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An amperometric H_2O_2 biosensor based on cytochrome c immobilized onto nickel oxide nanoparticles/carboxylated multiwalled carbon nanotubes/polyaniline modified gold electrode

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ABSTRACT

Cytochrome c was immobilized covalently onto nickel oxide nanoparticles/carboxylated multiwalled carbon nanotubes/polyaniline composite (NiO-NPs/cMWCNT/PANI) electrodeposited on gold (Au) electrode. An amperometric H_2O_2 biosensor was constructed by connecting this modified Au electrode along Ag/AgCl as reference and Pt wire as counter electrode to the galvanostat. The modified Au electrode was characterized by cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), scanning electron microscopy (SEM) and Fourier transform infra-red spectroscopy (FTIR). Cyclic voltammetric (CV) studies of the electrode at different stages demonstrated that the modified Au electrode had enhanced electrochemical oxidation of H_2O_2, which offered a number of attractive features to develop an amperometric biosensor based on split of H_2O_2. There was a good linear relationship between the current (mA) and H_2O_2 concentration in the range 3–700 μM. The sensor had a detection limit of 0.2 μM (S/N = 3) with a high sensitivity of 3.3 mA μM^{-1} cm^{-2}. The sensor gave accurate and satisfactory results, when employed for determination of H_2O_2 in different fruit juices.

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1. Introduction

The rapid and accurate determination of hydrogen peroxide (H_2O_2) is very important, as it is not only the product of the reactions catalyzed by many highly selective oxidases, but also employed in various fields such as food, pharmaceutical and environmental analysis [1–3]. H_2O_2, a powerful oxidizing agent, is usually utilized as an antimicrobial agent in food and sterilizing agent on the foil lining of aseptic packages containing fruit juices and milk products. The higher concentrations of H_2O_2 are associated with the diabetes, atherosclerosis and aging as it generates free hydroxyl radicals, which cause oxidative damage of the tissue components such as lipids and proteins beside DNA [4,5]. A number of analytical methods are available for determination of H_2O_2 such as titrimetry [2], colorimetry/spectrometry [3], chemiluminescence [6], high performance liquid chromatography (HPLC) and idiometry [7]. Electrochemical biosensing method is one of the promising approaches, because of its simplicity, rapidity and high sensitivity. In recent years, direct electrochemistry of metalloproteins and metalloenzymes has attracted the attention of many scientists', because of its potential application in the study of redox and electron transfer properties of biomolecules and in fabricating mediator-free or the third generation biosensors [8]. The proteins containing heme groups, such as hemoglobin, myoglobin and cytochrome c (Cyt c), possess peroxidase like catalytic activity for reduction of H_2O_2, due to the electroactive center of heme [9] and therefore employed for construction of H_2O_2 sensors [10,11].

Cyt c is a very basic redox heme protein, whose main function is to deliver electrons from Cyt c reductase to Cyt c oxidase. However, electron transfer between heme and bare electrode is usually slow and the protein is irreversibly denatured. Thus, it is necessary to search the way to develop a heme-modified electrode with well-behaved electrochemistry and good stability. The nanoparticles are known to adsorb redox enzymes and proteins, without loss of their biological activity. In addition, the electron transfer ability (direct electrochemistry) and biocatalytic activity of enzymes were increased, when they were adsorbed on nanomaterials. In recent years, nickle oxide nanoparticles (NiO-NPs) has attracted extensive interests due to its high surface to volume ratio, novel optical, electronic, magnetic, thermal, mechanical properties, quantum confinement effect and potential application as catalyst, in battery electrodes, gas sensors, electrochemic films and photoelectronic devices [12]. Carbon nanotubes (CNT) due to their unique physical properties, including high electrical conductivity, superior chemical and mechanical stability and large surface area, have established themselves as a good material for immobilization of enzymes, multiwalled carbon nanotubes (MWCNT) enhance the

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direct electron transfer between the enzyme's active sites and the electrode.

Among various conducting polymers, polyaniline (PANI) is unique, due to its relatively facile synthesis, conductivity and environmental stability. PANI/CNT composites have been prepared by electropolymerization of aniline or by in situ chemical polymerization [13].

We report herein the covalent immobilization of cytochrome c onto nickel oxide nanoparticles/carboxylated multiwalled carbon nanotubes/polyaniline composite electrodeposited on Au electrode for an amperometric determination of hydrogen peroxide.

2. Materials and method

2.1. Reagents and materials

Carboxylated multi-walled carbon nanotubes (cMWCNT) was obtained from Intelligent Materials Pvt. Ltd., Panchkula (Haryana) India. Nickle chloride was from Merck, Germany. Aniline and Cyt c were from SISCO Research Lab., Mumbai, India. All other chemicals were of analytical reagent (AR) grade. Au electrode (2 cm × 1 cm) was purchased from local market.

2.2. Apparatus

Potentiostat/galvanostat (Make: Autolab, model: AUT83785, manufactured by Eco Chemie) with a three electrode system composed of a Pt wire as an auxillary electrode, an Ag/AgCl electrode as reference electrode and Cyt c/NiONPs/MWCNT/PANI modified Au electrode as a working electrode. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Zeiss EV040). UV spectroscopy (Make: Shimadzu, Model 160 A), transmission electron micrography (TEM) (JEOL 2100 F) X-ray diffractometer (XRD), (Make: 122 Rigaku, D/Max2550, Tokyo, Japan) Fourier transform infra-red spectroscopy (FTIR) (Thermo Scientific, USA).

2.3. Construction of Cyt c/NiO-NPs/cMWCNT/PANI modified Au electrode

2.3.1. Preparation of NiO nanoparticles

NiO nanoparticles were synthesized by two step method [14]. Firstly 2.3 g $NiCl_2$ in 10 ml double distilled water (DDW) and 1.5 g $NaHCO_3$ in 10 ml DDW were dissolved separately in glass beakers. The $NiCl_2$ solution was stirred on a magnetic stirrer for 15 min and then $NaHCO_3$ solution was added to $NiCl_2$ solution drop wise under constant stirring. After 15 min, the products were collected by centrifugation and washed thoroughly with DDW and dried in air. The structural property of NiO-NPs was studied.

2.3.2. Electrodeposition of NiO-NPs/cMWCNT/PANI onto Au electrode

The surface of a Au electrode (2 cm × 1 cm) was polished manually by alumina slurry (diameter 0.05 μm) with a polishing cloth, followed by thorough washing with DDW, placed into ethanol, sonicated to remove adsorbed particles and washed finally with DDW. cMWCNT (1.0 mg) were suspended into 4 ml mixture of concentrated H_2SO_4 and HNO_3 in 3:1 ratio and ultrasonicated for 6–8 h to get its finely dispersed black colored solution. Aniline (200 μl) was added to 25 ml of 1N HCl and electropolymerized onto Au electrode through cyclic voltammetry by applying ten successive polymerization cycles at −0.2 to 0.8 V. NiO-NPs suspension (200 μl) and 1 ml of cMWCNT suspension (1:5 ratio) were added into 25 ml 1N HCl to get the NiO-NPs/cMWCNT mixture. Finally, electrodeposition of NiO-NPs and cMWCNT onto the Au electrode was carried out in an electrochemical cell system by applying five polymerization cycles at −0.2 to 0.8 V. During the electrochemical polymerization, the surface of Au wire became green gradually, indicating the deposition of NiO-NPs/cMWCNT/PANI film on Au wire (Fig. 1A). The resulting NiO-NPs/cMWCNT/PANI modified Au electrode was washed thoroughly with distilled water to remove unbound matter and kept in a dry Petri-plate at 4 °C.

2.3.3. Immobilization of cytochrome c onto NiO-NPs/cMWCNT/PANI modified Au electrode

To prepare Cyt c solution, 100 mg of Cyt c (oxidized) was dissolved in 8 ml of 10 mM potassium phosphate buffer (pH 7.0). Cyt c (oxidized) was reduced by adding 3–5 mg of sodium ascorbate. The excess of ascorbate was removed by dialyzing Cyt c solution against 10 mM of potassium phosphate buffer (pH 7.0) for 24 h at 4 °C with three changes of buffer. After dialysis, the volume of Cyt c solution was brought to 10 ml with the same buffer. This Cyt c remains reduced for several months, if stored at 4 °C. Cyt c (reduced) solution (2 ml) was placed onto surface of NiO-NPs/cMWCNT/PANI modified Au electrode and kept overnight at 4 °C for immobilization. The resulting electrode (with immobilized Cyt c) was washed 3–4 times with a 50 mM potassium phosphate buffer (pH 7.0) to remove residual/unbound Cyt c. Protein concentration was calculated in wash out solution to calculate conjugation yield. The resulting Cyt c/NiO-NPs/cMWCNT/PANI Au electrode was used as working electrode and stored at 4 °C, until use. This working electrode was characterized by SEM and FTIR at different stages of construction.

2.3.4. Cyclic voltammetric measurement and testing of H_2O_2 biosensor

Cyclic voltammogram (CV) of Cyt c/NiO-NPs/cMWCNT/PANI Au electrode was recorded in potentiostat–galvanostat from −0.1 to 0.9 V vs. Ag/AgCl as reference and Pt wire as counter electrode in a 25 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 100 μl (100 μM) of H_2O_2. The maximum response (current in mA) was observed at 0.28 V (Fig. 1B) and hence subsequent studies were carried out at this voltage. The following electrochemical reactions occurred during response measurement:

$$\text{Chemical reaction}: \ 2\text{cyt c-Fe(II)} + 2H^+ + H_2O_2 \rightarrow 2\,\text{cyt c-Fe(III)}| + 2H_2O$$

$$\text{Electrochemical reduction}: \ \text{cyt c-Fe(III)} + e^- \rightarrow \text{cyt c-Fe(II)}$$

2.4. Optimization of H_2O_2 biosensor

To optimize working conditions of the biosensor, effects of pH, incubation temperature, time and substrate (H_2O_2) concentration were studied on biosensor response. To determine optimum pH, the pH was varied between pH 4.5 and 9.0 at an interval of pH 0.5 using the following buffer, each at a final concentration of 0.01 M: pH 4.5–7.5 potassium phosphate buffers and pH 8.0–9.0 Tris–HCl buffers. Similarly to determine optimum temperature the reaction mixture was incubated at different temperature (20–50 °C) and time (1–20 s). The effect of H_2O_2 concentration on biosensor response was determined by varying the concentration of H_2O_2 in the range 3–700 μM.

2.5. Application of H_2O_2 biosensor in fruit juices

The sensor was employed for quantitative determination of hydrogen peroxide in commercially available juice of some fruits (green grapes, banana, papaya, mausmi, green apple) and sugarcane stem. Each juice was centrifuged at 8000 × g for 5 min and supernatant was used for an amperometric analysis. The fruit juice was analyzed for H_2O_2 as described for testing of biosensor under optimal conditions except that H_2O_2 solution was replaced by fruit juice. The H_2O_2 content in fruit juices was calculated from standard curve between H_2O_2 concentration vs. current in mA prepared under optimal assay conditions of Cyt c/NiO-NPs/cMWCNT/PANI Au electrode (Fig. 1C).

2.6. Evaluation

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

2.7. Interference study

It was carried out by measuring the sensor response before and after addition of some interferant (dopamine, ascorbic acid, L-cysteine, and glucose) into the 0.1 M phosphate buffer containing 100 μM H_2O_2 at their physiological concentration. The percentage of current ratio before and after addition of interferrant was calculated.

2.8. Reusability and storage stability of Cyt c/NiO-NPs/cMWCNT/PANI/Au electrode

The reusability and stability of the working electrode was studied by measuring its activity for six months at an interval of one week. The present electrode system was stored in dried condition at 4 °C when not in use.

3. Results and discussion

3.1. SEM studies of Au electrode during modification

The SEM images of the surfaces of the bare Au electrode, NiO-NPs/cMWCNTs/PANI/Au electrode and Cyt c/NiO-NPs/cMWCNT/PANI/Au electrode are shown in Fig. 2. The stepwise modification of electrode could be seen clearly from these SEM images. The SEM image of the bare Au electrode showed a smooth and featureless morphology Fig. 2(a). The NiO-NPs/cMWCNTs/PANI composite film showed net and porous structure, which provides larger surface area Fig. 2(b). On immobilization of Cyt c, the globular structures of Cyt c on the surface of NiO-NPs/cMWCNTs/PANI/Au was seen due to the covalent interaction between Cyt c and modified Au electrode Fig. 2(c).

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