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Effect of a Modification Site on the Electron-Transfer Reaction of Glucose Oxidase Hybrids Modified with Phenothiazine via a Poly(ethylene oxide) Spacer

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Glucose oxidase [GOx-(PT-PEO_{NH_2})] hybrids are synthesized by attaching phenothiazine (PT) groups to aspartic and glutamic acid residues on the enzyme surface via poly(ethylene oxide) (PEO) spacers of different molecular weights. A fast oxidation of $FADH_2$/FADH by PT^+ with the aid of the local motion of a hydrophilic, long, flexible PEO spacer is achieved for the GOx-(PT-PEO_{NH_2}) hybrids and yields greater electron-transfer (ET) rates than that for GOx-(PT_{NH_2}) hybrids, in which the PT groups are directly bonded to the GOx surface. The ET rate of GOx-(PT-PEO_{NH_2}) hybrids depends on the molecular weight of PT-PEO_{NH_2}, and the maximum is obtained at a molecular weight of 3000. The ET rates of GOx hybrids are compared in terms of the location of the PT modification and the length and structure of the spacer chain connection of the PT mediator to a surface amino acid residue. Greater ET rates are obtained for the modification at aspartic and glutamic acid residues than for the lysine modification when the PT groups are bonded directly or via a short PEO spacer chain. In contrast, no advantage of aspartic and glutamic acid residues over lysine residues in generating a fast oxidation of $FADH_2$/FADH by PT^+ is observed for GOx hybrids in which the PT groups are attached via longer PEO spacers. The long PEO spacer is able to compensate the disadvantage of lysine residues locating far from the FAD center in GOx hybrids whose mediation reactions are based on the so-called wipe mechanism.

Introduction

Direct electrochemical oxidation of the redox active site (FADH/$FADH_2$) of glucose oxidase (GOx) is difficult, because the redox center is insulated by thick protein shells.[1−4] Therefore, freely diffusing redox mediators such as ferrocene derivatives,[1,4−6] quinones,[6,7] octacyanotungstate,[8] and ruthenium complexes[9,10] have been generally used to reoxidize FADH/$FADH_2$ under an oxygen-free environment. The covalent immobilization of mediators to the GOx surface[12−23] or FAD enzymatic active site[24−27] is an effective method to establish the direct electron transfer (ET) between the buried FAD center and an electrode. Several research groups have investigated the mediation reactions of GOx with surface-modified ferrocene derivatives via several kinds of spacer chains.[12−14] Schuhmann et al. demonstrated that redox mediators bound via long, flexible, and hydrophilic spacer chains to the outer surface of GOx can transfer electrons to the electrode according to the so-called "wipe mechanism".[12]

We found that a rapid ET from $FADH_2$/FADH to PT^+ was achieved by attaching phenothiazine-labeled poly(ethylene oxide) (PT-PEO) spacers to the lysine residues on the GOx surface.[18−20] Those PEO spacers, which are hydrophilic, flexible, and much longer than the spacers used in the previous works, enable PT mediators to approach close to the cofactor. Particularly, the GOx hybrids prepared by attaching PT-PEO with a molecular weight of 3000 to surface lysine residues possess about 2000 times higher ET rate than the GOx hybrids with directly attached PT groups.[18−20]

The above studies suggest that the length and structure of a spacer chain and the number of mediators significantly

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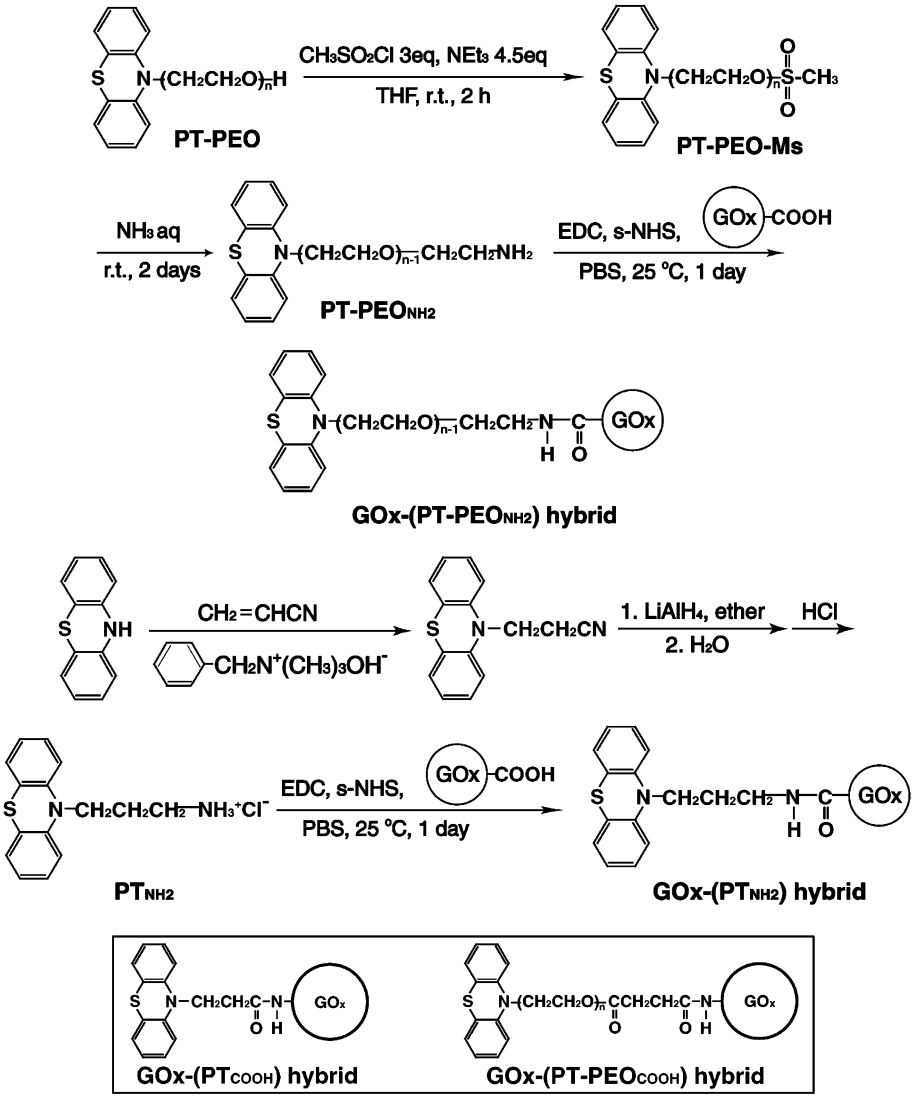
Scheme 1. Synthetic Procedures of GOx-(PT-PEO$_{NH_2}$) and GOx-(PT$_{NH_2}$) Hybrids and Chemical Structures of GOx-(PT-PEO$_{COOH}$) and GOx-(PT$_{COOH}$) Hybrids



affect the ET properties according to the wipe mechanism. In contrast, the effect of the location of modified mediators has not been systematically elucidated using a common mediator and spacer chain. In each GOx subunit, 10 of the acidic amino acid residues (aspartic acid and glutamic acid) are located less than 1.6 nm away from the active site FAD,[29] whereas none of the lysine residues are within 2.3 nm of the FAD. Therefore, the derivatization of GOx at acidic amino acid residues with PT-PEO is expected to yield hybrids where the PT groups are densely attached in the vicinity of the FAD site on the GOx surface and to achieve the mediated $FADH_2$ oxidation faster than that in the lysine-modified GOx hybrids.

The aim of the present study is to elucidate the effectiveness of the PEO spacer in generating a fast ET between the FAD center and the electrode for acidic amino acid residue-modified GOx hybrids, and to evaluate the effect of the location of mediator modification (acidic amino acid residues vs lysine residues) on the ET reaction of GOx hybrids. We newly synthesized two kinds of GOx hybrids, GOx-(PT-PEO$_{NH_2}$) and GOx-(PT$_{NH_2}$), in which the PT groups are covalently modified via a PEO spacer and directly to acidic amino acid residues on the GOx surface, respectively. The electrocatalytic properties of these GOx hybrids were investigated, and the obtained results were compared with those for the GOx hybrids where the PT groups were attached to lysine residues, in terms of the effect of the location of PT modification and the spacer length.

Experimental Section

Materials. GOx from *Aspergillus niger* (EC 1.1.3.4) and horseradish peroxidase (HRP; EC 1.11.1.7) were purchased from TOYOBO. Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), sodium acetate trihydrate, and phosphate buffer powder were from Wako Pure Chemicals. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was from Aldrich. A 40% aqueous solution of benzyltrimethylammonium hydroxide and β-D-glucose were from Tokyo Kasei. 3,3-Dimethoxybenzidine dihydrochloride (*o*-dianisidine) was from Sigma. *N*-Hydroxysulfosuccinimide (sulfo-NHS) was from Fluka. All reagents were used without further purification. Disposable ultrafiltration units (type USY-5; cutoff MW 50000) and chromatodisks were purchased from Advantec and GL Science, respectively. Three kinds of aqueous buffer solutions were prepared according to the methods in the previous work.[19]

Preparation of PT-PEO$_{NH_2}$ and GOx-(PT-PEO$_{NH_2}$) Hybrid[22] (Scheme 1**)**. PT-PEO synthesized according to the previous

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method[19] was dehydrated by azeotropic distillation and then lyophilization. To 150 mL of tetrahydrofuran (THF) containing PT-PEO (5 mmol) and triethylamine (22.5 mmol) was added dropwise methanesulfonyl chloride (15 mmol) in 25 mL of THF, with stirring. After the addition, the mixture was allowed to react at room temperature for 2 h. The produced salt was removed by filtration, and the filtrate was reprecipitated with diethyl ether. The precipitate was collected and dried under reduced pressure, yielding PT-PEO-Ms, yield 98%.

PT-PEO-Ms (1.6 mmol) in 70 mL of water was added dropwise to 500 mL of 28% ammonia water, and the mixture was stirred for 2 days. The reaction solution was dialyzed using dialysis membrane (cutoff MW 1000), lyophilized, and dried under reduced pressure, yielding PT-PEO_{NH_2}: yield 90%; ^{1}H NMR (δ from TMS in $CDCl_3$) 2.9 (2H, t, CH_2N), 3.1−3.9 (xH, m, CH_2-OCH_2), 4.0−4.1 (2H, t, $N_{arom}CH_2$), 6.9−7.2 (8H, m, H_{arom}). The number of protons at $\delta = 3.1-3.9$, x, is $4(n-1)$, where n is the degree of polymerization of PEO.

Acidic amino acid residues on the GOx surface were activated using sulfo-NHS esters. An appropriate amount of GOx, PT-PEO_{NH_2}, and equimolar quantities of EDC and sulfo-NHS with PT-PEO_{NH_2} were dissolved in 1/15 mol dm^{-3} phosphate buffer (pH 7.4), and the resulting solution was kept at 25 °C for 24 h. The produced GOx-(PT-PEO_{NH_2}) was separated from excess PT-PEO_{NH_2} by ultrafiltration. The yellow substances remaining on the ultrafiltration membrane were dissolved into 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) and filtered through a chromatodisk to remove impurities. The number of PT groups attached per GOx molecule was altered by varying the molar ratio of PT-PEO_{Am} against the GOx molecule from 200 to 1200 in the reaction mixture.

Preparation of GOx-(PT_{NH_2}) Hybrid (Scheme 1**)**. 3-(10-Phenothiazyl)propylamine hydrochloride (PT_{NH_2}) was prepared according to the reported procedure:[30] mp 229°C; ^{1}H NMR (δ from TMS in DMSO-d_6) 1.9 (2H, m, CH_2N), 2.8 (2H, t, CCH_2C), 3.9 (2H, t, $N_{arom}CH_2$) 6.9−7.2 (8H, m, H_{arom}), 8.0 (3H, m, NH_3).

The procedure for preparing GOx-(PT_{NH_2}) was almost identical to that for GOx-(PT-PEO_{NH_2}) except PT_{NH_2} and 0.15 mol dm^{-3} PIPES buffer were used instead of PT-PEO_{NH_2} and phosphate buffer, respectively.

A series of lysine-modified GOx hybrids were synthesized and purified according to the methods previously reported.[19] Scheme 1 also shows the chemical structures of GOx-(PT-PEO_{COOH}) hybrid and GOx-(PT_{COOH}) hybrid.

Measurements of the Number of Modified Mediators, Enzymatic Activity, and Redox Properties. The average number of modified PT groups per hybrid molecule was determined from a UV−vis absorption spectrum of the hybrids.[19] The activity of the GOx hybrid relative to that of native GOx was measured using the peroxidase−*o*-dianisidine assay.[31] We used a conventional three-electrode cell equipped with a glassy carbon working electrode (geometrical area 0.071 cm^2), a Ag|AgCl saturated KCl reference electrode, and a Pt wire auxiliary electrode. A glassy carbon electrode was polished with alumina powder and sonicated in pure water prior to use. A solution of GOx hybrid was introduced to the cell and gently deaerated by N_2 purge for 20 min. Cyclic voltammograms (CVs) were recorded from 0.3 to 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.

Results and Discussion

GOx Hybrids in Which PT Groups Are Bonded to Aspartic and Glutamic Acid Residues. The number of PT groups attached to GOx increases with increasing the molar ratio of PT-PEO_{NH_2} or PT_{NH_2} to GOx molecules in the feed, as shown in Figure 1. The slope of the plots for PT-PEO_{NH_2}, which corresponds to the reactivity of the modified group to aspartic and glutamic acid residues, does not reveal any strong dependence on the molecular weight, which is in contrast with the lysine modification where the slope tends to decrease with increasing molecular weight of PT-PEO_{COOH}.[19] The reactivity of PT-PEO_{NH_2} was comparable to that for the PT-PEO_{COOH} of molecular weight 1000 for the lysine modification, meaning that aspartic and glutamic acid residues have higher reactivity than lysine residues for the modification of PT-PEO groups. The number of modified PT groups was in proportion to the concentration of the pair of EDC and sulfo-NHS in the feed under constant concentrations of GOx and PT-PEO_{NH_2} or PT_{NH_2} (data not shown), suggesting that the number of modified PT groups is determined mainly by the activation step of acidic amino acid residues on the GOx surface. The hydrophilic NH_3^+ group at the PT-PEO_{NH_2} chain end seems not to be incorporated into a PEO coil and to electrostaticaly interact with the sulfonate group of sulfo-NHS modified on the GOx surface, resulting in the constant reactivity of PT-PEO_{NH_2} despite its molecular weight. The greater slope of the plot for PT_{NH_2} indicates that the PEO chain reduces the reactivity of PT-PEO_{NH_2} in comparison with that of PT_{NH_2}.

Figure 2 represents the dependence of the relative enzymatic activity of hybrids to native GOx on the number

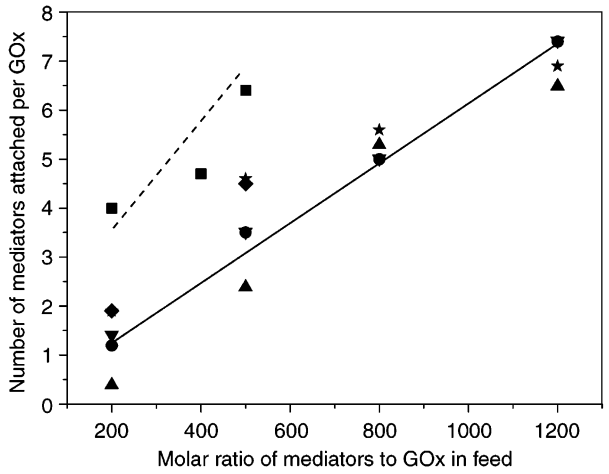

Figure 1. Number of mediators attached per GOx molecule in GOx-(PT-PEO_{NH_2}) or GOx-(PT_{NH_2}) hybrids (■) as a function of the molar ratio of mediator groups to GOx in the feed. The molecular weights of modified PT-PEO_{NH_2} groups are 1000 (◆), 2000 (★), 3000 (●), 4200 (▼), and 8000 (▲).

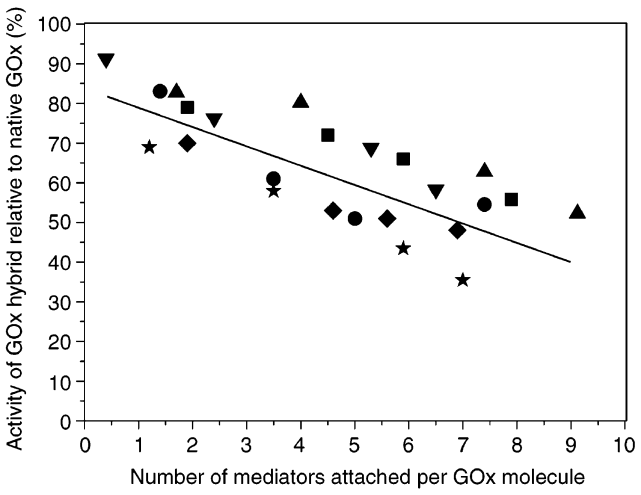

Figure 2. Relationship between the number of PT-PEO_{NH_2} or PT_{NH_2} (■) groups attached per GOx molecule and the enzyme activity of GOx hybrids relative to native GOx. The molecular weights of modified PT-PEO_{NH_2} groups are 1000 (◆), 2000 (★) 3000 (●), 4200 (▼), and 8000 (▲).

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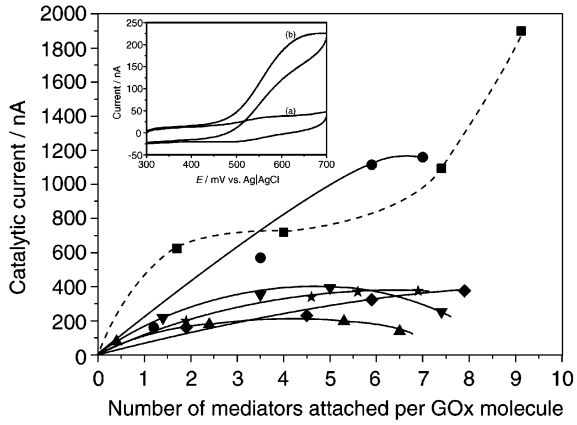

Figure 3. Relationship between the number of mediators attached per GOx molecule and the catalytic current of GOx hybrids modified with PT-PEO_{NH_2} of molecular weight 1000 (◆), 2000 (★) 3000 (●), 4200 (▼), and 8000 (▲) and PT_{NH_2} (■) at 0.62 V. The catalytic current was measured at a glassy carbon electrode in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) containing 0.05 mol dm^{-3} glucose. The inset shows CVs of the GOx-(PT-PEO_{NH_2} 8000)$_{6.5}$ hybrid measured at 10 mV s^{-1} at a glassy carbon electrode in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) in the absence (a) and presence (b) of 0.05 mol dm^{-3} glucose. The concentration of the hybrids was 9 μmol dm^{-3}.

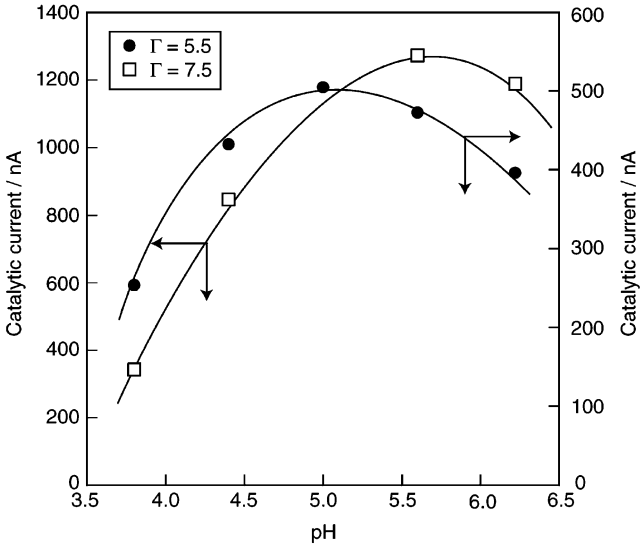

Figure 4. Dependence of the catalytic current on the buffer pH for GOx-(PT_{NH_2})$_{5.5}$ and GOx-(PT_{NH_2})$_{7.5}$ hybrids. The catalytic current was measured at 0.62 V (vs Ag|AgCl) at a glassy carbon electrode in 0.05 mol dm^{-3} sodium acetate buffer containing 0.05 mol dm^{-3} glucose. The concentration of the hybrids was 9 μmol dm^{-3}.

and molecular weight of the bonded PT groups. The magnitude of the decrease in the relative enzymatic activity of hybrids with increasing number of PT groups per GOx molecule does not significantly change with the molecular weight and structure of the spacer group. The relative activity of the GOx hybrid in the present work is, on the whole, lower than that of the lysine-modified GOx hybrids, implying that the activation step of surface residues is also responsible for the relative activity of the GOx hybrid.

Figure 3 shows the catalytic current (i_{cat}) measured under a diffusion-limited (at 0.620 V) and substrate saturation (using 0.05 mol dm^{-3} glucose) condition as a function of the number of PT groups per GOx molecule for GOx-(PT-PEO_{NH_2}) and GOx-(PT_{NH_2}) hybrids in 0.05 mol dm^{-3} acetate buffer at pH 5.1. In the absence of glucose,

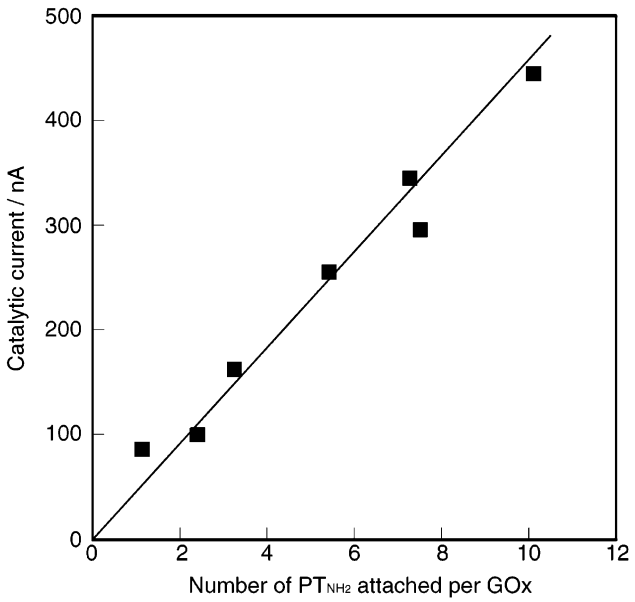

Figure 5. Relationship between the number of mediators attached per GOx molecule and the catalytic current for GOx-(PT_{NH_2}) hybrids (■). The catalytic current was measured at 0.62 V (vs Ag|AgCl) at a glassy carbon electrode in 0.05 mol dm^{-3} sodium acetate buffer (pH 3.8) containing 0.05 mol dm^{-3} glucose.

a pair of small peaks appeared around 0.55 V for GOx-(PT-PEO_{NH_2}) as shown in the inset CV (a) of Figure 3, which is attributed to the redox response of modified PT groups ($E^{\circ\prime}$(PT) = 0.54 V vs Ag|AgCl) attached to the GOx surface. A typical catalytic CV (b) was obtained after the addition of glucose, and the i_{cat} value increased with the number of modified PT-PEO_{NH_2}, showing that most of the modified PT groups are capable of mediating the ET reaction between the FAD center and the electrode. The manner in which i_{cat} increases depends on the molecular weight of modified PT-PEO_{NH_2}. Signs of leveling off appeared at the large number of PT groups for the i_{cat} of GOx hybrids with PT-PEO_{NH_2} of molecular weight 1000, 2000, and 3000. GOx hybrids with PT-PEO_{NH_2} of molecular weight 4200 and 8000 exhibited a maximum for i_{cat}, and the modification number at the i_{cat} maximum became smaller for the higher molecular weight of PT-PEO_{NH_2}.

GOx hybrids modified with PT-PEO_{NH_2} of molecular weight 3000 exhibited the largest i_{cat}, indicating the presence of the optimum PEO chain length in terms of the ET from $FADH_2$/FADH to PT^+ as in the case of lysine modification.[19,20] The i_{cat} maximum probably results from the simultaneous contribution of the following opposite factors: the increase in the probability of the access of PT groups toward FAD with increasing the PEO chain length and the overlap of the accessible area of PT groups on the GOx surface in the case of a too-long PEO chain. The leveling off or maximum of the plots in Figure 3, which was not observed for the previous lysine modification, suggests that the PT-PEO_{NH_2} groups are attached more densely to aspartic and glutamic acid residues that locate closer to the FAD center than lysine residues.

On the other hand, the GOx-(PT_{NH_2}) hybrid exhibits a large i_{cat} and an anomalously shaped plot of i_{cat} vs the number of PT groups per GOx molecule (Figure 3). Although the reason for this irregular behavior of the GOx-(PT_{NH_2}) hybrid is still not clear, the adsorption of the hybrid on the electrode is a possible reason, as indicated by Figure 4. Despite no difference in the pH dependence of the enzymatic activity, the pH dependence of i_{cat} is different between GOx-(PT_{NH_2}) hybrids with 5.5 and 7.5 PT_{NH_2}

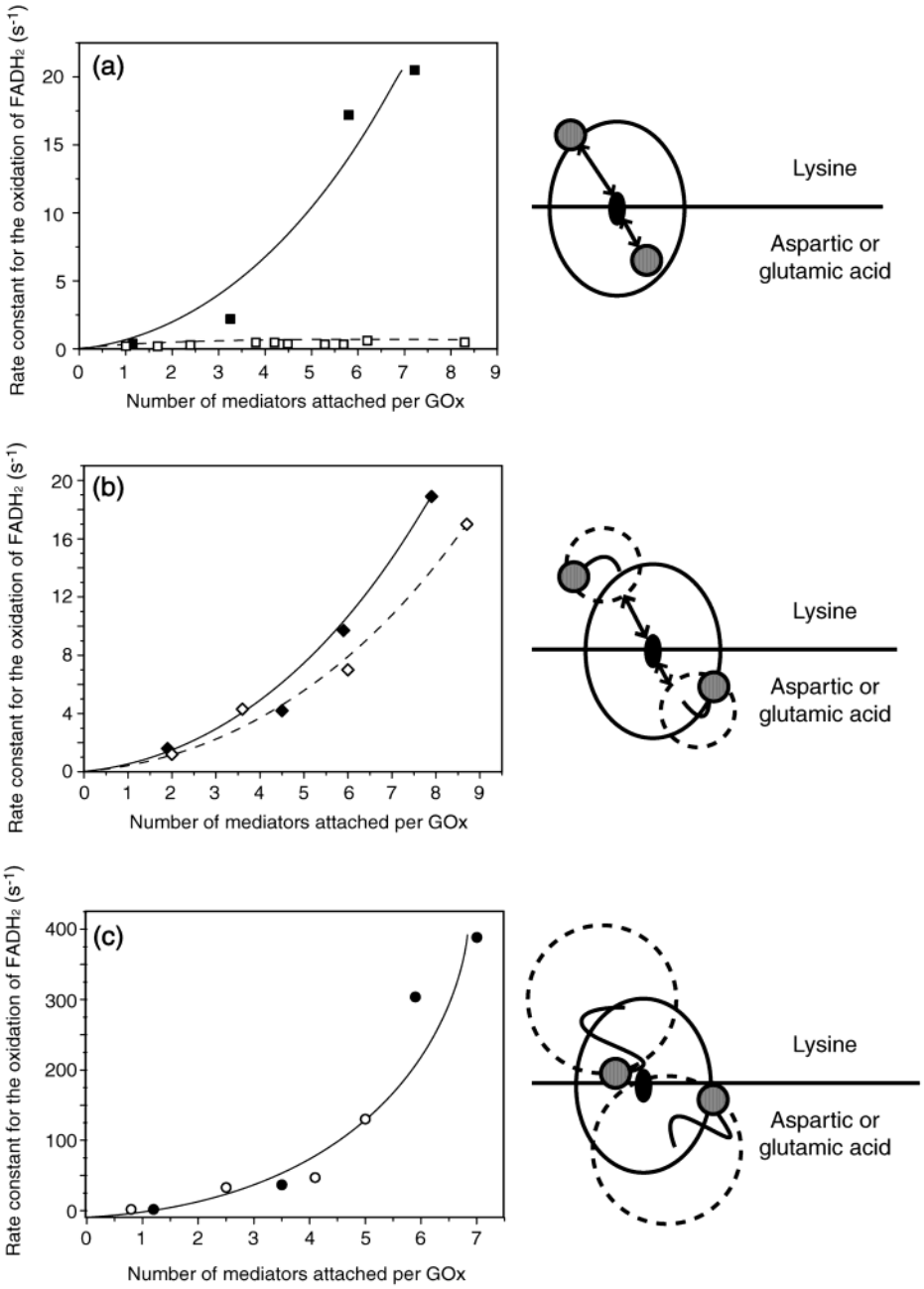

Figure 6. Relationship between the number of mediators attached per GOx molecule and the rate constants for the ET from $FADH_2$/FADH to PT^+ in GOx hybrids modified (a) without a spacer (PT_{NH_2}, ■; PT_{COOH}, □), (b) with a spacer of molecular weight 1000 (PT-PEO_{NH_2}, ◆; PT-PEO_{COOH}, ◇), and (c) with a spacer of molecular weight 3000 (PT-PEO_{NH_2}, ●; PT-PEO_{COOH}, ○). The ET distance (solid line arrow) and the accessible area of the PT mediator with PEO spacer (dotted circle) are schematically illustrated.

groups. With a greater number of PT_{NH_2} groups attached per GOx molecule, the maximum of i_{cat} shifted to a higher pH value. We would expect the protein to become more positively charged on modification, because negatively charged deprotonated acidic amino acid residues are exchanged for uncharged mediator groups, and this would have the enhancing effect of the isoelectric point (p*I*) for the GOx hybrids from 4.2 for native GOx. It is known that the solubility of the enzyme falls around p*I* and that the protein has a tendency to aggregate and adsorb onto surfaces due to increasing surface−surface interaction, as the net charge approaches zero.[32] Additionally, the modified PT_{NH_2} groups make GOx more hydrophobic due to the lack of a hydrophilic PEO chain. The contribution of the GOx-(PT_{NH_2}) hybrids adsorbed on the electrode to the i_{cat} presumably resulted in the anomalous behavior at pH 5.1 (Figure 3) and the pH dependence of i_{cat} in Figure 4.

To eliminate the adsorption effect, electrochemical measurements for GOx-(PT_{NH_2}) hybrids were performed at pH 3.8, which is far from the alkaline-shifted p*I*. As shown in Figure 5, a linear plot of i_{cat} vs the number of PT groups per GOx molecule was obtained at pH 3.8. Taking into account the fact that the enzymatic activity of the GOx hybrid at pH 3.8 is 67−75% of that at pH 5.1, it is surprising that the magnitude of i_{cat} for the GOx-(PT_{NH_2}) hybrid is comparable to that of the GOx hybrid having a PT-PEO_{NH_2} of molecular weight 1000. Despite the short alkyl spacer of PT_{NH_2}, the i_{cat} of the GOx-(PT_{NH_2}) hybrid increased with the number of PT groups, suggesting

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that most of the PT groups attached to acidic amino acid residues locate close to the FAD.

Effect of the Location of PT Modification and the Spacer Length on the Catalytic Reaction of GOx Hybrids. To analyze the effect of the modification site and the PEO spacer on the catalytic reaction of GOx hybrids, the average first-order rate constant for the oxidation of $FADH_2$/FADH by PT^+, k_{obs}, was calculated from the i_{cat} value using eq 1,[14]

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

where F is the Faraday constant, A is the electrode area, $D_{GOx\ hybrid}$ is the diffusion coefficient of the GOx hybrid, and $C_{GOx\ hybrid}$ is the concentration of the GOx hybrid. Under a glucose-saturated and diffusion-limited condition, i_{cat} is a function of the $D_{GOx\ hybrid}$ and k_{obs} values. There remains the possibility of the intermolecular mediation reaction to contribute to the i_{cat}. However, we confirmed the linear dependence of i_{cat} on the concentration of the GOx hybrid for the lysine-modified GOx hybrids in the concentration range up to 20 μmol dm^{-3}.[19] This indicates 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 present experimental condition ([GOx hybrid] = 9 μmol dm^{-3}). For the acidic amino acid residue-modified GOx hybrids, we have not checked how the i_{cat} value depends on $C_{GOx\ hybrid}$.

Figure 6 represents the dependence of k_{obs} for GOx hybrids on the modification site and the number and molecular weight of the attached mediators. The change in k_{obs} seems to show a quadratic dependence on the number of attached mediators. As shown in Figures 3 and 5, i_{cat} linearly increases with the number of attached PT groups, at least when the number is small. If eq 1 is valid for our systems, i_{cat} is proportional to the square root of k_{obs}, which results in the quadratic dependence of k_{obs} on the number of attached mediators. We think that this is the main reason for such a dependency.

When a PEO spacer is absent, as shown in Figure 6a, the GOx-(PT_{NH_2}) hybrid clearly possesses a larger k_{obs} than the GOx-(PT_{COOH}) hybrid at the same number of PT groups, although the pH condition of each is different. While all 15 lysine residues per GOx subunit are located more than 23 Å away from the FAD center, the distance from the FAD center is less than 16 Å for 10 of the 66 aspartic or glutamic acid residues.[29] It is highly probable that the ET distance from $FADH_2$/FADH to PT^+ is shorter for PT groups of the GOx-(PT_{NH_2}) hybrid than for those of the GOx-(PT_{COOH}) hybrid, because the distance between the modification site and the FAD center corresponds to the ET distance. In other words, the k_{obs} value strongly depends on the location of the PT modification.

In contrast, similar k_{obs} values were obtained for both GOx-(PT-PEO_{NH_2}) and GOx-(PT-PEO_{COOH}) hybrids in which PT groups are bonded to the GOx surface via a PEO of optimum length (molecular weight of 3000), as shown in Figure 6c. This suggests that the disadvantage in attaching PT groups to the sites far from the FAD center for the lysine modification can be compensated by using sufficiently long PEO spacers, which enable PT groups to access the broad area on the GOx surface. Therefore, the ET reaction depended only on the length of the PEO spacer, irrespective of the modification sites. The largest k_{obs} value, 388 s^{-1}, was obtained for the GOx-(PT-PEO_{NH_2}) hybrid with seven PT-PEO_{NH_2} groups of molecular weight 3000, which is greater than the so-far reported values for GOx with ferrocene-labeled long spacer chains attached to sugar or acidic amino acid residues on its surface.[12−14]

In the intermediate case between the two extreme cases described above, that is, in the case of the GOx hybrid having short PEO spacers (molecular weight of 1000), a slightly higher k_{obs} value was obtained for the acidic amino acid residue-modified GOx-(PT-PEO_{NH_2}) hybrids than for the lysine-modified GOx-(PT-PEO_{COOH}) hybrids. The k_{obs} value depends not only on the modification site but also on the PEO length (Figure 6b). The short PEO spacer cannot completely compensate the disadvantage in modifying amino acid residues far from the FAD center.

The differences in the ET distance (solid line arrow) and the accessible area of a PT mediator with a PEO spacer (dotted circle) are schematically illustrated in Figure 6 for the above three cases. For the hybrids without a PEO spacer, the ET distance is almost the same as the distance between the modified sites and the cofactor. On the other hand, the PEO spacer makes the ET distance shorter than the distance between the modified site and the FAD center due to the local motion of a hydrophilic and flexible PEO spacer.

The facile ET in the present GOx hybrids, especially when a PEO spacer length is optimized, may also be rationalized by taking into account multiple-electron transfer of PT groups at the electrode. Equation 1 is derived on the assumption that only one electron is transferred at the electrode in one oxidation event. We measured data that indicated that the oxidation of multiple PT groups occurred for the lysine-modified GOx hybrids (Figure 9 in ref 20). The oxidation current of attached PT groups linearly increased with their number, whereas it was independent of spacer PEO chain length.[20] The multiple-electron transfer would also contribute to the facile ET of the present GOx hybrids. However, this effect seems to be even for different modification sites and PEO spacer lengths in the GOx hybrids.

Conclusion

We have been electrochemically investigating the mediated ET reaction between the FAD center and the electrode by PT groups bonded to the GOx surface via a PEO spacer. The mediated ET occurs according to the wipe mechanism, in which the enzyme-bound mediators swing in and out of the active site. The present work demonstrated the effectiveness of PEO spacers, which are considerably longer than any spacer chains used in the previous works, in generating a fast mediated ET between the FAD center and the electrode for the modification of mediators at aspartic and glutamic acid residues as well as lysine residues. The comparison of the rate constant for the oxidation of $FADH_2$/FADH by PT^+ between the GOx hybrids in terms of the PT modification site and the length and structure of the spacer chain manifested that the local motion of long, hydrophilic, and flexible PEO spacers is able to compensate the disadvantage in attaching PT groups to sites far from the FAD center by making the ET distance much shorter than the distance between the modified site and the FAD center.

The modification of PEO on the enzyme surface was started for the purpose of dissolving enzymes into organic solvents in an active state.[33] We believe that our approaches using PT-PEO provide a new meaning to the generation of a fast self-mediated electrocatalytic reaction of oxidoreductase by the PEO modification on the enzyme surface.

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