, serum and urine

The results of quantitative analysis of D-amino acid serum and urine samples of apparently healthy and kidney infected patients and commercially available orange and pear juices are given as follows: In serum, 0.001–0.05 mM, mean 0.02 mM (healthy, $n = 25$), 0.117–0.499 mM, mean 0.22 mM (patients, $n = 25$); urine, 0.001–0.08 mM, mean 0.01 mM (healthy, $n = 25$), 0.08–1.108 mM, mean 0.38 mM (patients, $n = 25$); orange juice (0.81 mM in brand A, 0.68 mM in brand B and 0.75 mM in brand C) and pear juice (0.68 mM in brand A, 0.83 mM in brand B and 0.69 mM in brand C). Our results for serum and urine D-amino acid contents are in the normal established range.

Storage stability of DAO/NiHCNFe/PPy/GC electrode

The enzyme electrode lost 10 % of its initial activity after its 50 uses during the span of 2 months, when stored at 4 °C. This storage stability is higher than that of earlier electrodes (Table 3).

Table 3 A comparison of analytical properties of recent enzymic D-amino acid biosensors with the present biosensor

Properties	[1]	[19]	[18]	[9]	[10]	[14]	Present work
Source of DAAO	Porcine kidney	Porcine kidney	Porcine kidney	Porcine kidney	Porcine kidney	Rhodotorula gracilis	Goat kidney
Support of immobilization	Silica gel	Graphite–teflon electrode	Activated controlled pore glass	Egg shell membrane	Prussian blue and Nafion layer	Amberzyme oxirane support	NiHCNFe/PPy/GC
Methods for immobilization	Cross-linking	ND	Adsorption	Cross-linking	Adsorption	Cross-linking	Cross-linking
Type of transducer	Chemi luminescence	Amperometric	FIA system	DO metric	Amperometric	Amperometric	Amperometric
Working optimum pH	5.3	9.0	8.0	7.0	7.4	8.0	7.0
Optimum temp. (°C)	40	30	30	44	ND	30	35
Response time	ND	ND	ND	1 min	ND	10–15 min	<1 s
Linearity	1.11×10^{-6} to 2.1×10^{-4} M	5.0 to 50×10^{-4} M	0.1–1.0 mM	0.05–1.5 mM	50–200 μM	0.25–5.0 mM	20–500 μM
Detection limit	4.5×10^{-7} M	2.3×10^{-5} M	0.05 mM	30 μM	1 μM	50 μM	1.5 μM
Storage stability	ND	ND	1 week	2 month	10 days	2 weeks	2 months
K_m	0.5 mM	0.25 mM	ND	0.3 mM	0.41 mM	0.9 mM	–
Application	Serum sample	Food sample	Fish sauce	Serum sample	Fruit juices and milk sample	All D-amino acids	Fruit juices, urine, serum sample

Conclusion

The use of DAO/NiHCNFe/PPy film on GC electrode in presence of either Na^+ or K^+ ions led to improved analytical performance of D-amino acid biosensor in terms of sensitivity, i.e. detection limit (1.5 μM), high analytic recovery (98.80 %), precision (within and between batch CV = 1.36 and 2.77 %, respectively), stability (2 months at 4 °C) and no interference.

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