

# Comparison of Catalytic Electrochemistry of Glucose Oxidase between Covalently Modified and Freely Diffusing Phenothiazine-Labeled Poly(ethylene oxide) Mediator Systems

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The enzyme catalytic reaction is electrochemically compared under a substrate-saturated and diffusion-limited condition between native glucose oxidase (GOx) mixed with phenothiazine-labeled poly(ethylene oxide) (PT-PEO) and GOx-(PT-PEO) hybrid with PT-PEO covalently bonded to lysine residues on the enzyme surface. Although the catalytic current ( $i_{\text{cat}}$ ) increases with the ratio of [PT-PEO]/[GOx] in both systems, the dependence of the  $i_{\text{cat}}$  on the molecular weight of PT-PEO is totally different. In the mixed systems, the  $i_{\text{cat}}$  decreases with increasing the molecular weight of PT-PEO, which reflects the decreases in the diffusion coefficient ( $D$ ) of PT-PEO and the intermolecular electron transfer (ET) rate from  $\text{FADH}_2/\text{FADH}$  to  $\text{PT}^+$ -PEO. In the hybrids, on the other hand, the  $i_{\text{cat}}$  reveals a maximum at the molecular weight of 3000 (PT-PEO3000), which originates from the dependence of the intramolecular ET rate from  $\text{FADH}_2/\text{FADH}$  to  $\text{PT}^+$  on the molecular weight of PT-PEO. The greater ET rate is enough to make up for the smaller  $D$  of PT groups attached on GOx in the hybrid with PT-PEO3000, yielding to the slightly larger  $i_{\text{cat}}$  than that of the corresponding mixed system. The active local motion of the long and hydrophilic PEO chain, the higher local concentration of PT groups due to their immobilization on the GOx surface, and the simultaneous oxidation of multiple attached PT groups at the electrode are possible reasons for the large ET rate constant of the hybrid systems.

## 1. Introduction

Freely diffusing redox mediators including ferrocene (Fc) derivatives,<sup>1–3</sup> quinones,<sup>4</sup> octacyanotungstate,<sup>5</sup> and ruthenium complexes<sup>6</sup> have been frequently used to directly reoxidize glucose-reduced natural glucose oxidase (GOx) (EC 1.1.3.4) at electrodes in an oxygen-free environment, because flavin adenine dinucleotide (FAD) is located sufficiently far from the outermost surface of GOx to be electrically inaccessible. These mediator systems enable reduction of the influence of oxygen in the atmosphere and the electrochemical analysis of the catalytic reaction at lower potentials where interfering species such as ascorbic acid do not react.<sup>7</sup> The application of those low-molecular-weight mediators for enzymatic sensor systems, however, often leads to deterioration and instability of the systems because of the leakage of the mediators.<sup>8</sup> Instead of low-molecular-weight mediators, polymer redox mediators were used to avoid the leakage, but their use resulted in the reduction of catalytic current due to slow charge propagation and loss of the electrochemical activity induced by conformational changes.

Direct, not diffusionally mediated, electron transfer (ET) between FAD and electrodes has been achieved by covalently immobilizing electron relays to enzymes.<sup>9–18</sup> A 50-fold larger value was obtained for the ET rate constant from  $\text{FADH}_2/\text{FADH}$  to  $\text{Fc}^+$  in the Fc-modified FAD-reconstituted GOx compared with that in the randomly-Fc-modified GOx.<sup>9,10</sup> The immobilization of redox mediators to the outer surface of GOx via long and flexible spacer chains also realizes an effective ET from FAD to electrodes.<sup>11,12,17,18</sup> Because of a more than 1 order of magnitude lower diffusion of modified mediators, however, the immobilization of the mediators to enzymes did

not always bring about more efficient mediation reaction than the corresponding freely diffusing systems.<sup>13</sup>

We have previously reported that a series of GOx hybrids (GOx-(PT-PEO)), which were synthesized by covalently bonding phenothiazine (PT) to lysine residues on the enzyme surface via long poly(ethylene oxide) (PEO) chains, are capable of a direct electrical communication with electrodes.<sup>17,18</sup> Both the number of PT groups and the length of the PEO chain are revealed to be essential for realizing the fast oxidation rate of  $\text{FADH}_2/\text{FADH}$  by  $\text{PT}^+$  in the hybrids. The fast ET from  $\text{FADH}_2/\text{FADH}$  to  $\text{PT}^+$  makes up for the smaller diffusion of mediators attached to the GOx hybrid and resulted in catalytic current comparable to that for the corresponding freely diffusing mediator systems under the optimum conditions. We here quantitatively compare catalytic electrochemistry between GOx-(PT-PEO) hybrids and native GOx mixed with freely diffusing PT-PEO to clarify features and advantages of the hybrid systems.

## 2. Experimental Section

**Materials.** GOx from *Aspergillus niger* was purchased from TOYOBO. Sodium acetate trihydrate was from Wako Pure Chemicals. D-Glucose was from Junsei Chemicals.  $\beta$ -D-Glucose was from Tokyo Kasei Kogyo. *o*-Dianisidine was from Sigma. Sodium acetate buffer (0.05 mol  $\text{dm}^{-3}$ ) was prepared by dissolving an appropriate amount of sodium acetate trihydrate into deionized water and its pH was adjusted to 5.1 by adding 0.1 mol  $\text{dm}^{-3}$  HCl.

Five kinds of PT-PEO with different molecular weights and GOx hybrids modified with those PT-PEO groups were synthesized according to the methods previously reported.<sup>18</sup> The average number of modified PT-PEO groups per hybrid molecule was determined from a UV–visible absorption

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spectrum of the hybrid<sup>18</sup> and was altered from 0.2 to 8.8 by controlling the molar ratio of PT-PEO to GOx in the reaction mixture.

**Determination of the Relative Enzyme Activity.** The activity of the GOx-(PT-PEO) hybrid relative to that of native GOx was measured using the peroxidase-*o*-dianisidine assay<sup>19</sup> in O<sub>2</sub>-saturated 0.05 mol dm<sup>-3</sup> sodium acetate buffer (pH 5.1) at 25 °C. Although the relative enzyme activity of a hybrid to native GOx significantly depends not only on the molecular weight of PT-PEO but also on the number of attached PT-PEO groups, it was confirmed that all GOx hybrids used in this work retained more than 50% relative activity.<sup>18</sup>

**Electrochemical Measurements.** We used a conventional three-electrode cell equipped with a glassy carbon working electrode (geometrical area: 0.071 cm<sup>2</sup>), 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-(PT-PEO) hybrid or native GOx mixed with PT-PEO was introduced to the cell and deaerated by N<sub>2</sub> purge for 20 min. Cyclic voltammograms (CVs) were recorded from 0.3 to 0.7 V at the scan rate of 10 mV s<sup>-1</sup> in the absence and presence of 0.05 mol dm<sup>-3</sup> glucose using a BAS-CV-50W electrochemical analyzer.

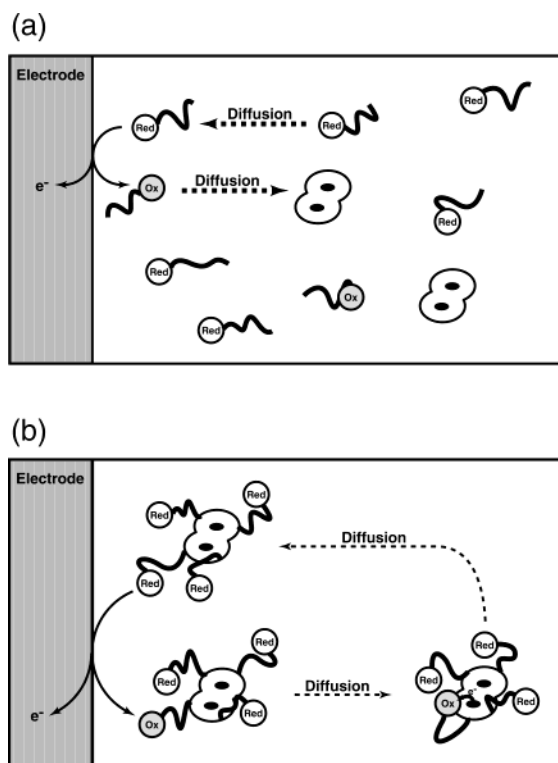
The diffusion coefficient (*D*) of PT-PEO was determined by chronoamperometry (CA) in 0.05 mol dm<sup>-3</sup> acetate buffer (pH 5.1) containing 1 mmol dm<sup>-3</sup> PT-PEO using the cell configuration, the electrodes, and the electrochemical analyzer that were the same as those in CV. The electrode potential was stepped from 0.3 to 0.7 V. Under Cottrell conditions, the *D* value of PT-PEO was determined using the Cottrell equation.

**Estimation of the Hydrodynamic Radius of PT-PEO.** For molecules diffusing in fluids, the *D* is correlated to the hydrodynamic radius (*r*):  $D = kT/6\pi\eta r$ , where *k* is the Boltzmann constant, *T* is the absolute temperature, and *η* is the viscosity. The hydrodynamic radius of PT-PEO was estimated from an amperometrically determined *D* value and the *η* value of water by using the above equation.

### 3. Results and Discussion

Figure 1 shows catalytic reaction schemes for native GOx mixed with PT-PEO (a) and GOx-(PT-PEO) hybrid (b). FAD is reduced to FADH<sub>2</sub> by glucose, and then FADH<sub>2</sub> is reoxidized to FAD through two steps of one-electron oxidation by electrochemically oxidized PT (PT<sup>+</sup>) groups. The diffusion-limited catalytic current (*i*<sub>cat</sub>) in the mixed systems was generated by the electrochemical oxidation of freely diffusing PT to PT<sup>+</sup> at the electrode, followed by bimolecular reaction between PT<sup>+</sup> and FADH<sub>2</sub>/FADH in the bulk solution. On the other hand, the *i*<sub>cat</sub> of the hybrids was generated by the electrochemical oxidation of PT attached to the surface of the GOx hybrid at the electrode, followed by the intramolecular reduction of PT<sup>+</sup> by FADH<sub>2</sub>/FADH of GOx at the enzyme surface. The main differences between the two systems are the kinetic order of the ET reaction from FADH<sub>2</sub>/FADH to PT<sup>+</sup> and the diffusion rate of PT moieties toward the electrode. The *D* value of the PT groups is more than 1 order of magnitude smaller in the hybrid systems because of the PT immobilization on the surface of high-molecular-weight GOx.

Figure 2 represents CVs of native GOx/PT-PEO with the molecular weight of 3000 (PT-PEO3000) mixed system ([PT]/[GOx] = 3.8) (1) and GOx hybrid with 3.8 groups of PT-PEO3000 (GOx-(PT-PEO3000)<sub>3.8</sub>) (2) in the absence (a) and



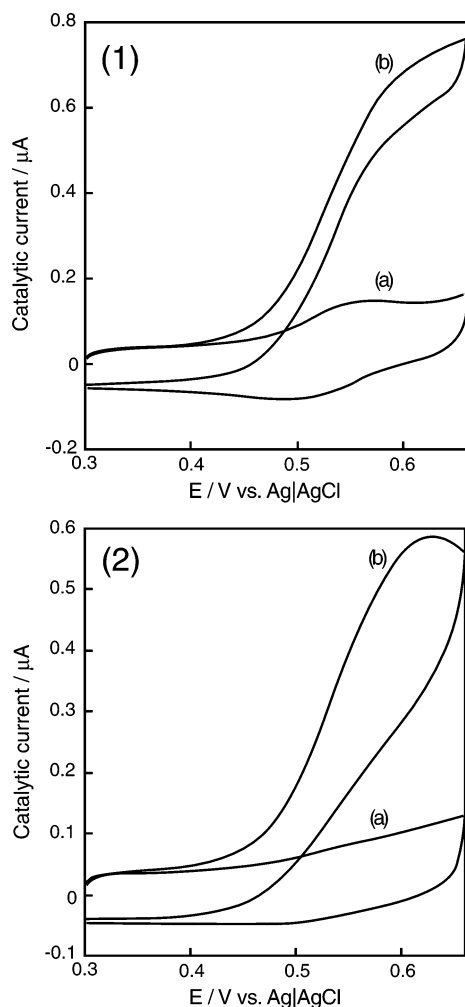
**Figure 1.** Enzymatic reaction schemes for GOx hybrids and native GOx mixed with freely diffusing PT-PEO.

presence (b) of 0.05 mol dm<sup>-3</sup> glucose. Although the mixed system exhibited a pair of small peaks around 0.54 V, which is attributed to the redox of a freely diffusing PT-PEO, no redox peak appeared for the GOx hybrid in the absence of glucose. This difference reflects a smaller *D* value of PT groups attached to the GOx hybrid surface compared with that of the freely diffusing PT-PEO. The retention of the electrochemical activity of the PT groups attached to the hybrid surface was confirmed by differential pulse voltammetry.<sup>18</sup> After the addition of glucose, the oxidation current increased at a potential more positive than 0.54 V for both systems, indicating the enzymatic reduction of PT<sup>+</sup>. The *i*<sub>cat</sub> increased with the glucose concentration and leveled off to a constant value above 10 mmol dm<sup>-3</sup> glucose in the two systems (data not shown).

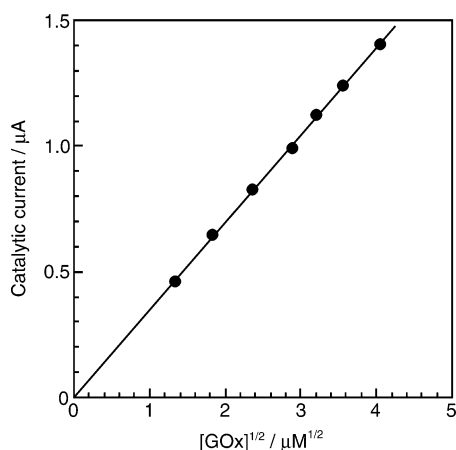
Under a glucose-saturated and diffusion-limited condition, eq 1 was proposed to describe the *i*<sub>cat</sub> of the mixed system:<sup>13,20</sup>

$$i_{\text{cat}} = FA(2D_{\text{PT-PEO}}k_{\text{cat(2nd)}}C_{\text{GOx}})^{1/2}C_{\text{PT-PEO}} \quad (1)$$

where *F* is the Faraday constant, *A* is the electrode area, *D*<sub>PT-PEO</sub> is the diffusion coefficient of PT-PEO, *k*<sub>cat(2nd)</sub> is the second-order rate constant of the ET from FADH<sub>2</sub>/FADH to PT<sup>+</sup>, *C*<sub>GOx</sub> is the concentration of native GOx, and *C*<sub>PT-PEO</sub> is the PT-PEO concentration. Figure 3 shows the relationship between the *i*<sub>cat</sub> measured at 0.62 V and the *C*<sub>GOx</sub> for the mixed systems. The *i*<sub>cat</sub> is proportional to the square root of the *C*<sub>GOx</sub> and also linearly depends on the *C*<sub>PT-PEO</sub> as shown in Figure 4, supporting the applicability of eq 1 to the mixed systems in the present work. The slope of the *i*<sub>cat</sub>–*C*<sub>PT-PEO</sub> plots (Figure 4) became smaller with increasing the molecular weight of PT-PEO. From these slopes, the *k*<sub>cat(2nd)</sub> values were estimated using eq 1 and chronoamperometrically determined *D*<sub>PT-PEO</sub> values (Table 1). Not only *D*<sub>PT-PEO</sub> but also *k*<sub>cat (second)</sub> decrease as the molecular weight of PT-PEO increases, indicating that the probability of encountering the FAD center is lower for a PT group of higher-



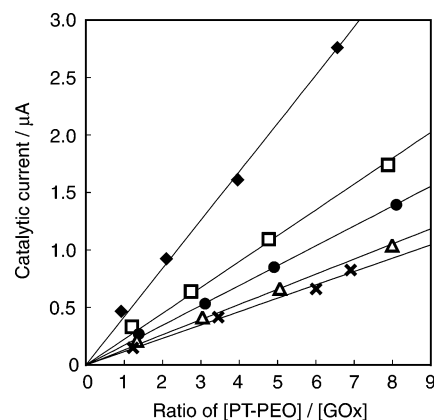
**Figure 2.** Cyclic voltammograms of PT-PEO3000 ( $33 \mu\text{mol dm}^{-3}$ )/native GOx ( $8.7 \mu\text{mol dm}^{-3}$ ) mixed system (1) and GOx-(PT-PEO3000)<sub>3.8</sub> hybrid ( $8.7 \mu\text{mol dm}^{-3}$ ) (2) measured at a glassy carbon electrode at a scan rate of  $10 \text{ mV s}^{-1}$  in  $0.05 \text{ mol dm}^{-3}$  sodium acetate buffer (pH 5.1) with (b) and without (a) of  $0.05 \text{ mol dm}^{-3}$  glucose.



**Figure 3.** Catalytic current of native GOx mixed with PT-PEO3000 as a function of the enzyme concentration measured at  $0.62 \text{ V}$  at a glassy carbon electrode in  $0.05 \text{ mol dm}^{-3}$  sodium acetate buffer (pH 5.1) containing  $0.05 \text{ mol dm}^{-3}$  glucose. The concentration of PT-PEO3000 was  $50 \mu\text{mol dm}^{-3}$ .

molecular-weight PT-PEO because of the steric hindrance of a coiled long PEO chain.

The  $i_{\text{cat}}$  of the GOx hybrids, on the other hand, is a function of the diffusion coefficient of the hybrid ( $D_{\text{GOx-hybrid}}$ ), the first-order rate constant of the ET from  $\text{FADH}_2/\text{FADH}$  to  $\text{PT}^+$

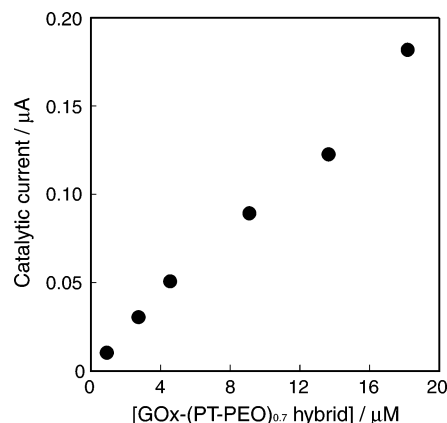


**Figure 4.** Catalytic current of native GOx mixed with PT-PEO as a function of the concentration and the molecular weight of PT-PEO measured at  $0.62 \text{ V}$  at a glassy carbon electrode in  $0.05 \text{ mol dm}^{-3}$  sodium acetate buffer (pH 5.1) containing  $0.05 \text{ mol dm}^{-3}$  glucose. The molecular weights of PT-PEO are 1000 ( $\blacklozenge$ ), 2000 ( $\square$ ), 3000 ( $\bullet$ ), 4200 ( $\triangle$ ), and 8000 ( $\times$ ). The concentration of GOx was  $9 \mu\text{mol dm}^{-3}$ .

**TABLE 1: Diffusion Coefficients ( $D_{\text{med}}$ ) and Hydrodynamic Radius ( $r$ ) of PT-PEO and Rate Constants ( $k_{\text{cat(2nd)}}$ ) of Electron Transfer from  $\text{FADH}_2/\text{FADH}$  to  $\text{PT}^+$ -PEO in GOx/PT-PEO Mixed Systems**

| mediator   | $D_{\text{med}}$<br>( $\times 10^6 \text{ cm}^2 \text{ s}^{-1}$ ) <sup>a</sup> | $r$<br>( $\text{\AA}$ ) <sup>b</sup> | $k_{\text{cat(2nd)}}$<br>( $\times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ ) <sup>c</sup> |
|------------|--|--------------------------------------|--|
| PT-PEO1000 | 2.4  | 10                                   | 5.2  |
| PT-PEO2000 | 1.7  | 14                                   | 1.9  |
| PT-PEO3000 | 1.3  | 18                                   | 1.5  |
| PT-PEO4200 | 0.93   | 26                                   | 1.3  |
| PT-PEO8000 | 0.71   | 34                                   | 1.2  |

<sup>a</sup> Determined from chronoamperometric measurements. <sup>b</sup> Calculated using the Stokes–Einstein equation. <sup>c</sup> Calculated using eq 1.

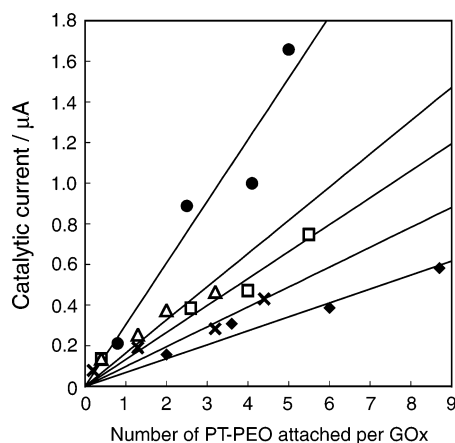


**Figure 5.** Catalytic current of  $\text{GOx}-(\text{PT-PEO8000})_{0.7}$  as a function of the hybrid concentration measured at  $0.62 \text{ V}$  at a glassy carbon electrode in  $0.05 \text{ mol dm}^{-3}$  sodium acetate buffer (pH 5.1) containing  $0.05 \text{ mol dm}^{-3}$  glucose.

( $k_{\text{cat(1st)}}$ ), and the hybrid concentration ( $C_{\text{GOx-hybrid}}$ ) as described in eq 2:<sup>13</sup>

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

Actually, the  $i_{\text{cat}}$  is in proportion to the  $C_{\text{GOx-hybrid}}$  as shown in Figure 5, and the intermolecular ET between  $\text{FADH}_2/\text{FADH}$  and  $\text{PT}^+$  on another GOx surface can be neglected under the present experimental condition ( $C_{\text{GOx-hybrid}} = 9 \mu\text{mol dm}^{-3}$ ). Figure 6 represents the  $i_{\text{cat}}$  of several GOx hybrids measured at  $0.62 \text{ V}$  under a substrate-saturated condition ( $0.05 \text{ mol dm}^{-3}$  glucose) as a function of the number of modified PT-PEO



**Figure 6.** Catalytic current of the GOx hybrids as a function of the number of modified PT-PEO per GOx hybrid molecule and the molecular weight of PT-PEO measured at 0.62 V at a glassy carbon electrode in 0.05 mol dm<sup>-3</sup> sodium acetate buffer (pH 5.1) containing 0.05 mol dm<sup>-3</sup> glucose. The molecular weights of PT-PEO are 1000 (◆), 2000 (□), 3000 (●), 4200 (△), and 8000 (×). The concentration of GOx hybrids was 9 μmol dm<sup>-3</sup>.

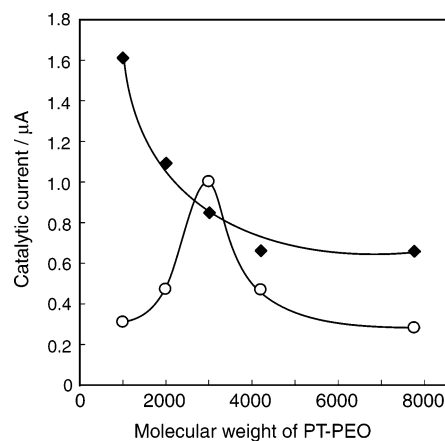
**TABLE 2: Comparison of Rate Constants of Electron Transfer from FADH<sub>2</sub>/FADH to PT<sup>+</sup> between GOx/PT-PEO Mixed Systems ( $k_{\text{cat}(2\text{nd})}[\text{PT-PEO}]$ ) and GOx Hybrids ( $k_{\text{cat}(1\text{st})}$ )**

| mediator   | $k_{\text{cat}(2\text{nd})}[\text{PT-PEO}]$<br>(s <sup>-1</sup> ) <sup>a</sup> | $k_{\text{cat}(1\text{st})}$<br>(s <sup>-1</sup> ) <sup>b</sup> | [PT-PEO]/[GOx] |
|------------|--|---|----------------|
| PT-PEO1000 | 17   | 17  | 3.6            |
| PT-PEO2000 | 4.4  | 19  | 4.0            |
| PT-PEO3000 | 3.4  | 47  | 4.1            |
| PT-PEO4200 | 3.7  | 12  | 3.2            |
| PT-PEO8000 | 3.5  | 7.5   | 3.2            |

<sup>a</sup> The  $k_{\text{cat}(2\text{nd})}$  was converted to a pseudo-first-order rate constant,  $k_{\text{cat}(2\text{nd})}[\text{PT-PEO}]$ . The concentration of GOx was 9 μmol dm<sup>-3</sup>. The [PT-PEO]/[GOx] ratio of the mixed systems was three. <sup>b</sup> The  $k_{\text{cat}(1\text{st})}$  values were corrected for the relative enzymatic activity. The concentration of GOx hybrid was 9 μmol dm<sup>-3</sup>.

groups. For all GOx hybrids, the  $i_{\text{cat}}$  increased with the number of attached PT groups, demonstrating that most of the PT groups bonded to the GOx surface can act as mediators for the ET reaction from the FAD center to the electrode. A noteworthy difference from the mixed systems is that a maximum in the slope of the  $i_{\text{cat}}$  vs number of attached PT groups plots appeared for the GOx-(PT-PEO3000) hybrids, suggesting the existence of an optimum PEO chain length for the intramolecular ET from FADH<sub>2</sub>/FADH to PT<sup>+</sup> in the hybrids. Assuming that the  $D$  value of native GOx ( $4.1 \times 10^{-7}$  cm<sup>2</sup> s<sup>-1</sup> at 25 °C)<sup>6</sup> is applicable to all of the GOx hybrids, the  $k_{\text{cat}(1\text{st})}$  was calculated using eq 2. To compare the ET rate constants from FADH<sub>2</sub>/FADH to PT<sup>+</sup> between the two systems, the second-order rate constant,  $k_{\text{cat}(2\text{nd})}$ , of the mixed systems was converted to pseudo-first-order rate constant,  $k_{\text{cat}(2\text{nd})}[\text{PT-PEO}]$  (Table 2). It should be emphasized that the  $k_{\text{cat}(1\text{st})}$  of GOx-(PT-PEO) hybrid is one to twelve times greater than the  $k_{\text{cat}(2\text{nd})}[\text{PT-PEO}]$  under the same ratio of [PT]/[GOx]. The higher local concentration of PT groups due to the immobilization on the GOx surface and the active local motion of the long, flexible, and hydrophilic PEO chain<sup>21–24</sup> are responsible for the larger  $k_{\text{cat}}$  values of the hybrids.

Figure 7 compares the  $i_{\text{cat}}$  between the mixed and hybrid systems as a function of the molecular weight of PT-PEO. With increasing the molecular weight of PT-PEO, the  $i_{\text{cat}}$  of the mixed system decreases steeply and levels off at the molecular weight of PT-PEO more than 4200. The reduction of both  $D_{\text{PT-PEO}}$  and  $k_{\text{cat}(2\text{nd})}$  values with the molecular weight of PT-PEO is responsible for the  $i_{\text{cat}}$  decrease, although the participation of

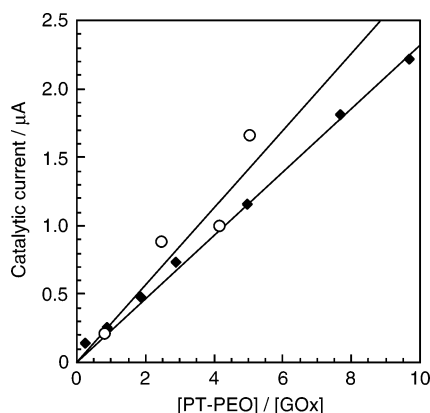


**Figure 7.** Dependence of the catalytic current on the molecular weight of PT-PEO measured for native GOx mixed with PT-PEO (◆) and GOx-(PT-PEO) (○). The catalytic current of the mixed systems was measured at the [PT-PEO]/[GOx] ratio of three. The number of modified PT-PEO per GOx monomer is 3.6 for 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 concentration of GOx hybrids was 9 μmol dm<sup>-3</sup>.

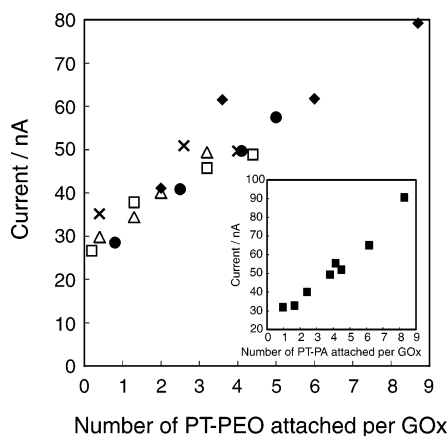
the latter is more pronounced (Table 1). In contrast, the  $i_{\text{cat}}$  of the GOx hybrid exhibits a maximum around the molecular weight of 3000. Because the  $D_{\text{GOx-hybrid}}$  values of all of the hybrids are almost the same as mentioned below, the dependence of the  $i_{\text{cat}}$  on the PT-PEO molecular weight reflects the change in the  $k_{\text{cat}}$  of the hybrids. It is interesting that the dependence of the  $k_{\text{cat}}$  on the molecular weight of PT-PEO is totally different between the hybrid and the mixed systems.

In the mixed system, PEO dissolved in a buffer solution is in a coil shape and the hydrophobic PT group seems to be wrapped with the hydrophilic PEO chain, leading to the decrease in the  $k_{\text{cat}}$  with increasing the molecular weight of PT-PEO. In the hybrid, on the other hand, PEO chains are attached by one end to the GOx surface. The structure and molecular motion of PEO chains tethered on a substrate surface depend on its surface density and molecular weight.<sup>22,23</sup> With increasing its surface density and/or molecular weight, the conformation of the tethered PEO chains alters from isolated mushrooms to dense polymer brushes, in which PEO chains are extended much further than their natural dimensions by the need to escape their neighbors. Anne et al. observed a CV response of the terminal ferrocene (Fc) group of two asymmetrical Fc-PEO chains with markedly different lengths that are attached to the surface of glassy carbon electrodes.<sup>23</sup> Despite the difference in the PEO chain conformation (mushroom vs polymer brush), Fc units of the two polymers gave similar peak currents in CV at high potential scan rates, indicating that the flexibility of terminally attached PEO-Fc chains is almost identical. The density of PT-PEO chains attached to the GOx hybrid surface is not clear because of the uncertainty of the modification site of each PT-PEO. Until the diameter of the PEO coil exceeds the distance separating terminally attached PEO chains (isolated mushroom), however, the accessible area of PT groups becomes large, and the probability of the PT-mediated ET between the FAD center and the electrode will increase with increasing the molecular weight and modification number of PT-PEO. In the hybrids with a high modification number and/or higher molecular weight of PT-PEO (weakly overlapping mushroom or polymer brush), although the spin-label ESR<sup>24</sup> and electrochemical<sup>23</sup> measurements demonstrated that the molecular mobility of the end of the surface confined PEO chain is maintained, the overlap of the excluded volume of PEO chain reduces the accessible area





**Figure 8.** Comparison of the catalytic current vs [PT-PEO]/[GOx] ratio relationship between GOx-(PT-PEO3000) hybrid (○) and native GOx mixed with PT-PEO3000 (◆) measured at 0.62 V at a glassy carbon electrode in 0.05 mol dm<sup>-3</sup> sodium acetate buffer (pH 5.1) containing 0.05 mol dm<sup>-3</sup> glucose. The concentration of native GOx or GOx hybrid was 9 μmol dm<sup>-3</sup>.



**Figure 9.** Relationship between the number of modified PT-PEO groups and the oxidation current at 0.62 V of GOx hybrids (9 μmol dm<sup>-3</sup>) modified with PT-PEO of molecular weight 1000 (◆), 2000 (□), 3000 (●), 4200 (△), and 8000 (×). The oxidation current was measured by cyclic voltammetry at a glassy carbon electrode in 0.05 mol dm<sup>-3</sup> sodium acetate buffer (pH 5.1) without glucose. The inset shows a similar relationship for GOx-(PT-PA) hybrids.

of PT group on the GOx surface. This yields the maximum for the  $k_{\text{cat}(1\text{st})}$  value of the hybrids.<sup>17,18</sup> The  $k_{\text{cat}(1\text{st})}$  would also be affected by other factors including the relative positions of mediators, entropic and enthalpic factors associated with chain mobility and penetration, and interactions of PT-PEO with glycan and other PT-PEO chains.

The GOx-(PT-PEO3000) hybrid exhibited a larger  $i_{\text{cat}}$  than native GOx mixed with freely diffusing PT-PEO3000 at all [PT]/[GOx] ratios up to 5, as shown in Figure 8. Although the  $D$  value of the GOx-(PT-PEO3000) hybrid is ca. one-third of that of PT-PEO3000, the  $k_{\text{cat}}$  is 10 times greater in the hybrid than in the mixed system. These results indicate that the fast reoxidation of FADH/FADH<sub>2</sub> by PT<sup>+</sup> in the hybrids demonstrated by relatively greater  $k_{\text{cat}}$  values is enough to compensate for the smaller diffusion of PT mediators attached to GOx.

Figure 9 shows the oxidative CV current at 0.62 V for GOx hybrids under a glucose-free condition, which is the sum of the oxidation current of attached PT groups and the double-layer charging current, as a function of the number of modified PT-PEO groups. It should be mentioned that the oxidation current linearly increased with the number of attached PT groups, and a single plot of current vs number of modified PT-PEO was obtained for all GOx hybrids despite the difference in the

molecular weight of modified PT-PEO. This indicates that all PT groups attached on the GOx surface are electrochemically active and the diffusion coefficient of hybrids is almost constant irrespective of both the number and molecular weight of modified PT-PEO. This fact supports the validity of employing the same  $D$  value for all hybrids in the estimation of ET rate constants. From the slope of the plot, the diffusion coefficient of PT-PEO, i.e.,  $D_{\text{GOx-hybrid}}$ , was estimated to be  $1.3 \times 10^{-7}$  cm<sup>2</sup> s<sup>-1</sup> at 25 °C. It seems reasonable that the  $D_{\text{GOx-hybrid}}$  is slightly smaller than the  $D$  values reported for native GOx and Fc-modified GOx via shorter spacer chains.<sup>6</sup> In addition, the GOx-(PT-PA) hybrids,<sup>18</sup> in which PT groups are immobilized without PEO spacer, also revealed a linear current vs number of the modified PT plot as shown in the inset of Figure 9, and a slightly larger  $D_{\text{GOx-hybrid}}$  value ( $2.2 \times 10^{-7}$  cm<sup>2</sup> s<sup>-1</sup> at 25 °C) was calculated from the slope of the plot. It was predicted that the ET to or from electrodes of polymers containing a number of equivalent, noninteracting redox centers will yield a voltammetric wave with a shape matching that of the corresponding molecule with a single electroactive center but with a magnitude determined by the total number of redox centers present.<sup>25</sup> The predicted behavior has been observed for several synthetic polymers.<sup>25</sup> It is interesting that the noninteracting multiple type ET was observed for the PT groups immobilized on the surface of high molecular-weight enzymes such as GOx, although the surface PT groups look like nonequivalent. Adsorption of GOx hybrids on the electrode did not appear, because no catalytic current was detected for the electrode which was transferred into a buffer solution with only 0.05 M glucose after a series of CV measurements in a buffer containing both hybrid and glucose. The fact that the multiple ET was observed also for the GOx-(PT-PA) hybrids suggests that the multiple ET of the hybrids occurs through the multiple collisions of GOx hybrid with the electrode rather than the intramolecular electron exchange reactions between the PT relays. This multiple ET might be one of the possible reasons for the increase in  $k_{\text{cat}(1\text{st})}$  with the number of PT-PEO, because eq 2 was derived by assuming that only one electron is transferred at the electrode in one oxidation event, and under this situation, the oxidation current in Figure 9 should be constant or decrease with the number of PT-PEO for all the GOx hybrids.

#### 4. Conclusion

The molecular weight of PT-PEO differently affects the  $i_{\text{cat}}$  of GOx in the mixed and hybrid systems. For the freely diffusing PT-PEO in the mixed system, the  $i_{\text{cat}}$  decreases with increasing the molecular weight of PT-PEO, which reflects the decreases in the  $D$  of PT-PEO and the intermolecular ET rate from FADH<sub>2</sub>/FADH to PT<sup>+</sup>-PEO. For the covalently attached PT-PEO on the GOx surface, the  $i_{\text{cat}}$  reveals a maximum for PT-PEO with the molecular weight of 3000 (PT-PEO3000), which originates from the dependence of the intramolecular ET rate from FADH<sub>2</sub>/FADH to PT<sup>+</sup> on the molecular weight of PT-PEO. It seems that the structure and molecular motion of the PEO chains affect the ET rate from FADH<sub>2</sub>/FADH to PT<sup>+</sup>. The active local motion of the long and hydrophilic PEO chain covalently attached on the GOx surface contributes significantly to the large ET rate constant of the hybrid systems, which is enough to make up for the smaller  $D$  of PT groups attached on the GOx surface and brought about a slightly larger  $i_{\text{cat}}$  of the hybrid modified with PT-PEO3000 than that of the corresponding mixed system. The present and our previous results<sup>26</sup> suggest that the disadvantages in the mediator immobilization to the enzyme surface can be compensated by optimizing the modi-

fication parameters including the molecular weight, the number, and the location of attached PT-PEO groups. The use of optimized hybrids could be a possible method for fabricating all-in-one sensor systems, in which both an enzyme and mediators are immobilized on the electrode surface.

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