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An amperometric uric acid biosensor based on multiwalled carbon nanotube–gold nanoparticle composite

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ABSTRACT

An amperometric uric acid biosensor was fabricated by immobilizing uricase (EC 1.7.3.3) onto gold nanoparticle (AuNP)/multiwalled carbon nanotube (MWCNT) layer deposited on Au electrode via carbodiimide linkage. Determination of uric acid was performed by oxidation of enzymically generated H_2O_2 at 0.4 V. The sensor showed optimal response within 7 s at 40 °C in 50 mM Tris–HCl buffer (pH 7.5). The linear working range of the biosensor was 0.01–0.8 mM. The limit of detection (LOD) was 0.01 mM. The sensor measured uric acid levels in serum of healthy individuals and persons suffering from gout. The analytical recoveries of the added uric acid, 10 and 20 mg L^{-1}, were 98.0% and 96.5%, respectively. Within- and between-batch coefficients of variation were less than 5.6% and less than 4.7%, respectively. A good correlation ($r = 0.998$) was obtained between serum uric acid values by the standard enzymic colorimetric method and the current method. A number of serum substances had practically no interference. The sensor was used in more than 200 assays and had a storage life of 120 days at 4 °C.

Determination of uric acid in serum is very important in laboratory medicine and for routine clinical investigations [1,2]. The normal level of uric acid in serum is between 0.13 and 0.46 mM (2.18–7.7 mg dl^{-1}) [3]. An increase in the concentration of uric acid reflects disorders of purine metabolism, most notably gout and hyperuricemia or Lesch–Nyhan syndrome [4,5], leukemia, and pneumonia [6]. Among the various methods available for determination of uric acid, such as chemical [7], enzymic colorimetric [8], chemiluminescence [9], fluorescence [10], voltammetric–colorimetric [11], enzymatic–spectrophotometric [12], and high-performance liquid chromatography (HPLC)[1] [13,14], biosensing methods are simple, rapid, economic, highly sensitive and specific. Recently, the biocompatible nanomaterials have opened a new area toward the development of third-generation biosensors based on direct electron transfer between the enzyme and the electrode [15,16]. Several attempts had been made to fabricate amperometric biosensors for uric acid determination employing immobilized uricase onto various modified electrodes such as polyaniline-deposited Pt electrode [17], Ir-modified carbon electrode [18], polypyrrole film on Pt surface [19], ZnO nanorods-modified glassy carbon (GC) electrode [20], polyaniline deposited on indium tin oxide (ITO)-coated glass plate [21], polypyrrole and polyaniline [22], and polyaniline/carboxylated multiwalled carbon nanotubes (c-MWCNTs) deposited on ITO-coated glass plate [23].

The discovery of CNTs has generated enormous interest in exploring their unique properties for various applications [24,25]. A promising application of CNTs is their use in biosensors and nanoscale electronic devices. CNTs have been used to promote electron transfer reactions of desired biomolecules such as dopamine [26] and B1 nicotinamide adenine dinucleotide (NADH) [27]. Gold nanoparticles (AuNPs) are an ideal material for biosensors, especially those with electrochemical detection, because the Au surface area is suitable for binding of biomolecules and the metal facilitates direct and fast electron transfer [28]. Immobilization of proteins in a functional state on AuNPs is of critical importance in practical applications, especially biosensing and biolabeling [29–32]. This article deals with a novel approach to immobilization of uricase onto AuNP/c-MWCNT-modified Au electrode and its application in the construction of an amperometric biosensor for determination of uric acid.

Materials and methods

Materials

Uric acid and Sephadex G-100 from Sigma–Aldrich and sodium citrate dehydrate, hydrogen tetrachloroaurate trihydrate, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC), and

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; GC, glassy carbon; ITO, indium tin oxide; c-MWCNT, carboxylated multiwalled carbon nanotube; NADH, nicotinamide adenine dinucleotide; AuNP, gold nanoparticle; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide; NHS, *N*-hydroxysuccinimide; UV, ultraviolet; TEM, transmission electron microscope; SAM, self-assembled monolayer; SEM, scanning electron microscopy; FTIR, Fourier transform infrared.

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N-hydroxysuccinimide (NHS) from Sisco Research Laboratories (Mumbai, India) were used. c-MWCNTs (functionalized MWCNTs, 12 walls, 15–30 μm length, 90% purity, nil metal content) from Intelligent Materials (Panchkula, India) were used. A uric acid kit for enzymatic colorimetric determination manufactured by Transasia Bio-medicals (Solan, India) was obtained from Scientific Emporium (Rohtak, India). Au wire (1.5 × 0.05 cm, 23 carat) was purchased from a local market. All other chemicals were of analytical reagent grade. Double distilled water was used throughout the experiments.

Electrochemical experiments were performed with a potentiostat–galvanostat (Autolab, Eco Chemie, Utrecht, Netherlands, model AUT83785) with a conventional three-electrode cell.

Purification of uricase

Uricase was prepared by dissolving 3.0 mg of powder of enzymatic reagent 1 of the uric acid kit into 1 ml of 0.02 M sodium phosphate buffer (pH 7.0) and loading it on a Sephadex G-100 column (24 × 1 cm) preequilibrated with 0.02 M sodium phosphate buffer (pH 7.0). The column was run in the same buffer at a flow rate of 0.5 ml min^{-1}. The fractions (2 ml each) were collected after passing the void volume. The fractions were tested for protein by the Lowry method. Fractions showing the presence of protein were pooled and treated as purified enzyme and tested for its activity as given below.

Assay of free uricase

The assay of free uricase was carried out as described previously [33] with slight modification and based on a decrease in A_{293} due to uric acid. The reaction mixture contained 3.0 ml of 50 mM Tris–HCl buffer (pH 8.5), 0.1 ml of uric acid (10 mM) and 50 μl of uricase (1.6 mg ml^{-1}). The blank contained 3.05 ml of 50 mM Tris–HCl buffer (pH 8.5) and 0.1 ml of 10 mM uric acid. The control consisted of 3.0 ml of 50 mM Tris–HCl buffer (pH 8.5), 0.1 ml of 10 mM uric acid, and 50 μl of heat-denatured uricase. The decrease in A_{293} was read in an ultraviolet (UV) spectrophotometer for 4 min at an interval of 1 min. The activity of enzyme was calculated as follows:

$$\text{unit activity} = \frac{(\Delta A_{293} \text{ in test solution} - \text{blank}) \times \text{test volume}}{12.6 \times \text{volume of enzyme taken}},$$

where 12.6 is the extinction coefficient of uric acid, ΔA_{293} is the change in absorbance at 293 nm, test volume = 3.15 ml and volume of enzyme = 0.1 ml. One unit of enzyme is defined as the amount of uricase required to convert 1.0 μmol of uric acid to allantoin per minute per milliliter at pH 8.5 and 25 °C.

Preparation of AuNPs

AuNPs were prepared as described previously [34] with slight modification. Here 20 ml of $HAuCl_4$ solution (1 mg 5 ml^{-1}) was taken in a 50-ml flask kept on a stirring hot plate. A magnetic stir bar was added, and the solution was boiled. To this boiling solution, 2 ml of 1% trisodium citrate dehydrate was added dropwise. An AuNP suspension gradually formed as the citrate reduced the gold. Change of color of the solution from blackish to wine red confirmed aggregation of nanoparticles. The colloidal gold solution was then stored in dark bottles at 4 °C after cooling. The morphological characterization of the AuNPs was carried out by a transmission electron microscope (TEM) study in the Department of Anatomy at the All India Institute of Medical Sciences (AIIMS, New Delhi).

Preparation of AuNP/MWCNT/Au electrode

An Au wire was first polished with alumina slurry and cleaned ultrasonically with ethanol and double distilled water. Au electrode was then immersed in an ethanol solution containing 1 mM cysteamine for 10 h to give a cysteamine self-assembled monolayer (SAM). Then 1 g of c-MWCNT was suspended in a 1 ml mixture of concentrated H_2SO_4 and HNO_3 in a 3:1 ratio and ultrasonicated for 2 h to get a finely dispersed black-colored solution of c-MWCNTs. A mixture of 100 mM EDC and 100 mM NHS was prepared by mixing 1.0 ml each of 200 mM EDC and 200 mM NHS, and its pH was adjusted to 6.0. Then 0.1 ml of this mixture was added to a 0.1-ml dispersed solution of c-MWCNT and kept for 1 h at room temperature to convert the carboxyl groups of the shortened c-MWCNTs into active carbodiimide esters. The cysteamine-modified Au electrode was placed in the above active MWCNT solution (adjusting the pH at 8.5 with NaOH) for 10 h, during which time the amines at the terminus of the SAM formed amide bonds with the active carbodiimide esters of the tubes [35]. The electrode was washed with distilled water thoroughly and then immersed in AuNP solution for 12 h at 4 °C. The electrode was washed thoroughly with distilled water to remove unbound matter and kept in a dry Petri plate at 4 °C.

Preparation of enzyme electrode

The enzyme electrode was prepared by dropping a 10-μl uricase solution (2 mg ml^{-1}) on the AuNP/MWCNT/Au electrode and keeping it at 4 °C for 24 h. It was rinsed clearly. The enzyme electrode was dried and stored in reaction buffer solution at 4 °C before use.

SEM study of uricase/AuNP/c-MWCNT/Au electrode

Scanning electron microscopy (SEM) studies of bare Au electrode, AuNP/c-MWCNT/Au, and uricase/AuNP/c-MWCNT/Au electrode were carried out in the Department of Chemistry at our University to confirm immobilization of uricase. The electrodes were cut into small pieces and placed on a copper disc of 2 cm diameter. The Au particles were deposited on its surface using a spray gun and electron micrographs were taken.

Electrochemical characterization of uricase/AuNP/c-MWCNT/Au electrode

Cyclic voltammetry studies were carried out using a three-electrode system composed of uricase/AuNP/c-MWCNT/Au electrode as working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode. To discern the role of individual components, cyclic voltammograms of bare Au electrode, MWCNT/Au electrode, and AuNP/MWCNT/Au electrode were recorded in 50 mM Tris–HCl buffer (pH 8.5) containing 0.1 mM H_2O_2 at a scan rate of 0.0 to +1 V s^{-1} at an interval of 50 mV s^{-1}. To measure the response of working electrode/sensor, the three-electrode system was immersed in 15 ml of 50 mM Tris–HCl buffer (pH 8.5) and the reaction was started by adding 0.1 ml of uric acid (10 mM), which was oxidized to allantoin, producing an electroactive H_2O_2. Formation of H_2O_2 was detected by its oxidation to generate electrons (i.e., current at the electrode) as follows:

$$\text{uric acid} + H_2O \overset{\text{uricase}}{\rightarrow} \text{allantoin} + H_2O_2 + CO_2$$
$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-.$$

The flow of electron (i.e., current) was measured in milliamps (mA) at 0.4 V. To optimize the working conditions of the electrode, the pH of 50 mM Tris–HCl buffer was varied from pH 5.0–10.0 at an interval of pH 0.5. Similarly, the incubation temperature was

varied from 20 to 50 °C at an interval of 5 °C and the current response was measured at 2–12 s at an interval of 1 s. The cyclic voltammetry studies were carried out at different uric acid concentrations ranging from 0.01 to 0.8 mM. The K_m value for uric acid was calculated from a Lineweaver–Burk plot. The amperometric response was also measured in the presence of potential interfering compounds and metabolites such as glucose, cholesterol, ascorbic acid, urea, pyruvate, bilirubin, $CuSO_4$, KCl, FAD, NaCl, $ZnSO_4$, NADH, $CaCl_2$, EDTA, NEM, riboflavin, $MnCl_2$ and FMN at a final concentration of 1 mM.

Serum uric acid determination by uricase/AuNP/c-MWCNT/Au electrode

Fresh serum samples from apparently healthy individuals (both male and female) and persons suffering from gout (of different sex and age groups) were collected from the hospital at the local Pt. BDS Post Graduate Institute of Medical Science (Rohtak) and analyzed for uric acid using the current electrode. The procedure was the same as described for response measurement of electrode except that uric acid was replaced by serum. The uric acid concentration was extrapolated from a standard curve of uric acid concentrations prepared under optimal conditions.

Reusability and storage stability of uricase/AuNP/c-MWCNT/Au electrode

To reuse the enzyme 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 4 months, when uricase/AuNP/c-MWCNT/Au electrode was stored dry in a refrigerator at 4 °C. The activity of electrode was measured every 5 days.

Results and discussion

AuNPs synthesized in this study were essentially very fine and monodisperse with a mean diameter of approximately 19.25 nm, as seen from the TEM image in Fig. 1. The immobilization of uricase onto AuNP/MWCNT/Au electrode was confirmed from SEM images of the surfaces of the bare electrode and uricase immobilized onto AuNP/c-MWCNT/Au electrode. High-resolution SEM images of AuNP/c-MWCNT/Au surface with immobilized enzyme had folds

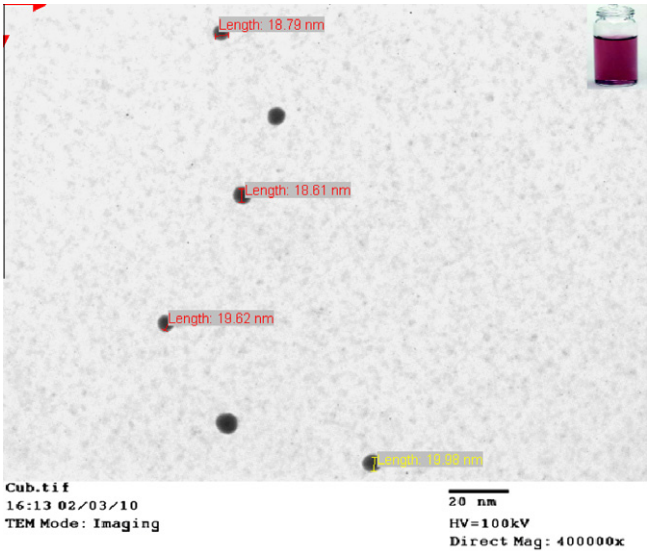


Fig.1. TEM image of AuNPs.

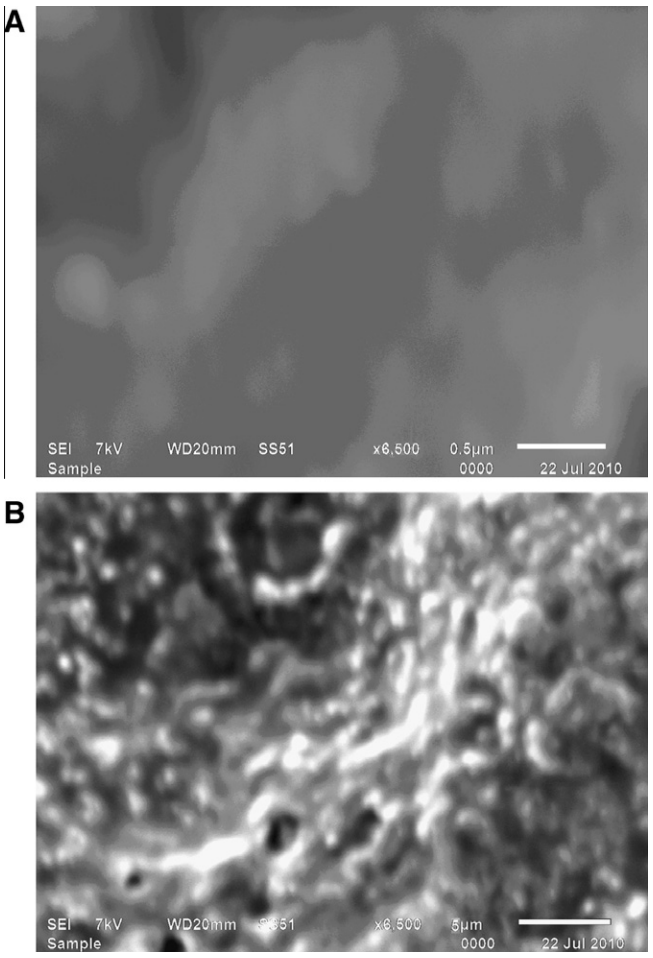


Fig.2. SEM images of bare Au electrode (A) and uricase/AuNP/MWCNT modified Au electrode (B).

and clusters along some beaded structures that were not observed in the electrode without immobilized enzyme (Fig. 2). A change in surface morphology of the support after the immobilization process is evidence of enzyme immobilization.

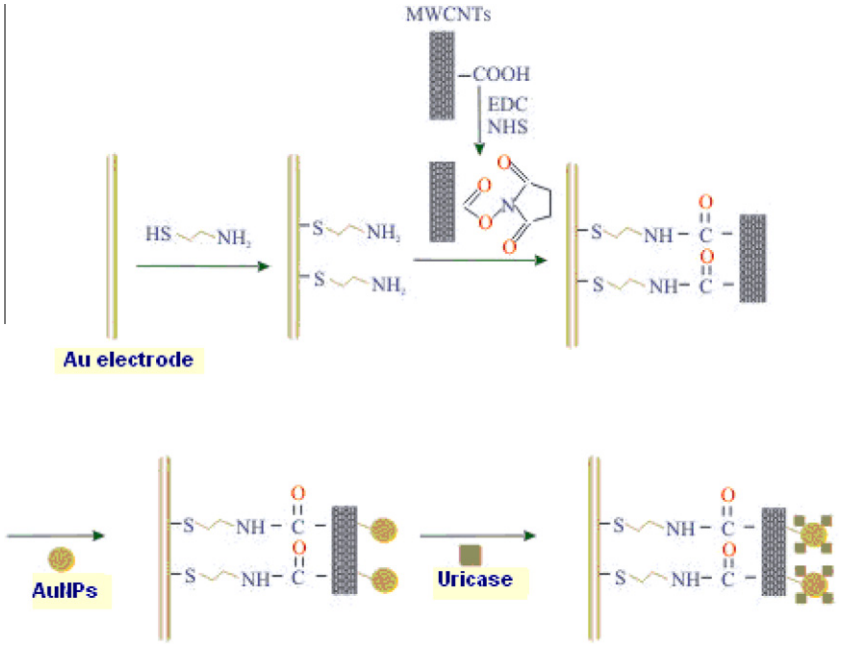
Construction of uric acid biosensor

The construction of the uric acid biosensor by immobilizing uricase onto AuNP/c-MWCNT-modified Au electrode is summarized in Scheme 1. An amine-functionalized SAM was formed on modified electrode surface with cysteamine. c-MWCNTs were solubilized after refluxing and sonication in concentrated sulfuric acid and nitric acid. Carboxylic acids moieties at defect sites located at the sidewalls of the nanotubes were converted to carbodiimide esters after treatment with EDC and NHS. The CNTs were then immobilized onto the electrode surface by forming amide bonds with amines at the terminus of the SAM.

The AuNPs used in this work, dispersed on the surface of MWCNTs, might provide a large available surface and enhance the electrocatalytic activity for H_2O_2 electrooxidation. AuNPs were absorbed onto the surface of MWCNTs by the physical absorption method [35].

FTIR spectral studies

Fourier transform infrared (FTIR) spectrum for c-MWCNT/Au electrode discerned the attachment of c-MWCNTs to the bare Au electrode through cysteamine, as shown by the peak at



Scheme 1. Schematic representation of construction of uricase/AuNP/MWCNT/Au electrode.

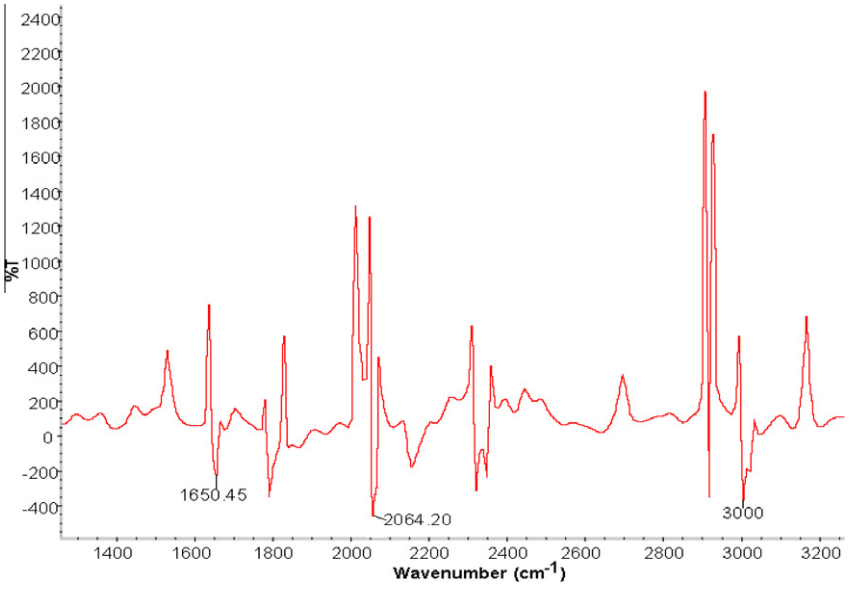


Fig.3. FTIR measurements of uricase/AuNP/c-MWCNT/Au electrode. T, transmittance.

1650.45 cm^{-1}, which was due to the amide bond between SAM of cysteamine-modified Au electrode and the active carbodiimide esters of the MWCNTs (Fig. 3).

Electrochemical characteristics and optimization of AuNP/MWCNT/Au electrode

Fig. 4 shows current responses of Au electrode to the addition of 0.1 mM H_2O_2 at different stages of construction. Bare electrode yielded a very small current response to H_2O_2 (trace a) that was not detectable due to the H_2O_2 not being electrooxidized at the bare electrode surface when a potential of 0.0–1.5 V was applied. The small current indicated that the direct oxidation of H_2O_2 at the Au electrode was inefficient. At the MWCNT/Au electrode (trace b), a slight increase in current response to H_2O_2 was observed compared with the bare Au electrode and this may be ascribed to the fact that the relative surface area of the electrode was increased after immobilization of MWCNTs on it. The significant increase in the current response of the AuNP/Au electrode can be ascribed to the good catalytic activity of AuNPs to the electrooxidation of H_2O_2. The introduction of AuNPs into the MWCNT/Au electrode dramatically improved the current response (trace c). An anodic current was found to increase from 0.079 to 0.150 mA with MWCNT/AuNPs. In particular, it amplified the H_2O_2 current by two times compared with MWCNT/Au electrode and four times compared with bare Au electrode. This phenomenon could be ascribed to the increase in effective electrode surface due to immobilization of MWCNTs and AuNPs on the Au electrode.

The experimental conditions affecting the biosensor response were studied in terms of the effects of pH, incubation temperature, time and substrate (uric acid) concentration. The optimal

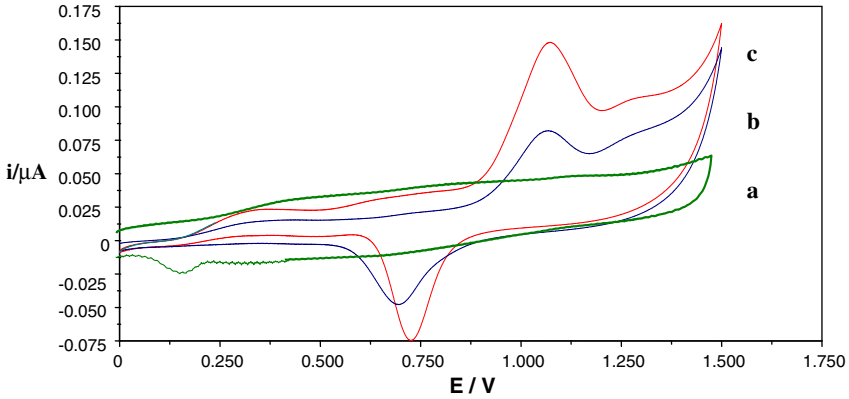


Fig.4. Cyclic voltammograms of AuNP/c-MWCNT/Au electrode: (a) bare Au electrode; (b) modified Au electrode with c-MWCNTs; (c) after absorption of AuNP/MWCNT/Au electrode in 50 mM Tris–HCl buffer (pH 7.5).

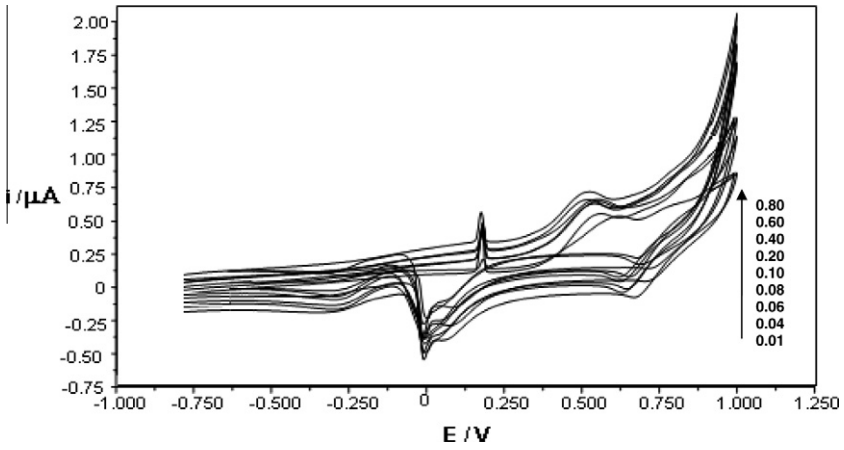


Fig.5. Cyclic voltammograms of uricase/AuNP/c-MWCNT/Au electrode in Tris buffer (50 mM, pH 7.5) at a scan rate of 50 mV s^{-1} at different concentrations of uric acid (in mM).

current was obtained within 7 s at pH 7.5, which is higher than that of free enzyme (pH 7.0) [36]. The change in optimal pH of enzyme after immobilization might be due to an improved stability at that pH, the prevention of dissociation, an internal pH different from the external pH, and/or even a chemical modification of the enzyme. The immobilized enzyme had an optimal temperature of 40 °C, which is slightly higher than that of free enzyme (37 °C) [36]. Uricase/AuNP/c-MWCNT/Au-modified electrode achieved 95% of steady state currents within 7 s, which is lower than 60 s [36], 70 s [22], and 100 s [37] reported previously. There was a linear relationship between biosensor response and uric acid concentration up to a final concentration of 0.8 mM (Fig. 5). The Lineweaver–Burk plot between $1/I$ (mA) and 1/[uric acid] (see Supplementary Fig. 1 in supplementary material) yielded a K_m of 0.5 mM, which is higher than that for free enzyme (0.40 mM) [35] and previous enzyme electrodes 0.34 mM [21], 0.13 mM [38], and but lower than polyaniline-polypyrrole modified electrode (1.57 mM) [22], indicating increased affinity of enzyme toward substrate (uric acid) after immobilization, which might be due to enhanced diffusion of uric acid through AuNP/MWCNT composite.

Evaluation of uricase biosensor

Linearity, sensitivity and analytical recovery

There was a linear relationship between biosensor response (i.e., current) and uric acid concentration ranging from 0.01 to 0.8 mM in Tris–HCl buffer (pH 7.5), which is better than previous reports of 0.025–0.1 mM [36], 0.0–0.30 mM [37], and 0.05–0.5 mM [38]. The calibration curve is linear up to 0.8 mM with a sensitivity of 0.44 mA mM^{-1}, which is higher than that reported previously (0.024 mA mM^{-1}) [39]. Analytical recoveries of exogenously added uric acid in serum, 10 and 20 mg L^{-1}, in the method were 98% and 96.5%, respectively, showing the reliability of the method. These recoveries are better than previous recoveries of 93.6 ± 2.34 and 87.18 ± 3.17% [36] and 94.3% and 89.8% [38], respectively.

Detection limit

The detection limit of the biosensor was 0.01 mM at a signal-to-noise ratio of 3, which is lower than that of the previously reported uric acid biosensor of 0.05 mM [38] but higher than 0.005 mM [23].

Precision study

To study the repeatability and reproducibility of the method, the uric acid content in same serum sample was determined five 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 nearly consistent and that within- and between-batch coefficients of variation for serum uric acid determination were less than 5.6 ± 0.6% and less than 4.7 ± 0.4%, respectively, showing the good reproducibility and reliability of the method. These results are better than those reported previously (6.5% and <5.0%, respectively) [36].

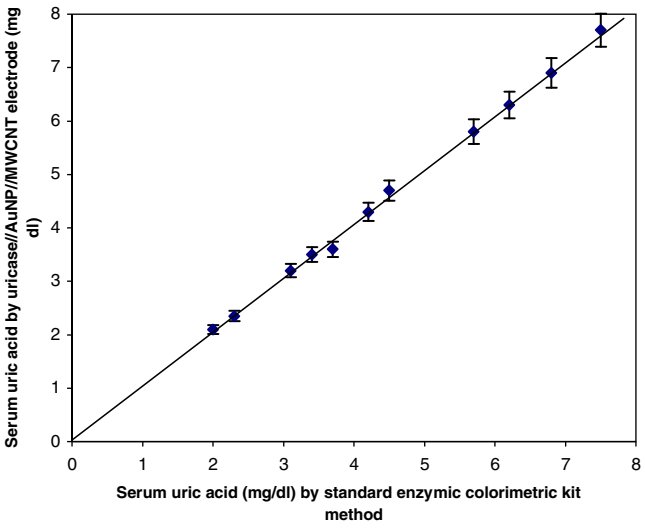


Fig.6. Correlation between serum uric acid values determined by standard enzymic colorimetric method and uricase/AuNP/c-MWCNT/Au electrode.

Accuracy

To study the accuracy of the current method, the uric acid levels in 11 serum samples from apparently healthy persons as determined by the current method (y) were compared with those obtained by the standard enzymic colorimetric method (x). The value obtained by the uricase/AuNP/MWCNT/Au electrode was $r = 0.998$ (Fig. 6), which is better than those in previous reports of $r = 0.98$ [36] and $r = 0.98$ [38].

Determination of serum uric acid

The uric acid in sera of apparently healthy individuals and diseased individuals suffering from gout of both sexes, as measured by the current biosensor, was in the range of 2.6–6.7 mg dl^{-1} ($n = 10$) in apparently healthy persons and 6.9–11.9 mg dl^{-1} ($n = 10$) in gout patients (Table 1).

No interference was found with glucose, cholesterol, ascorbic acid, urea, pyruvate, bilirubin, $CuSO_4$, KCl, FAD, NaCl, $ZnSO_4$, NADH, $CaCl_2$, EDTA, NEM, riboflavin, $MnCl_2$ and FMN, each at a final concentration of 1 mM. However, ascorbic acid showed some interference at a physiological concentration (7% inhibition at 3.4 mM), similar to our previous report [23]. A previous uric acid biosensor had ascorbic acid interference, causing 56% decrease in activity [36].

Table 1
Serum uric acid levels in apparently healthy adults and gout patients, as measured by uric acid biosensor based on uricase AuNP/MWCNT/Au electrode.

Serum uric acid (mg dl^{-1}) (means ± SE)					
Sex	Age (years)	Healthy persons	Sex	Age (years)	Gout patients
M	21	2.60 ± 0.03	F	38	7.28 ± 0.02
M	25	3.80 ± 0.04	F	40	6.90 ± 0.05
F	32	4.80 ± 0.02	F	41	9.58 ± 0.03
F	27	6.70 ± 0.02	M	37	8.62 ± 0.03
F	35	5.70 ± 0.05	F	39	7.40 ± 0.05
M	33	3.10 ± 0.05	M	45	8.90 ± 0.01
F	22	5.40 ± 0.05	M	48	10.0 ± 0.01
M	34	4.30 ± 0.01	M	55	11.9 ± 0.02
M	23	3.70 ± 0.03	F	49	8.50 ± 0.04
F	37	5.73 ± 0.01	F	57	8.95 ± 0.02

Note. SE, standard errors; M, male; F, female.

Stability of enzyme electrode

The stability of the enzyme electrode under storage conditions (50 mM Tris–HCl buffer, pH 7.5, 4 °C) was investigated, and the activity was measured every 5 days for 4 months. The enzyme electrode lost 45% of the initial response after a storage period of 4 months. With the storage period prolonged, the response current was decreased. After 45 days, the response current still retained 75% of the initial response, indicating a higher stability of electrode than the previously reported 30 days [22], 60 days [36], 60 days [38] and 3 months [37]. The loss of the current response of the enzyme electrode might result from the decrease of enzyme activity during storage, but it is not due to the loss of the enzyme because no significant uricase activity was observed in the storage solution. The relatively good storage stability implies that MWCNT film immobilized by covalent linkage and AuNPs absorbed onto the electrode surface was very stable. Moreover, AuNPs are compatible with the immobilized enzyme and facilitate maintaining the activity of uricase [35].

Conclusions

A more stable and reproducible deposition of c-MWCNT film on Au electrode was achieved using the covalent linkage method. This uricase/AuNP/c-MWCNT/Au electrode showed improved performance in terms of response time (7 s), sensitivity (0.44 mA mM^{-1} in 0.01–0.8 mM uric acid), reusability (more than 200 times), and stability (4 months). There is further scope of improvement in the current biosensor in terms of storage stability and reusability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.02.007.

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