Amperometric Detection of Superoxide Dismutase at Cytochrome *c*-Immobilized Electrodes: Xanthine Oxidase and Ascorbate Oxidase Incorporated Biopolymer Membrane for *in-vivo* Analysis

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Amperometric measurement of superoxide dismutase (SOD) was carried out at cytochrome *c*-immobilized monolayers and ascorbate oxidase (AOD)/xanthine oxidase (XOD)/cytochrome *c*- and (AOD, XOD)/cytochrome *c*-multilayers. Cytochrome *c* was covalently immobilized on mercaptopropionic acid-containing self-assembled monolayers on gold. A biopolymer membrane of poly-L-lysine confining XOD and AOD was cast on the monolayer of cytochrome *c*. While both the cytochrome *c*-immobilized monolayer and multilayer electrodes show anodic current responses to the generation of superoxide radical, the sensitivity of the multilayer system for the detection of superoxide radical was high relative to that of the monolayer system. In the case of the cytochrome *c*-multilayer electrodes, the generation of superoxide radical near the sensing element, cytochrome *c*, resulted in high sensitivity for the detection of superoxide. The use of a XOD and AOD-incorporated poly-L-lysine membrane enabled the detection of the generation of superoxide radical in the presence of L-ascorbic acid. Though L-ascorbic acid could scavenge superoxide radical, the biopolymer membrane confined with AOD will oxidize any L-ascorbic acid that permeated into the membrane. By using the multilayer electrodes, one could measure the activity of SOD in the presence of L-ascorbic acid.

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Introduction

Superoxide radical is highly reactive and short-lived and is formed in all aerobic cells. The superoxide radical generated during the "respiratory burst" of phagocytic cells plays an important role in the destruction of microorganisms invading into our body. But it may be involved in oxidative damage to tissues and organisms if it exceeds the level at which the systems are able to provide defence. The ubiquitous presence of SOD enzyme throughout the evolutionary chain emphasizes the importance of superoxide radical in cell function and survival. SOD plays a major protective role in living cells and has been widely used as a pharmacological tool in the study of pathophysiological mechanisms.¹⁻⁶ Detection of superoxide and SOD has been intensively pursued by spectrophotometric and electrochemical methods.⁷⁻¹⁶ Electrochemical sensors are of great interest due to their advantages, which include microfabrication and *in-vivo* and *in-vitro* applications.^{17,18} The hemoprotein, cytochrome *c* (cyt *c*), undergoes facile reductive reaction with superoxide. Electrochemical biosensors for superoxide with cyt *c* as the sensing element were constructed employing a suitable electron-transfer promoter.¹¹⁻¹⁴ SOD is a selective scavenger of superoxide, and the best way for the detection of SOD thus could be the estimation of superoxide. Superoxide undergoes spontaneous disproportionation to produce H₂O₂, an electroactive species. Thus, the electrical response of the superoxide sensor system for interfering

compounds is a very important limitation in its application. We recently reported a selective detection of superoxide and SOD at cyt *c*/alkanethiol electrodes, where the response due to interferents was suppressed by the use of a mixed-monolayer of alkanethiols for anchoring cyt *c*; but the presence of L-ascorbic acid, a potential scavenger of superoxide existing ubiquitously in our body, was detrimental to the detection of superoxide and SOD.¹⁹ The detection of superoxide and consequently SOD in the presence of L-ascorbic acid was established using XOD/cyt *c*-bilayer electrodes, where the poly-L-lysine: polystyrenesulfonate complex membrane employed for the immobilization of XOD restricted the permeation of L-ascorbic acid across the membrane and consequently suppressed the scavenging of the superoxide produced at the membrane-bound XOD by L-ascorbic acid.²⁰ The XOD/cyt *c*-bilayer electrodes could detect SOD in the presence of L-ascorbic acid; however, the sensitivity of XOD/cyt *c*-bilayer electrodes for SOD was rather low (detection limit, 0.2 µg ml⁻¹) relative to that of cyt *c*-monolayer electrodes (detection limit, 3 ng ml⁻¹);^{19,20} the permeation of SOD across the polyion complex membrane confining XOD was suppressed in the case of the bilayer electrodes, which resulted into a low sensitivity for the detection of SOD. In the present investigation, the cyt *c*-immobilized monolayer electrodes were prepared by covalently-immobilizing cyt *c* on mixed-monolayers of 3-mercaptopropionic acid (MPA) with the coadsorbate 3-mercaptopropanol (MP); a biopolymer membrane consisting of XOD and AOD was formed with poly-L-lysine on the monolayer of cyt *c* to fabricate the cyt *c*-immobilized multilayer electrodes. The SOD sensor activity of the cyt *c*-immobilized monolayer and multilayer electrodes was pursued. We emphasize here, the features of the XOD and AOD-

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incorporated biopolymer membrane for enhanced sensor activity and *in-vivo* analysis. The fabricated cyt *c*-multilayer electrodes show rapid, large current response for the superoxide generated by xanthine-XOD enzymatic reaction. The SOD sensor activities of the cyt *c*-immobilized electrodes were pursued in the presence of L-ascorbic acid.

Experimental

Reagents

Cyt *c* (from horse heart; ferric form), poly-L-lysine (MW 100000), SOD, AOD and catalase were purchased from Sigma and were used as supplied. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Nacalai), XOD (Wako; 5 U ml⁻¹), MPA (TCI), MP (TCI) and glutaraldehyde (glu; Aldrich) were used as received. Xanthine, L-ascorbic acid and supporting electrolytes were of analytical-reagent grade and were purchased from Kanto Chemical Co. Deionized water, purified by passage through a Millipore Milli-Q filtration system, was used to prepare all aqueous solutions.

Fabrication of cyt *c*-immobilized monolayer electrodes

Polycrystalline Au electrodes of 1.6 mm diameter (BAS) were polished with aqueous slurries of successively finer alumina powder (down to 0.06 µm) on polishing microcloth (BAS) and were sonicated in water. Electrodes were etched for 3 min in 1:3:6 (in volume) mixture of conc. HNO₃/conc. HCl/water and sonicated in water. Electrodes were then cleaned electrochemically in 0.05 M H₂SO₄ by potential cycling in the range of -0.2 to 1.5 V at the potential sweep rate of 10 V s⁻¹ for 10 min or until the cyclic voltammogram (CV) characteristic of a clean Au electrode was obtained. The real surface area of the Au electrodes was determined by cyclic voltammetry from the charge required to reduce the surface oxide layer²¹ and was used in the calculations of surface coverage values. The surface roughness factor of the Au electrodes thus pretreated, calculated as the ratio of the real surface area to the geometric one, was 2.7 ± 0.3. The Au electrodes thus pretreated were dipped for 12 h in an ethanolic solution of 1.5 mM MPA + 8.5 mM MP, then rinsed sequentially with copious amounts of ethanol and water. They are denoted as Au/MPA + MP. The surface coverage (Γ) of alkanethiols bound to Au electrodes was determined using the reductive desorption experiment, as described elsewhere,^{22,23} in 0.5 M KOH to be (9.2 – 9.9) × 10⁻¹⁰ mol cm⁻². This is equivalent to a monolayer coverage of alkanethiols on gold surface.²²⁻²⁴ Cyt *c* was covalently-immobilized on the MPA + MP mixed-monolayer electrode according to the reported procedure.^{19,20} For covalent immobilization of cyt *c*, Au/MPA + MP electrode was incubated at 25°C in aqueous 4 mM sodium phosphate buffer (SPB; pH 7.0) containing 50 µM cyt *c* for 20 min, and the water-soluble condensing agent, EDC, was then added to give a final concentration of 2 mg ml⁻¹ EDC. After 1 h, the electrodes were rinsed with 4 mM SPB and the resulting electrode was defined as Au/cyt *c*. The amount of surface-immobilized cyt *c* was determined from the cyclic voltammetric responses as charge in the cathodic wave (*Q_p^c*) by the cut and weigh method.

Fabrication of cyt *c*-immobilized multilayer electrodes

On the cyt *c*-immobilized monolayer electrodes, a biopolymer membrane consisting of XOD and AOD was formed by adding a few microliters of each XOD (5 U ml⁻¹; 3.5 µl), AOD (2 U µl⁻¹; 2 µl), poly-L-lysine (25 mM in monomer unit; 2.5 µl) and glutaraldehyde (0.05%; 2 µl) on top of the cyt *c*-immobilized

Fig. 1 CVs of Au/cyt *c* (a – c) and Au/MPA + MP (d) recorded in aqueous 4 mM SPB (pH 7.0). Potential sweep rates were 100 (a), 50 (b, d) and 20 mV s⁻¹ (c). The MPA + MP Au-alkanethiolate layer was formed from an ethanol solution of 1.5 mM MPA + 8.5 mM MP.

monolayer electrodes and drying at 4°C for 2 h, followed by drying at reduced pressure for 1 h. The resulting electrode was denoted as Au/cyt *c*/PL:(XOD, AOD). Au/cyt *c*/PL:(XOD) electrode was prepared by excluding the addition of AOD in the above process. On Au/cyt *c*/PL:(XOD) electrode, AOD was then immobilized using poly-L-lysine as in the above process or using glutaraldehyde alone; the resulting electrodes were defined as Au/cyt *c*/PL:(XOD)/PL:(AOD) and Au/cyt *c*/PL:(XOD)/AOD_{glu}, respectively. The amounts of XOD and AOD added in the biopolymer membrane were 17 mU and 4 U, respectively. The fabricated bi- and tri-layer electrodes were dipped in 0.1 M SPB (pH 7.0) for 30 min prior to the measurements.

Measurements

Electrochemical measurements were performed in a two-compartment three-electrode cell using a Cypress CS-1090 electrochemical measurement system. The working electrode and the counter electrode (a Pt spiral wire) were separated by a porous glass, and an NaCl saturated Ag|AgCl electrode was used as the reference electrode. Stock solutions of SOD (10 U µl⁻¹ in 10 mM SPB) and xanthine (15 mM in 50 mM NaOH) were prepared just before the measurements. Superoxide was generated by xanthine-XOD enzymatic reaction in oxygen-saturated solutions (Eq. (1)).²⁵

Electrolyte solutions (SPB; pH 7.0) were deaerated or oxygen-saturated by bubbling argon or oxygen gas, respectively, for at least 30 min prior to electrochemical measurements; during the measurements, argon or oxygen gas was passed over the electrolyte solution.

Results and Discussion

Electrochemical characteristics of cyt *c* immobilized on Au-alkanethiolate electrodes

Figure 1 shows the typical CVs of Au/cyt *c* and Au/MPA +

Fig. 2 Current-time curves of Au/cyt *c*/PL:(XOD) recorded at the applied potential of 0.15 V on successive additions of 0.15 mM xanthine (a) and on the additions of 0.75 mM xanthine and 300 U ml⁻¹ SOD (b). Electrolyte solution was 0.1 M SPB (pH 7.0).

MP recorded in deaerated aqueous 4 mM SPB at various potential sweep rates (20 – 100 mV s⁻¹). The CV of Au/MPA + MP shows only a double-layer charging current in the region of 400 to –200 mV (Fig. 1(d)). The cyt *c*-immobilized electrode shows a reversible redox wave with the formal potential, E° (taken as $(E_p^c + E_p^a)/2$), of –0.01 V. The redox response of Au/cyt *c* is stable on consecutive potential cycling and is consistent with that anticipated for an electrochemically reversible reaction involving surface-immobilized species. For example, the peak currents vary linearly with the potential sweep rate (ν) in the range of 20 to 500 mV s⁻¹, and the ratio of I_p^c to I_p^a at a given sweep rate is unity. The E° of Au/cyt *c*, –0.01 V, is equal to those reported for cyt *c* adsorbed or covalently-bound to the COOH-terminated Au-alkanethiolate layers.^{26–28} The faradaic charge Q_p^c associated with the cathodic wave of cyt *c* was 0.69 $\mu\text{C cm}^{-2}$ (taking the real surface area of the Au electrode into account) independent of ν in the range of 20 to 500 mV s⁻¹; the Γ of cyt *c* was determined to be 7.2×10^{-12} mol cm⁻². The determined Γ value of cyt *c* is a little higher than the theoretical monolayer surface coverage value of cyt *c*, 6.8×10^{-12} mol cm⁻², calculated using the unit cell dimensions of cyt *c* (5.83, 5.83 and 4.18 nm) and the area of a cyt *c* molecule (2400 Å²).^{29,30} Thus, the fabricated Au/cyt *c* electrode possesses a monolayer coverage of cyt *c* covalently-immobilized on the Au-alkanethiolate layer.

Amperometric measurement of superoxide radical and SOD

The activity of the cyt *c*-immobilized monolayer and multilayer electrodes for monitoring the superoxide generated by xanthine-XOD system was examined chronoamperometrically at 0.15 V. The current-time curves of Au/cyt *c*/PL:(XOD) electrode recorded at an applied potential of 0.15 V for the superoxide generated on the additions of xanthine to the oxygen-saturated electrolyte solution of 0.1 M SPB are shown in Fig. 2. The O₂⁻ radical can undergo spontaneous dismutation into O₂ and H₂O₂ in aqueous solution; the counterbalance between the generation of the O₂⁻ radical and its disproportionation will result in a constant equilibrium concentration of the O₂⁻ radical. Before the addition, a stable back-ground current of ~10 nA cm⁻² was recorded under the applied potential condition. Immediately after the addition of 0.15 mM xanthine, the current was increased anodically and reached a plateau within 20 s (Fig.

Table 1 Current responses of the cyt *c*-immobilized electrodes for superoxide and SOD against the potential interferent and scavenger of superoxide, L-ascorbic acid, observed in 0.1 M SPB at the applied potential of 0.15 V

Electrode	Current ^a /μA cm ⁻²		
	O ₂ ^{-b}	SOD ^c	L-Ascorbic ^d
Bare Au			10.0
Au/cyt <i>c</i> ^e	0.15	0.14	< 0.1
Au/cyt <i>c</i> /PL:(XOD)	2.10	0.18	
Au/cyt <i>c</i> /PL:(XOD, AOD)	1.90	0.18	< 0.1
Au/cyt <i>c</i> /PL:(XOD)/PL:(AOD)	1.90 ^f	0.17 ^f	
Au/cyt <i>c</i> /PL:(XOD)/AOD _{glu}	1.40	0.07	
	1.50	0.07	

a. The reported values were reproducible within the limits of ± 5%.

b. Anodic current observed for the addition of 0.75 mM xanthine.

c. Decrease in the anodic current for the addition of 3 U ml⁻¹ SOD.

d. Anodic current observed for the addition of 50 μM L-ascorbic acid.

e. Electrolyte solution contained 60 μM ml⁻¹ XOD.

f. Current observed in the presence of 50 μM L-ascorbic acid.

2(a)); on successive additions of xanthine, the current response increased stepwise anodically. The magnitude of the anodic current for the addition of 0.75 mM xanthine recorded at Au/cyt *c*/PL:(XOD) was 2.1 μA cm⁻² (Fig. 2(b)). During the generation of superoxide radical, though the concentration of coproduced H₂O₂ and uric acid will increase with time (Eq. (1)), the observation of a steady-state current reveals that both H₂O₂ and uric acid interfere negligibly in the observed electrical response. The current-time curve of Au/cyt *c* electrode recorded in an oxygen-saturated solution of 0.1 M SPB containing 60 μM ml⁻¹ XOD showed an anodic current on the addition of 0.10 mM xanthine and attained a plateau within 10 s (data not shown); the magnitude of the anodic current was 0.15 μA cm⁻² (Table 1). In contrary to Au/cyt *c*/PL:(XOD) electrode, the current response of Au/cyt *c* did not increase anodically further on a successive addition of 0.15 mM xanthine. The anodic current response recorded for the O₂⁻ radical generated by the enzymatic reaction at Au/cyt *c*/PL:(XOD) is very high relative to that recorded at Au/cyt *c*. This observation clearly suggests that the immobilization of XOD in the vicinity of the sensing element, cyt *c*, enhanced the sensitivity for the detection of O₂⁻ radical. Moreover, it further reveals that the O₂⁻ radical generated near the electrode surface only would undergo electrochemical oxidation reaction at the electrode surface, leaving the O₂⁻ radical generated far from the electrode surface and in bulk electrolyte solution to undergo mainly a disproportionation process. In a control experiment, Au/cyt *c*/PL:(XOD) electrode fabricated without the presence of XOD in the biopolymer membrane gave no current response for the addition of xanthine. Moreover, the addition of SOD, a selective scavenger of O₂⁻ radical, caused the anodic current to decrease by >85% within 20 s (Fig. 2(b)). All the observations clearly demonstrate that only the O₂⁻ radical generated by the xanthine-XOD system was giving the anodic current response. Here, the residual anodic current observed in the presence of SOD (Fig. 2(b)) would be due to the electrocatalytic oxidation of the O₂⁻ radical that had not been accessed by SOD, because of the generation of O₂⁻ radical in the proximity of the surface-immobilized cyt *c*.

The cyt *c*-immobilized electrodes were examined for the detection of O₂⁻ radical in the presence of L-ascorbic acid. L-

Fig. 3 (a) Current-time curves of Au/cyt *c*/PL:(XOD, AOD) on the additions of 0.75 mM xanthine and 15, 30, 45 and 60 U ml⁻¹ SOD; (b) current-time curves of Au/cyt *c*/PL:(XOD, AOD) on the additions of 50 μM L-ascorbic acid, 0.75 mM xanthine and 15, 30, 45 and 60 U ml⁻¹ SOD. Other conditions are the same as in Fig. 2.

Ascorbic acid is available in blood at concentrations of ~50 μM.^{31,32} Figure 3 shows the current-time curves of Au/cyt *c*/PL:(XOD, AOD) observed at 0.15 V for the O₂⁻ radical generated on the addition of xanthine in the presence and absence of 50 μM L-ascorbic acid. In the presence of L-ascorbic acid, O₂⁻ radical will be quenched by a reductive reaction (Eq. (2)) and thus the amperometric response of the O₂⁻ radical would diminish.

The steady-state back-ground current of Au/cyt *c*/PL:(XOD, AOD) was ~10 nA cm⁻². The addition of 50 μM L-ascorbic acid gave a very small anodic current response, <0.05 μA cm⁻² (Fig. 3(b)). The observed anodic current could be associated with the electrooxidation of L-ascorbic acid. At a bare Au electrode, 50 μM L-ascorbic acid gave an anodic current of 10 μA cm⁻² at 0.15 V in 0.1 M SPB (Table 1). The anodic currents observed for L-ascorbic acid at the cyt *c*-immobilized multilayer electrodes were enormously lower than that observed at a bare Au electrode. On the addition of xanthine, while no anodic current response was observed at Au/cyt *c* in the presence of L-ascorbic acid (Table 1), an anodic current of 1.9 μA cm⁻² was recorded at Au/cyt *c*/PL:(XOD, AOD) (Fig. 3) irrespective to the presence or absence of L-ascorbic acid. At Au/cyt *c*/PL:(XOD, AOD), the ascorbate oxidase enzyme existing in the biopolymer membrane oxidized the L-ascorbic acid molecules that permeated into the membrane and thus the scavenging of superoxide radical by L-ascorbic acid has been resisted. As one example of the above discussion, the cyt *c*-multilayer electrode fabricated without the presence of AOD in the poly-L-lysine membrane, Au/cyt *c*/PL:(XOD), showed an anodic current of 1.1 μA cm⁻² (Table 1) for the addition of xanthine in the presence of 50 μM L-ascorbic acid, which is considerably lower than that recorded, 2.1 μA cm⁻², in the absence of L-ascorbic acid (Fig. 2(b)). The anodic currents recorded for the generation of superoxide radical at Au/cyt *c*/PL:(XOD)/PL:(AOD) and Au/cyt *c*/PL:(XOD)/AOD_{glu} are lower than that recorded at Au/cyt *c*/PL:(XOD, AOD) (Table 1).

Fig. 4 Decrease in the anodic current response for the superoxide radical generated by XOD-xanthine enzymatic reaction observed at Au/cyt *c*/PL:(XOD, AOD) was plotted against the amount of SOD enzyme added. Electrolyte solutions were 0.1 M SPB (pH 7.0) (solid circles) and + 50 μM L-ascorbic acid (rectangles), and the applied potential was 0.15 V.

Fig. 5 Schematic illustration of the reactions which took place at the SOD sensing cyt *c*-immobilized multilayer electrodes. Ferricytochrome *c*, SOD and L-ascorbic acid react competitively with the superoxide generated in the biopolymer membrane of the multilayer electrode.

Among the cyt *c*-immobilized electrodes examined (Table 1), Au/cyt *c*/PL:(XOD, AOD) is preferable for the detection of superoxide in the presence of L-ascorbic acid.

The current responses of Au/cyt *c*/PL:(XOD, AOD) for the addition of different amounts of SOD were recorded (Fig. 3) both in the presence and absence of L-ascorbic acid. The anodic current response of the O₂⁻ radical decreases on the addition of SOD. Immediately after the addition of SOD, the steady-state anodic current of the O₂⁻ radical decreases and attains a steady-state within 20 s. The amount of decrease in the anodic current was plotted against the amount of SOD added (Fig. 4). The magnitude of the current response for the addition of SOD did not differ considerably by the presence of L-ascorbic acid (50 μM) in the electrolyte solution. At Au/cyt *c*/PL:(XOD, AOD), thus, the SOD enzyme could be detected amperometrically, irrespective of the presence of as much as 50 μM of L-ascorbic acid. The different reactions took place at the cyt *c*-multilayer electrodes are given in Fig. 5. The O₂⁻ radical generated in the biopolymer membrane would be electrocatalytically oxidized by

the immobilized cyt *c* to give an anodic current response. Superoxide radical diffusing out of the biopolymer membrane undergoes disproportionation, SOD-assisted dismutation and quenching with L-ascorbic acid and thus could not contribute in the amperometric response. The quenching of superoxide radical by L-ascorbic acid and SOD permeated into the membrane produces a current response. In the detection of SOD, the O_2^- radical generated by the XOD-xanthine enzymatic reaction in the biopolymer membrane will undergo a competitive reaction with cyt *c*, L-ascorbic acid and SOD. Au/cyt *c*/PL:(XOD, AOD) responds to the addition of SOD in the concentration range of 0.5 to 300 U ml⁻¹ and thus could be used for the detection of SOD as low as 0.5 U ml⁻¹ (~50 ng ml⁻¹; signal-to-noise ratio = 3) in the presence of as much as 50 μM of L-ascorbic acid.

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References

1. M. Tarr and F. Samson, "Oxygen Free Radicals in Tissue Damage", **1993**, Birkhauser, Boston.
2. J. T. Hancock, *Br. J. Biomed. Sci.*, **1997**, *54*, 38.
3. J. A. Badwey, J. T. Curnette, and M. L. Karnovsky, *N. Eng. J. Med.*, **1979**, *300*, 1157.
4. A. M. Michelson, J. M. McCord, and I. Fridovich, "Superoxide and Superoxide Dismutases", **1977**, Academic Press, London.
5. D. K. Das and N. Maulik, *Methods Enzymol.*, **1994**, *233*, 601.
6. B. Halliwell and J. M. C. Gutteridge, *Biochem. J.*, **1984**, *219*, 1.
7. J. M. McCord and I. Fridovich, *J. Biol. Chem.*, **1969**, *244*, 6049.
8. I. Fridovich, *Adv. Enzymol.*, **1986**, *58*, 61.
9. B. M. Babior, R. S. Kipnes, and J. T. Curnette, *J. Clin. Invest.*, **1973**, *52*, 742.
10. K. Tanaka, F. Kobayashi, Y. Isogai, and T. Iizuka, *Bioelectrochem. Bioenerg.*, **1991**, *26*, 413.
11. P. Manning, C. J. McNeil, J. M. Cooper, and E. W. Hillhouse, *Free Rad. Biol. Med.*, **1998**, *24*, 1304.
12. J. M. Cooper, K. R. Greenough, and C. J. McNeil, *J. Electroanal. Chem.*, **1993**, *347*, 267.
13. F. Lisdat, B. Ge, E. E. Forster, and F. W. Scheller, *Anal. Chem.*, **1999**, *71*, 1359.
14. W. Scheller, W. Jin, E. E. Forster, B. Ge, F. Lisdat, R. Buttemeier, U. Wollenberger, and F. W. Scheller, *Electroanal.*, **1999**, *11*, 703.
15. S. Goldstein, C. Michel, W. Bors, M. Saran, and G. Czapski, *Free Rad. Biol. Med.*, **1988**, *4*, 295.
16. H. Ukeda, A. K. Sarker, D. Kawana, and M. Sawamura, *Anal. Sci.*, **1999**, *15*, 353.
17. F. Mizutani and M. Asai, in "Bioinstrumentations", ed. D. L. Wise, **1990**, Butterworths, Boston, 317.
18. A. Heller, *Acc. Chem. Res.*, **1990**, *23*, 128.
19. K. V. Gobi and F. Mizutani, *J. Electroanal. Chem.*, **2000**, *484*, 172.
20. K. V. Gobi, Y. Sato, and F. Mizutani, *Electroanal.*, in press.
21. E. Gileadi, E. K. Eisner, and J. Penciner, "Interfacial Electrochemistry", **1975**, Addison-Wesley, Massachusetts, 437.
22. C. A. Widrig, C. Chung, and M. D. Porter, *J. Electroanal. Chem.*, **1991**, *310*, 335.
23. K. V. Gobi, T. Okajima, K. Tokuda, and T. Ohsaka, *Langmuir*, **1998**, *14*, 1108.
24. S. Imabayashi, M. Iida, D. Hobara, Z. Q. Feng, K. Niki, and T. Kakiuchi, *J. Electroanal. Chem.*, **1997**, *428*, 33.
25. J. S. Olson, D. P. Ballou, G. Palmer, and V. Massey, *J. Biol. Chem.*, **1974**, *249*, 4350.
26. M. J. Tarlov and E. F. Bowden, *J. Am. Chem. Soc.*, **1991**, *113*, 1847.
27. T. Ruzgas, L. Wong, A. K. Gaigalas, and V. L. Vilker, *Langmuir*, **1998**, *14*, 7298.
28. S. Arnold, Z. Q. Feng, T. Kakiuchi, W. Knoll, and K. Niki, *J. Electroanal. Chem.*, **1997**, *438*, 91.
29. G. W. Bushnell, G. V. Louie, and G. D. Brayer, *J. Mol. Biol.*, **1990**, *214*, 585.
30. R. E. Dickerson, T. Takano, D. Eisenberg, O. B. Kallai, L. Samson, A. Cooper, and E. Margoliash, *J. Biol. Chem.*, **1971**, *246*, 1511.
31. M. Nishikimi, *Biochem. Biophys. Res. Comms.*, **1975**, *63*, 463.
32. R. Maidan and A. Heller, *Anal. Chem.*, **1992**, *64*, 2889.