Direct Electrical Response between Glucose Oxidase and Poly(mercapto-*p*-benzoquinone) Films

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Glucose oxidase was immobilized in a conductive redox polymer, poly(mercapto-*p*-benzoquinone), by means of an electropolymerization of mercaptohydroquinone in the presence of the enzyme. A glassy carbon electrode coated with the resulting polymer film functioned well as a direct response type of glucose sensor, where the polymer chain served as a conductive molecular chain between the active center (FAD) in the enzyme and the substrate electrode. The sensor showed excellent selectivity and possessed good durability.

Electrical response between redox centers of enzymes and metal electrodes must be excellent in amperometric biosensors and in electrodes used for selective electrochemical synthesis of biochemicals. For almost all the flavoenzymes, direct electrical communication between the redox center of the enzymes, oxidized flavin-adenine dinucleotide (FAD), and electrode is prevented by bulky thick insulating proteins that surround the redox center. For the purpose of getting over these obstacles, appropriate redox mediators such as ferrocene derivatives,[1—4] metal complexes,[5,6] and quinones[7—10] have been used.

Recently, direct electrical communication between the buried redox center of the enzymes and electrodes was achieved either by covalently binding of electron relays to the protein of the enzymes[11—14] or by electrode-attached conductive polymers that penetrate the enzymes sufficiently deeply for electron exchange.[15—18] We previously reported a new conductive redox polymer, poly(mercaptohydroquinone/mercapto-*p*-benzoquinone) (SQ) film which was prepared by an electropolymerization of mercaptohydroquinone (H_2QSH).[19] We have attempted the immobilization of glucose oxidase (GOD), a typical flavoenzyme, in the polymer film to evaluate glucose sensitivity and selectivity of the resulting polymer film on a glassy carbon electrode (GCE) by covering with a dialysis membrane.[20] But the presence of the membrane failed to get rapid responses to glucose. When the GCE surface was electroplated with Au prior to the preparation of the SQ film, the GOD-immobilized GCE possessed good durability even in the absence of the membrane and could respond rapidly to glucose. Characteristics of the enzyme electrode prepared here are shown in this paper.

Experimental

Material. GOD (EC 1.1.3.4, *Aspergillus niger*, 200 $U\,mg^{-1}$) was purchased from Boehringer Mannheim and used as received. H_2QSH was synthesized as previously reported.[21] All other chemicals were of reagent grade and were used without further purification. Glucose solutions were made from a stock solution that had been left to mutarotate overnight. Doubly distilled water was used as a solvent.

Electrodes. A representative electrode was prepared as follows: An electrode consisting of a GC rod (3 mm diameter, Furuuchi Kagaku), fixed in a glass tube with epoxy resin, was polished first with 0.3 μm alumina and then 0.05 μm alumina, sonicated, rinsed with water, and dried in air. The surface of the electrode was electroplated with gold at a constant potential of −0.1 V vs. Ag/AgCl for 5 min in 0.2 M (1 M=1 $mol\,dm^{-3}$) potassium chloride solution containing 1 mM tetrachloroauric acid to prevent the detachment of the SQ film from the GCE surface.

Apparatus. Electropolymerization of H_2QSH and electrochemical measurements were carried out in a three-compartment cell using the gold-electroplated GC working electrode, a Pt wire counter electrode, and an Ag/AgCl reference electrode. All potentials were referred to Ag/AgCl electrode. All measurements were carried out at 20 °C with use of a potentiostat (Hokuto Denko HA-301), a function generator (Hokuto Denko HB-104), and a potential sweeper (Yanako p-1000).

Immobilization of GOD. Immobilization of GOD in the SQ film was carried out by electropolymerization of H_2QSH in the presence of GOD. The electropolymerization was accomplished at 0.5 V for 1 h in 1/15 M phosphate buffer solution (pH 5.6, 1 cm^3) containing 5 mM H_2QSH and 60 mg GOD, using the gold-electroplated GCE as a substrate electrode. The GOD-immobilized electrode was rinsed by immersion in the phosphate buffer for 1 h to remove loosely trapped GOD and dried at 5 °C in a dark place for 12 h. The resulting enzyme electrode is denoted here as GOD/SQ/Au/GCE. An approximate scheme for the electropolymerization of H_2QSH is shown in Fig. 1. Postulated chemical structures of the oxidized form and the reduced form of the SQ film are shown in **1** and **2**, respectively.

Results and Discussion

Voltammetric Characterization of GOD/SQ/Au/GCE. Figure 2 shows voltammograms of the GOD/SQ/Au/GCE in the presence and absence of glucose measured in an N_2 atmosphere. In the absence of glucose, the voltammogram displays a typical oxidation current of the SQ film. In the presence of glucose, the anodic current increased largely with the increase in glucose concentration at more positive potential than 0.2 V where the SQ film is the oxidized form, **1**. It can be said that H_2QSH trapped in the enzyme during the electropolymerization can not serve as an elec-

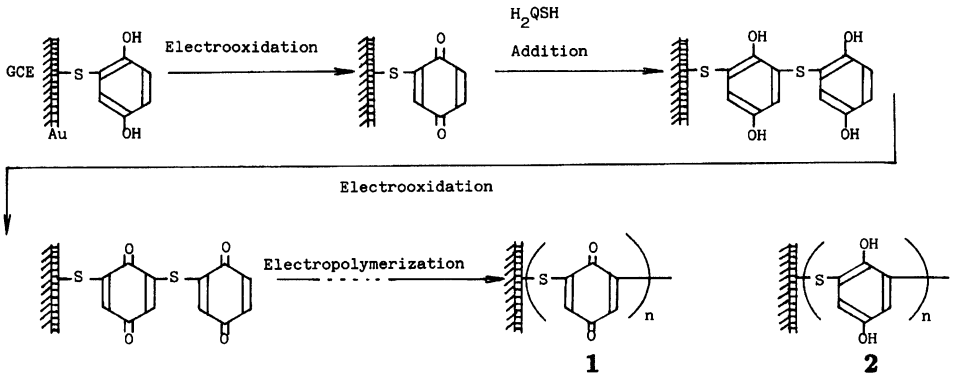

Fig. 1. Scheme of electropolymerization of H_2QSH on Au/GCE.

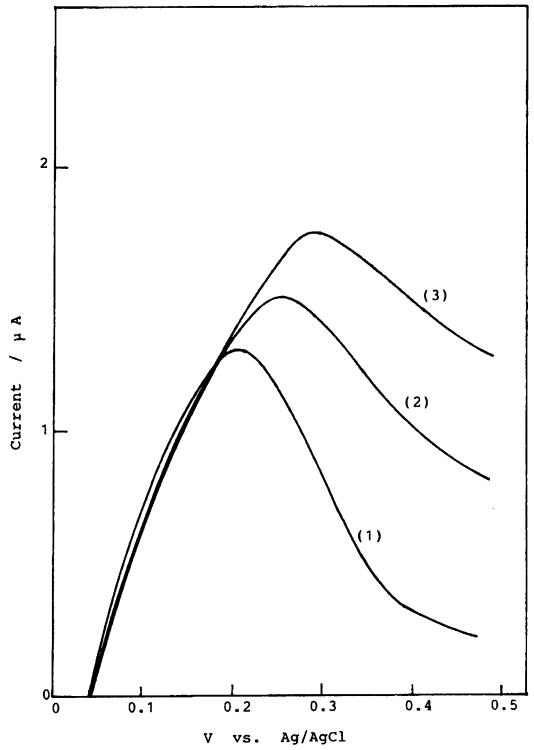

Fig. 2. Voltammograms of the GOD/SQ/Au/GCE in 1/15 M phosphate buffer solution (pH 5.6) containing glucose at a scan rate of 1 $mV\,s^{-1}$ in N_2 atmosphere. Glucose concentration: (1) 0, (2) 10 mM, (3) 20 mM.

tron mediator between the redox center of the enzyme and quinone moieties of the SQ chain, because H_2QSH will easily attach to quinone rings when electron transfer occurs between H_2QSH and quinone moieties of the polymer, as shown in Fig. 1. As a result, the anodic current based on the enzymatic reaction should decreased rapidly. The decrease in current was not observed on the GOD/SQ/Au/GCE. This fact suggests that the SQ polymer chain may penetrate the insulating protein of the enzyme sufficiently deeply and can act as an effective electron-transferring chain between the enzyme's redox center and the substrate electrode. In order to ascertain such "direct communication" via the conductive polymer chain, the SQ film was prepared by an electropolymerization of H_2QSH in the absence of GOD. The resulting SQ polymer-coated electrode was rinsed with methanol for 10 min to remove unreacted H_2QSH and then washed with distilled water, and then immersed in the 1 cm^3 phosphate buffer containing 60 mg GOD for ca. 20 h at 5 °C in a dark place under N_2 bubbling. The resulting SQ-coated electrode (abbreviated as (GOD)/SQ/Au/GCE) was submitted to the amperometric measurement of glucose. Results for the two electrodes, GOD/SQ/Au/GCE and (GOD)/SQ/Au/GCE, are shown in Fig. 3. Well-defined but small current responses to glucose were obtained on the (GOD)/SQ/Au/GCE in which the H_2QSH is scarcely present. The result strongly supports the suggestion that direct electron transfer takes place between the active center ($FAD/FADH_2$) of the enzyme and the

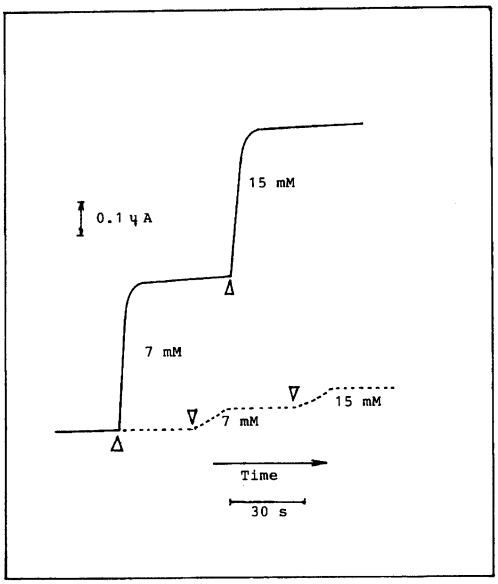

Fig. 3. Typical responses of the GOD/SQ/Au/GCE and the (GOD)/SQ/Au/GCE to addition of glucose at 0.3 V in N_2 atmosphere in the phosphate buffer solution. Each triangle indicates the time when 100 mM glucose solution was injected into the test solution. —: GOD/SQ/Au/GCE, ---: (GOD)/SQ/Au/GCE.

substrate electrode via the conductive polymer chain. The overall electron-transfer reaction may be written as follows:

$$\text{GOD(FAD)} + \text{glucose} \longrightarrow \text{GOD(FADH}_2\text{)} + \text{gluconolactone} \quad (1)$$

$$\text{GCE/Au/SQ(Q)} + \text{GOD(FADH}_2\text{)} \longrightarrow \text{GCE/Au/SQ(H}_2\text{Q)} + \text{GOD(FAD)} \quad (2)$$

$$\text{GCE/Au/SQ(H}_2\text{Q)} \longrightarrow \text{GCE/Au/SQ(Q)} + 2\text{H}^+ + 2\text{e} \quad (3)$$

where Q and H_2Q are *p*-benzoquinone and hydroquinone moieties of the SQ film, respectively. The electron-transfer from the enzyme to the substrate electrode denoted in Eq. 3 is performed by the propagation of the hydroquinone/*p*-benzoquinone redox reaction in the SQ film.

Constant Potential Measurements. Glucose response of the GOD/SQ/Au/GCE was measured by following procedure: At first, the working electrode potential was maintained at 0.3 V in 10 cm^3 of the phosphate buffer containing 0.1 M NaCl to obtain background currents, until the anodic current decayed to a constant value, and then 0.1 cm^3 of a stock solution of glucose was added to the buffer solution under slow N_2 bubbling. In case of the GOD/SQ/Au/GCE having no dialysis membrane, the time required to reach the steady-state current was less than 5 s after the addition of glucose sample. A typical relationship between steady-state current density at 0.3 V and glucose concentration is shown in Fig. 4. The calibration curve of glucose was approximately linear up to 10 mM, and glucose can be determined up to ca. 30 mM though the curve is not linear. Reproducibility of the steady-state current density was ±2% (coefficient of variation) when measured with the same electrode, and ±7% with different electrodes prepared by the procedure described above. The apparent Michaelis–Menten constant (K_M^{App}) for the GOD/SQ/Au/GCE, which is not an intrinsic property of the enzyme but rather of the system as a whole, can be determined from Eadie–Hofstee plots.[22] The K_M^{App} value for the enzyme electrode was estimated as 13 mM, which is a little higher than the value of 9.0 mM reported by Yoneyama et al. who used hydroquinonesulfonate as an electron-transfer mediator in polypyrrole film.[7]

Influence of Dissolved Oxygen. For fabricating the glucose sensor, interference by dissolved oxygen must be minimized. The interference of oxygen was examined by comparing the glucose response of the GOD/SQ/Au/GCE measured in air-saturated solution with that measured in N_2-saturated solution at a constant potential of 0.3 V. It was found that there was no appreciable difference in the steady-state current between the two cases, as shown in Fig. 4. This result clearly indicates that the glucose response obtained by the enzyme electrode is not affected in the presence of atmospheric oxygen. Figure 5 shows anodic voltammograms of the SQ/Au/GCE (no GOD) in the presence and absence of hydrogen peroxide in the phosphate buffer solution. Anodic currents generated by the electrooxidation of hydrogen peroxide appeared at potentials more positive than 0.7 V, indicating that the response currents measured at 0.3 V included no oxidation current of hydrogen peroxide. Thus it is clear that the response current of the enzyme electrode is entirely due to electrocatalytic oxidation of glucose.

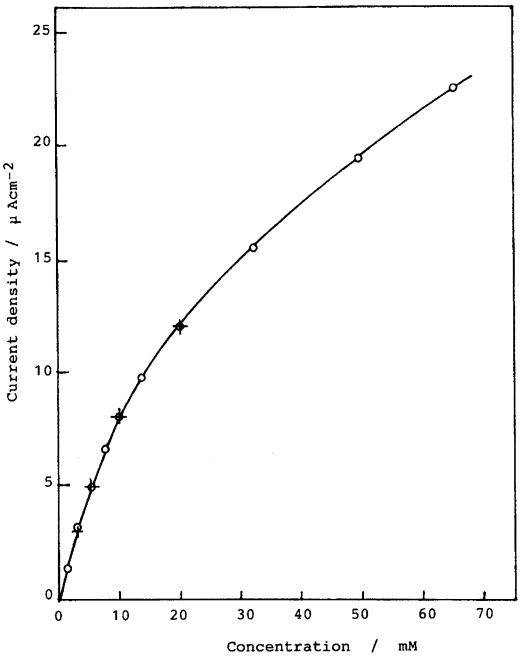

Fig. 4. Calibration curve of glucose at the GOD/SQ/Au/GCE obtained at 0.3 V. Measurements were carried out under the same conditions as in Fig. 3. ○: N_2-saturated solution, +: Air-saturated solution.

Dependence of Film Thickness on Response. The amount of the SQ film, which can be represented by the quinone moieties in the film, was de-

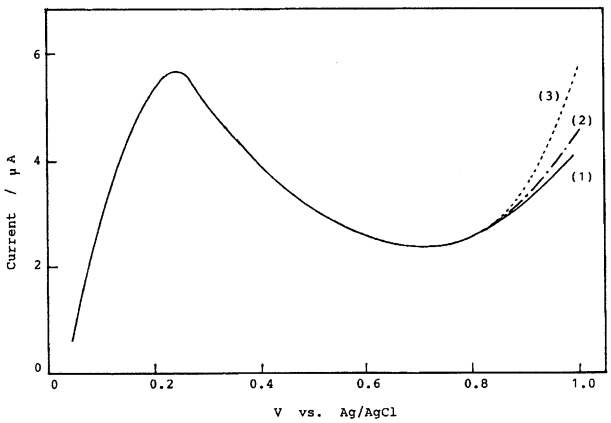

Fig. 5. Oxidation currents of H_2O_2 on the SQ/Au/GCE in the phosphate buffer solution at 5 $mV\,s^{-1}$. (1) 0, (2) 0.1 mM, (3) 1 mM.

termined by the charge required for the electroreduction of the quinone moieties to the hydroquinone moieties in the film. Figure 6 shows plots of the response current of the GOD/SQ/Au/GCE to 10 mM glucose as a function of the amount of quinone moieties on the Au/GCE. The response current increased with the increase in the amount of quinone moieties up to 1.6×10^{16} QU cm^{-2} (1 QU≡one quinone moiety unit) and above the value decreased slightly, while the time required to reach the steady-state current showed a tendency to prolong with the increase in film thickness. A closely packed monolayer of 4-methyl-1,2-dihydroxybenzene lying flat on a perfectly flat carbon surface corresponds to ca. 3.6×10^{14} molecules cm^{-2}.[23] The SQ polymer chain may elongate from the electrode to solution through alternate electrooxidation and addition reactions of H_2QSH, as shown in Fig. 1. Thus it may be safely said that the polymer chain consisting of ca. 1.2×10^{16} QU cm^{-2}, which was prepared by 1 h electropolymerization of H_2QSH under the conditions described above, is long enough to reach the active center in the GOD (a radius of ca. 50 Å). The decrease in response current observed in the case of thick SQ film probably is due to additional resistance against diffusion of glucose into the enzyme embedded in the thick film.

Selectivity and Stability. The selectivity of the GOD/SQ/Au/GCE was examined by holding the potential at 0.3 V, while other sugars such as xylose, arabinose, mannose, galactose, fructose, lactose, mannitol, trehalose, sorbitol, and saccharose were added separately at concentrations of 10 mM. No response current was observed for these sugars examined here by the current sensitivity used for the glucose measurement (>1 nA). The excellent selectivity suggests that the GOD immobilized in the SQ film retains its native selectivity, and that the enzyme conformation is not much changed by the immobilization in the SQ film. Figure 7 shows the result obtained to investigate the storage stability of the GOD/SQ/Au/GCE. The enzyme electrode was stored in atmospheric conditions at 5 °C for over 10 d. The response test on each day was carried out at least 10 times at ca. 5-min intervals using the same sample solution. Even with no dialysis membrane, the current response on the GOD/SQ/Au/GCE retained an almost constant value, 7.5 μA, for the long period. On the other hand, the response current decreased remarkably when the GC surface was not electroplated with gold. These results indicate that the decrease in the response current may be due to undesired detachment of the SQ film having GOD from the GC surface and that the detachment can be depressed by the gold-electroplating. The same enzyme electrode was provided to evaluate the consecutive response measurements in a large amount of the phosphate buffer solution containing 10 mM glucose, where detectable change in glucose concentration does not occur during the measurement. The initial response current density, 7.5 μA cm^{-2}, at 0.3 V showed little decrease (less than ca. 2%) even after 10 h. It may be safely said that the GOD/SQ/Au/GCE fabricated here displays excellent storage characteristics

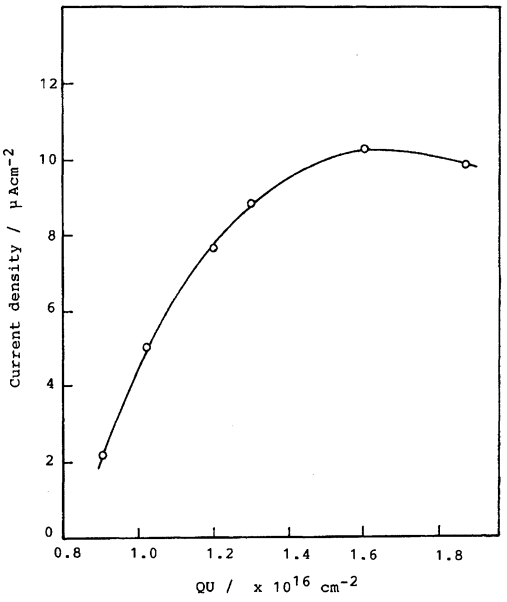

Fig. 6. Relationship between steady-state currents at 0.3 V and QU in the GOD/SQ/Au/GCE measured in the phosphate buffer solution containing 10 mM glucose. Other experimental conditions were the same as in Fig. 3.

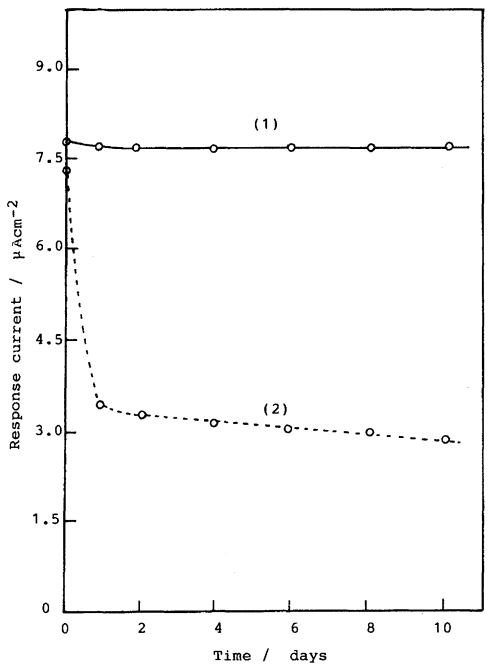

Fig. 7. Comparison of storage stability between the GOD/SQ/Au/GCE and the GOD/SQ/GCE. Amperometric response currents were measured at 0.3 V in the phosphate buffer solution containing 10 mM glucose. —O—: GOD/SQ/Au/GCE, ---O---: GOD/SQ/GCE.

and has good durability in consecutive measurements.

Conclusion

Electropolymerization of mercaptohydroquinone in the presence of GOD gave a conductive redox polymer where GOD can be immobilized. The Au-electroplated GCE coated with the resulting polymer film functioned as a direct response type of glucose sensor, where the polymer chain served as a conductive molecular chain between the substrate electrode and the active centers in the enzyme. The enzyme electrode can be prepared easily and showed excellent selectivity and possessed good stability for 10 d of storage and for continuous measurements. In addition, dissolved air or oxygen did not interfere with the glucose response of the enzyme electrode. Further investigation is in progress to extend these studies to other flavoenzyme systems.

References

1) S. M. Zakeeruddin, D. M. Fraser, M. K. Nazeerunddin, and M. Gratzel, *J. Electroanal. Chem. Interfacial Electrochem.*, **337**, 253 (1992).
2) I. Taniguchi, S. Miyamoto, S. Tomiura, and F. M. Hawkridge, *J. Electroanal. Chem. Interfacial Electrochem.*, **240**, 333 (1988).
3) P. D. Hale, T. Inagaki, H. Karan, Y. Okamoto, and T. A. Skotheim, *J. Am. Chem. Soc.*, **111**, 3482 (1989).
4) A. E. G. Cass, G. Davis, M. J. Green, and H. A. O. Hill, *J. Electroanal. Chem. Interfacial Electrochem.*, **190**, 117 (1985).
5) S. M. Zakeeruddin, D. M. Fraser, M. K. Nazeeruddin, and M. Gratzel, *J. Electroanal. Chem. Interfacial Electrochem.*, **337**, 253 (1992).
6) B. A. Gregg and A. Heller, *Anal. Chem.*, **62**, 258 (1990).
7) Y. Kajiya, H. Sugai, C. Iwakura, and H. Yoneyama, *Anal. Chem.*, **63**, 49 (1991).
8) T. Ikeda, T. Shibata, and M. Senda, *J. Electroanal. Chem. Interfacial Electrochem.*, **261**, 351 (1989).
9) T. Ikeda, H. hamada, K. Miki, and M. Senda, *Agric. Biol. Chem.*, **49**, 541 (1985).
10) N. K. Cenas, A. K. Pocius, and J. Kulys, *Bioelectrochem. Bioenerg.*, **12**, 583 (1984).
11) W. Schuhmann, T. J. Ohara, H. -L. Schmidt, and A. Heller, *J. Am. Chem. Soc.*, **113**, 1394 (1991).
12) Y. Degani and A. Heller, *J. Am. Chem. Soc.*, **110**, 2615 (1988).
13) Y. Degani and A. Heller, *J. Phys. Chem.*, **91**, 1285 (1987).
14) P. N. Bartlett, R. G. Whitaker, M. J. Green, and J. Frew, *J. Chem. Soc., Chem. Commun.*, **1987**, 1603.
15) P. D. Hale, L. I. Boguslavsky, T. Inagaki, H. I. Karan, H. S. Lee, T. A. Skotheim, and Y. Okamoto, *Anal. Chem.*, **63**, 677 (1991).
16) B. A. Gregg and A. Heller, *Anal. Chem.*, **62**, 258 (1990).
17) Y. Degani and A. Heller, *J. Am. Chem. Soc.*, **111**, 2357 (1989).
18) H. Shinohara, T. Chiba, and M. Aizawa, *Sens. Actuators*, **13**, 79 (1988).
19) G. Arai and M. Furui, *Nippon Kagaku Kaishi*, **1984**, 673.
20) G. Arai, M. Masuda, and I. Yasumori, *Chem. Lett.*, **1992**, 1791.
21) W. Alcolay, *Helv. Chim. Acta*, **30**, 578 (1947).
22) P. J. Debenedetti, *Chem. Eng. Sci.*, **42**, 2203 (1987).
23) C. Degrand and L. L. Miller, *J. Am. Chem. Soc.*, **102**, 5728 (1980).