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A sensor for superoxide in aqueous and organic/aqueous media based on immobilized cytochrome *c* on binary self-assembled monolayers

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Abstract

A method for the electrochemical detection of superoxide radical was developed, based on cytochrome *c* (cyt *c*) immobilized on the binary self-assembled monolayers (SAMs) of thioctic acid (T-COOH) and thioctic amide (T-NH_2) on gold electrode. The sensor works by electrochemically detecting cyt *c* reduced by the superoxide radical generated by a xanthine–XOD system. The electrochemical properties of immobilized cyt *c* were investigated in aqueous buffer and in a mixture of aqueous and organic solvents. The interaction of superoxide radical with the modified electrode was characterized in phosphate buffer solution (PBS) and in the mixtures of both PBS and dimethyl sulfoxide (DMSO) and PBS and glycerol (Gly). The results showed that the sensors responded immediately to superoxide radical in PBS and gave a steady-state anodic current within 10 s during the generation of superoxide radical. In 40% DMSO and in 30% Gly solution, the current response reached a steady-state anodic current within 20 s. The sensor could also be used to estimate superoxide dismutase (SOD).

Keywords: Superoxide sensor; Self-assembled monolayers; Cytochrome *c*; Gold electrode; Organic solvents

1. Introduction

Superoxide radical belongs to the group of reactive oxygen species produced in biological respiration and metabolism, and it may damage organisms if it exceeds the level at which these organisms are able to protect themselves from its effects. Superoxide radical is believed to be closely implicated in a number of biological phenomena, such as aging (Mannino et al., 1999; Pinzino et al., 1999; Hensley and Floyd, 2002; Youdim and Joseph, 2001) and diseases (Hancock, 1997). The detection of superoxide radical is thus essential if we are to better understand the radical's role in degenerative processes and more accurately diagnose the diseases in which it is involved.

The quantitative detection of superoxide radical concentration in biological models is very difficult due to its fleeting existence and low concentration. Most techniques for the detection of superoxide radical use indirect spectroscopic methods (Fridovich, 1997), such as spectrophotometric measurement (Haseloff et al., 1991), chemiluminescence method (Reichl et al., 2001), and electron spin resonance spectroscopy (Harbour and Hair, 1978). However, these strategies are ex situ detection techniques with poor selectivity or sensitivity.

A new amperometric sensor technique has been developed to detect superoxide radical both in vitro and in vivo. It is based on a promotor-modified gold electrode on which cyt *c* is immobilized (Lisdat et al., 1999; McNeil et al., 1995). Superoxide radical reduces the immobilized cyt *c*, which can be immediately reoxidized by the electrode at a suitable potential.

This electrochemical strategy offers specific advantages, including the possibility of on-line measurement, minimum disturbance of chemical/enzymatic interventions, measurement in vivo, and low equipment cost.

Recently, single component COOH monolayers (Lisdat et al., 1999; Scheller et al., 1999) and mixed-monolayers of short alkanethiols (Gobi and Mizutani, 2000) with immobilized cyt *c* gold electrodes have been employed in the electrochemical detection of superoxide radical. However, amperometric sensors are limited both by the amount of cyt *c* immobilized in the SAMs and the rate of electron transfer between cyt *c* and electrode.

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Therefore, the development of a new sensor surface giving an increase in protein loading and a more efficient electron transfer is very important.

Recently, we have developed a new type of binary SAMs composed of thioctic acid (T-COOH) and thioctic amide (T-NH_2) to modify gold electrode (Ji et al., 2006). This binary SAMs is superior for attaching cyt *c*. T-COOH and T-NH_2, with disulfide-containing bases, have distinct advantages for gold electrode modification. The disulfide-containing base yields two gold–sulfur bonds and gives additional stability, compared with the single gold–sulfur bond formed by alkanethiols with gold surface. Furthermore, the components of the terminal groups of the SAMs composed of T-COOH/T-NH_2, carboxyl and amino, match the residual groups (–NH_2 and –COOH) of protein, making the interaction between the SAMs and cyt *c* strong and the amount of adsorbed cyt *c* increase, due to the electrostatic interaction and steric effect between SAMs and cyt *c*.

The use of protein-based sensors has mostly been limited to studies in aqueous buffer. In order to broaden the range of applications, sensors have also been developed for use not only in aqueous buffer but also in mixtures of the aqueous and organic solvents of a system (Beissenhirtz et al., 2003).

In our study, a sensor based on the direct electron transfer of cyt *c* immobilized on binary SAMs was used to detect superoxide radical in PBS and in the mixtures of both PBS and dimethyl sulfoxide (DMSO) and PBS and glycerol (Gly). The effect of the mixtures of PBS and organic solvents on the electrochemical behavior of cyt *c* immobilized on T-COOH/T-NH_2 modified electrode was investigated in this study. The influence of hydrogen peroxide (H_2O_2) and uric acid on the sensor signal was also investigated. Additionally, the sensor was used to estimate of SOD activity.

2. Experimental

2.1. Materials, buffers, and mixture solutions

Superoxide dismutase (SOD), Dimethylsulfoxide (DMSO), potassium ferricyanide, xanthine (Xa) and hydrogen peroxide (H_2O_2, 30%) were purchased from Wako Pure Chemical, Co. Ltd., and used as received. Glycerol (Gly) and tetraethylammonium perchlorate (TEAP), a polarographic grade product, were purchased from Nacalai Tesque, Inc. Xanthine oxidase (XOD) was provided by Sigma. Other chemicals were of analytical reagent grade. Horse heart cyt *c* and other materials were the same as those used in a previous study (Ji et al., 2006).

Phosphate buffer solutions (PBS) were also prepared by the same method described in the paper referred to above. Four mM PBS, pH 7.0, was used for the preparation of cyt *c* electrode, and 10 mM PBS, pH 7.0, served to record cyclic voltammograms, 10 mM PBS, containing 1.0 mM EDTA, pH 7.5, was used in the generation of superoxide radical. In order to ensure the high conductivity of all mixtures, TEAP was added to DMSO and Gly, leading to a final concentration of 30 mM. The mixture solutions were prepared freshly in the cell by adding the desired volume of organic solvents (containing 30 mM TEAP) to 10 mM PBS (pH 7.5).

2.2. Preparation of modified electrodes and electrochemical measurement

The gold disk electrodes were prepared and cyclic voltammetry was carried out in the same way as reported in the previous paper (Ji et al., 2006). All potential values given below are referenced to Ag/AgCl/3 M NaCl. The surface concentration (Γ) of cyt *c* on the modified gold electrode can be calculated by the slope of the line. Amperometric measurements were taken using a potentiostat/galvanostat combined with an arbitrary function generator (HOKUTO DENKO) and an x-y recorder. The current–time data were recorded by applying a potential of +150 mV on a stirred cell.

3. Results and discussion

3.1. Electrochemistry of cyt c immobilized on binary SAMs

The electrochemical behavior of immobilized cyt *c* was investigated by cyclic voltammetry in our previous study (Ji et al., 2006). A pair of well-defined quasi-reversible redox peaks occurred. The formal potential, $E^{\circ\prime}$, taken as the average of E_{pc} and E_{pa}, was −0.032 V, with ΔE_p, the peak-to-peak potential separation of anodic and cathodic waves, 0.010 V at a scan rate of 40 mV s^{-1}, as shown in Fig. 1. The redox peak currents (the ratio of the anodic and cathodic peak currents was close to 1.0) were directly proportional to the potential scan rates at low scan rates.

The surface concentration (Γ) and the heterogeneous electron transfer rate constant (k) of cyt *c* on the modified gold electrode were 9.2×10^{-12} mol cm^{-2} and 15.8 ± 0.6 s^{-1} at a ratio of T-COOH to T-NH_2 of 3:2.

The blockage of the binary monolayer of T-COOH and T-NH_2 modified electrode for potentially interfering substances was examined. Fig. 2 illustrates the efficient blockage of SAMs modified electrode surface for interfering substances. The bare Au electrode in hexacyanoferrate solution (1 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ in 0.1 M PBS, pH 7.5) showed a

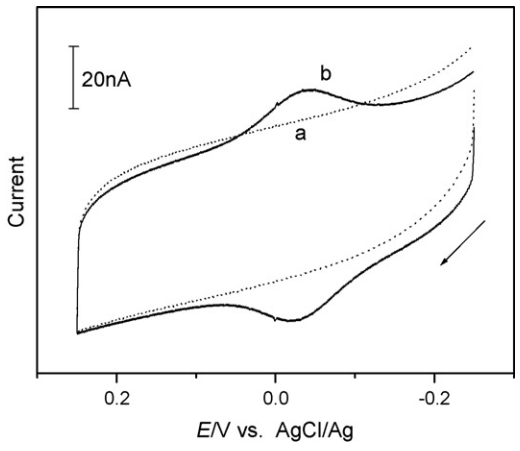

Fig. 1. Cyclic voltammograms of the cyt *c*/T-COOH/T-NH_2/Au electrode (T-COOH:T-NH_2 = 3:2 in adsorption solution) in 10 mM PBS (pH 7.0) before (a) and after (b) adsorption from the solutions of 0.05 mg mL^{-1} cyt *c*. Scan rate: 40 mV s^{-1}.