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A SOD-Based Amperometric Biosensor for Superoxide Ion

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Abstract. A superoxide dismutase (SOD)-based superoxide ion (O_2^-) sensor was fabricated by immobilizing SOD on a self-assembled monolayer (SAM) of 3-mercaptopropionic acid (MPA) which was prepared on a gold electrode. The SAM of MPA was found to function as an effective promoter for the electrode reaction of SOD. The amperometric response to O_2^- was monitored at 300 mV and -100 mV vs. Ag/AgCl in 5 mM phosphate buffer solution containing KO_2. The sensor was proved to have a high sensitivity, selectivity and short response time (< 5 s) and negligible interference.

Key words : Superoxide ion, Biosensor, Superoxide dismutase, 3-mercaptopropionic acid, Self-assembled monolayer

1. Introduction

In our recent papers[1-4], we have proposed a novel third-generation superoxode ion (O_2^-) biosensor in which superoxide dismutase (SOD) enzyme is stably confined on a self-assembled monolayer (SAM) of cysteine prepared on gold electrode and its direct electrode reaction is significantly promoted by the SAM of cysteine in neutral aqueous media even though no redox response can be obtained at the conventional bare electrodes. This sensor has been expected to be very promising for the durable and reliable measurement of O_2^- in biological systems.

In this study we tried to prepare a similar SOD-based O_2^- sensor using the SAM of 3-mercaptopropionic acid (MPA) as an electron-transfer promoter. We could successfully prepare the SOD-modified gold electrode and found that it can also function as O_2^- sensor.

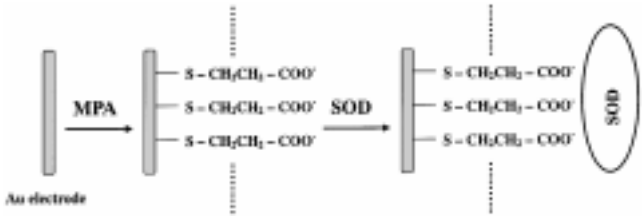
2. Experimental

2.1. Reagents

MPA (Cica-reagent) was obtained from Kanto Chemicals Co. (Tokyo, Japan) and prior to use its aqueous solution was freshly prepared and deoxygenated by bubbling pure nitrogen for at least 30 min. Copper-zinc SOD(EC.1.15.1.1) was purchased form Wako Pure Chemical Industries, Ltd. and used without further purification. KO_2 was purchased from Sigma Chemicals Co. and used as supplied. Other reagents were of analytical reagent grade and used as received. A stock solution of KO_2 was prepared by adding KO_2 to DMSO (stored together with molecular sieve 4 A (Wako Pure Chemical Industries, Ltd.)), sonicating the solution for 5 min and then putting additional molecular sieve 4 A into it to remove a trace of H_2O. All aqueous solutions were prepared with deionized water (Milli-Q system, Millipore, Japan).

2.2. Fabrication of SOD/MPA-immobilized gold electrodes

Polycrystalline gold electrodes (1.6 mm in diameter) were polished with aqueous slurries of successively finer alumina powder (down to 0.06 μm) on a polishing microcloth, sonicated in Milli-Q water for 10 min and rinsed with water. The electrodes were then electropolished by potential cycling in 0.05 M H_2SO_4 solution in the potential range of -0.2 to 1.5 V at the potential scan rate of 10 Vs^{-1} until the cyclic voltammogram characteristic for a clean Au electrode was obtained. According to the standard procedure[5] for preparing the SAMs of thiols and disulfides on gold electrodes, MPA-modified Au electrodes were prepared by dipping the cleaned Au electrodes in 1 mM MPA solution for 10 min and rinsed with water to remove the non-chemisorbed MPA. The SOD was immobilized on the MPA-modified Au electrode by soaking it in 5 mM phosphate buffer solution containing 0.20 mM SOD for 30 min (Scheme 1). The thus-prepared SOD/MPA-immobilized Au electrode was rinsed with water and stored at 4°C while not used.



Scheme 1

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2.3. Electrochemical measurements

Electrochemical measurements were performed at 25± 0.5°C in a conventional two-compartment three-electrode electrochemical cell using a computer-controlled electrochemical analyzer (BAS 100B/W). The MPA- and SOD/MPA-immobilized Au electrodes, abbreviated as MPA/Au and SOD/MPA/Au, respectively, were used as the working electrode, a platinum spiral wire as the auxiliary electrode and a Ag/AgCl electrode (saturated with KCl) as the reference electrode. The working electrode and the auxiliary electrode were separated by a porous glass. The electrochemical measurements were carried out in 5 mM phosphate buffer solution (PBS, pH 7.4). The O_2^- solutions were prepared by the addition of aliquots of KO_2 stock solution to PBS (N_2-saturated) and their concentrations were estimated using the method reported by Ge and Lisdat.[6)]

3. Results and Discussion

3.1. Electrochemical behavior of SOD/MPA/Au

Fig. 1 shows typical cyclic voltammograms (CVs) of the MPA/Au and SOD/MPA/Au in 5 mM PBS under N_2 atmosphere. One couple of well-defined redox peaks with formal potential, E^{01}, of 205 mV was observed at the SOD/MPA/Au, while no voltammetric response was observed at the MPA/Au. It is well known that the direct electron transfer of SOD at bare gold electrode is very slow and has not been observed, but it could be significantly facilitated by use of a MPA monolayer. This demonstrates that the SAM of MPA functions as an effective promoter for the electrode reaction of SOD. Typical cyclic voltammograms of the SOD/MPA/Au in 5 mM PBS at various potential scan rates are shown in Fig. 2. We found that both the anodic and the cathodic peak currents (I_p^a and I_p^c) vary linearly with potential scan rate (v) in the range of 20-800 mVs^{-1} (data not shown here). Moreover, the CVs remained essentially unchanged on consecutive potential cycling at 100 mV s^{-1} for at least 30 min, indicating that SODs are stably confined on the SAM of MPA. The current ratio of I_p^a to I_p^c (ca. 0.8 at 100 mVs^{-1}) and the separation between the cathodic and anodic peak potentials (ca. 150 mV at 100 mVs^{-1}) indicate that the electrode reaction of SOD confined on the SAM of MPA on Au electrode is quasi-reversible.

His and Liedberg have proved that the -SH group of MPA is active enough for chemisorption on a gold electrode using infrared reflection absorption spectroscopy, X-ray photoelectron spectroscopy and static secondary ion mass spectrometry[7)]. In our previous work[1)], we have concluded that the SOD confined on the cysteine-SAM electrode can be expected to possess its inherent enzymatic activity for the dismutation of O_2^-, i.e., $-NH_2$ and -COOH groups of cysteine do not coordinate to the Cu^{2+} moiety of SOD. Similarly, the functional group -COOH of MPA is also considered not to interact with the Cu^{2+} moiety of SOD[8)]. Since Bovine SOD has a net negative charge at pH 7.4 (pI = 4.9)[9)] and the pKa value of MPA adsorbed on gold electrode surface is about 6.0 in pH 7.4 solution[10)], in contrast with electrochemistry of cytochrome c at Au electrodes modified with COOH-terminated SAMs, the orientation of SOD on the gold electrode through the SAM of MPA and the resulting facilitation of its electron transfer may be considered to be not only simply due to an electrostatic interaction between SOD molecule and the $-COO^-$ group of MPA but also due to a unique interaction on a molecular level.

3.2. Responses of SOD/MPA/Au to O_2^-

SOD efficiently catalyzes the dismutation of O_2^- to O_2 and H_2O_2 via a redox cycle of the $Cu^{+/2+}$ moiety[11)]. During this

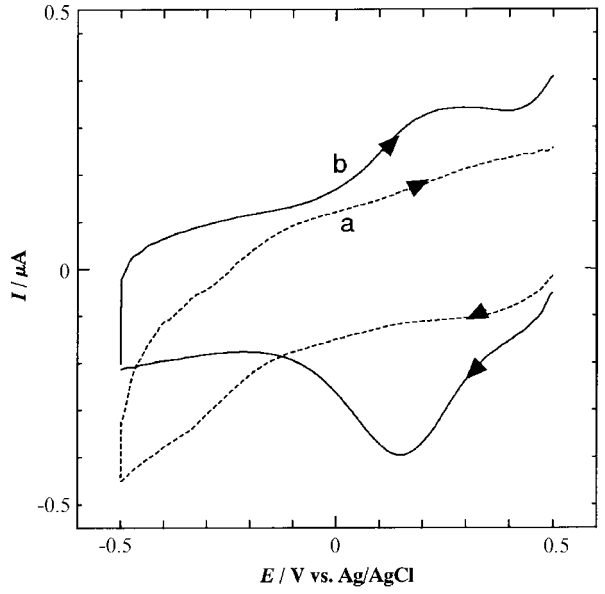

Fig. 1. CVs obtained at (a) MPA/Au and (b) SOD/MPA/Au in 5 mM PBS (pH 7.4) at scan rate 100mVs^{-1}. The surface coverages of MPA and SOD were 8.1× 10^{-10} and 1.0× 10^{-11} mol cm^{-2}, respectively.

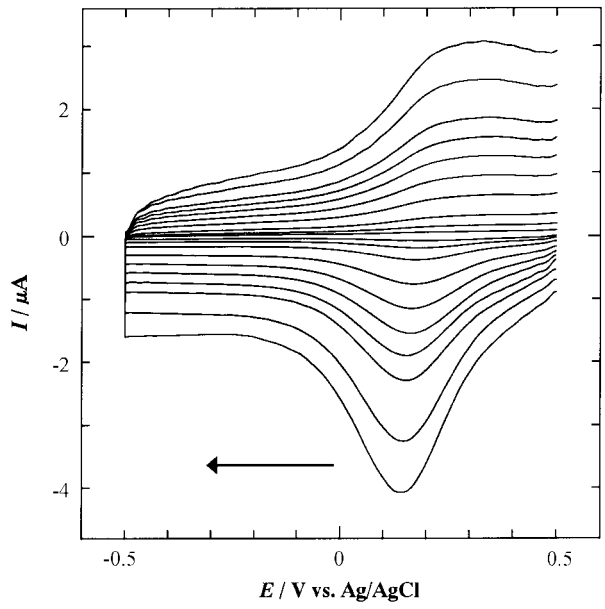

Fig. 2. CVs obtained at SOD/MPA/Au in 5 mM PBS (pH 7.4) at different scan rates: 800, 600, 500, 400, 300, 200, 100, 50, 20 mVs^{-1}.

dismutation, two O_2^- ions are stoichiometrically converted to one O_2 molecule and one H_2O_2 molecule by consuming of two H^+ ions. Namely, one O_2^- reduces the SOD [Cu (II)] to produce O_2 and the SOD [Cu (I)], while another O_2^- oxidizes the SOD [Cu (I)] to produce H_2O_2 and the SOD [Cu (II)]. Consider these two redox processes separately by fabricating two electrodes on which each reaction occurs separately, and in which SOD is immobilized on the electrode, as illustrated in our previous paper[3,4]. In the case of reduction, the redox reaction between O_2^- and SOD [Cu (I)] takes place to produce H_2O_2 and SOD [Cu (II)]. The generated SOD [Cu (II)] can be reduced at the electrode. So the increase of reduction peak current is observed. On the other hand, in the case of oxidation, O_2^- reduces SOD [Cu (II)] to produce SOD [Cu (I)], which can be reoxidized at the electrode and thus the oxidation current was enhanced. Therefore, we can detect O_2^- as its oxidation or reduction current by suitably choosing the applied potential for current measurements by taking into account the potential interferences.

Amperometric responses of SOD/MPA/Au to successive concentration changes of O_2^- were examined at the applied potentials of 300 and -100 mV, and the obtained current-time responses are shown in Fig. 3. The generated O_2^- can undergo spontaneous dismutation into O_2 and H_2O_2 under the experimental conditions. The cathodic and anodic current responses increased stepwise with successive addition of KO_2 and the steady-state current response was obtained within 5 s. The calibration plots obtained from Fig. 3 are depicted in Fig. 4. The steady-state currents at 300 and -100 mV were proportional to O_2^- concentration in the examined range of ~13-195 nM and ~13-234 nM, respectively. The sensitivity of SOD/MPA/Au was found to be 19 and 25 nA cm^{-2}/nM at 300 and -100 mV, respectively (N = 30) and detection limit was 5 and 6.4 nM at 300 at -100 mV, respectively.

3.3. Stability, reproducibility and selectivity

For the stability test, the anodic and cathodic responses of SOD/MPA/Au for O_2^- were recorded six times every day and the current responses were found to be constant for at least 10 days. Between experiments, the sensors were kept in phosphate buffer at room temperature, which was found to be more satisfactory than storage at 4°C, as suggested recently[12]. In addition, we have found that the standard deviation of the current responses of SOD/MPA/Au did not exceed 6% (N = 8).

The main purpose for the development of O_2^- sensor lies in detecting O_2^- in biological systems. As reported previously[13], there are a variety of interferences coexisting in biological samples, suggesting that the sensor used for the practical measurements should have significant selectivity against potential interferences. The interferences from H_2O_2, uric acid (UA), ascorbic acid (AA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were investigated at 300 and -100 mV, in which the concentrations of the interferences approximate their ECF levels[14,15]. At 300 mV, the interference level of 22% was obtained for AA and it did not exceed 10% for UA. On the other hand, no response of AA and UA was observed at -100 mV. The interference of H_2O_2 and DOPAC was found to be negligible at both 300 and -100 mV.

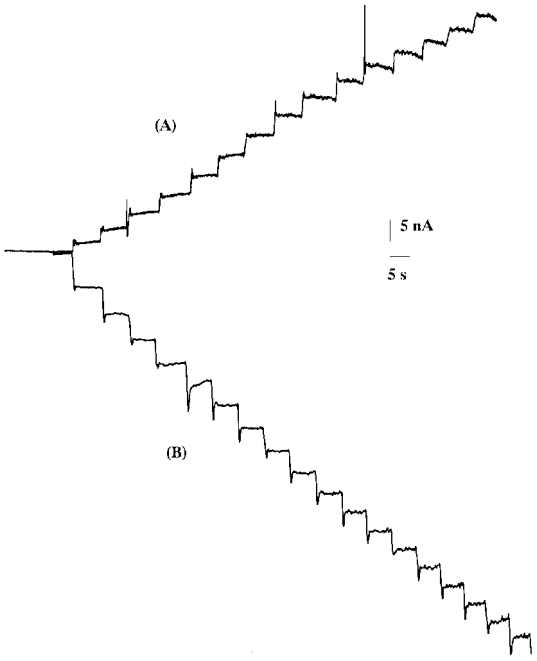

Fig. 3. Typical steady-state current-time responses of SOD/MPA/Au at (A) 300 mV and (B) -100 mV in PBS (N_2-saturated, pH 7.4) upon successive addition of 52 nM KO_2. The solution was stirred with a magnetic stirrer at 800 rpm.

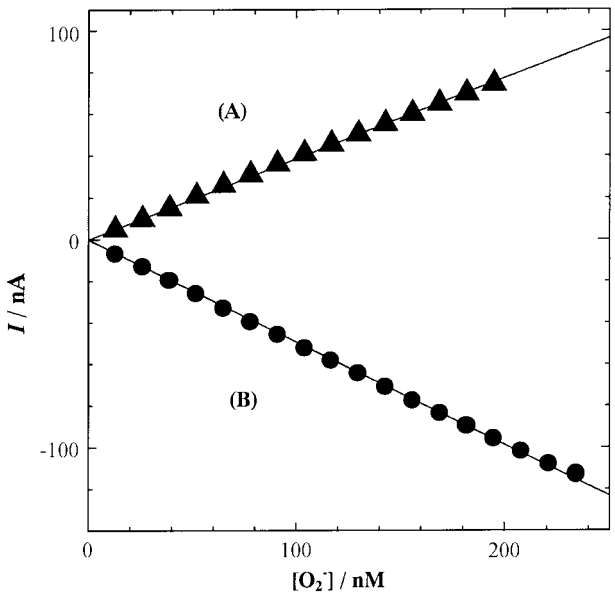

Fig. 4. Calibration plots of the anodic (A) and cathodic (B) steady-state currents against the concentration of O_2^-. The data (A) and (B) were taken from Figure 3(A) and (B), respectively.

4. Conclusion

In this study, we have demonstrated the fabrication of a SOD-based O_2^- sensor in which SOD is immobilized on the

SAM of MPA formed on the Au electrode and the SAM of MPA functions as an effective promoter for the electrode reaction of SOD. The present sensor had good sensitivity and selectivity, a rapid response time, excellent linearity at nanomolar O_2^- concentrations and a low detection limit. We are performing detailed studies concerning the sensor characteristics and its miniaturization and the results will be reported in near future.

Acknowledgements

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