International Journal of Biological Macromolecules 50 (2012) 112–118

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

# Construction of amperometric uric acid biosensor based on uricase immobilized on PBNPs/cMWCNT/PANI/Au composite

Rachna Rawal, Sheetal Chawla, Nidhi Chauhan, Tulika Dahiya, C.S. Pundir*

*Department of Biochemistry, M D University, Rohtak 124 001, India*

ARTICLE INFO

*Article history:*
Received 5 August 2011
Received in revised form 4 October 2011
Accepted 7 October 2011
Available online 15 October 2011

*Keywords:*
Uric acid
Uricase
Prussian blue nanoparticles
Multiwalled carbon nanotubes
Polyaniline

ABSTRACT

A chitosan–glutaraldehyde crosslinked uricase was immobilized onto Prussian blue nanoparticles (PBNPs) absorbed onto carboxylated multiwalled carbon nanotube (c-MWCNT) and polyaniline (PANI) layer, electrochemically deposited on the surface of Au electrode. The nanohybrid-uricase electrode was characterized by scanning electron microscopic (SEM), Fourier transform infrared spectroscopy (FTIR) and cyclic voltammetry. An amperometric uric acid biosensor was fabricated using uricase/c-MWCNT/PBNPs/Au electrode as working electrode, Ag/AgCl as standard and Pt wire as auxiliary electrode connected through a potentiostat. The biosensor showed optimum response within 4 s at pH 7.5 and 40 °C, when operated at 0.4 V vs. Ag/AgCl. The linear working range for uric acid was 0.005–0.8 mM, with a detection limit of 5 μM. The sensor was evaluated with 96% recovery of added uric acid in sera and 4.6 and 5.4% within and between batch of coefficient of variation respectively and a good correlation ($r = 0.99$) with standard enzymic colorimetric method. This sensor measured uric acid in real serum samples. The sensor lost only 37% of its initial activity after its 400 uses over a period of 7 months, when stored at 4 °C.



## 1. Introduction

Uric acid is the excreted end product of purine catabolism in primates, birds and some other animals. In most mammals and many other vertebrates, uric acid is further degraded to allantoin by the action of uricase or urate oxidase. A healthy adult human excretes uric acid at a rate of 0.6 g/24 h; the excreted product arises in part from turnover of the purine nucleotide of nucleic acid [1]. The normal level of uric acid in serum is between 0.13 mM and 0.46 mM (2.18–7.7 mg $dl^{-1}$) [2,3]. An increase in level of uric acid in serum leads to gout, arthritis [4], cardiovascular diseases [5] and neurological diseases [6]. Consequently, uric acid determination is of paramount importance in the diagnosis and medical management of diseases caused by disorder of purine metabolism. Various methods such as colorimetric [7], electrochemical [8], high performance liquid chromatography (HPLC) [9,10], chemiluminescence [11] and fluorescence methods [12] have been reported for the measurement of uric acid. However, these methods have certain drawbacks such as time-consuming, labor-intensive, expert handling, pretreatment of sample and also oxidation of certain amounts of uric acid during the processing [7]. Biosensing methods are considered better for routine analysis, because of their simplicity, quick response time, ease of procedure, higher specificity and sensitivity.

A number of uricase biosensors have been reported based on luminol-copolymer [13], poly(2-aminophenol) [14], NiO thin film [15]. However these supports (film/membranes) had few drawbacks such as poor stability, reusability and slow electron transfer. Some uric acid biosensor based on ZnO nanotube arrays [16], ZnO nanowires [17], multiwalled carbon nanotube-gold nanoparticle composite [18] biosensors have been reported but these also suffered from limited electron communication, complexity of immobilization and stability of enzyme.

Nanoparticles modified electrode present unusual advantages in electroanalysis such as catalysis, enhancement of electron transport and high effective surface area [19–21]. The composite based on integration of carbon nanotubes (CNTs) and some other materials to possess properties of individual components with a synergistic effect have also gained interest. Materials for such purposes include conducting polymer [22,23], redox mediators [24], metal and metal oxide nanoparticles [25,26]. The Prussian blue nanoparticles (PBNPs) are efficient redox mediator for selective detection of hydrogen peroxide in the presence of oxygen and other interferents and has been applied in the construction of a large number of enzyme-based biosensors [27–29]. Chitosan has been found to be an interesting biopolymer for immobilization of desired biomolecules, because of excellent film forming ability, high permeability, mechanical strength, non toxicity, biocompatibility and low cost [30]. Carbon nanotubes (CNTs) possess many unique

* Corresponding author. Tel.: +91 9416492413; fax: +91 126274640.
*E-mail addresses:* pundircs@rediffmail.com, pundircs@yahoo.com, pundircs@gmail.com (C.S. Pundir).

0141-8130/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijbiomac.2011.10.002

properties such as good electrical conductivity, strong adsorptive ability and excellent bioconsistency and thus have ability to promote the electron transfer of hydrogen peroxide. Polyaniline (PANI), a conducting polymer, has become the most attractive one in formation of CNTs based composite, because of its superior transducing ability and good environmental stability. PANI/CNT composites synthesized chemically or electrochemically and have improved the electrical conductivity, electrochemical capacitance and electrocatalytic properties of polymers.

In this report, a uric acid biosensor was constructed by immobilizing uricase in crosslinked chitosan network through glutaraldehyde, onto PBNPs/c-MWCNT/PANI/Au modified electrode. The resulted uric acid biosensor was employed to measure uric acid in human serum and evaluated.

## 2. Materials and methods

### 2.1. Reagents

Uric acid, Sephadex G-100, distilled aniline and glutraldehyde (25%) from Sigma (Aldrich). Potassium ferricyanide ($K_3[Fe(CN)_6]$), ferrous sulfate ($Fe_2(SO_4)_3$), polyvinylpyrollidone (PVP) and chitosan were from SRL, Mumbai were used. Carboxylated multiwalled carbon nanotubes (functionalized MWCNT) (12 walls, length: 15–30 μm, purity: 90%, metal content: nil) from Intelligent Materials Pvt. Ltd., Panchkula (Haryana) India were used. The kit for enzymatic colorimetric determination of uric acid was from Transasia Bio-medicals Ltd. (Solan, HP). Gold wire (1.5 cm × 0.05 cm) (23 carat) was purchased from local market. All other chemicals were of analytical reagent (AR) grade. Double distilled water was used throughout the experiments. Fresh serum samples of healthy individuals were collected from Hospital of Pt. BDS University of Health & Medical Science, Rohtak and stored at −20 °C until use.

### 2.2. Apparatus

All electrochemical experiments were performed on an Autolab PGSTAT 30 electrochemical workstation (Eco Chemie BV, The Netherlands) with the GPES 4.9 software. A modified gold electrode of 1.0 mm diameter, a KCl-saturated Ag/AgCl electrode and a Pt electrode served as the working, reference and counter electrodes respectively, in the electrochemical experiments. All potentials were measured with respect to the reference electrode. The FTIR spectra in FTIR spectrophotometer (Thermo Scientific, USA) were recorded. Scanning electron microscopic (SEM) (Model Joel JSM-6510, Japan) images were taken at Dept. of Chemistry, M.D.U. Rohtak. All experiments were carried out at room temperature.

### 2.3. Preparation of uricase

Uricase was prepared by dissolving 3.0 mg of reagent1 of kit for enzymic colorimetric method for uric acid (consisting of uricase, buffer and chromogen) in 1 ml of 20 mM sodium phosphate buffer (pH 7.0) and loading on a Sephadex G-100 column (24 cm × 1 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The column was run in the same buffer at a flow rate of 0.5 ml/min. The fractions (each of 2 ml) were collected after passing the void volume and tested for protein using Lowry method. All the fractions showing the presence of protein were pooled and treated as purified enzyme. The enzyme was tested for activity of uricase as given below.

### 2.4. Assay of free uricase

The assay of free uricase was carried out as described [31] with slight modification and based on decrease in $A_{293}$ due to uric acid. The reaction mixture contained 3.0 ml of 50 mM Tris–HCl buffer (pH 8.5) and 0.1 ml of 10 mM uric acid and 50 μl (1.6 mg/ml) of uricase. The blank contained 3.05 ml of buffer and 0.1 ml uric acid. The decrease in $A_{293}$ was read in a UV spectrophotometer for 4 min at an interval of 1 min. The activity of enzyme was calculated as follows:

$$\text{Unit activity} = \frac{(\Delta A_{293/\text{min}} \text{ in test solution} - \text{blank}) \times \text{test volume}}{12.6 \times \text{volume of enzyme}}$$

where 12.6 is extinction coefficient of uric acid, $\Delta A$ is change in absorbance at 293 nm; test volume = 3.0 ml; volume of enzyme = 0.1 ml. One enzyme unit is defined as amount of enzyme required to convert 1.0 μmol of uric acid into allantoin per min per ml at pH 8.8 at 25 °C.

### 2.5. Preparation of PBNPs/c-MWCNT/PANI/Au electrode

#### 2.5.1. Electropolymerization of PANI onto Au electrode and construction of c-MWCNT/PANI/Au electrode

Prior to the surface modification, the Au electrode was cleaned with piranha solution ($H_2SO_4$:$H_2O_2$ in ratio 3:1) for 20 min and then rinsed thoroughly with distilled water. Prior to electrodeposition, Au electrode was polished with alumina slurry. The electrochemical polymerization of 50 μl aniline mixed with 10 ml of 1 N HCl onto Au electrode (working electrode) was carried out through cyclic voltammetry by applying 10 polymerization cycles at 0.0–1.5 V as described [32]. One gram c-MWCNT was suspended in 1 ml mixture of concentrated $H_2SO_4$ and $HNO_3$ in 3:1 ratio and ultrasonicated for 24 h to get a finely dispersed black colored solution of c-MWCNT. The polyaniline (PANI) modified electrode was dipped into the c-MWCNT solution for 12 h. The resulting c-MWCNT/PANI/Au electrode was washed thoroughly with distilled water to remove unbound matter and kept in dry petri plate at 4 °C.

#### 2.5.2. Electrodeposition of PBNPs onto the c-MWCNT/PANI/Au electrode

The electrodeposition of PVP protected PBNPs onto the c-MWCNT/PANI/Au electrode was carried out as follow. A mixture was prepared by adding $K_3[Fe(CN)_6]$ solution into a solution of $Fe_2(SO_4)_3$ containing PVP, to achieve a final concentration of 1 mM for $K_3[Fe(CN)_6]$ + 0.5 mM for $Fe_2(SO_4)_3$ + 10 mM PVP with 0.1 M KCl and 0.01 M HCl [33,34]. PBNPs/c-MWCNT/PANI modified Au electrode was constructed by electrodeposition in the resulting solution by potential cycling between 0.0 and 1.5 V (vs. Ag/AgCl) for 10 cycles at a scan rate of 0.1 V $s^{-1}$. The PBNPs/c-MWCNT/PANI modified Au electrode was rinsed with double distilled water and dried in air.

#### 2.5.3. Preparation of enzyme electrode (uricase/PBNPs/c-MWCNT/PANI/Au)

One microliter of uricase was mixed with 1 ml of chitosan solution (1%), followed by addition of 1 ml of 2.5% glutaraldehyde. The mixture was hand-mixed completely. The enzyme electrode was constructed by coating the resulting solution (100 μl) onto the modified electrode, and allowing it to be left for 24 h at 4 °C [35]. The hybrid electrode was stored at 4 °C when not in use.

### 2.6. Response measurement of uricase/PBNPs/c-MWCNT/PANI/Au electrode and its optimization

Cyclic voltammetric studies were carried out using a three-electrode system comprising of uricase/PBNPs/c-MWCNT/PANI/Au electrode as working electrode, Ag/AgCl as a reference electrode and Pt wire as auxiliary electrode. To discern the role of individual components, cyclic voltammograms (CV) of bare

gold electrode, PANI/Au electrode, c-MWCNT/PANI/Au electrode, PBNPs/cMWCNT/PANI/Au electrode and uricase/PBNPs/c-MWCNT/PANI/Au electrode were recorded in 50 mM Tris–HCl buffer pH 8.5 containing 10 mM uric acid at a potential range of 0 V to +1.5 V $s^{-1}$. To measure the response of working electrode/sensor, the three electrode system was immersed into 15 ml of 50 mM Tris–HCl buffer (pH 8.5) and the reaction was started by adding 0.1 ml uric acid (10 mM), which was oxidized to allantoin producing an electroactive $H_2O_2$. Formation of hydrogen peroxide was detected by its oxidation to generate electrons i.e. current at the electrode. The flow of electron i.e. current was measured in mA at 0.6 V. The following electrochemical reactions occur during the response measurement.

$$\text{Uric acid} + O_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{High potential}} 2H^+ + O_2 + 2e^-$$

$$2e^- \xrightarrow{\text{PBNPs/c-MWCNT/PANI}} \text{Gold electrode}$$

Amperometric response of the working electrode was optimized by studying analytic and kinetic properties like determination of optimum pH, temperature and response time. To determine optimum pH, pH of reaction buffer was varied from pH 5.0 to 10.0 using different buffer each at a final conc. to 50 mM as follow. Sodium succinate in pH range 4.0–5.5, sodium phosphate in pH range 5.5–6.5 and Tris–HCl buffer in range pH 7.0–9.0. Similarly, the optimum temperature was studied by incubating the reaction mixture from 20 °C to 50 °C at an interval of 5 °C. Optimum response time was studied by measuring current at different time scales ranging from 2 s to 12 s. The effect of substrate concentration was studied at different uric acid concentration ranging from 0.005 mM to 0.8 mM at potential cycling between 0.0 and 1.5 V (vs. Ag/AgCl) for 10 cycles at a scan rate of 0.1 V $s^{-1}$. The amperometric response was also measured in the presence of potential interfering compounds such as glucose, urea, ascorbic acid, pyruvate, bilirubin, cholesterol, KCl, NaCl, $CaCl_2$, $MgCl_2$, $CaSO_4$ and $ZnSO_4$.

### 2.7. Evaluation

The following criteria were studied to evaluate the performance of this biosensor e.g. linearity, analytical recovery, detection limit, precision and correlation with standard method.

### 2.8. Serum uric acid determination by uricase/PBNPs/c-MWCNT/PANI/Au electrode

The procedure for serum uric acid determination was the same as that described for response measurement of the electrode, except that uric acid was replaced by serum. The uric acid concentration was extrapolated from the standard plot, between different uric acid concentrations vs. respective current (μA). Results were evaluated using different statistical parameters and correlation with standard enzymic colorimetric method.

### 2.9. Reusability and storage stability of uricase/PBNPs/c-MWCNT/PANI/Au electrode

To reuse the working electrode, it was washed by dipping it in a series of test tubes containing 2 ml of reaction buffer. The long-term storage and stability of the biosensor was investigated over 7-months, when uricase/PBNPs/c-MWCNT/PANI/Au electrode was stored in a refrigerator at 4 °C.

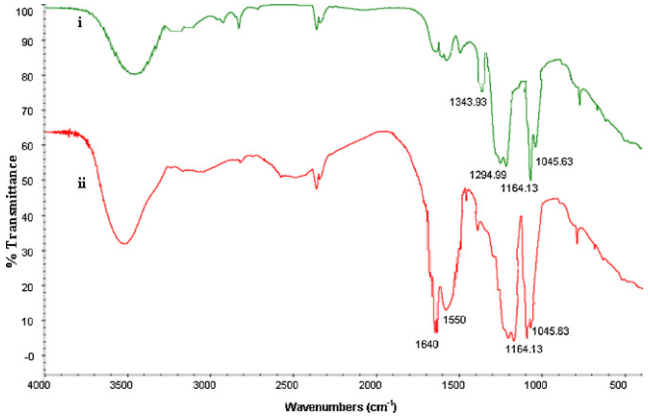



**Fig. 1.** FTIR spectra of (i) c-MWCNT/PANI and (ii) uricase/PBNPs/c-MWCNT/PANI/Au electrode.

## 3. Results and discussion

### 3.1. Characterization of uricase/PBNPs/MWCNT/PANI/Au electrode by FTIR and SEM

Fig. 1 displayed the FTIR spectra of (i) c-MWCNT/PANI and (ii) uricase/PBNPs/c-MWCNT/PANI/Au. Curve i shows the peak of C–O bond of free –COOH groups present at 1294.99 and 1343.93 $cm^{-1}$ and C–N bonds of c-MWCNT immobilized on PANI at 1045.63 and 1164.13 $cm^{-1}$. It reveals that the –COOH group at one end of c-MWCNT gets attached to $-NH_2$ groups of PANI through –CO–NH– bonds, while it is free at another end [36]. The uricase/PBNPs/MWCNT/PANI/Au electrode (curve ii) showed characteristic peaks at 1550 and 1640 $cm^{-1}$ attributed to the primary and secondary amide linkages of enzyme molecules. Peaks at 1615–1700 $cm^{-1}$ confirmed the presence of C=N bond. The SEM images of the surfaces of the bare Au electrode, c-MWCNT/PANI/Au electrode and uricase immobilized onto PBNPs/c-MWCNTs/PANI/Au electrode are shown in Fig. 2A, B and C, respectively, which confirm the stepwise immobilization of PANI, c-MWCNT, PBNPs and uricase onto the Au electrode.

The fabrication of the uricase biosensor based on PBNPs electrodeposited onto c-MWCNT/PANI modified Au electrode is summarized in Scheme 1. Firstly PANI was electrodeposited on to Au electrode with free $-NH_2$ group at their end, which gets linked to –COOH groups of c-MWCNT through amide bond (–CO–NH). Then, PBNPs are electrochemically deposited on the surface of PANI/MWCNT with the diameter nanoscaled. These PBNPs have negative charge [37] and when protected by polyvinylpyrrolidone (PVP) act as steric stabilization substances for the nucleation and growth of PBNPs [38]. Enzyme, when treated with glutaraldehyde and chitosan solution leads to formation of covalent bond (C=N). This pretreated enzyme when applied on to PBNPs/c-MWCNT/PANI/Au electrode, there occurs electrostatic interaction between negatively charged PB nanoparticles and positively charged chitosan.

Fig. 3A shows comparative cyclic voltammograms of (a) PANI modified Au electrode (b) c-MWCNT-PANI modified Au electrode (c) PBNPs/c-MWCNT/PANI modified Au electrode immersed in 50 mM of Tris–HCl buffer solution pH 8.5 at 50 mV $s^{-1}$. The cyclic voltammogram of PANI modified Au electrode exhibited a low background current with two redox peaks for oxidation of aniline to radical cation and then to radical dication [39]. In Fig. 3A (b) and (c), a couple of stable and well-defined redox peaks were observed and the formal potential was calculated at around 300 mV (vs. Ag/AgCl) which is comparable to earlier reports (250 mV) [40].

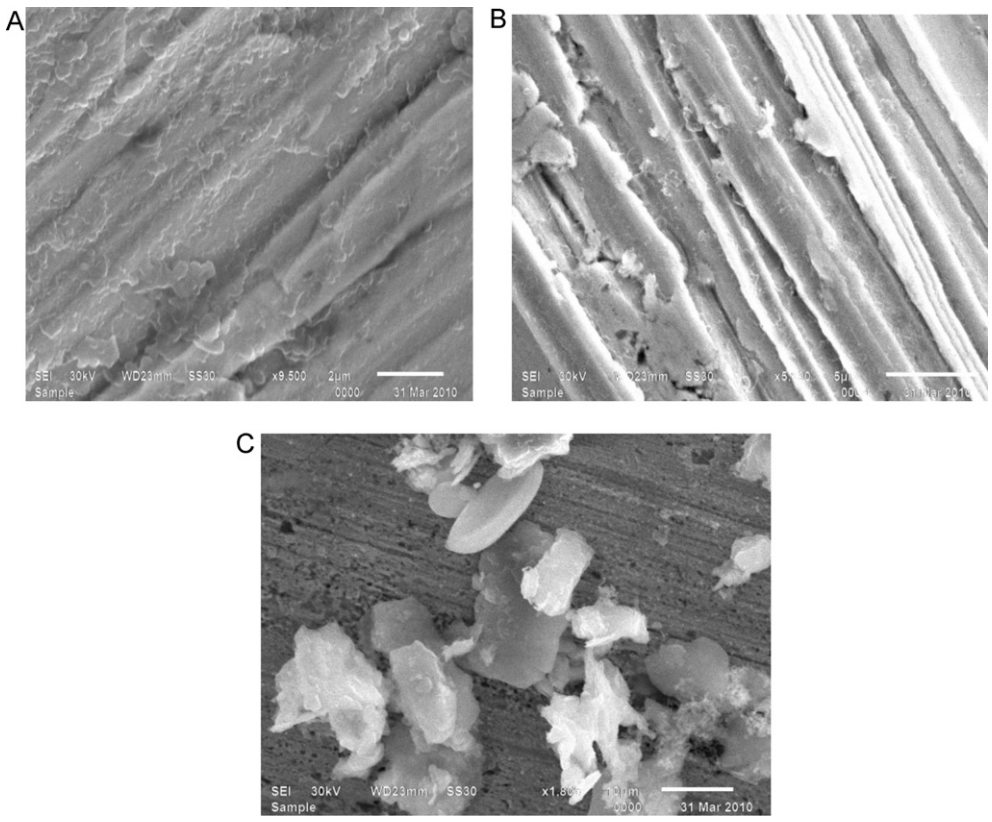
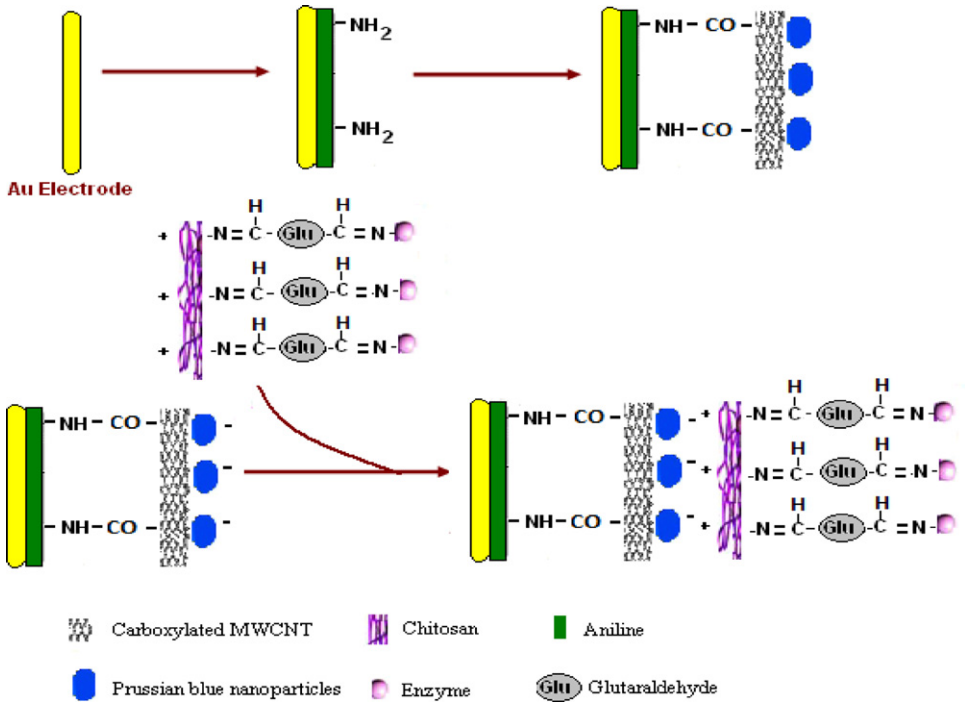



**Fig. 2.** SEM image of (A) bare electrode, (B) electrode modified with c-MWCNT and PAN1, c-MW, and (C) enzyme immobilized onto c-MWCNT, PAN1 and PBNPs modified Au electrode.

Obviously, these redox peaks were attributed to the reversible electrochemistry of PB. However, the background current and redox peak currents of PBNPs/c-MWCNT/PANI modified Au electrode were much higher than those of the c-MWCNT/PANI modified Au electrode. The increasing currents were due to the presence of c-MWNT, which provided a three-dimensional electroconductive network and had a large surface to volume ratio. So, c-MWNT played an important role in improving the redox of PBNPs and accelerating the electron transfer between the PBNPs and the Au electrode [41].




**Scheme 1.** Representation of uricase immobilized onto modified Au electrode (Uricase/PBNPs/c-MWCNT/PANI/Au).

### 3.2. Response towards uric acid at the uricase/PBNPs/MWCNT/PANI/Au electrode

To evaluate the catalytic activity of uricase at the PBNPs/MWCNT/PANI/Au electrode, the modified electrode was characterized by a cyclic voltammogram in the presence of uric acid in the potential range, 0 V to +1.5 V. Fig. 3B shows cyclic voltammograms of PBNPs/MWCNT/PANI/Au electrode in an unstirred 0.1 M KCl solution (20 ml) and 0.1 M Tris–HCl buffer, pH 8.5 (5 ml) without (curve a) and with (curve b) uric acid solution at scan rate 100 mV $s^{-1}$. It could be seen that with the addition of 10 mM of uric acid, the oxidation peak current was increased while reduction current suppressed, which showed the catalytic properties of modified electrode towards the oxidation of uric acid.

### 3.3. Optimization of uric acid biosensor

Cyclic voltammetric peak height of the modified electrode consisting immobilized enzyme depends strongly on the type of supporting electrolyte. Among different electrolytes, tested such as HCl (1 N) and KCl (1 N), only KCl gave well-defined reduction peak and hence used in the subsequent amperometric studies. The optimum pH of the biosensor/immobilized uricase was 7.5, which is slightly lower than that of free enzyme (pH 8.0), enzyme bound to polyethylene terephthalate (PET) membrane (8.5) [42] but higher than that of PANI/MWCNT/ITO electrode (7.0) [43]. The decrease in optimum pH after immobilization might be either due to the presence of negatively charged matrix in surroundings (c-MWCNT) or alerted conformation of enzyme after immobilization. The change in optimum pH of the immobilized uricase towards the neutral side makes it more efficient in quantitative determination of uric acid in biological fluids like serum (pH 7.4), as there will be no need to pre-treat the samples to adjust their pH to electrode optimal range. The optimum temperature of biosensor/immobilized uricase was 40 °C, which is higher than that of free enzyme (30 °C). Polyaniline (PANI) being a good thermal insulator for enzymes might have provided the microenvironment to protect the enzyme from environmental and thermal variations, which favors the repeated use of PANI-enzyme electrode [44,45]. The optimum response of the present electrode was observed within 4 s. A fast response of present biosensor might be due to improved electronic and ionic transport capacity of CHIT/c-MWCNT/PBNPs nanocomposite film, as c-MWCNT provided a three-dimensional electro-conductive network, which extended throughout the ion-conductive matrix of CHIT [46] and that the PBNPs obtained larger catalytic specific surface area and c-MWCNT could facilitate the electron transport of PBNPs [47]. Also, there might be a synergistic catalytic effect between PBNPs and c-MWCNT towards the reduction of $H_2O_2$.

The response of the present electrode system in relation to the change in uric acid concentration was found linear in the range of 0.005–0.8 mM (Fig. 4) with an apparent $K_m$ value of 0.058 mM. The decrease in $K_m$ value for uric acid after immobilization indicated increased affinity of immobilized enzyme towards its substrate. This $K_m$ value was lower than that of polyaniline–polypyrrole film modified platinum electrode (1.57 mM) [48], self-assembled monolayer on Au electrode (0.90 mM) [49] and polyaniline (7.83 mM) [50].

The effect of biological components on the response of the biosensor was evaluated by exposing the biosensor to some potential interferents in the reaction buffer at pH 7.5. The decrease in the current was calculated as uric acid concentrations equivalence (i.e. the concentration of uric acid that can produce the same decrease as the interferents). Negligible interference was observed with ascorbic acid, urea, cholesterol, EDTA, Lactic acid, glucose and pyruvate (Table 1), while no interference was observed with bilirubin, KCl, NaCl, $CaCl_2$, $MgCl_2$, $CaSO_4$ and $ZnSO_4$.

### 3.4. Evaluation of the biosensor

There was a linear relationship between current and uric acid concentration ranging from 0.005 to 0.8 mM in reaction mixture (Fig. 5). The detection limit of the present electrode system was 5 μM (S/N = 3). The percentage recovery of added uric acid in serum

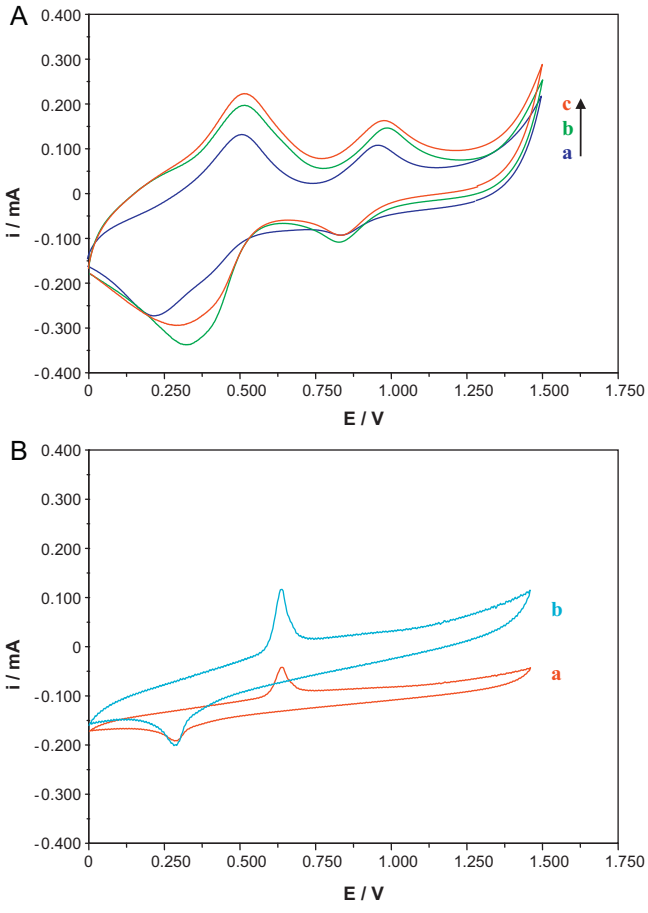



**Fig. 3.** Cyclic voltammograms of (A) (a) PANI modified gold electrode, (b) c-MWCNT-PANI modified electrode, and (c) PBNPs/c-MWCNT/PANI modified electrode and (B) Uricase/PBNPs/c-MWCNT/PANI/Au electrode (a) without and (b) with 10 mM uric acid.

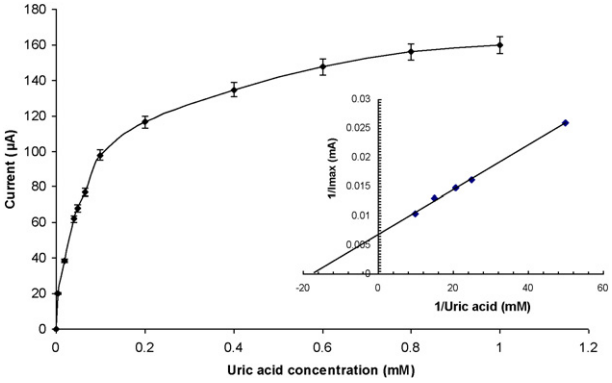



**Fig. 4.** Effect of substrate concentration on PBNPs/c-MWCNT/PANI modified Au electrode. Inset: Lineweaver–Burk plot. Error bars represent the standard deviation for three independent measurements.

**Table 1**
Effect of interferents on the amperometric response of uricase bound PBNPs/c-MWCNT/PANI modified Au electrode.

| Compound added | Concentration (physiological conc. in serum) | % Relative response[a] |
|---|---|---|
| None | – | 100.00 ± 0.01 |
| Ascorbic acid | 3.40 mM | 88.40 ± 0.64 |
| Urea | 0.10 g/l | 92.10 ± 0.51 |
| Cholesterol | 2.00 g/l | 84.70 ± 0.35 |
| EDTA | 2.00 mM | 79.33 ± 0.57 |
| Lactic acid | 0.50 mM | 91.60 ± 0.72 |
| Glucose | 0.90 g/l | 89.45 ± 0.36 |
| Pyruvate | 0.01 g/l | 93.90 ± 0.30 |

[a] The values are mean of 3 replications.

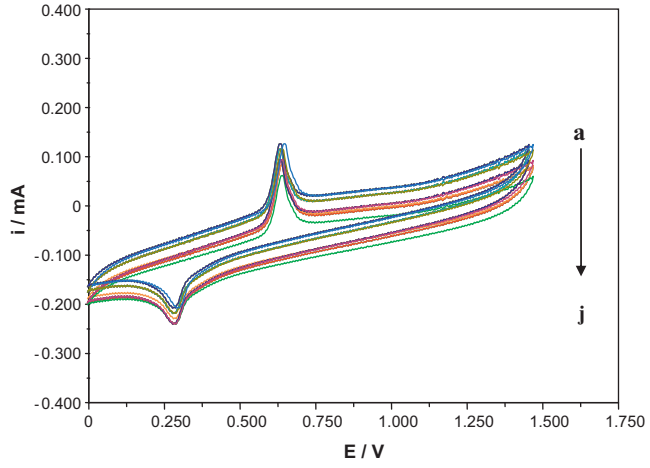



**Fig. 5.** Cyclic voltammograms recorded at different concentration of uric acid from (a) 0.005 mM, (b) 0.02 mM, (c) 0.04 mM, (d) 0.06 mM, (e) 0.08 mM, (f) 0.1 mM, (g) 0.2 mM, (h) 0.4 mM, (i) 0.6 mM, and (j) 0.8 mM (scan rate: 100 mV $s^{-1}$).

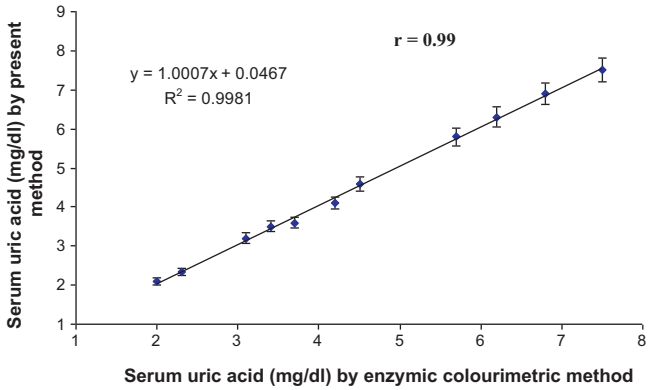



**Fig. 6.** Correlation between serum uric acid values determined by standard colorimetric method employing free enzyme (*x*-axis) and the present by biosensor employing uricase/PBNPs/c-MWCNT/PANI/Au electrode method (*y*-axis). Error bars represent the standard deviation for three independent measurements.

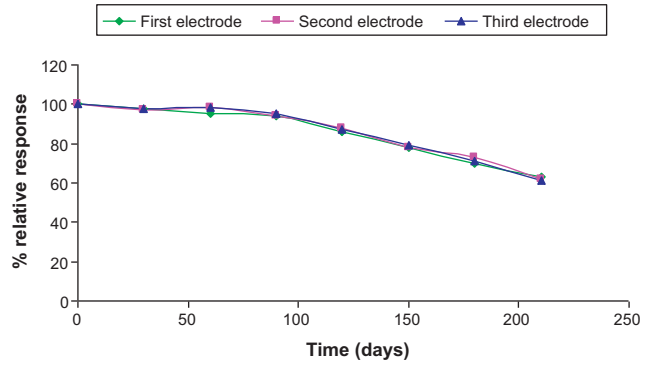



**Fig. 7.** Effect of storage at 4 °C on the response of three similar uricase/PBNPs/c-MWCNT/PANI/Au electrodes.

(10 mg/l and 20 mg/l) was 95% ± 0.4 and 97% ± 0.4 (mean ± S. D; $n = 6$), respectively, in the reaction mixture showing the efficiency of the biosensor. The within and between batch coefficient of variation (CV) for uric acid determination in serum by the present biosensor were 4.6% and 5.4% respectively indicating the reproducibility and reliability of the method. The accuracy of the present method was tested by comparing uric acid values in 11 serum samples by the present method ($y$) with those obtained by kit for enzymic colorimetric method ($x$). The values obtained by both methods showed good correlation with $r = 0.99$ with regression equation, $y = 0.984x - 0.119$ (Fig. 6). These results indicate the better analytical performance of the present biosensor in biological samples as compared to earlier biosensors [45].

### 3.5. Reusability and storage stability of uricase/PBNPs/c-MWCNT/PANI/Au electrode

Uricase/c-MWCNT/PBNPs/Au electrode storage stability was tested by measuring activity of biosensor every week over 7 months. The uricase/PBNPs/c-MWCNT/PANI/Au electrode showed practically 15% of their initial activity, after their regular use for 400× over a period of 4 months when stored at 4 °C. Thereafter a gradual but low decrease in its response was observed up to 7 months (Fig. 7). The enzyme electrode was constructed thrice and effect of storage at 4 °C was studied on the response of these three similarly constructed electrodes. The results showed practically no variation in storage stability of the three electrodes. The better shelf-life of present uricase/PBNPs/c-MWCNT/PANI/Au electrode over previously reported sensors may be attributed to the

**Table 2**
A comparison of present uric acid biosensor with earlier reported amperometric biosensors.

| Properties | Zhang et al. [38] | Cete et al. [51] | Arora et al. [52] | Arslan. [48] | Bhambi et al. [43] | Chauhan and Pundir [18] | Present |
|---|---|---|---|---|---|---|---|
| Support for immobilization | ZnO nanorods | PPy | PANI | PPy & PANI | PANI/MWCNT | AuNPs/MWCNT | PBNPs/c-MWCNT/PANI |
| Method of immobilization | Crosslinking | Crosslinking | Glutaraldehyde coupling | Entrapment | Covalent | Covalent | Crosslinking & adsorption |
| Linearity (mM) | 0.005–1 | 0.005–1 | 0.01–0.6 | 0.002–0.08 | 0.005–0.6 | 0.01–0.8 | 0.005–0.8 |
| Detection limit (μM) | 2 | 5 | 10 | | 5 | 10 | 5 |
| Response time (Sec) | ND | 330 | 60 | 70 | 8 | 7 | 4 |
| Stability (days) | 20 | 35 | 126 | 28 | 90 | 120 | 210 |

PPy: polypyrrole; PBNPs: Prussian blue nanoparticles; PANI: polyaniline; c-MWCNT: carboxylated multiwalled carbon nanotubes; ND: not detected.

presence of carbon nanotubes with a large surface area and strong affinity to bind with the enzyme.

A comparison of analytical and kinetic properties of present uric acid biosensor with those of earlier uric acid biosensors is given in Table 2.

## 4. Conclusion

A uric acid biosensor was constructed employing chitosan glutaraldehyde cross-linked uricase immobilized on PBNPs/c-MWCNT/PANI hybrid nanocomposites modified Au electrode. This uricase/c-MWCNT/PBNPs/Au electrode showed improved performance in terms of response time (4 s), detection limit (5 μM) (S/N = 3), reusability (more than 400×) and stability (7 months). The PBNPs/MWCNT/PANI nanohybrid film can be potentially used for improved performance of other amperometric biosensor also.

## References

[1] D.L. Nelson, M.M. Cox, Biosynthesis of Amino Acids, Nucleotides and Related Molecules, Lehninger Principles of Biochemistry, 4th ed., Freeman and Company, New York, 2004.
[2] S.H. Huang, Y.C. Shih, C.Y. Wu, C.J. Yuan, Y.S. Yang, Y.K. Li, T.K. Wu, Biosens. Bioelectron. 19 (2004) 1627–1633.
[3] C.R. Raj, T. Ohsaka, J. Electroanal. Chem. 540 (2003) 69–77.
[4] R.L. Wortmann, E. Braunwald, A.S. Fauci, D.L. Kasper, S.L. Hauser, D.L. Longo, J.L. Jameson, Development and Analytical Application Uric Acid Biosensor, Harrison's Principles of Internal Medicine, 15th ed., McGraw-Hill, New York, 2001.
[5] U.M. Khosla, S. Zharikov, J.L. Finch, T. Nakagawa, C. Roncal, W. Mu, Krotova, J.F. Baker, E. Krishnan, L. Chen, H.R. Schumacher, Am. J. Med. 118 (2005) 816–826.
[6] H.J. Moallem, G. Taningo, C.K. Jiang, R. Hishhorn, S. Fikrig, Clin. Immunol. 105 (2002) 75–80.
[7] A.K. Bhargava, H. Lal, C.S. Pundir, J. Biochem. Biophys. Methods 39 (1999) 125–136.
[8] F. Zhang, X. Wang, S. Ai, Z. Sun, Q. Wan, Z. Zhu, Y. Xian, L. Jin, Yamamoto, J. Wang, T. Golden, P. Tuzhi, Anal. Chem. 59 (1987) 740–744.
[9] M.L. Greenberg, M.S. Hershfield, Anal. Biochem. 176 (1989) 290–293.
[10] D. Yao, A.G. Vlessidis, N.P. Evmiridis, Anal. Chim. Acta 478 (2003) 23–30.
[11] J. Galban, Y. Andreu, M.J. Almenara, S. de Marcos, J.R. Castillo, Talanta 54 (2001) 847–854.
[12] D. Yao, A.G. Vlessidis, N.P. Evmiridis, Anal. Chim. Acta 467 (2002) 133–144.
[13] J. Ballesta-Claver, I.F. Díaz Ortega, M.C. Valencia-Mirón, L.F. Capitán-Vallvey, Anal. Chim. Acta 702 (2011) 254–261.
[14] S. Kurşun, B.Z. Ekinci, A. Paşahan, E. Ekinci, J. Appl. Poly. Sci. 120 (2011) 406–410.
[15] K. Arora, M. Tomar, V. Gupta, Biosens. Bioelectron. (2011), doi:10.1016/j.bios.2011.09.026.
[16] S.M. Usman Ali, Z.H. Ibupoto, C.O. Chey, O. Nur, M. Willander, Chem. Senses 1 (2011) 19–23.
[17] S.M. Usman Ali, N.H. Alvi, Z. Ibupoto, O. Nur, M. Willander, B. Danielsson, Sens. Actuators B: Chem. 152 (2011) 241–247.
[18] N. Chauhan, C.S. Pundir, Anal. Biochem. 413 (2011) 97–103.
[19] C.M. Welch, R.G. Compton, Anal. Bioanal. Chem. 384 (2006) 601–619.
[20] X.H. Kang, Z.B. Mai, X.Y. Zou, P.X. Cai, J.Y. Mo, Anal. Biochem. 363 (2007) 143–150.
[21] X.L. Luo, J.J. Xu, Y. Du, H.Y. Chen, Anal. Biochem. 334 (2004) 284–289.
[22] H. Zengin, W. Zhou, J. Jin, R. Czerw, D.W. Smith Jr., L. Echegoyen, D.L. Carroll, S.H. Foulger, J. Ballato, Adv. Mater. 14 (2002) 1480–1483.
[23] M. Cochet, W.K. Maser, A.M. Benito, M.A. Callejas, M.T. Martínez, J.M. Benoit, J. Schreiber, O. Chauvet, Chem. Commun. 16 (2001) 1450–1451.
[24] Z.F. Li, J.H. Chen, W. Li, K. Chen, L.H. Nie, S.Z. Yao, J. Electroanal. Chem. 603 (2007) 59–66.
[25] G.Y. Gao, D.J. Guo, C. Wang, H.L. Li, Electrochem. Commun. 9 (2007) 1582–1586.
[26] S. Qu, J. Wang, J.L. Kong, P.Y. Yang, G. Chen, Talanta 71 (2007) 1096–1102.
[27] A.A. Karyakin, O.V. Gltelmacher, E.E. Karyakina, Anal. Chem. 67 (1995) 2419–2423.
[28] A.A. Karyakin, E.E. Karyakin, Anal. Chem. 72 (2000) 1720–1723.
[29] J.P. Li, T.Z. Peng, Y.Q. Peng, Electroanalysis 15 (2003) 1031–1037.
[30] R. Khan, A. Kaushik, P.R. Solanki, A.A. Ansari, M.K. Pandey, B.D. Malhotra, Anal. Chim. Acta 616 (2008) 207–213.
[31] K. Yosuki, T. Ichikawa, E. Nakano, J. Biochem. 120 (1996) 969–973.
[32] J.S. Ye, H.F. Cui, Y. Wen, W.D. Zhang, G.Q. Xu, F.S. Sheu, Microchim. Acta 152 (2006) 267–275.
[33] T. Uemura, S. Kitagawa, J. Am. Chem. Soc. 125 (2003) 7814–7815.
[34] T. Uemura, M. Ohba, S. Kitagawa, Inorg. Chem. 43 (2004) 7339–7345.
[35] H. Yao, N. Li, J.Z. Xu, J.J. Zhu, Chin. J. Chem. 23 (2005) 275–279.
[36] N. Chauhan, J. Narang, C.S. Pundir, Analyst 136 (2011) 1938–1945.
[37] A.A. Karyakin, Electroanalysis 13 (2001) 813–819.
[38] L.Z. Zhang, J. Ling, T. Mu, J. Du, Z. Liu, B. Han, J. Chen, Colloids Surf. A: Physicochem. Eng. Aspects 243 (2004) 63–66.
[39] D.L. Feldheim, C.A Foss Jr. (Eds.), In: 'Metal Nanoparticles: Synthesis, Characterization and Applications', Marcel Dekker, New York, 2002.
[40] K.B. Wu, Y.Y. Sun, S.S. Hu, Sens. Actuators B: Chem. 96 (2003) 658–662.
[41] X. Zhai, W. Wei, J. Zeng, X. Liu, S. Gong, Anal. Lett. 39 (2006) 913–926.
[42] K. Phaugat, M. Bhambi, C.S. Renu, Pundir, J. Mol. Catal. B: Enzyme 62 (2010) 27–31.
[43] M. Bhambi, G. Sumana, B.D. Malhotra, C.S. Pundir, Artif. Cells Blood Substit. Immobil. Biotechnol. 38 (2010) 178–185.
[44] S. Iijima, Nature 354 (1991) 56–58.
[45] Z. Liu, X. Lin, J.Y. Lee, W. Zhang, M. Han, L.M. Gan, Langmuir 18 (2002) 4054–4060.
[46] M.G. Zhang, W. Gorski, J. Am. Chem. Soc. 127 (2005) 2058–2059.
[47] Q. Zhao, M.B. Nardelli, W. Lu, J. Bernholc, Nano Lett. 5 (2005) 847–851.
[48] F. Arslan, Sensors 8 (2008) 5492–5500.
[49] I.S. Kuwabata, T.S. Nakminami, H. Yoneyama, Sens. Actuator B 52 (1998) 72–77.
[50] Y. Jiang, A. Wang, J. Kan, Sens. Actuator B 124 (2007) 529–534.
[51] S. Cete, A. Yasar, F. Arslan, Artif. Cells Blood Substit. Immobil. Biotechnol. 34 (2006) 367–380.
[52] K. Arora, G. Sumana, V. Saxena, R.K. Gupta, S.K. Gupta, J.V. Yakhmi, M.K. Pandey, S. Chand, B.D. Malhotra, Anal. Chim. Acta 594 (2007) 17–23.