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Electrical Communication between Glucose Oxidase and Electrodes Mediated by Phenothiazine-Labeled Poly(ethylene oxide) Bonded to Lysine Residues on the Enzyme Surface

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A series of glucose oxidase (GOx) hybrids (GOx−phenothiazine-labeled poly(ethylene oxide) (PT-PEO)) capable of direct electrical communication with electrodes is synthesized by covalently modifying PT-PEO to lysine residues on the enzyme surface. The length of the PEO chain and the number of PT groups are systematically altered. After the PT-PEO modification, all the hybrids maintain more than 50% of enzyme activity relative to that of native GOx, although loss of the activity becomes greater with increasing PEO chain length. The catalytic current, i_{cat}, is observed at a potential more positive than 0.55 V after the addition of glucose, due to the intramolecular electron transfer (ET) from reduced forms of flavin adenine dinucletide ($FADH_2$/FADH) to PT^+ that are electrogenerated at the electrode. The i_{cat} value increases with the number of PT groups, indicating that most of the modified PT groups act as mediators. The magnitude of the i_{cat} increase depends on the PEO chain length and reveals a maximum for PT-PEO with the molecular weight of 3000. In contrast, the i_{cat} is almost constant for GOx−2-(10-phenothiazyl)propionic acid (PT-PA) hybrids with more than two PT groups synthesized by covalently modifying PT-PA to surface lysines, indicating that only a few key PT groups function as mediators. The maximum rate constant (130 s^{-1}) for the ET from $FADH_2$/FADH to PT^+ is obtained for the GOx hybrid modified with five PT-PEO groups with the molecular weight of 3000.

Glucose oxidase (GOx; EC 1.1.3.4) is a dimeric glycoprotein of 186 kDa containing two tightly bound flavin adenine dinucleotide (FAD) cofactors, which catalyze the electron transfer (ET) from glucose to oxygen accompanying the production of gluconolactone and hydrogen peroxide.[1] The direct electrical communication between the redox center (FAD) and electrodes is prevented because the redox center is located too far from the outermost surface. Therefore, freely diffusing redox mediators with a more positive redox potential than that of the redox center have been frequently used to shuttle electrons between FAD and the electrode under an oxygen-free condition.[2−7]

In terms of the application to enzymatic sensor systems[8] and the electrochemical control of an enzymatic function, enzymes capable of a direct electron exchange with electrodes have been arousing great interest. One possible way to provide electrochemical activity for enzymes is the covalent immobilization of an electron relay to the FAD unit[9,10] or on the surface of GOx.[11−16] Heller et al.[11] and Schuhmann[12] reported the synthesis and electrochemical properties of GOx with ferrocene (Fc) derivatives attached to the sugar or acidic amino acid residues on its surface via spacer chains of different lengths. These studies demonstrated that redox mediators bound via long, flexible, and hydrophilic spacer chains to the outer surface of GOx can transfer electrons to the electrode surface according to the so-called "wipe mechanism" with the enzyme-bound mediators swinging in and out of the active site of the enzyme. Although the number and the location of mediators and the length of spacer chains are decisive factors to achieve efficient mediation properties, they have not been systematically controlled in the previous studies and their

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effect on the mediation electrochemistry of GOx hybrids still remains ambiguous.

We synthesized a series of new biopolymer conjugates (GOx−phenothiazine-labeled poly(ethylene oxide) (PT-PEO)) by covalently bonding phenothiazine (PT) mediators to lysine residues on the enzyme surface via PEO chains and electrochemically investigated the ET from $FADH_2$/FADH to PT^+ (oxidized PT group) as a function of the number of attached PT groups and the length of the PEO chain under a substrate-saturated and diffusion-limited condition. PEO is a nontoxic, flexible, water-soluble polymer, and PEO covalently attached to the protein surface does not denature proteins or hinder the approach of other small molecules in the case of low modification numbers.[17] PT is expected to have higher ability to mediate the ET between FAD and electrodes than Fc due to its higher redox potential. We found that all the PT groups bonded to lysine residues effectively mediate the ET reaction and an optimum length of the PEO chain exists in terms of the ET rate. We also synthesized GOx−2-(10-phenothiazyl)propionic acid (PT-PA) hybrids, in which PT-PA is directly bound to lysine residues on the GOx surface, and compared its catalytic electrochemistry with that of GOx−(PT-PEO) hybrids to gain further insight into the effect of the PEO spacer on the catalytic ET reaction of GOx hybrids.

EXPERIMENTAL PROCEDURES

Materials. GOx from *Aspergillus niger* and horseradish peroxidase (HRP; EC 1.11.1.7.) were purchased from Toyobo; succinic anhydride, *N*,*N*′-dicyclohexyl carbodiimide (DCC), sodium acetate trihydrate, piperazine-1,4-bis(2-ethansulfonic acid) (PIPES), and phosphate buffer powder were from Wako Pure Chemicals; diethylene glycol dimethyl ether, D-glucose, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC), and phenothiazine were from Junsei Chemicals; 40% aqueous solution of benzyltrimethylammonium hydroxide and *β*-D-glucose were from Tokyo Kasei; *N*-hydroxysuccinimide (NHS) was from Aldrich; *o*-dianisidine was from Sigma; *N*-hydroxysulfosuccinimide (sulfo-NHS) was from Fluka; and *N*-ethyl-*N*-(2-hydroxy- 3-sulfopropyl)-*m*-toluidine (EHSPT) was from Dojindo Laboratories. All the reagents were used without further purification. Disposable ultrafiltration units (type, USY-5; cutoff MW, 50 000) and chromato disks were purchased from Advantec and GL Science, respectively.

Sodium acetate buffer (0.05 mol dm^{-3}) was prepared by dissolving an appropriate amount of sodium acetate trihydrate into deionized water and adjusting its pH to 5.1 by adding 0.1 mol dm^{-3} HCl. PIPES buffer (0.15 mol dm^{-3}) was prepared by dissolving an appropriate amount of PIPES powder into deionized water, and then its pH was adjusted to 7.4 by adding 0.1 mol dm^{-3} NaOH. Phosphate buffer ($^1/_{15}$ mol dm^{-3}, pH 7.4) was prepared by dissolving a bag of phosphate buffer powder into 1 dm^3 of deionized water.

Synthesis of PT-PEO (Scheme 1a). Phenothiazine (0.1 mol) was dissolved in dried ethylene glycol dimethyl ether (110 mL) and then dried potassium hydroxide (0.064 mol) was added as a catalyst. After this mixture was introduced into an autoclave, the atmosphere in the autoclave was replaced with N_2 by repeated evacuation and N_2 gas purge. Under the evacuated condition, an equivalent molar ratio of ethylene oxide to phenothiazine was admitted from a tank. While the reaction mixture was stirred at 110 °C for 2 h, the pressure inside the autoclave was reduced to the initial value, indicating that 1 molar equiv of ethylene oxide was added to phenothiazine. The appropriate amount of ethylene oxide to obtain the desired molecular weight of PT-PEO was gradually introduced into the autoclave as the inside pressure was maintained at (1.47−1.96) × 10^5 Pa at 120 °C. After confirming the end of the polymerization reaction indicated by the reduction of the inside pressure, ethylene glycol dimethyl ether was completely removed under reduced pressure.

Scheme 1. Synthetic Procedures for GOx−(PT-PEO) and GOx−(PT-PA) Hybrids

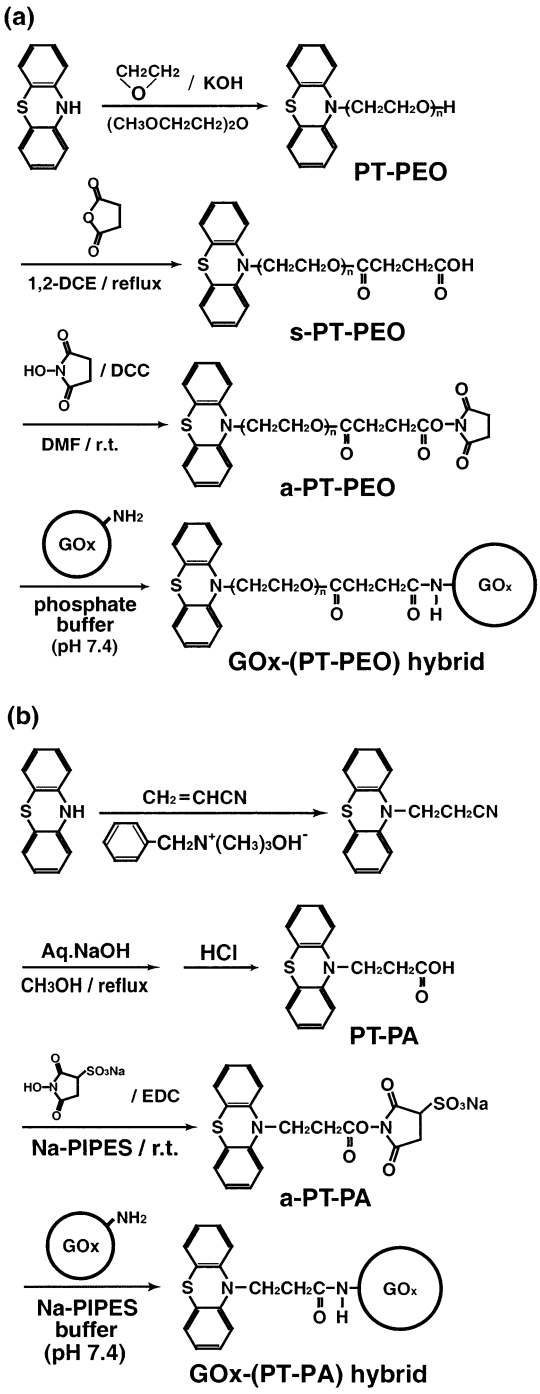

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Table 1. Number-Average Molecular Weight and Hydrodynamic Properties of PT-PEO

PT-PEO	number-average mol wt: OH titration	number-average mol wt: EA	M_w/M_n[a]	D[b] ($\times 10^6$ cm^2 s^{-1})	r[c] (nm)
PT-PEO1000	1050	1050	1.20	2.4	1.0
PT-PEO2000	2050	2030	1.09	1.7	1.4
PT-PEO3000	2970	2860	1.05	1.3	1.8
PT-PEO4200	4170	4360	1.58	0.93	2.6
PT-PEO8000	7779	8750	1.20	0.71	3.4

[a] Measured by GPC using tetrahydrofuran as a carrier solvent and calibrated by poly(ethylene glycol) standards. [b] Diffusion coefficient at 25 °C in 0.05 mol dm^{-3} sodium acetate buffer (pH5.1) determined by chronoamperometry. [c] Hydrodynamic radius calculated from the Stokes−Einstein equation.

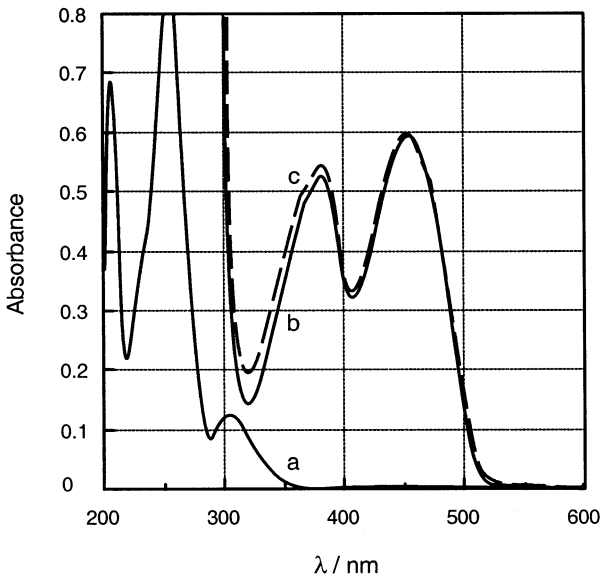

Figure 1. UV−visible spectra of PT-PEO (a), native GOx (b), and GOx−(PT-PEO) hybrid (c).

The reaction mixture remaining in the bottom of the autoclave was taken and neutralized by adding sulfuric acid until its pH became 6. To remove ionic impurities, the mixture was stirred with an acid adsorbent and then with a base adsorbent for 30 min, respectively, and then filtered. The filtrate was evaporated to dryness at 100 °C for 1 h under reduced pressure. The structure and number-average molecular weight of obtained PT-PEO were characterized by a combination of ^{1}H NMR, hydroxyl group titration, gel permeation chromatography (GPC), and elemental analysis (EA), as listed in Table 1. The number-average molecular weight was calculated from the content of nitrogen in EA and the end hydroxyl group titration, for both assuming the structure of PT-PEO as shown in Figure 1. The reasonable agreement between the values determined by the hydroxyl group titration and EA reveals that PT-PEO molecules synthesized in this work have the structure shown in Figure 1. The M_w/M_n values close to unity also support that PT-PEO can be prepared by the ring-opening anionic polymerization.

Preparation of GOx−(PT-PEO) Hybrids (Scheme 1a). PT-PEO (5 mmol) and succinic anhydride (25 mmol) were dissolved in 1,2-dichloroethane (100 mL) containing a small amount of pyridine (2 mL). After refluxing at 65 °C for 10 h under nitrogen atmosphere, the reaction mixture was evaporated and the remaining solid was redissolved into pure water. After addition of diethyl ether, the aqueous phase was collected and mixed with chloroform. The chloroform layer was collected, evaporated, and dried in vacuo, yielding s-PT-PEO; yield, 75%: ^{1}H NMR (δ from TMS in $CDCl_3$) 2.66 (4H, s, CH_2COO), 3.50−3.80 (xH, m, CH_2OCH_2), 3.85 (2H, t, $NCCH_2O$), 4.10 (2H, t, NCH_2), 4.24 (2H, t, $OCOCH_2$), 6.85−7.2 (8H, m, H_{arom}). The number of protons at δ = 3.50−3.80, x, is 4(n − 1), where n is the degree of polymerization of PEO.

s-PT-PEO (3.7 mmol) and *N*-hydroxysuccinimide (4.4 mmol, NHS) were dissolved in 50 mL of dimethylformamide (DMF), and the resulting solution was cooled to 0 °C. To this solution, a DMF solution of DCC (4.4 mmol) was added dropwise at 0 °C under stirring. The mixture was allowed to react at room temperature for 24 h. After the reaction, the mixture was again cooled to 0 °C and filtered to remove DCC−urea. The solid obtained by evaporating the filtrate was dissolved in benzene, and insoluble impurities were removed by filtration. The filtrate was dropped into petroleum ether. The precipitate was collected and dried under reduced pressure, yielding a-PT-PEO; yield, 70%: ^{1}H NMR (δ from TMS in $CDCl_3$) 2.70−3.0 (4H, t, CH_2COO; 4H, m, H_{NHS}), 3.50−3.80 (xH, m, CH_2OCH_2), 3.86 (2H, t, $NCCH_2O$), 4.10 (2H, t, NCH_2), 4.27 (2H, t, $OCOCH_2$), 6.86−7.20 (8H, m, H_{arom}).

GOx and a-PT-PEO were dissolved in $^1/_{15}$ mol dm^{-3} phosphate buffer (pH 7.4) and kept at 25 °C for 24 h. The produced GOx−(PT-PEO) hybrid was separated from excess PT-PEO by ultrafiltration. The yellow substances remaining on the ultrafiltration membrane were dissolved into sodium acetate buffer (pH 5.1) and filtered through a chromato disk to remove impurities. The resulting sodium acetate solution of GOx−(PT-PEO) hybrid was used for the electrochemical measurements.

Preparation of GOx−(PT-PA) Hybrid (Scheme 1b). Cyanoethylation of phenothiazine resulted in 2-(10-phenothiazyl)-propionitrile,[18] followed by its hydrolysis in an alkaline solution. Repeated recrystallization of the obtained solid from ethanol yielded PT-PA as a white solid; yield, 30%; mp, 164 °C: ^{1}H NMR (δ from TMS in DMSO-d_6) 2.7 (2H, t, CH_2CO), 4.14 (2H, t, NCH_2), 6.9−7.3 (8H, m, H_{arom}), 12.35 (1H, s, COOH).

An appropriate amount of PT-PA (7.4−129 μmol) was dissolved in 0.15 M Na-PIPES buffer (pH 7.4) aided by sonication, and the resulting solution was cooled to 0 °C. Equimolar quantities of EDC and sulfo-NHS were added to the PT-PA solution to activate PT-PA, and then GOx was added to the buffer solution 30 min after the activation. The reaction mixture was kept at 25 °C for 24 h. Unreacted PT-PA and impurities were removed from the reaction mixture by ultrafiltration. The yellow substances remaining on the ultrafiltration membrane, GOx−(PT-PA) hybrids, were dissolved in a sodium acetate buffer (pH 5.1), which was employed for the electrochemical measurements.

Estimation of the Number of Modified Mediators and the Relative Enzymatic Activity. Figure 1 shows typical UV−visible absorption spectra of PT-PEO (a), native GOx (b), and GOx−(PT-PEO) hybrid (c) recorded by a Shimadzu UV-160A spectrophotometer. The concentration of the GOx hybrid can be estimated using the extinction coefficient of native GOx (ϵ_{452} = 21 600 M^{-1} cm^{-1})[19] from the absorbance at 452 nm, where there

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is no absorption of PT-PEO or PT-PA. The difference in the absorbance between the GOx hybrid and native GOx at 320 nm corresponds to the absorption of PT-PEO or PT-PA groups attached to the GOx surface and can be converted to their concentration using their ϵ_{320} values.[20] The concentration ratio of the PT groups to GOx corresponds to the average number of modified mediators per GOx molecule. At present, we have no information about the distribution of the number of attached PT groups among GOx hybrids or about the location of lysine residues on the GOx surface responsible for the binding. The absorption arising from the PT groups was not observed when the PT-PEO or PT-PA modification was carried out without carbodiimide and NHS or its derivatives, confirming the formation of amide bonding between lysine residues on the GOx surface and a carboxylic acid group of PT-PEO or PT-PA.

The enzymatic activity relative to that of native GOx was determined using the peroxidase−*o*-dianisidine assay[21] for GOx−(PT-PEO) hybrids and the 4AA−EHSPT assay[22] for GOx−(PT-PA) hybrids in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) at 25 °C under O_2 saturation. The relative activity equals the proportion of active FAD to all FAD groups in the hybrids.

Electrochemical Measurements. A conventional three-electrode cell was used with a glassy carbon working electrode (geometrical area, 0.071 cm^2), a Ag|AgCl|3 M KCl reference electrode, and a Pt wire auxiliary electrode. A glassy carbon electrode was polished with alumina powder (0.05-μm diameter) and sonicated in pure water prior to use. A solution of GOx hybrids was introduced to the cell and gently deaerated by N_2 purge for 20 min. Cyclic voltammograms (CVs) were recorded under N_2 atmosphere between 0.3 and 0.7 V at a scan rate of 10 mV s^{-1} in the absence and presence of 0.05 mol dm^{-3} glucose using a BAS-CV-50W electrochemical analyzer. The differential pulse voltammogram (DPV) was measured from 0.3 to 0.7 V at a scan rate of 1 mV s^{-1}, a pulse amplitude of 50 mV, and a sampling time of 50 ms in the absence of glucose using the same electrochemical analyzer.

The diffusion coefficient of PT-PEO was determined by chronoamperometry (CA) in 0.05 mol dm^{-3} acetate buffer (pH 5.1) containing 1 mmol dm^{-3} PT-PEO. The electrode potential was stepped up from 0.3 to 0.7 V. The electrochemical analyzer, the cell configuration, and the electrodes used in CA were the same as those in CV.

RESULTS AND DISCUSSION

Number of Modified Mediators and Relative Activity of the GOx Hybrid. The number of mediators attached per GOx molecule was controlled by varying the molar ratio of PT-PEO or PT-PA to the GOx molecule in the reaction mixture. Figure 2 shows the relation between the number of bonded PT-PEO or PT-PA groups per GOx and the molar ratio of a-PT-PEO or a-PT-PA to GOx in the hybrid preparation. For all the hybrids, the number of attached PT groups increases with the molar ratio of a-PT-PEO or a-PT-PA to GOx. In the case of GOx−(PT-PEO) hybrids, the reactivity of a-PT-PEO to lysine residues tends to decrease with increasing molecular weight except PT-PEO with a molecular weight of 8000 (PT-PEO8000). Due to the steric hindrance of a longer PEO chain, it is considered that the probability of encountering lysine residues becomes lower for the activated carboxylic acid group of a-PT-PEO with higher molecular weight. The maximum number of modified PT-PEOs, which is 9, was obtained for PT-PEO1000. This value is comparable to those in the previous reports[7,13] obtained for the lysine modification without denaturants, which are used to modify amino acid residues inside enzymes. Considering that some of the 30 lysine residues per GOx molecule are not located on the surface,[23] the maximum modification number seems to be reasonable.

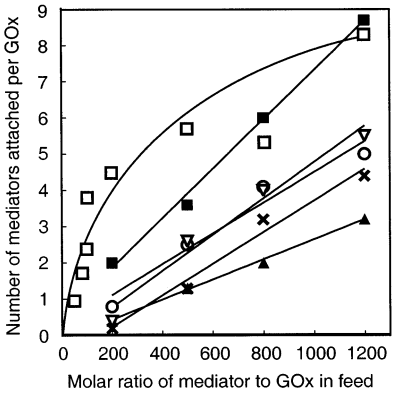

Figure 2. Number of mediators attached per GOx molecule in GOx−(PT-PEO) and GOx−(PT-PA) (□) hybrids as a function of the molar ratio of activated PT-PEO or PT-PA to GOx in feed. The molecular weights of modified PT-PEO groups are 1000 (■), 2000 (▽), 3000 (○), 4200 (▲), and 8000 (×).

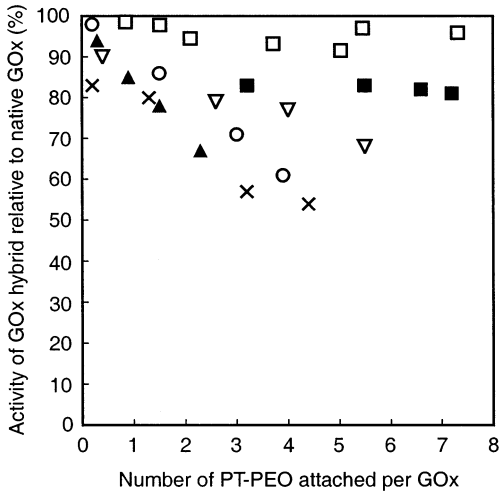

Figure 3. Relationship between the number of PT-PEO or PT-PA (□) groups attached per GOx molecule and the enzyme activity of GOx hybrids relative to native GOx. The molecular weights of modified PT-PEO groups are 1000 (■), 2000 (▽), 3000 (○), 4200 (▲), and 8000 (×).

Figure 3 represents the dependence of the relative enzymatic activity of hybrids to native GOx on the number and the molecular weight of the attached PT-PEO groups. The relative enzymatic

(19) Nakamura, S.; Fujiki, S. *J. Biochem.* **1968**, *63*, 51−58.

(20) The ϵ_{320} value measured for each PT-PEO ranges from 2390 to 2870 M^{-1} cm^{-1} and depends on the molecular weight of PT-PEO. The ϵ_{320} value of PT-PA is 2900 M^{-1} cm^{-1}.

(21) Keesey, J. *Biochemical Information*; Boehringer Mannheim Biochemicals: Indianapolis, IN, 1987; p 27.

(22) *Enzyme Catalog: Toyobo Enzymes*; Toyobo Co., Ltd.: Osaka, Japan, 1998; p 112.

(23) Hecht, H. J.; Schomburg, D.; Kalisz, H.; Schmid, R. D. *Biosens. Bioelectron.* **1995**, *8*, 197−203.

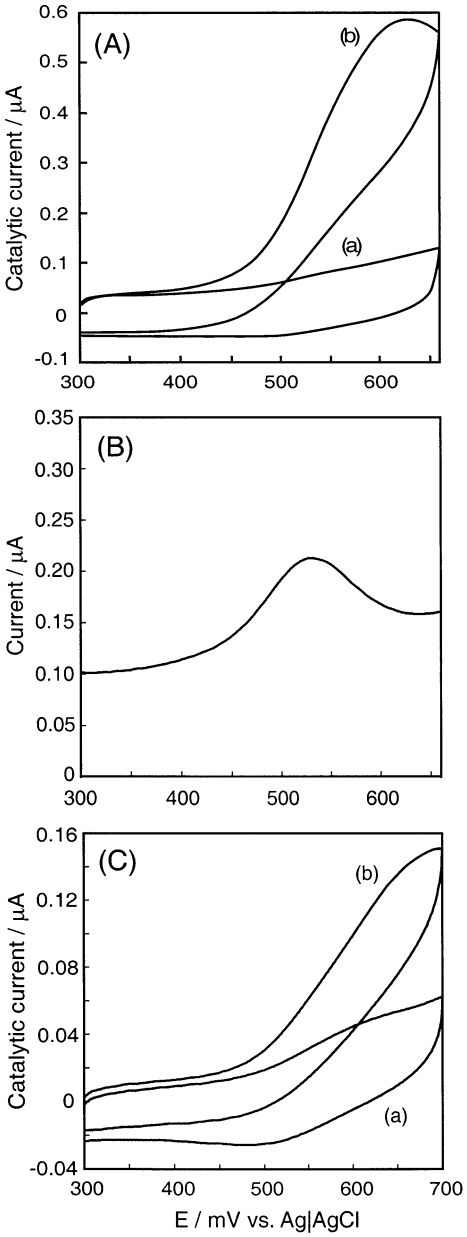

Figure 4. Cyclic voltammograms of GOx−(PT-PEO3000)$_{3.8}$ hybrid (8.9 μmol dm^{-3}) (A) and GOx−(PT-PA)$_{5.3}$ hybrid (9.7 μmol dm^{-3}) (C) at a glassy carbon electrode in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) at a scan rate of 10 mV s^{-1} in the absence (a) and presence (b) of 0.05 mol dm^{-3} glucose. Differential pulse voltammogram (B) of GOx−(PT-PEO3000)$_{3.8}$ hybrid (8.9 μmol dm^{-3}) measured at a glassy carbon electrode in the same buffer at a scan rate of 1 mV s^{-1} in the absence of glucose is also shown.

activity of hybrids seems to significantly depend not only on the molecular weight of PT-PEO but also on the number of attached PT-PEO groups. The modification of PT-PEO with low molecular weight little affects the relative enzymatic activity of hybrids. Although the modification with a large number of PT-PEO groups brought about a decline in the relative activity of the GOx hybrid, all GOx hybrids used in this work retained more than 50% relative activity. NHS esters have high reaction selectivity for lysine residues and are effective in preventing cross-linking among the GOx molecules.[24] We think that the use of NHS or its derivative for the carbodiimide-promoted amide bond formation between

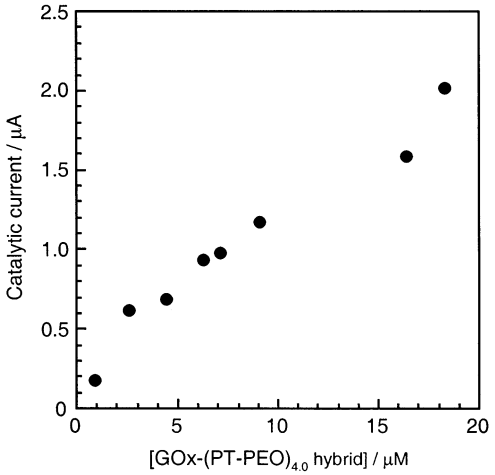

Figure 5. Catalytic current of GOx−(PT-PEO3000)$_{4.0}$ as a function of the hybrid concentration measured at 0.62 V at a glassy carbon electrode in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) containing 0.05 mol dm^{-3} glucose.

a-PT-PEO or a-PT-PA and lysine residues on GOx explains why the high relative activity of GOx was maintained after the mediator modification. Actually, SDS−PAGE measurements of the hybrids revealed the absence of cross-linked GOx.

Electrochemical Measurements. Figure 4A shows CVs of GOx−(PT-PEO3000)$_{3.8}$ hybrid (the average number of modified PT-PEO3000 is 3.8) in the absence (a) and presence (b) of 0.05 mol dm^{-3} glucose. Although no apparent redox peak appeared in the CV in the absence of glucose, PT groups attached to the GOx surface retained redox activity at 0.53 V, as shown in a DPV of GOx−(PT-PEO3000)$_{3.8}$ hybrid (Figure 4B). The GOx−(PT-PA)$_{5.3}$ hybrid also exhibited a pair of small peaks around 0.55 V in the absence of glucose, which is attributed to the redox of PT groups attached to the GOx surface (Figure 4C(a)). On the other hand, the oxidation current of GOx hybrids (Figure 4A(b) and C(b)) greatly increased at a potential higher than the redox potential of PT groups in the presence of glucose, indicating the enzymatic reduction of the electrochemically oxidized PT (PT$^+$). This means that PT groups bound to the GOx surface can function as electron mediators between the electrode and the FAD center of GOx. Despite a similar number of modified PT groups, the GOx−(PT-PEO) hybrid showed greater catalytic current i_{cat} than that of the GOx−(PT-PA) hybrid in the same potential range.

The i_{cat} measured at 0.62 V for GOx−(PT-PEO) or GOx−(PT-PA) hybrid increased with the glucose concentration and leveled off above 0.02 mol dm^{-3} glucose (data not shown). The i_{cat} linearly increased with the concentration of GOx−(PT-PEO) hybrid up to 20 μmol dm^{-3} as shown in Figure 5, indicating that molecular interactions between hybrids are not significant and the intramolecular ET from FADH$_2$/FADH to PT$^+$ groups on the same GOx molecule mainly occurs in the GOx−(PT-PEO) at the hybrid concentration used (9 μmol dm^{-3}).

Figure 6 represents the i_{cat} of the hybrids measured at 0.62 V under a substrate-saturated condition (0.05 mol dm^{-3} glucose) as functions of the number and the molecular weight of the modified PT-PEO groups. For all the GOx−(PT-PEO) hybrids, the i_{cat} increased with the number of attached PT groups, demonstrating

(24) Brinkley, M. *Bioconjugate Chem*. **1992**, *3*, 2−13.

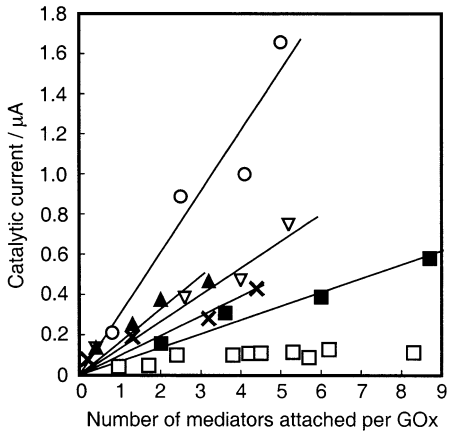

Figure 6. Relationship between the number of mediators attached per GOx molecule and the catalytic current for GOx hybrids modified with PT-PEO of molecular weight 1000 (■), 2000 (▽), 3000 (○), 4200 (▲), and 8000 (×), and GOx−(PT-PA) hybrids (□) at 0.62 V at a glassy carbon electrode in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) containing 0.05 mol dm^{-3} glucose. The concentration of the hybrids was 9 μmol dm^{-3}.

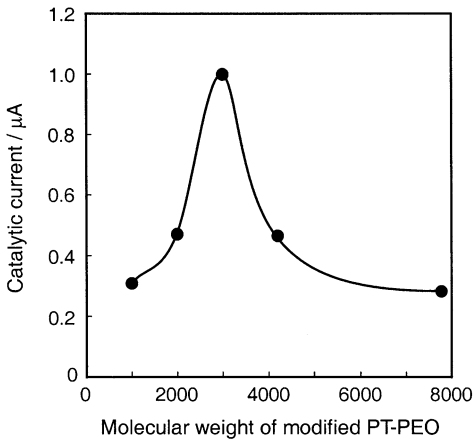

Figure 7. Dependence of the catalytic current of GOx hybrids (9 μmol dm^{-3}) on the molecular weight of modified PT-PEO. The number of modified PT-PEO per GOx molecule is 3.6 for GOx−(PT-PEO1000), 4.0 for GOx−(Pt-PEO2000), 4.1 for GOx−(PT-PEO3000), 3.2 for GOx−(PT-PEO4200), and 3.2 for GOx−(PT-PEO8000). The catalytic current was measured at 0.62 V at a glassy carbon electrode in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) containing 0.05 mol dm^{-3} glucose.

the participation of most of the PT groups bonded to the GOx surface in the ET reaction between the electrode and FAD. Further, it is interesting to note that the magnitude of the i_{cat} of a hybrid with a similar number of attached PT depends on the molecular weight of PT-PEO and reveals a maximum for GOx−(PT-PEO3000) hybrids, as shown in Figure 7. This suggests the existence of an optimum PEO chain length in terms of the ET from $FADH_2$/FADH to PT^+ groups in the GOx−(PT-PEO) hybrids. It has been reported by some research groups that the intramolecular ET rate between the redox center and mediators was enhanced with an increase in the chain length linking mediators to the enzyme.[25] The length of linkers in those works was, however, less than 15 methylene units and considerably shorter than those in the present work. The use of longer PEO spacers resulted in the finding of optimum spacer length for the direct electron exchange between the FAD center and mediators.

In contrast to GOx−(PT-PEO) hybrids, GOx−(PT-PA) hybrids with more than two PT groups revealed an almost constant i_{cat} (Figure 6). Only a few key PT groups function as mediators for the ET reaction between electrodes and the FAD center in GOx−(PT-PA) hybrids due to the short and hydrophobic PA spacer.

Estimation of the Rate Constants of ET from $FADH_2$/FADH to PT^+ Groups. Under the oxygen-free environment, the intramolecular ET reactions from $FADH_2$/FADH to PT^+ in GOx hybrids are described as follows:

$$\mathrm{GOx(FAD)\text{-}(PT)}_n + \text{glucose} \rightarrow \mathrm{GOx(FADH_2)\text{-}(PT)}_n + \text{gluconolactone} \quad (1)$$

$$\mathrm{GOx(FADH_2)\text{-}(PT)}_n \rightarrow \mathrm{GOx(FADH_2)\text{-}(PT)}_{n-1}(\mathrm{PT^+}) + \mathrm{e^-} \quad (2)$$

$$\mathrm{GOx(FADH_2)\text{-}(PT)}_{n-1}(\mathrm{PT^+}) \xrightarrow{k_1} \mathrm{GOx(FADH)\text{-}(PT)}_n + \mathrm{H^+} \quad (3)$$

$$\mathrm{GOx(FADH)\text{-}(PT)}_n \rightarrow \mathrm{GOx(FADH)\text{-}(PT)}_{n-1}(\mathrm{PT^+}) + \mathrm{e^-} \quad (4)$$

$$\mathrm{GOx(FADH)\text{-}(PT)}_{n-1}(\mathrm{PT^+}) \xrightarrow{k_2} \mathrm{GOx(FAD)\text{-}(PT)}_n + \mathrm{H^+} \quad (5)$$

Under a glucose-saturated condition, the diffusion-limited i_{cat} for the intramolecular mediation reaction of GOx hybrid is given by[13]

$$i_{cat} = 2FA(D_{GOx-hybrid}\, k_{obs})^{1/2} C_{GOx-hybrid} \quad (6)$$

where i_{cat} is the catalytic current, F is the Faraday constant, A is the electrode area, $D_{GOx-hybrid}$ is the diffusion coefficient of the GOx hybrid, k_{obs} is the average rate constant for the oxidation of $FADH_2$/FADH by PT^+, and $C_{GOx-hybrid}$ is the concentration of the GOx hybrid. The k_{obs} is expressed in the form of $k_1k_2/(k_1^{1/2} + k_2^{1/2})^2$, where k_1 and k_2 are the rate constants for the oxidation of $FADH_2$ and FADH by PT^+, respectively. As described by eq 6, the i_{cat} is a function of $D_{GOx-hybrid}$, k_{obs}, and $C_{GOx-hybrid}$. Because preliminary light scattering measurements indicate that the difference in D between native GOx and GOx hybrids is small, the value for native GOx (4.1×10^{-7} cm^2 s^{-1} at 25 °C)[7] was used for all GOx hybrids to estimate k_{obs} values.

The obtained k_{obs} values (Table 2) are more than 2 orders of magnitude greater for GOx−(PT-PEO) hybrids than for GOx−(PT-PA) hybrids. Similarly to the i_{cat}, k_{obs} increases with the number of PT groups in GOx−(PT-PEO) hybrids and is almost independent of the number of PT groups in GOx−(PT-PA) hybrids, indicating that the difference in the i_{cat} mainly comes from the difference in the k_{obs}. Since the through-space distance from the FAD center is more than 2.3 nm for all the 30 lysine residues per GOx, it is considered that the approach of the PT groups close to FAD is not achieved by the short alkyl chain but by the long and flexible PEO chain.

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Table 2. Rate Constants for ET from $FADH_2$/FADH to PT^+ in GOx−(PT-PEO) and GOx−(PT-PA) Hybrids with a Different Number of Modified Mediators

GOx hybrids	n^a	k_{obs} (s^{-1})^b
GOx−$(PT\text{-}PA)_n$	1.0	0.063
	2.4	0.24
	4.5	0.31
	5.7	0.25
GOx−$(PT\text{-}PEO1000)_n$	2.0	4.6
	3.6	17
	6.0	28
	8.7	68
GOx−$(PT\text{-}PEO2000)_n$	0.4	0.82
	2.6	10
	4.0	19
	5.5	52
GOx−$(PT\text{-}PEO3000)_n$	0.8	1.8
	2.5	33
	4.1	47
	5.0	130
GOx−$(PT\text{-}PEO4200)_n$	0.4	0.76
	1.3	1.7
	2.0	7.1
	3.2	12
GOx−$(PT\text{-}PEO8000)_n$	0.2	0.3
	1.3	1.9
	3.2	7.5
	4.4	19

[a] The number of PT-PEO or PT-PA groups attached per GOx hybrid molecule. [b] Rate constants were calculated using the equation $i_{cat} = 2FA(D_{GOx-hybrid}\, k_{obs})^{1/2} C_{GOx-hybrid}$ and corrected for the relative enzymatic activity.

The k_{obs} of GOx−(PT-PEO) alters from 0.3 to 130 s^{-1} depending on the molecular weight and number of PT-PEO groups. For GOx with Fc attached to sugar or acidic amino acid residues on the surface via long spacer chains, k_{obs} values comparable to or greater than the values for GOx−(PT-PEO) hybrids were observed.[11,12] However, direct comparison among these values is difficult because of the difference in the modified positions and the ambiguity of the number of modified mediators in the previous reports. Although many factors participate in the presence of the optimum PEO chain for the ET from $FADH_2$/FADH to PT^+, the motion of the PEO chains is one important factor. It is considered that the end PT group of surface-tethered PT-PEO moves around the bonding site in an arc. The hydrodynamic radius of PT-PEO, which was calculated from its chronoamperometrically determined diffusion coefficient using the Stokes−Einstein relation, increases with the molecular weight. It ranges from 1.0 nm for PT-PEO1000 to 3.4 nm for PT-PEO8000 (Table 1). With an increase in the molecular weight of PT-PEO in the hybrids, the possibility of access of PT groups close to FAD increases and a faster ET rate from $FADH_2$/FADH to PT^+ can be achieved. A slight difference in the distance between $FADH_2$/FADH and PT^+ at the closest approach may yield significant difference in k_{obs}, since the ET rate decays exponentially with the distance between a donor and an acceptor.[26] Factors in reducing the ET rate, however, must exist in the case of too long PEO chains. Overlap of the accessible space of PT groups on the GOx surface with each other and interference in the access of the PT group to FAD from a bulky long PEO seem to be included in the factors reducing the ET rate.

The dependence of the k_{obs} on the number of attached PT groups includes the influence of the location of PT-PEO modification with respect to the FAD center, since the reactivity to a-PT-PEO and the distance from FAD are not equivalent among the lysine residues. We examined the electrochemical properties of GOx hybrids prepared by the systematic modification of PT-PEO to surface glutamic acid and aspartic acid residues,[27] some of which are located closer to FAD than the lysine residues.[1] Comparing the k_{obs} values for glutamic acid- and aspartic acid-modified hybrids with those for the lysine-modified hybrids at a similar number of modified PT groups, greater values were obtained for the former hybrids in the case of shorter PEO chains.[27] This suggests that the PT-PEO modification closer to FAD is effective to achieve fast ET, and a long PEO spacer is possible to compensate for the disadvantage in the location of PEO modification.

The independence of the i_{cat} on the number of modified PT-PA suggests that the location of modified mediators is critical in GOx−(PT-PA) hybrids due to their short spacer chains. We prepared GOx−(PT-PA) hybrids with 0.96 and 1.70 PT groups by incubating GOx with 50- and 80-fold excess PT-PA at 25 °C for 3 h, respectively. Almost half of the i_{cat} for those of GOx−(PT-PA) hybrids with more than two PT groups was observed for these two hybrids. Taking into account that one GOx molecule consists of two subunits with the same structure, the constant i_{cat} for GOx−(PT-PA) hybrids with more than two PT groups might imply that the PT groups bonded to the most reactive lysine residues in the PT-PA modification became the most effective key mediators.

CONCLUSION

We have found here that PT groups bonded to lysine residues on the GOx surface via the PEO chain effectively mediate fast ET between the electrode and the FAD center. This fast ET is primarily attributed to the fast oxidation of $FADH_2$/FADH by PT^+ in GOx−(PT-PEO) hybrids. While the ET rate from $FADH_2$/FADH to PT^+ increased with the number of PT-PEO groups, the optimum molecular weight, that is, the optimum spacer length of PT-PEO, was found with respect to the ET from $FADH_2$/FADH to PT^+. This is the first finding of the optimum spacer length for direct electron exchange with electrodes in GOx with covalently modified mediators via spacer chains. The optimum spacer length would be determined also by many factors unexamined in this work: the relative positions of mediators, entropic and enthalpic factors associated with chain mobility and penetration, and interactions of PT-PEO with glycan chains and other PT-PEOs. Elucidation of the relative importance of these factors resulting in the optimum spacer length is left for future studies. The comparison of catalytic electrochemistry between GOx−(PT-PEO) and GOx−(PT-PA) hybrids has shed light on the importance of a long, hydrophilic and flexible PEO spacer for generating fast ET from $FADH_2$/FADH to PT^+. The fast ET enables GOx−$(PT\text{-}PEO3000)_{5.0}$ to exhibit a catalytic current comparable to that for the corresponding freely diffusing PT-PEO3000 system despite the smaller diffusion coefficient of mediators attached to the GOx hybrid.[28] Additionally, the effect of dioxygen on the catalytic

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current of this GOx hybrid was negligible. These results suggest the possibility of synthesizing GOx hybrids that realize a specific glucose sensing without perturbation by dioxygen.

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