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A superoxide dismutase-modified electrode that detects superoxide ion

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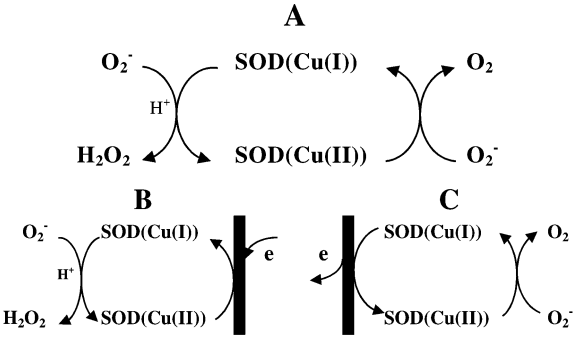
A superoxide dismutase (SOD)-modified electrode, in which SOD is oriented on the gold electrode *via* a self-assembled monolayer of cysteine so as to allow its direct electrode reaction, possesses a bi-directional electrocatalysis for both the oxidation of superoxide ion (O_2^-) to O_2 and the reduction of O_2^- to H_2O_2 and functions as a third generation O_2^- biosensor.

Superoxide ion, O_2^-, is the primary species of the so-called reactive oxygen species (ROSs) that are ubiquitously produced in biological respiration and metabolism, and it is believed that superoxide ion is closely implicated in a number of biological phenomena, such as aging, diseases, ischemia—reperfusion and inflammation.[1] Therefore, the detection of superoxide ion has become a major interest to scientists from many fields.

Up to the present day, a number of the techniques for assaying O_2^- have been demonstrated, including EPR spin trapping,[2] spectrophotometry (using cytochrome *c*),[3] chemiluminescence[4] and electrochemical methods.[5] Since electrochemical methods have more advantages, such as direct detection, high sensitivity, measurement *in vivo* and so on, there are many papers about the electrochemical measurements of O_2^-, typically based on the direct oxidation of O_2^-,[5a] the oxidation of O_2^- mediated by redox catalysts such as cytochrome *c* and hemin[3b,5b–f] and the oxidation of H_2O_2,[5f–l] the product of O_2^- dismutation which is catalyzed by superoxide dismutase (SOD). Here, a novel SOD-modified electrode that detects O_2^- is proposed.

The concept of the present technique for detecting O_2^- can be illustrated using as an example copper, zinc superoxide dismutase (Cu,Zn-SOD) in Scheme 1, together with the schematic mechanism of O_2^- dismutation catalyzed by SOD. SOD efficiently catalyzes the dismutation of O_2^- to O_2 and H_2O_2 *via* a redox cycle of the copper complex moiety [*i.e.* the Cu(I/II) couple] of Cu,Zn-SOD.[1] During this dismutation, two O_2^- ions are stoichiometrically converted to one O_2 molecule and one H_2O_2 molecule with consumption 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)]. Now, 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 surface. In the case (B) in Scheme 1, 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. On the other hand, in the case (C), O_2^- reduces SOD [Cu(II)] to produce SOD [Cu(I)], which can be reoxidized at the electrode. Thus, by measuring the oxidation or reduction current at the SOD-modified electrode in the presence of O_2^-, we can detect O_2^-; that is, the present SOD-modified electrode, which is based on the specificity and selectivity of SOD for its substrate O_2^- as well as its direct electrode reaction, can be defined as a novel third generation biosensor for O_2^-. Therefore, the present SOD-modified electrode essentially differs from the previously reported SOD-based O_2^- sensors,[4,5h–m] which are based on indirect electroanalysis of the product (H_2O_2) resulting from the dismutation of O_2^- catalyzed by SOD.



Scheme 1 (A) O_2^- dismutation catalyzed by SOD; (B) the reduction of O_2^- to H_2O_2; and (C) the oxidation of O_2^- to O_2 mediated by the SOD [Cu(I/II)] redox couple confined on the electrode surface.

According to the standard procedure for preparing the self-assembled monolayers (SAMs) of thiols and disulfides on gold electrodes,[6] the Au electrodes modified with the SAM of cysteine – cysteine-modified Au electrodes – were prepared by dipping the previously electropolished Au electrodes (surface area 2.0 mm^2) into a 1 mM cysteine aqueous solution for 10 min and rinsing with water to remove the non-chemisorbed cysteine. Then, Cu,Zn-SOD (EC.1.15.1.1, Wako Pure Chemicals, Ltd., Osaka, Japan) was immobilized on the cysteine-modified Au electrode by soaking it in a 25 mM phosphate buffer solution (PBS) containing 0.56 mM SOD for 30 min.[5o] The thus-prepared SOD-modified Au electrode was rinsed with water and stored in a refrigerator while not being used. The preparation of these modified electrodes was reproducible, which could be confirmed from the cyclic voltammetric estimation of surface coverages of cysteine and SOD.

Fig. 1 compares the cyclic voltammograms (CVs) obtained at the SOD-modified electrode in 25 mM PBS in the absence and presence of O_2^-. In the absence of O_2^- the well-defined redox wave was observed (Fig. 1a) and the current response was stable; for example, it remained essentially unchanged when the potential scan was continuously repeated at 100 mV s^{-1} for 20 min. The redox response was observed neither at the cysteine-modified electrode in the same solution as in Fig. 1a (Fig. 1d) nor at the bare Au electrode in 25 mM PBS containing 0.56 mM SOD (Fig. 1c). These results demonstrate that the observed redox response is ascribable to the SOD confined on the SAM of cysteine on the Au electrode and at the same time that the SAM of cysteine functions as an effective promoter for the direct electron transfer of the SOD.[5n,o] The formal redox potential ($E^{0\prime}$) of SOD [exactly $E^{0\prime}$ of the Cu(I/II) complex moiety] was estimated to be (65 ± 3) mV *vs.* Ag/AgCl. In the presence of O_2^- (2.6 μM min^{-1})[7] both the cathodic and anodic peak currents corresponding to the redox reaction of the SOD confined on the electrode are significantly increased (Fig. 1b), compared with its absence (Fig. 1a). The observed increase in the anodic and cathodic peak current can be, as expected from

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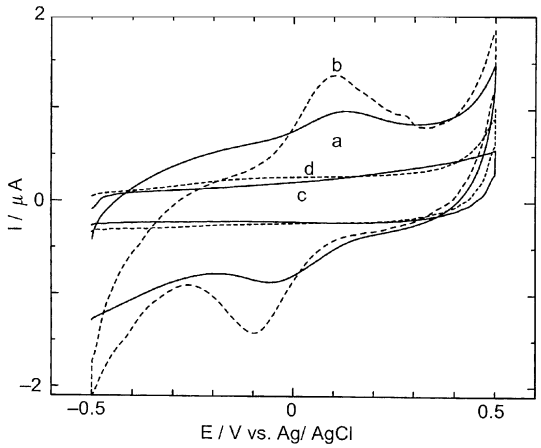

Fig. 1 CVs obtained at (a, b) SOD-modified, (c) bare and (d) cysteine-modified Au electrodes (surface area 2.0 mm^2) in 25 mM PBS (pH 7.4) saturated by N_2 (a, c, d) or O_2 (b). Solutions (b) and (c) contain 0.002 U ml^{-1} XOD and 25 μM xanthine (the rate of O_2^- generation is 2.6 μM min^{-1}) and 0.56 mM SOD, respectively. Potential scan rate 100 mV s^{-1}.

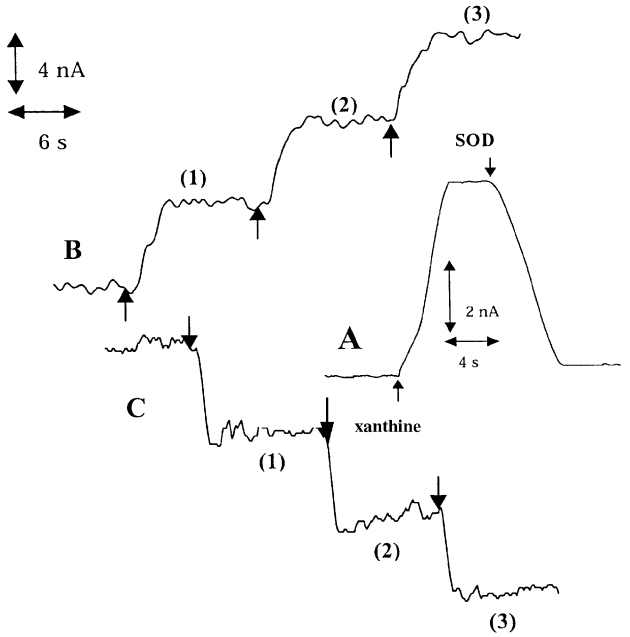

Fig. 2 (A) Anodic current response of the SOD-modified Au electrode at 0.3 V on the addition of xanthine and SOD into 25 mM PBS (pH 7.4, 4 ml, O_2-saturated) containing 0.002 U ml^{-1} XOD. The concentrations of xanthine and SOD were 50 nM and 5 μM, respectively. The addition of 50 nM xanthine generates O_2^- at 13 nM min^{-1}. (B, C) Typical current–time response curves of the SOD-modified Au electrode to successive additions (20 μl) of 10 μM xanthine at the applied potentials of (B) 0.3 and (C) −0.2 V. The rates of O_2^- generation: (1) 13, (2) 26 and (3) 39 nM min^{-1}. The solution was stirred with a magnetic stirrer at 200 rpm. The arrows indicate the positions of the addition of xanthine or SOD.

Scheme 1B and C, considered to result from the oxidation and reduction of O_2^-, respectively, which are effectively mediated by the SOD confined on the electrode. The thing to be noted here is that the $E^{0\prime}$ values of the O_2/O_2^- and O_2^-/H_2O_2 redox couples are −0.35 and 0.68 V *vs*. Ag/ AgCl at pH 7.4 and thus the SOD [Cu(I/II)] with $E^{0\prime}$ = 65 mV can mediate both the oxidation of O_2^- to O_2 and the reduction of O_2^- to H_2O_2. Such a bi-directional electromediation (electrocatalysis) by the SOD-modified electrode is essentially based on the inherent specificity of the SOD enzyme which catalyzes the dismutation of O_2^- to O_2 and H_2O_2 *via* a redox cycle of its Cu(I/II) complex moiety (Scheme 1A).[1]

The activity of the SOD-modified electrode as an O_2^- sensor was examined in 25 mM PBS by *in situ* generation of O_2^- based on the xanthine–xanthine oxidase (XOD) system[7] (Fig. 2A). Before the addition of xanthine, a stable background response current of *ca*. 70 pA was observed at the applied potential of 0.3 V which is a sufficient potential for the oxidation of the SOD [Cu(I)] to the corresponding oxidized state. Immediately after the addition of xanthine (which generates O_2^- at 13 nM min^{-1}),[7] the current was increased anodically and reached a steady-state value within 4 s. The addition of 5 μM SOD caused, as expected, the anodic current to decrease by >96%, because SOD is the most effective scavenger of O_2^-.[1] These data clearly indicate that the anodic current response observed after the addition of xanthine is due to the oxidation of O_2^- *via* the SOD confined on the electrode surface. As demonstrated above, the present sensor allows us to detect O_2^- as its oxidation or reduction current by suitably choosing the applied potential for current measurements. The typical data are shown in Fig. 2B and C. The cathodic (and anodic) current response increased stepwise with successive additions of the aliquots of xanthine and the steady-state current response was obtained within 4 s. The current response increased in proportion to the rate of O_2^- generation. We found that the steady-state currents at 0.3 and −0.2 V are proportional to the rate of O_2^- generation in the examined range of 13–130 nM min^{-1} and the detection limit is 6 nM min^{-1}. No observable degradation in the sensor sensitivity (both the anodic and cathodic responses) for O_2^- could be found after the sensors were tested for 7 consecutive days (at least 4 times each day). In addition, the interference of H_2O_2 and uric acid, which are coproduced during the generation of O_2^- using the xanthine–XOD system,[7] was found to be neglible. A detailed study on the sensor characteristics in biological samples is currently underway.

In conclusion, we have realized the selective oxidation (and reduction) of O_2^- *via* its redox reaction with SOD confined on the electrode, and consequently the present SOD-modified Au electrode has been proved to function as a third generation O_2^- biosensor.

Notes and references

1 B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford, 1989 and refs. cited therein; *Reactive Oxygen Species in Biological Systems: An Interdisciplinary Approach*, D. L. Gilbert and C. A. Colton, ed., Kluwer Academic/Plenum Publishers, New York, 1999.

2 R. P. Mason and K. T. Knecht, *Methods Enzymol*., 1994, **233**, 112; J. Vasquez-Vivar, N. Hogg, K. A. Pritchard, Jr., P. Martasek and B. Kalyanaraman, *FEBS Lett*., 1997, **403**, 127.

3 (*a*) I. Fridovich, *J. Biol. Chem*., 1997, **272**, 18 515 and refs. cited therein; (*b*) M. Pontie and F. Bdioui, *Analysis*, 1999, **27**, 564.

4 Y. Ohara, T. E. Peterson and D. G. Harrison, *J. Clin. Invest*., 1993, **91**, 2546.

5 (*a*) K. Tanaka, F. Kobayashi, Y. Isogai and T. Iizuka, *Bioelectrochem. Bioenerg*., 1991, **26**, 413; (*b*) F. Lisdat, B. Ge, E. Ehrentreich-Forster, R. Reszka and F. W. Scheller, *Anal. Chem*., 1999, **71**, 1359; (*c*) J. Xue, X. Xian, X. Ying, J. Chen, L. Wang and L. Jin, *Anal. Chim. Acta*, 2000, **405**; (*d*) W. Scheller, W. Jin, E. Ehrentreich-Forster, B. Ge, F. Lisdat, R. Buttemeier, U. Wollenberger and F. W. Scheller, *Electroanalysis*, 1999, **11**, 703; (*e*) K. V. Gobi and F. Mizutani, *J. Electroanal. Chem*., 2000, **484**, 172; (*f*) C. J. McNeil, D. Athey and W. O. Ho, *Biosens. Bioelectron*., 1995, **10**, 75 and refs. cited therein; (*g*) V. Lvovich and A. Scheeline, *Anal. Chem*., 1997, **69**, 454; (*h*) L. Campanella, L. Persi and M. Tomassetti, *Sens.Actuators, B*, 2000, **68**, 351; (*i*) S. Mesaros, Z. Vankova, A. Mesarosova, P. Tomcik and S. Grunfeld, *Bioelectrochem. Bioenerg*., 1998, **46**, 33; (*j*) S. Mesaros, Z. Vankova, S. Grunfeld, A. Mesarosova and T. Malinski, *Anal. Chim. Acta*, 1998, **358**, 27; (*k*) L. Campanella, G. Favero, L. Persi and M. J. Tomassetti, *Pharm. Biomed. Anal*., 2000, **23**, 69; (*l*) M. I. Song, F. F. Bier and F. W. Scheller, *Bioelectrochem. Bioenerg*., 1995, **38**, 419; (*m*) S. Descroix and F. Bedioui, *Electroanalysis*, 2001, **13**, 524; (*n*) X. Wu, X. Meng, Z. Wang and Z. Zhang, *Chem. Lett*., 1999, 1271; (*o*) Y. Tian, M. Shioda, S. Kasahara, T. Okajima, L. Mao, T. Hisaboli and T. Ohsaka, *Biochim. Biophys. Acta*, 2002, **1569**, 151.

6 A. Ulman, *An Introduction to Ultrathin Organic Films: From Langmuir–Blodgett to Self-Assembly*, Academic Press, San Diego, 1991.

7 J. S. Olson, D. P. Ballou, G. Palmer and V. Massey, *J. Biol. Chem*., 1974, **249**, 4350.