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Amperometric determination of serum total cholesterol with nanoparticles of cholesterol esterase and cholesterol oxidase

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ABSTRACT

We describe the preparation of glutaraldehyde cross-linked and functionalized cholesterol esterase nanoparticles (ChENPs) and cholesterol oxidase nanoparticles (ChOxNPs) aggregates and their co-immobilization onto Au electrode for improved amperometric determination of serum total cholesterol. Transmission electron microscope (TEM) images of ChENPs and ChOxNPs showed their spherical shape and average size of 35.40 and 56.97 nm, respectively. Scanning electron microscope (SEM) studies of Au electrode confirmed the co-immobilization of enzyme nanoparticles (ENPs). The biosensor exhibited optimal response at pH 5.5 and 40 °C within 5 s when polarized at +0.25 V versus Ag/AgCl. The working/linear range of the biosensor was 10–700 mg/dl for cholesterol. The sensor showed high sensitivity and measured total cholesterol as low as 0.1 mg/dl. The biosensor was evaluated and employed for total cholesterol determination in sera of apparently healthy and diseased persons. The analytical recovery of added cholesterol was 90%, whereas the within-batch and between-batch coefficients of variation (CVs) were less than 2% and less than 3%. There was a good correlation ($r = 0.99$) between serum cholesterol values as measured by the standard enzymic colorimetric method and the current method. The initial activity of ENPs/working electrode was reduced by 50% during its regular use (200 times) over a period of 60 days when stored dry at 4 °C.

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Enzyme nanoparticles (ENPs)[1] are the aggregated form of enzyme molecules in nanometer scale within the dimensions 10–100 nm. These ENPs exhibit unique optical, electrical, electronic, thermal, chemical, and catalytic properties besides increased surface area. Direct immobilization of enzymes onto nanomaterials may cause denaturation, leading to loss of activity. This problem was solved by aggregating the enzyme molecules to form their nanoparticles (NPs) and cross-linking within them in a controlled manner. This has resulted in promising strategies for development of improved biosensors in terms of limit of detection as well as current response [1]. ENPs aggregates of a few enzymes such as horseradish peroxidase (HRP) [2], glucose oxidase (GOD) [3,4], uricase [5], and cholesterol oxidase (ChOx) [6] have been immobilized onto the surface of metal electrodes during the past decade to construct improved biosensors for H_2O_2, glucose, uric acid, and cholesterol, respectively.

Measurement of total cholesterol level in serum is very important due to its presumed relationship to atherosclerosis and cardiovascular diseases, nephritis, myxedema, obstructive jaundice, diabetes mellitus, and cerebral thrombosis [7]. However, the recently reported cholesterol oxidase nanoparticles (ChOxNPs)-based cholesterol biosensor requires the pretreatment of serum samples with 0.1 ml of cholesteryl esterase (5 mg/ml) at 37 °C for 10 min prior to analysis, which complicates the procedure and renders it expensive and time-consuming. This problem was overcome in the current work by co-electrodepositing cholesterol esterase nanoparticles (ChENPs) with ChOxNPs onto Au electrode. To the best of our knowledge, there has been no report on preparation of ChENPs and their co-immobilization/electrodeposition with ChOxNPs onto an electrode so far. The co-electrodeposited enzymes form a single-step procedure; hence, activity is dependent on this one step as compared with multiple steps in case of

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[1] *Abbreviations used*: ENP, enzyme nanoparticle; NP, nanoparticle; HRP, horseradish peroxidase; ChOx, cholesterol oxidase; ChOxNP, cholesterol oxidase nanoparticle; ChENP, cholesterol esterase nanoparticle; ChE, cholesterol esterase; DW, double-distilled water; TEM, transmission electron microscope; SEM, scanning electron microscope; PB, phosphate buffer; CV, cyclic voltammetry; MWCNT, multiwall carbon nanotube.

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individual enzymes. Furthermore, co-electrodeposition provides close proximity of enzymes, which leads to easy diffusion of intermediates between different enzymes in the reaction medium [8]. Thus, co-electrodeposition of ChENPs and ChOxNPs onto Au electrode is expected to provide better ENPs electrode for total cholesterol determination.

Materials and methods

Reagents

4-Aminophenazone, HRP from Sigma–Aldrich, phenol, cholesterol, ChOx from microorganism sp. (130 U/mg), cholesterol esterase (ChE) from porcine pancreas (15 U/mg), Triton X-100, glutaraldehyde, alumina slurry, chitosan, isopropanol, cholesteryl acetate from SRL Mumbai, and ethanol from Changshu Yangyuan Chemical (China) were used. Gold wire (1.5 × 0.05 cm, 23 karat) was purchased from a local market. All other chemicals were of analytical reagent (AR) grade. Double-distilled water (DW) was used throughout the experiments.

Instruments

Autolab PGSTAT 30 electrochemical workstation (Eco Chemie, The Netherlands) with GPES 4.9 software, a transmission electron microscope (TEM; JEOL 2100F, Japan), and a scanning electron microscope (SEM; JSM-6510, JEOL) at Jawaharlal Nehru University (New Delhi, India). A modified Au electrode, a KCl-saturated Ag/AgCl electrode, and a Pt electrode served as the working, reference, and counter electrodes, respectively, in all electrochemical experiments. All current responses were measured at room temperature against Ag/AgCl reference electrode. DW was used throughout the experiments. TEM and SEM images of ENPs and ENPs modified electrode were taken at Advanced Instrument Research Facility (AIRF) of Jawaharlal Nehru University.

Dissolution of enzymes

Both ChE and ChOx were dissolved individually in 0.02 M sodium phosphate buffer (PB, pH 7.0) at a concentration of 1.0 mg/ml and stored at 4 °C until use.

Assay of ChE

The assay of ChE was carried out according to Ref. [9] with some modifications. The reaction mixture, consisting of 1.7 ml of PB (0.05 M, pH 7.0) containing 50 mmol/L of tauro-deoxycholate, 0.1 ml of ChE solution, and 0.1 ml of ChOx solution, was preincubated at 37 °C for 2 min. The reaction was started by adding 0.1 ml of cholesteryl acetate solution (500 mg/dl). Then, 1.0 ml of color reagent was added, and the reaction mixture was kept in the dark at 37 °C for 10 min to develop the color. Next, A_{520} of the color was read against control in the Spectronic 20D, and the content of H_2O_2 generated in the reaction was calculated from the standard curve between H_2O_2 concentration and A_{520}. One unit of ChE is defined as the amount of enzyme protein required to generate 1.0 μmol of H_2O_2/min/ml. This H_2O_2 was generated from oxidation of cholesterol by ChOx, which in turn was produced from hydrolysis of cholesteryl acetate by ChE.

Preparation of cholesteryl acetate solution

Cholesteryl acetate (50 mg) was dissolved in 1.0 ml of Triton X-100 by slowly heating and stirring until the solution was clear. PB (0.05 M, pH 7.0) was added to get a final concentration of 50 mg/dl and stored at 4 °C until use [10].

Preparation of color reagent

The color reagent was prepared by dissolving 50 mg of 4-aminophenazone, 100 mg of phenol, and 1.0 mg of HRP in 100 ml of PB (0.4 M, pH 7.0) and stored in an amber-colored bottle at 4 °C and prepared fresh every week.

Assay of ChOx

Assay of ChOx was carried out in a 15-ml test tube wrapped with black paper according to Ref. [9] with modification. The reaction mixture, consisting of 1.8 ml of PB (0.05 M, pH 7.0) containing 0.4% Triton X-100, 0.1 ml of cholesterol solution, and 0.1 ml of ChOx solution, was incubated for 10 min at 37 °C. Color reagent (1.0 ml) was added and kept at 37 °C for 15 min to develop the color. Then, A_{520} was read, and the content of H_2O_2 was interpolated from the standard curve between the H_2O_2 concentration and A_{520}.

One unit of ChOx is defined as the amount of enzyme protein required to generate 1 μmol of H_2O_2 from cholesterol/min/ml.

Preparation and characterization of ChENPs

NPs of ChE were prepared separately by desolvation with ethanol and subsequent cross-linking with glutaraldehyde [4]. First, 2 ml of enzyme solution (2 mg/ml) was transformed into ENPs by continuously pipetting 4 ml of desolvating agent (i.e., ethanol) into 2 ml of enzyme solution under continuous stirring at 500 rpm and at a dropping rate of 0.1 ml/min. After the desolvation process, 1.8 ml of 2.5% glutaraldehyde was added to these ENPs, and the suspension was stirred for 24 h to ensure the cross-linking of ENPs and, thus, forming ENPs. The resulting ENPs were purified by three cycles of differential centrifugation (14,000 *g*, 10 min) at 4 °C, and a pellet was redispersed to the original volume in DW. Each redispersion step was performed in an ultrasonication bath over 5 min. Amino (–NH_2) groups were introduced on ENPs by adding 0.12 g of cysteamine dihydrochloride with constant stirring for 5–6 h. Lastly, functionalized ENPs were separated from free enzyme solution by centrifugation at 1200 rpm at 4 °C for 10 min, followed by dispersion in PB (0.1 M, pH 7.0), and stored at 4 °C. The shape and size of ChENPs was studied by taking their images in with a TEM at the Advanced Instrument Research Facility on a commercial basis.

Preparation and characterization of ChOxNPs

NPs of ChOx were also prepared and characterized as described above for ChENPs.

Co-electrodeposition of ChENPs + ChOxNPs on Au electrode: construction of enzyme electrode

Prior to the surface modification, Au wire was cleaned with piranha solution (H_2SO_4/H_2O, 3:1) for 20 min and then rinsed thoroughly with DW. The cleaned Au electrode was then polished with silica slurry. The mixture of ChENPs + ChOxNPs suspension in a 1:1 ratio was electrochemically deposited onto polished Au electrode with cyclic voltammetry (CV) by immersing it into a mixture of 23 ml of 2.5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1) and 2 ml of ChENPs + ChOxNPs mixture and then applying a potential between −0.2 and + 0.6 V (vs. Ag/AgCl) for 20 cycles at a scan rate

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