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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