Appl Biochem Biotechnol (2014) 174:1683–1694
DOI 10.1007/s12010-014-1097-6

Construction of an Uricase Nanoparticles Modified Au Electrode for Amperometric Determination of Uric Acid

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Published online: 21 August 2014

Abstract A method is described for preparation of uricase nanoparticles (100 nm in size) and their direct immobilization onto the Au electrode. The enzyme electrode along with Ag/AgCl as reference and Pt as auxiliary electrode were connected through potentiostat/galvanostat to construct an amperometric uric acid biosensor. The enzyme electrode was characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, and electrochemical impedance spectroscopy (EIS). The enzyme electrode detected uric acid level as low as 5.0 μM at a signal-to-noise ratio of 3, within 7 s at pH 8.5 and 40 °C. The biosensor showed a linear working range, 0.005 to 0.8 mM for uric acid with a sensitivity of 0.03 mA μM^{-1} cm^{-2}. The biosensor was evaluated. The biosensor lost only 15 % of its initial activity over a period of 7 months, when stored at 4 °C. The fabricated biosensor was successfully employed for determination of uric acid in human serum and urine.

Keywords Uricase · Uricase nanoparticles · Uric acid · Uric acid biosensor · Serum · Urine

Introduction

Uric acid (2,6,8-trihydroxypurine) is the principal end product of purine metabolism in human [1]. Monitoring uric acid in blood or urine or both is very important, as it is a powerful indicator of early signs of kidney and metabolic disorders. The normal level of uric acid in serum is between 0.13 and 0.46 mM (2.18–7.7 mg dl^{-1}) [2, 3]. However, abnormal uric acid level in a human body could be caused by several diseases, such as gout, hyperuricemia, Lesch–Nyhan syndrome, cardiovascular, and chronic renal disease [2–5]. Furthermore, a few other diseases, such as leukemia and pneumonia, are also related with the uric acid levels. Consequently, serum/urine uric acid measurement is routinely required for diagnosis and treatment of these diseases [6]. Among the various methods available for determination of uric acid, such as colorimetric [7], enzymic colorimetric [8], enzymic spectrophotometric [9],

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capillary electrophoresis [10], chemiluminescence method [11], fluorescence [12], and voltammetric–calorimetric [13], biosensing methods based on immobilized uric acid oxidase (uricase) are comparatively more simple, rapid, specific, sensitive, cheaper, and require relatively economic equipment, and user-friendly operation [14]. Researchers have shown that the response time could be decreased to a few seconds (4–10 s) using enzyme immobilized onto nanoparticles (NPs) rather than on a membrane or hydrogel [15, 16]. The small response time, high sensitivity, and stability have been attributed to the unique properties of nanostructures such as small size (normally in the range of 1–100 nm in diameter) and high surface-to-bulk ratio leading to interesting optical, electric, chemical, mechanical, physical, and catalytic properties, which are not present in their respective bulk material [17]. Furthermore, the method of immobilization of enzyme onto these nanomaterials, i.e., adsorption, cross-linking, or covalent binding, has a profound effect on activity of the enzyme as well as the shelf life of enzyme electrode. We have fabricated the improved glucose and cholesterol biosensors employing glucose oxidase NPs and cholesterol oxidase NPs, respectively [18, 19]. The present work describes the preparation of uricase NPs and their direct immobilization onto Au electrode through gold–thiol bond for improved amperometric determination of uric acid.

Materials and Methods

Recombinant uricase from *Candida* species expressed in *Escherichia coli*, 4-aminophenazone, glutaraldehyde, and cysteamine dihydrochloride was from Sigma Aldrich Co., St. Louis, USA. Uric acid Enzo kit, manufactured by Transasia Bio-Medicals Ltd (Solan, HP, India) and uric acid from SISCO Research Laboratory, Mumbai, India were used. Gold wire (1.5 cm× 0.05 cm) (23 carat) was purchased from a local market. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used throughout the experiments. Fresh serum and first morning urine samples of healthy individuals and diseased persons (gout patients) were collected from the hospital of Pt. BDS University of Health & Medical Science, Rohtak and stored at −20 °C until use.

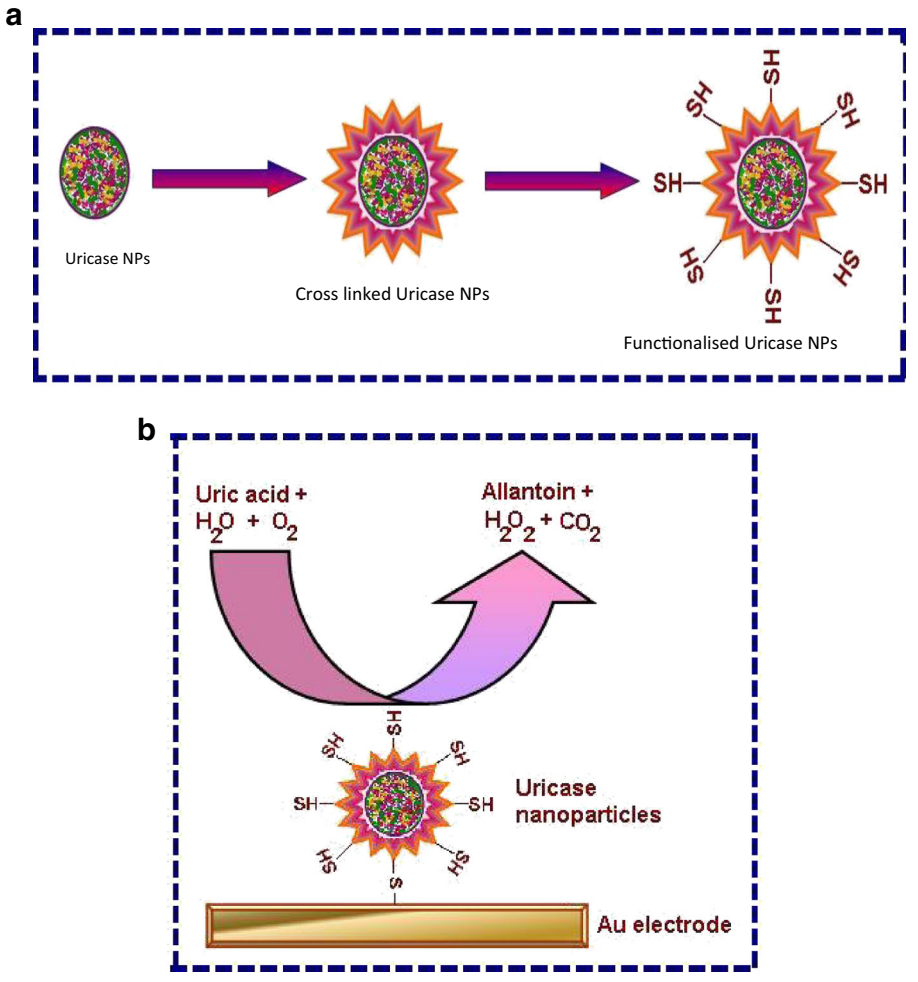
Apparatus Used

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed on a Potentiostat/Galvanostat (Autolab, Eco Chemie, The Netherland, Model: AUT83785) with a three-electrode system. Scanning electron microscopic (SEM) images of enzyme electrode in a scanning electron microscope (Model Joel JSM 6510, Japan) were taken at the Department of Chemistry, M.D. University, Rohtak. Transmission electron microscopy (TEM) of uricase NPs was carried out at AIRF, Jawaharlal Nehru University, New Delhi. All experiments were carried out at room temperature (30±5 °C).

Preparation of Uricase Nanoparticles (NPs)

The uricase NPs were synthesized by desolvation method of Liu et al. [20]. To uricase solution (1 mg ml^{-1}), 5 ml of absolute ethanol was added dropwise at a rate of 0.1–0.2 ml min^{-1} under continuous stirring at a speed of 8,000×*g*. The desolvating agent encouraged the enzyme/protein molecules to aggregate into small particles (NPs) by removing in between water molecules, thus reducing the distance between the enzyme molecules. This was followed by an addition of 1 ml, 1 %

glutaraldehyde solution in the above solution under the similar stirring condition at 4 °C for 24 h to ensure complete cross-linking of uricase NPs. Such a high concentration of glutaraldehyde is likely to provide intermolecular cross-linking of uricase molecules through Schiff base. The enzyme NPs thus formed were thiol functionalized by adding 0.02 g ml^{-1} of cysteamine solution (0.02 g ml^{-1}) to enzyme NPs suspension under constant stirring for 5–6 h. Enzyme NPs were separated from free enzyme by centrifuging NPs suspension at 15,000×*g* for 10 min at 4 °C, followed by redispersion of uricase NPs in 50 mM of phosphate buffer (pH 7.4) and sonication for 5 min. It is assumed that alpha $–NH_2$ group of cysteamine reacts with excess unreacted –CHO groups from the glutaraldehyde cross-linked uricase molecules to form Schiff base. Thus, the glutaraldehyde cross-linked uricase nanoparticles (NPs) get functionalized with –SH groups. These –SH functionalized uricase NPs were stored at 4 °C, until use (Scheme 1A) [21].



Scheme 1 **a** Schematic illustration of the stepwise synthesis of functionalized uricase NPs. **b** Scheme of reaction mechanism of uricase NPs/Au working electrode

Preparation of Uricase NPs Modified Au Electrode

Thiol groups on the surface of uricase NPs provide a facile method for attaching the NPs on the surface of Au electrode to prepare an enzyme NP-based enzyme electrode and thus circumventing complications associated with solution system. Before the experiment, the potential of the cleaned bare Au electrode was scanned over the +0.5 to +1.5 V range in freshly prepared 0.2 M H_2SO_4 until the voltammogram characteristic of the clean polycrystalline Au was established. The polycrystalline Au electrode was placed in the uricase NP suspension under mild stirring at 4 °C for 12 h to provide a nanoparticle self-assembled uricase NP layer. The uricase NPs/Au electrode was rinsed with 50 mM of phosphate buffer (PB, pH 7.4) carefully and stored in a PB buffer at 4 °C, when not in use [20]. The thiol-functionalized cross-linked uricase NPs get bound to polycrystalline Au electrode through Au–thiolate bond through the reduction process as follows:

$$\underset{\text{Polycrystalline-AuE}}{Au_n\text{–}AuO} + \underset{\text{Functionalized UOxNPs}}{2(UOxNPs)\text{-}SH} \xrightarrow{\text{Reduction}} \underset{\text{UOx-E(Uricase- electrode)}}{Au_n\,(Au\text{-}S\text{-}UOxNPs)_2} + H_2O$$

Response Measurement of Uricase NPs Modified Au Electrode and Its Optimization

Cyclic voltammetric studies were carried out using a three-electrode system comprising uricase NPs modified Au electrode as a working electrode, Ag/AgCl as a reference electrode, and Pt wire as auxiliary electrode connected through potentiostat/galvanostat. To discern the role of individual components, CV of bare Au electrode and uricase NPs modified Au electrode were recorded in 50 mM Tris–HCl buffer pH 8.5 containing 10 mM of uric acid at a potential range of 0.0 V to +1.5 V s^{-1}. The maximum current was generated at 0.2 V; hence, in subsequent electrochemical studies, the enzyme electrode was polarized at 0.2 V.

To measure the response of enzyme electrode/biosensor, 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 of uric acid (10 mM), which was oxidized to allantoin producing an electroactive H_2O_2 (Scheme 1B). Formation of hydrogen peroxide was detected by its electro-oxidation under high voltage to generate electrons, i.e., current at the electrode. The flow of electron, i.e., current was measured in milliampere at +0.2 V. The following electrochemical reactions occurred during the response measurement:

$$\text{Uric acid} + O_2 + H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + H_2O_2$$

$$H_2O_2 \xrightarrow{0.2\ \text{V}} 2H^+ + O_2 + 2e^-$$

Amperometric response of the enzyme electrode was optimized by studying its kinetic properties like determination of optimum pH, incubation temperature, and response time. To determine optimum pH, pH of reaction buffer was varied from pH 4.0 to 9.0 using different buffers each at a final concentration to 50 mM as follows: sodium succinate in pH range 4.0–5.5, sodium phosphate in pH range 6.0–8.0, and Tris–HCl buffer in pH range 8.5–9.0. Similarly, the optimum temperature was studied by incubating the reaction

mixture from 20 to 50 °C at an interval of 5 °C. Optimum response time was studied by measuring current at different time scales ranging from 2 to 12 s at an interval of 1 s. The effect of substrate concentration was studied at different uric acid concentrations ranging from 0.005 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}. Apparent K_m and I_{max} were calculated from LB plot between reciprocals of uric acid concentration and biosensor's current response. 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 $ZnSO_4$ at their physiological concentration.

Serum and Urine Uric Acid Determination by Uricase NPs Modified Au Electrode

An amperometric method for serum and urine uric acid determination was developed using the present biosensor. It was the same as described for response measurement of the electrode, except that uric acid was replaced by serum/urine and uric acid concentration was interpolated from the calibration plot, between different uric acid concentrations (mM) and respective current (mA).

Evaluation of Electrode

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

Reusability and Storage Stability of Uricase NPs Modified 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 NPs modified Au electrode was stored dry in a refrigerator at 4 °C.

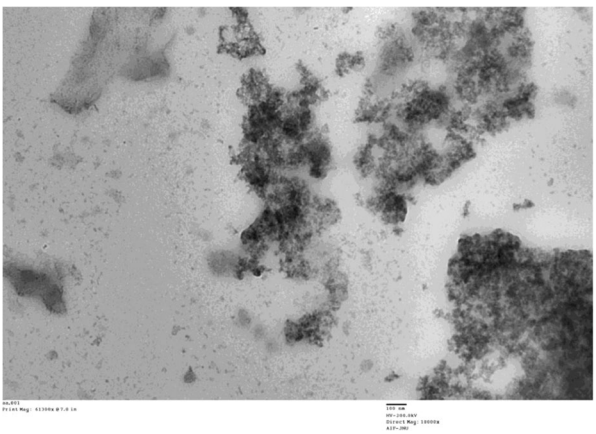


Fig. 1 TEM image of uricase NPs

Results and Discussion

Characterization of Uricase NPs

Thiol groups on the surface of uricase NPs provide a facile method for attaching the uricase NPs onto the surface of Au electrode to develop an enzyme electrode. A typical high-resolution TEM image of the free-standing uricase NPs is shown in Fig. 1. The seemingly white NPs, which result from the transparency of the protein to the electron beam, have a diameter around 100 nm, indicating that single uricase NPs were synthesized and composed of 5–7 cross-linked enzyme molecules (diameter around 14–18 nm) [22].

Scanning Electron Microscopic (SEM) Study

The SEM images of the surfaces of the bare Au electrode and uricase NPs modified Au electrode were taken. SEM of the bare Au electrode (Fig. 2a) showed a smooth and featureless morphology. However, granular and globular shapes on the electrode surfaces (Fig. 2b) display presence of an enzyme NPs layer after immobilization. The SEM images clearly show the immobilization of uricase NPs onto the Au electrode.

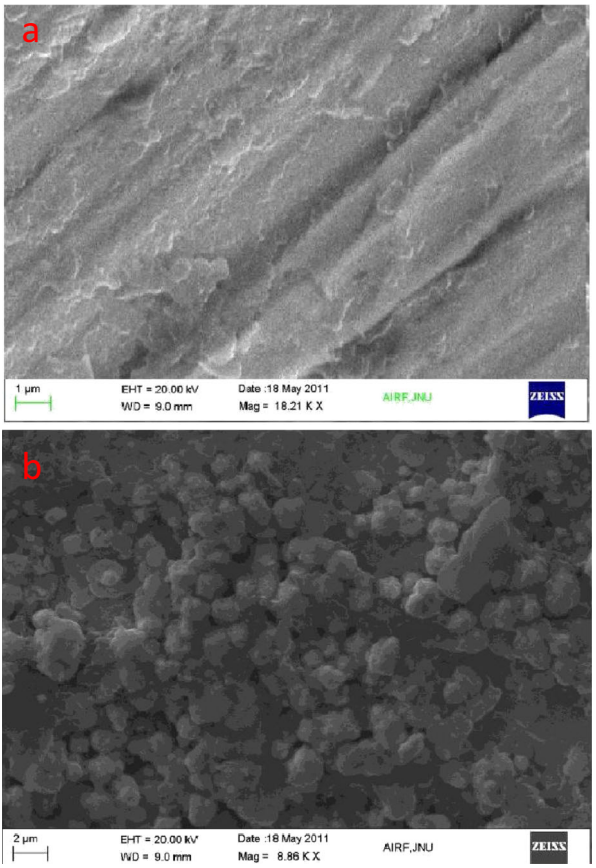


Fig. 2 SEM image of **a** bare Au electrode and **b** uricase NPs modified Au electrode

Electrochemical Characteristics of Uricase NPs onto Au Electrode

Direct electrochemical reactions of redox protein and enzyme at solid electrodes might bring new insights into biological electron transfer processes as well as enable new classes of reagentless biosensors. The resulting uricase NPs/Au interface was characterized by CV to observe whether the enzyme retains its redox activity after cross-linking and self-assembly onto Au surface. CV measurements were carried out in an unstirred electrochemical cell. The voltammogram of the surface-confined enzyme NPs in 50 mM of phosphate buffer (pH 7.4) is shown in Fig. 3a. A pair of stable and well-defined redox peaks with regard to conversion of H_2O_2 into $2H^+ + O_2 + 2e^-$ were observed, with a cathodic peak at +0.19 V and the corresponding anodic peak at +0.31 V, which might be due to electrochemical oxidation-reduction of enzyme. Control experiments, performed with a Au electrode (Fig. 3a), displayed no redox activity at electrode. Both the anodic and cathodic peak currents of uricase NPs were increased with the increase of the scan rate in the range from 10 to 100 mV s^{-1} (Fig. 3b). The ratio of the cathodic to the anodic peak current was near unity. Peak currents varied linearly with the scan rate, as shown in the inset to Fig. 3b, indicating that the electrode reaction was a typical surface-controlled quasi-reversible process.

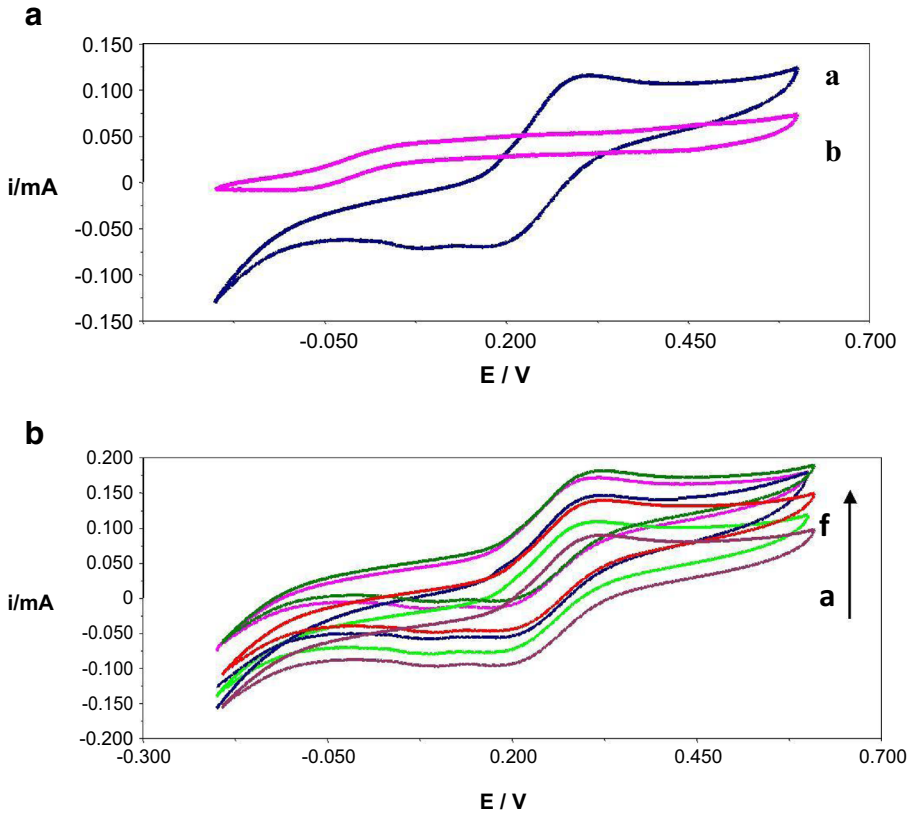


Fig. 3 **a** Cyclic voltammograms of the Au electrode modified by uricase NPs (**a**) and polycrystalline Au electrode (**b**) in 50 mM of phosphate buffer, scan rate 100 mV s^{-1}. **b** Cyclic voltammograms of uricase NPs modified Au electrode in 50 mM of phosphate buffer (pH 7.4) at 10, 20, 40, 60, 80, and 100 mV s^{-1} (from *a* to *f*)

Optimization of Working Conditions of Biosensor

The optimum pH of the biosensor/immobilized uricase NPs was 8.5, which is slightly higher than that of free enzyme (pH 8.0), similar to enzyme bound to polyethylene terephthalate (PET) membrane (8.5) [1], and higher than that on PANI/MWCNT/ITO electrode (7.0) [23]. The increase in optimum pH after immobilization might be due to altered conformation of enzyme NPs due to immobilization. The change in optimum pH of the immobilized uricase toward the basic 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 NPs was 40 °C, which is higher than that of free enzyme (30 °C). The response of the present electrode/immobilized uricase NPs in relation to the change in uric acid concentration was linear in the range of 0.005–0.8 mM (Fig. 4). Apparent K_m value of enzyme electrode/immobilized uricase NPs was 0.058 mM (Fig. 5a). This K_m value was lower than that of polyaniline–polypyrrole film-modified platinum electrode (1.57 mM) [23], self-assembled monolayer on Au electrode (0.90 mM) [24], and polyaniline (7.83 mM) [25]. The decrease in K_m value for uric acid after immobilization indicated increased affinity of immobilized enzyme toward its substrate.

Evaluation of Uricase NPs Modified Au Electrode

There was a linear relationship between current (mA) and uric acid concentration ranging from 0.005 to 0.8 mM with a sensitivity of 0.03 mA μM^{-1} cm^{-2} in Tris–HCl buffer pH 8.5 for uricase NPs bound electrode (Fig. 4), which is better than earlier reported electrodes, 0.025 to 0.1 mM [26] and 0.0 to 0.30 mM [27]. The minimum detection limit of biosensor was 5.0 μM at a signal-to-noise ratio of 3, which is better/lower than that of any other earlier electrochemical uric acid biosensor (0.05 mM) [1]. Analytical recovery of exogenously added uricase in serum (10 and 20 mg l^{-1}) in the method was 99.57 and 98.77 %, respectively, showing the reliability of the method. This analytical recovery was better than 93.6±2.34 and 87.18± 3.17 % [26] and 94.3 and 89.8 % [1]. In order to check the repeatability and reproducibility of the method, the uric acid content in the 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 almost consistent, and within and between batch coefficients of variation (CVs) for serum uric acid determination were <5.6 and <4.7 %,

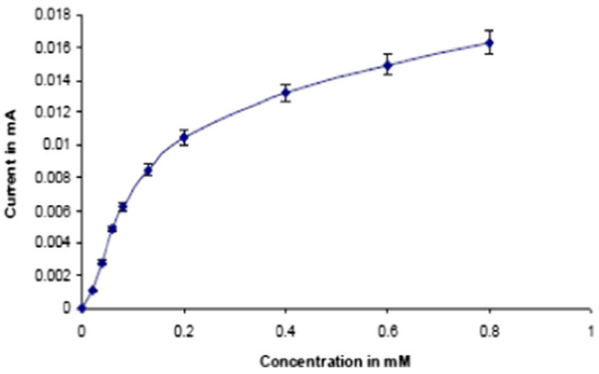


Fig. 4 Effect of uric acid concentration on response of uric acid biosensor based on uricase NPs modified Au electrode

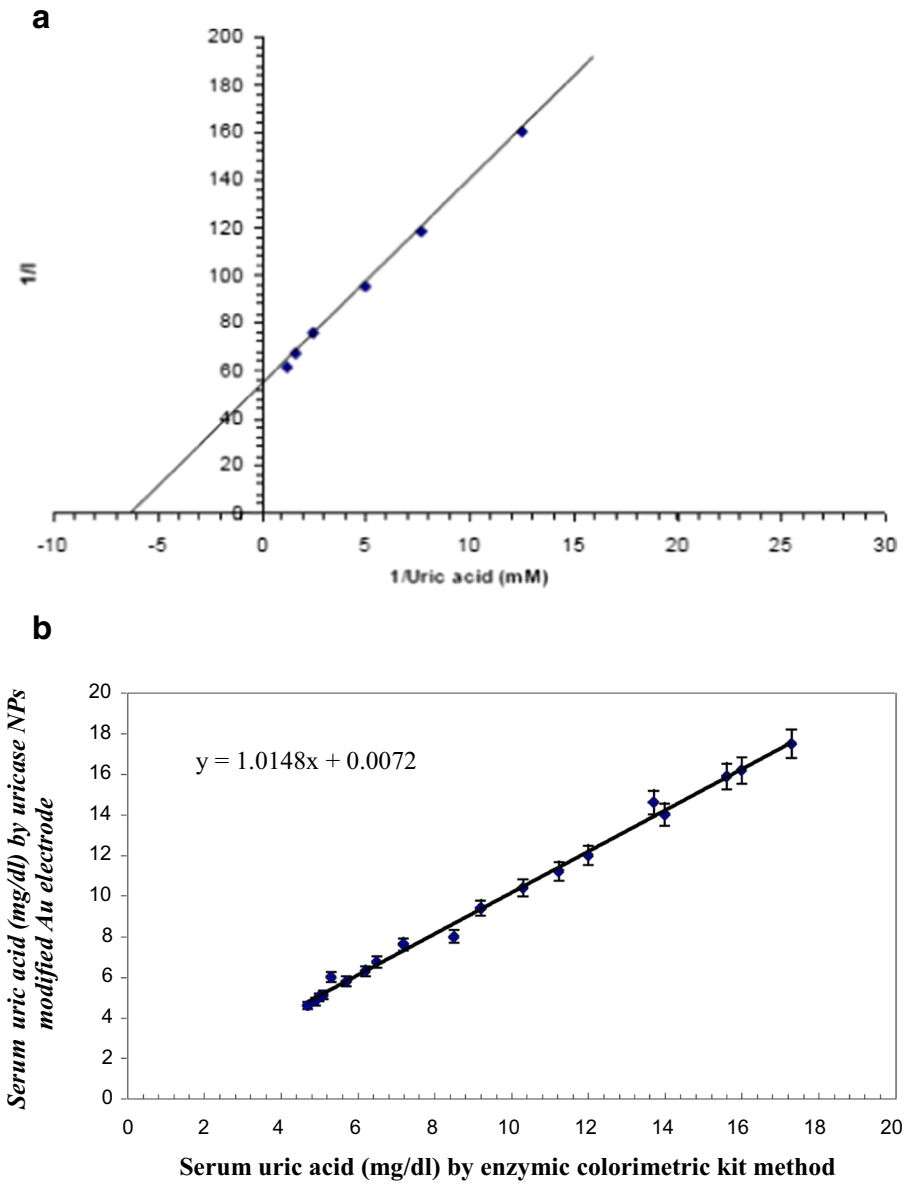


Fig. 5 **a** Lineweaver–Burk plot of the effect of uric acid concentration on response of uric acid biosensor based on uricase NPs modified Au electrode. **b** Correlation between serum uric acid values determined by standard enzymic colorimetric method (x) and the present uricase NPs modified Au electrode (y)

showing the good reproducibility and reliability of the method. This precision is better than previous reports (<6.5 and <5.0 %) [26]. The serum uric acid values obtained by uricase NPs modified Au electrode showed a good correlation (r=0.998) with those by standard enzymic colorimetric method (Fig. 5b), showing the high accuracy of method. This accuracy was better than earlier reports, r=0.98 [28] and r=0.98 [1].

Interference Studies

No interference was observed 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 at their physiological concentration. Bhargava et al. [8] earlier observed lesser, i.e., 2 % decrease in activity,of alkylamine glass bead-bound uricase by NaCl, but Pundir and Bhargava [29] observed no interference by $CaCl_2$ and NaCl in the activity of immobilized porcine liver uricase onto alkylamine glass beads.

Measurement of Serum and Urine Uric Acid

The biosensor was employed to measure uric acid content in serum and urine. The serum uric acid content in healthy adults was from 2.6 to 5.73 mg dl^{-1} with a mean of 4.58 mg dl^{-1}, which is in normal established range (2.18–7.7 mg dl^{-1}), and in diseased persons suffering from gout, leukemia, toxemia of pregnancy, and nephrolithiasis ranged from 6.9 to 11.9 mg dl^{-1} with a mean of 8.803 mg dl^{-1} (n=10) using uricase NPs modified Au electrode (Table 1). The urine uric acid content in healthy adults as measured by present biosensor ranged from 2.7 to 5.4 mg dl^{-1} with a mean of 3.68 mg dl^{-1} (n=10) and 7.42 to 12.91 mg dl^{-1} with a mean of 10.20 mg dl^{-1} for diseased persons (Table 2).

Reusability and Storage Stability of Uricase NPs Modified Au Electrode

Uricase NPs modified Au electrode storage stability was tested by measuring its activity every week over a period of 7 months. The uricase NPs modified Au electrode lost only 15 % of its initial activity, after its 200 regular use over a period of 7 months, when stored at 4 °C. 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 NPs modified Au electrode over previously reported sensors

Table 1 Serum uric acid level in apparently healthy individuals and diseased persons as measured by biosensor based on uricase NPs modified Au electrode

S. no.	Sex	Serum uric acid (mg dl^{-1}) in apparently healthy individuals	Serum uric acid (mg dl^{-1}) in diseased persons
1	M	2.6	7.28
2	M	3.8	6.9
3	F	4.8	9.58
4	F	6.7	8.62
5	F	5.7	7.4
6	M	3.1	8.9
7	F	5.4	10.0
8	M	4.3	11.9
9	M	3.7	8.5
10	F	5.73	8.95
		Mean=4.58	Mean=8.803

Table 2 Urine uric acid level in apparently healthy individuals and diseased persons as measured by biosensor based on uricase NPs modified Au electrode

S. no.	Sex	Urine uric acid (mg dl^{-1}) in apparently healthy individuals	Urine uric acid (mg dl^{-1}) in diseased persons
1	M	3.6	14.22
2	M	3.8	8.91
3	F	3.8	8.58
4	F	2.7	11.62
5	F	3.7	7.42
6	M	3.1	8.90
7	F	5.4	11.00
8	M	4.3	12.91
9	M	3.7	9.50
10	F	2.73	8.95
		Mean=3.68	Mean=10.20

[14, 30] may be attributed to the presence of uricase NP aggregates with a large surface area and strong affinity to bind with the electrode.

Conclusion

A novel amperometric biosensor for uric acid was developed based on adsorption of uricase NPs modified Au electrode. This uricase NPs modified Au electrode reveal improved performance in terms of response time (7 s), sensitivity (0.03 mA μM^{-1} cm^{-2} in 0.005–0.8 mM uric acid), low apparent K_m (0.058 mM), reusability (more than 100 times), and stability (7 months).

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