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Activated direct electron transfer of nanoAu bioconjugates of cytochrome c for electrocatalytic detection of trace levels of superoxide dismutase enzyme

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ABSTRACT

A novel amperometric biosensor for the detection of trace levels of superoxide dismutase enzyme is developed with the use of nanoAu bioconjugates of cytochrome c (Cyt c). NanoAu particles of finite size were synthesized by borohydride reduction in the presence of alkanethiols having —COOH and —OH end groups. The nanoAu particles were characterized by transmission electron microscopic analysis, and the mean size of the nanoparticles is determined to be 2.96 nm diameter. The heme protein, Cyt c, is bound to the nanoAu particles, and the resulting nanoAu bioconjugates are investigated by cyclic voltammetric and rotating-disk voltammetric experiments. Cyt c has become electrodically reactive by binding with the nanoAu particles, and Cyt c-bound nanoAu particles exhibited a reversible, mass-transport limited electron-transfer reaction. Reversible redox peaks are observed with the formal redox potential of +0.05 V (vs. Ag|AgCl) at bare GC and also at Au|alkanethiolate monolayer electrodes. Cyt c is highly reactive to superoxide radical, and electrocatalytic oxidation of superoxide occurs at the applied potential of +0.15 V in the presence of the nanoAu bioconjugates. Steady state current–time curves show a sharp increase in the anodic current to the generation of superoxide radical and attain a plateau in ca. 6 min. The structure and morphology of the alkanethiolate layer at the nanoAu-Cyt c interface tremendously influences the electrocatalytic current for superoxide. The electrocatalytic current observed for superoxide radical varied sharply by the presence of superoxide dismutase. From the dependence of the electrocatalytic current for superoxide on the concentration of superoxide dismutase, a low-detection-limit of as low as 0.25 U mL^{-1} (~50 ng mL^{-1}) has been established.

1. Introduction

Superoxide radical is highly reactive and is continuously generated during cell respiration and metabolism. It plays an important role in the destruction of microorganisms invading into our body. It is however frequently observed that high amounts of superoxide formation can exceed the antioxidant abilities of cells and may be involved in oxidative damage to tissues, nerve cells, etc. Such excessive generation of superoxide leads to the development of various pathologies such as cardiovascular dysfunction, arteriosclerosis, ischemia, and critical neurodegenerative diseases. Superoxide dismutase enzyme (SOD) plays a major protective role in living cells and has been widely used as a pharmacological tool in the study of pathophysiological mechanisms. Ubiquitous presence of SOD throughout the evolutionary chain emphasizes its importance, and the detection of SOD enzyme is essential if we are to better control the degenerative processes and to diagnose more accurately the diseases in which it is involved [1–4].

SOD is a potential and selective scavenger of superoxide, and the best way for the detection of SOD thus could be the analysis of superoxide radical. Electrochemical methods are of great importance in the development of sensors because of its potential advantages such as portable instrumentation, in vivo analysis with microprobes, on-line measurement, miniaturization and low equipment cost [5]. Biorecognition elements impart high selectivity and sensitivity to sensors, enabling direct detection of the analyte of interest from complex sample matrices without any purification or pre-concentration steps. Cytochrome c (Cyt c) would undergo facile reductive reaction with superoxide radical, and thus electrochemical sensors for detection of superoxide were constructed with Cyt c as biorecognition element using suitable electron-transfer promoters [6–9]. Mediatorless third-generation biosensors have been fabricated for the detection of superoxide and SOD with the use of monolayer-based functional electrodes. Self-assembled monolayers of alkanethiols with covalently bound Cyt c were investigated for the detection of superoxide radical and SOD. To improve the sensor performances, nanoAu particles of rod-like, spherical, and pyramidal structures [10], nano NiO powder [11], etc. were investigated as the base electrode surface. However, the biorecognition element is limited merely up to a

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monolayer level. Thus, three-dimensional macroporous materials [12], sol–gel matrix [13,14], and meticulously fabricated layer-by-layer multilayer assembly [15] have been investigated to increase the active surface area and thus the sensitivity.

Gold nanoparticles have emerged as a new kind of inspiring materials and have been widely used in the modification of various electrodes and in the fabrication of different kinds of biosensor platforms. They could act as electron-transfer relays and thus could promote the heterogeneous electron transfer between the biorecognition element and the electrode [16–20]. Thus, such nanoAu bound biorecognition elements would be immensely useful in fabricating high-performance three-dimensional matrix based electrochemical sensors and would provide added advantages of mediatorless sensors and three-dimensional bulk matrices.

In this investigation, finite nanogold particles passivated with short chain alkanethiols having carboxyl and hydroxyl end groups are synthesized, and Cyt c is bound to the —COOH groups present around of the nanogold particles. The electrochemical characteristics of the nanoAu-Cyt c conjugates are investigated by cyclic voltammetric and rotating-disk voltammetric experiments. Electrocatalytic activity of the nanoAu-Cyt c conjugates with superoxide radical is investigated for the detection of SOD enzyme. Influence of the surface morphology of the nanogold particles on the electrocatalytic activity and thus on the sensor performance are discussed.

2. Experimental

2.1. Reagents

Horse heart cytochrome c (Cyt c), superoxide dismutase (from bovine erythrocytes; 4600 U mg^{-1}) xanthine oxidase (XOD), hydrogen tetrachloroaurate, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) were received from Sigma, stored at −20 °C and used as supplied. Sodium borohydride, xanthine, 3-mercaptopropionic acid (MPA), 3-mercaptopropanol (MPO), 2-aminoethanothiol (AET), disodium hydrogen phosphate, sodium dihydrogen phosphate, and solvents used in this study were of analytical grade and were used as supplied from Tokyo Chemical Industry without further purification. Distilled deionized water filtered at last with 0.2 μm pore filter cartridge (resistance = 18 MΩ) was used for all the aqueous solutions.

2.2. NanoAu-Cyt c conjugate preparation

NanoAu particle was prepared by borohydride reduction of gold (III) chloride in non-aqueous solvent according to the Brust method reported elsewhere [21]. Briefly, 0.16 g hydrogen tetrachloroaurate (III) (0.38 mmol) was dissolved in 7 mL water, and 450 mL of methanol, 0.12 g mercaptopropionic acid (MPA; 1.13 mmol), and acetic acid (3 mL) were added to the Au (III) solution. The solution was cooled under stirring in an ice-bath. Sodium borohydride (0.3 g, 8 mmol) dissolved in 10 mL water was added in small portions to the resultant yellowish Au (III) solution under stirring in a period of 90 s, during which the solution turned to dark black-brown color. The mixture was kept under stirring at 5 °C for 3 h and centrifuged (18,000 rpm) to precipitate the nanoAu particles protected with a monolayer of the alkanethiol (MPA). The precipitate was redistributed in methanol and centrifuged to remove the unreacted compounds. The resulting nanoAu particles were dissolved in 25 mM sodium phosphate buffer (pH 8.0) and filtered through nanoporous cellulose filter membrane (Millipore 25 nm cut-off membrane) to remove aggregated large nanoparticles. The nanoAu particles thus prepared was denoted hereafter as nanoAu/MPA. The surface morphology of the nanoparticles was changed by treating nanoAu/MPA with 3-mercaptopropanol (MPO) in PBS for 1 h, and the resultant mixture was dialyzed to prepare nanoAu particles protected with a mixed binary layer of alkanethiols. The nanoAu particles thus prepared are denoted as nanoAu/MPA + MPO. The nanoAu particles were imagined with transmission electron microscope (TEM) analysis. A dilute solution of the nanoparticle in 25 mM PBS (pH 8.0) was spread on amorphous carbon support, dried and rinsed with PBS. The nanoparticles were imagined with a Hitachi HF-2000 TEM analyzer at the magnification of 1.5 million times.

Cyt c was bound to the nanogold particles through electrostatic interaction by adding 12 mg of cytochrome c to 10 mL nanogold solution, and the resultant mixture was filtered through MWCO membrane (30,000 Da; Whatman) to remove excess unbound cytochrome c. The resultant nanogold-Cyt c conjugate in PBS was treated with EDAC (0.3 mg mL^{-1}) for 1 h to bind Cyt c covalently with the nanogold particles. The concentration of Cyt c bound to the nanoAu particles was estimated by determining the amount of Cyt c adsorbed using UV–vis spectroscopy. The resultant covalently bound nanoAu-Cyt c bioconjugates were denoted hereafter as nanoAu/MPA/Cyt c and nanoAu/MPA + MPO/Cyt c.

2.3. Voltammetric and electrocatalytic experiments

Cyclic voltammetric and rotating-disk voltammetric experiments were carried out with a BAS 100B/W electrochemical analyzer using a conventional two-compartment three-electrode cell. GC and Au electrodes of 5.0 mm and 1.6 mm diameter act as working electrodes, a platinum coil as counter electrode and an Ag|AgCl (NaCl satd.) as reference electrode, and the experimental solutions were deaerated with nitrogen for 15 min. Phosphate buffer solution (PBS; pH 7.0) of 25 mM concentration was used as the electrolyte solution. The working electrodes were polished with aqueous slurries of alumina (10 μm, 1 μm, and then 0.05 μm), washed and cleaned with ultra-sonication for 2 min in water. Au working electrodes were then electrochemically polished by potential scanning (scan rate = 10 V s^{-1}) in 0.05 M sulfuric acid from −0.2 V to 1.5 V for 10 min. The resulting Au electrodes were modified with an alkanethiol monolayer by dipping into an ethanol solution of 10 mM alkanethiol (AET, MPA, etc.) for 1 h and rinsed well with ethanol and water. The resulting electrodes were denoted as Au/AET, Au/MPA, etc.

Superoxide radical for electrocatalytic experiments was generated by xanthine-XOD enzymatic reaction to maintain a steady-state concentration of the superoxide radical. In electrocatalytic experiments, the electrolyte solution was oxygen-saturated by bubbling oxygen for 15 min and stirred with magnetic stirrer gently at 200 rpm to perform at hydrodynamic conditions.

3. Results and discussion

3.1. Synthesis of nanoAu particles and TEM analysis

NanoAu particles have been synthesized in this study according to a modified Brust reaction performed in a single-phase non-aqueous solvent. They are covered with a monolayer or mixed-monolayer of alkanethiols having functional end groups —COOH and —OH, which prevents aggregation of the nanoparticles and also promotes the dispersion of the nanoparticles in solution. A dilute solution of the nanoparticles (0.6 mg mL^{-1}) in PBS (pH 7.0) was spread on an amorphous carbon support, and the substrate was dried and rinsed gently with water. The TEM image of nanoAu/MPA bound on an amorphous carbon support is obtained with a magnification of 1.5 million times and is shown in Fig. 1(A). Sphere-shaped discrete nanoparticles of approximately uniform size are seen all throughout the image. The dark black nature of the circular dots