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Electron Transfer Reactions of Glucose Oxidase at Au(111) Electrodes Modified with Phenothiazine Derivatives

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The catalytic reaction of glucose oxidase (GOx) mediated by 3-(10-phenothiazyl)propionic acid (PT-PA) and phenothiazine-labeled poly(ethylene oxide) (PT-PEO1000) that are covalently bonded to Au(111) electrodes has been investigated. The PT-PA and PT-PEO1000 are reacted with 2-aminoethanethiol (AET), followed by the formation of a self-assembled monolayer (SAM) onto the Au surface. The PT group immobilized on the SAM of AET acts as an effective mediator for the electron transfer (ET) between the electrode and the FAD center of freely diffusing GOx in solution. The ET rate constant estimated from the catalytic current using a newly derived equation is larger by 1 order of magnitude for the PT-PA-modified system (1.1×10^5 dm^3 mol^{-1} s^{-1}) than for the PT-PEO1000 system (1.4×10^4 dm^3 mol^{-1} s^{-1}). The order of the magnitude of the ET rate constant clearly contrasts with the GOx hybrid systems that we previously investigated (*Anal. Chem.* 2003, *75*, 910−917), in which the presence of the PEO spacer enhances the ET reaction rate. The reduction in the apparent PT concentration at the electrode interface due to the high mobility of the PEO chain, leading to low efficiency in the formation of an enzyme-mediator complex, is a possible reason for the lower mediation ability of PT-PEO1000 than that of PT-PA for the ET between the FAD group and PT$^+$ immobilized on the electrode. Inhibition of the penetration of GOx molecules into the monolayer and of the accessibility of some part of PT groups to GOx molecules could also be reasons for the lower mediation ability of PT-PEO1000 thickly modified on the electrode.

Direct electrical communications between the redox center of enzymes and electrodes are generally inhibited by an electronically insulating protein shell surrounding the electroactive site of the enzymes. To achieve efficient electron transfer (ET) between the redox enzymes and electrodes, the use of freely diffusing electron mediators,[1−6] the covalent attachment of redox relays to the surface[7−17] or redox center of the enzyme,[18−22] and the encapsulation of biocatalytic systems in a redox polymer[23,24] have been proposed. We realized the rapid ET from $FADH_2$/FADH to electrodes by attaching phenothiazine-labeled-poly(ethylene oxide) (PT-PEO) to the lysine[13,15,16] or glutamic and aspartic acid[14,17] residues on the surface of glucose oxidase (GOx). The long, hydrophilic, and flexible PEO spacers enable the PT mediators to approach close to the cofactor. In particular, PT-PEO with the molecular weight of 3000 provided a 2000-times higher ET rate, as compared with the short-chain PT mediators.[13−17] Those results suggest that the length of a spacer chain decisively affects the mediation of ET according to the wipe-mechanism with the

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enzyme-bound mediators swinging in and out of the active site of the enzyme.[25]

The enzyme electrodes in which enzymes and mediators are co-immobilized at a monolayer or submonolayer level over the transducer have been examined.[26−31] Those systems have advantages over the enzyme electrodes consisting of enzymes and mediators incorporated in a thick polymer film in terms of the control of the molecular architecture for the recognition interface and of the efficiency of the substrate and mediator supply. Binding through ω-functionalized self-assembled monolayers (SAMs),[28,29] avidin−biotin binding,[31−33] and antigen−antibody binding[31,34,35] has been generally used to fabricate enzyme monolayer-modified electrodes. Anicet et al. employed the latter two techniques to construct enzyme monolayer integrated systems, in which GOx and ferrocene (Fc) molecules are bound via PEO linear chains.[31] Due to the high mobility of PEO spacers, it was proved that the transport of Fc heads to the enzyme prosthetic group is not the rate-determining step for ET. We are interested in the mediation ability and the effect of spacer chain length in the mediation reaction of GOx when the PT-PEO mediators are immobilized on the electrode surface.

Electroactive groups immobilized on the electrode surface via SAMs undergo electron exchange with redox agents in solution. The properties of surface-confined groups determine the exchange rate, the chemical specificity, and the direction of current flow.[36−38] Investigation of the redox reactions of freely diffusing enzymes at redox-active monolayer-modified electrodes is an important step toward the realization of enzyme-monolayer electrodes in which both enzymes and mediators are bound to electrodes. However, most of the redox solutes examined in the previous studies are restricted to low-molecular-weight species, and only a few preliminary reports for the enzyme appeared.[39,40]

In this study, we prepared two kinds of mediator-modified electrodes by immobilizing 2-(10-phenothiazyl)propionic acid (PT-PA)[13,15] or PT-PEO with the molecular weight of 1000 (PT-PEO1000)[13,15,16] through amide linkage to a SAM of 2-aminoethanethiol (AET) on gold electrodes. The ET reactions between the redox center of GOx in a buffer solution and the surface-immobilized PT groups were investigated by cyclic voltammetry. The obtained data were analyzed by an equation derived for the steady-state catalytic current. The difference in the ET rate between the PT-PA- and PT-PEO1000-modified electrodes is discussed from the viewpoint of the spacer length.

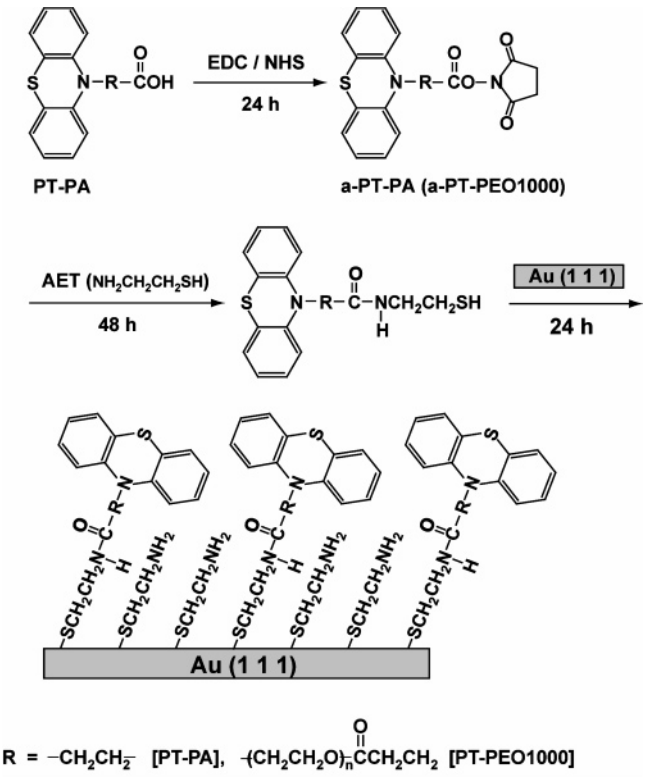

Figure 1. Schematic representation for the preparation procedure of thiol-terminated PT mediator-immobilized Au electrodes.

EXPERIMENTAL SECTION

Materials. GOx from *Aspergillus niger* was purchased from Toyobo, 2-aminoethanethiol hydrochloride (AET) and D-glucose were from Tokyo Kasei, *N*-hydroxysuccinimide (NHS) was from Fluka, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was from Junsei Chemicals, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) was from Dojindo Laboratories, and sodium acetate trihydrate was from Wako Pure Chemicals. All of the reagents were used without further purification. Water was distilled and purified with a Milli-Q system (Millipore Co.). PT-PA and PT-PEO1000 activated by EDC and NHS (a-PT-PA and a-PT-PEO1000), whose chemical structures are shown in Figure 1, were synthesized according to the previous paper.[15]

Sodium acetate buffer (0.05 mol dm^{-3}, pH 5.1) was prepared as previously reported.[15] HEPES buffer (0.01 mol dm^{-3}, pH 7.0) was prepared by dissolving an appropriate amount of HEPES powder into deionized water, and then its pH was adjusted to 7.0 by adding 0.1 mol dm^{-3} NaOH.

Electrochemical Measurements. Cyclic voltammetric (CV) measurements were carried out on a BAS CV-50W electrochemical analyzer with a conventional three-electrode system in which a mediator-immobilized Au electrode (geometrical area, 0.50 cm^2), a Ag|AgCl|saturated KCl electrode, and a Pt wire were used as the working, reference, and auxiliary electrodes, respectively. All

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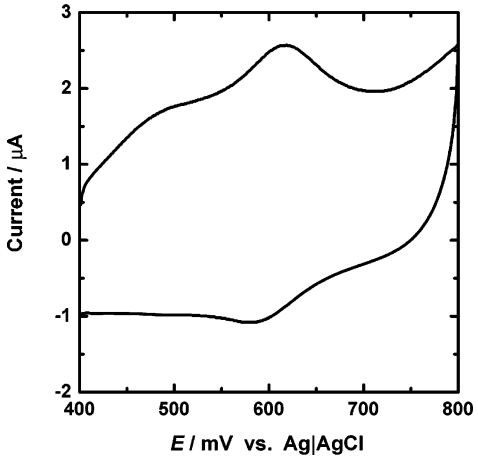

Figure 2. Cyclic voltammogram of AET and thiol-terminated PT-PA co-immobilized Au electrode in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) at a scan rate of 10 mV s^{-1}.

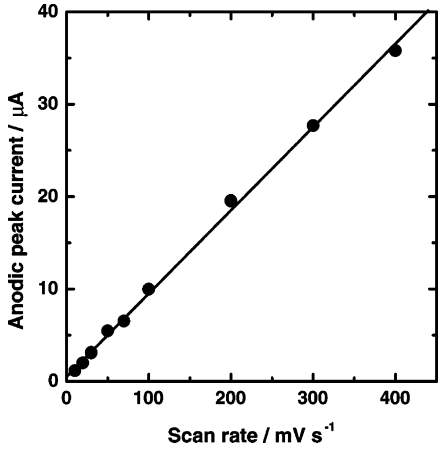

Figure 3. Scan rate dependence of the anodic peak current for AET and thiol-terminated PT-PA co-immobilized Au electrode.

the measurements were performed at room temperature under Ar atmosphere.

Preparation of Mediator-Immobilized Au Electrodes. Gold substrates were prepared by vapor deposition of gold (99.99% purity) onto freshly cleaved mica.[41] Figure 1 represents the procedure for preparing mediator-modified Au electrodes. The carboxylic groups (5 mmol dm^{-3}) of PT-PA were activated by EDC (17 mmol dm^{-3}) and NHS (17 mmol dm^{-3}) for 24 h in a HEPES buffer/DMF mixed solution (1/1 v/v at room temperature (rt)) at pH 7.0. To this solution, AET (5 mmol dm^{-3}) was added, and the condensation reaction was allowed to proceed for 48 h at ambient temperature, yielding a mixture of thiol-terminated PT-PA and unreacted AET molecules. Similarly, a mixture of thiol-terminated PT-PEO1000 and AET molecules was obtained from the condensation reaction of a-PT-PEO1000 (5 mmol dm^{-3}) with AET (5 mmol dm^{-3}) for 48 h. Immersion of a gold substrate in those mixed thiol solutions resulted in mixed SAMs of the thiol-terminated PT-PA or PT-PEO1000 and AET on the Au electrode. The resulting mediator-modified electrode was washed with DMF, ethanol, and then water.

RESULTS AND DISCUSSION

Redox Response of Mediator-Modified Au Electrode and Its Application to GOx Electrocatalytic Reaction. A CV peak for the reductive desorption (RD) of the SAM obtained from an AET and thiol-terminated PT-PA mediator mixture appeared at −0.7 V, which agrees with the peak potential for the RD of a single-component SAM of AET. The surface coverage of thiol molecules calculated from the CV peak area is 6.3×10^{-10} mol cm^{-2}, which is slightly smaller than the monolayer coverage of alkanethiols (7.8×10^{-10} mol cm^{-2}).[42] Those results propose two possibilities: AET molecules are solely adsorbed or the thiol-terminated PT-PA has the same desorption potential as that of AET.

The amount of immobilized thiol-terminated PT-PA molecules in the SAM was estimated by measuring a redox response of PT groups in 0.05 M sodium acetate buffer (pH 5.1) (Figure 2). A pair of CV peaks around 0.6 V is assigned to the PT groups, and a value of 1.5×10^{-10} mol cm^{-2} was obtained from the anodic peak area. Figure 2 clearly shows that the SAM is composed of AET and thiol-terminated PT-PA mediator. The single RD peak results from the identical desorption potential between AET and PT-PA mediator. The difference in the number of molecules estimated from the RD and the redox response of PT groups of the monolayer indicates that ~1/4 of the thiol molecules on the Au electrode surface are thiol-terminated PT-PA. Surface immobilization of the mediator is also confirmed by a linear dependence ($R^2 = 0.999$) of the peak current for the PT oxidation on the scan rate from 1 to 400 mV s^{-1}, as shown in Figure 3. A similar result showing that ~1/5 of the adsorbed thiol molecules are thiol-terminated PT-PEO1000 molecules (1.3×10^{-10} mol cm^{-2}) was obtained for the mixed SAM of AET and thiol-terminated PT-PEO1000. The linear dependence of the anodic peak current on the scan rate was also observed for the PT-PEO1000 immobilized electrode between 1 and 400 mV s^{-1}. The shape of the PT oxidation peak depends on the electrolyte solution used. The peak becomes sharper when the electrolyte solution changes from 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) to 0.1 mol dm^{-3} n-Bu_4NClO_4 in MeCN, which coincides with the previous work reporting the redox response of ferrocene attached to a glassy carbon electrode via a short or long PEO spacer.[43]

Figure 4 shows CVs of the PT-PA immobilized Au electrode measured in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) containing 0.05 mol dm^{-3} D-glucose in the absence (a) and presence (b) of GOx. Although a redox peak of PT groups immobilized on the Au electrode appears at around 0.63 V in the absence of GOx, the oxidation current of PT groups greatly increases in the presence of GOx, indicating that ET from $FADH_2$/FADH in GOx to the electrochemically oxidized PT (PT^+) at the electrode surface occurs. The catalytic current increased with the concentration of D-glucose and leveled off at above 5 mmol dm^{-3} ($K_m = 0.64$ mmol dm^{-3}), indicating that 0.05 mol dm^{-3} of D-glucose corresponds to a substrate-saturated condition. A catalytic current was also observed at the PT-PEO1000 immobilized Au electrode under conditions similar to those in Figure 4. These results demonstrate that the PT groups immobilized on the Au electrode are able to function as electron mediators between the electrode

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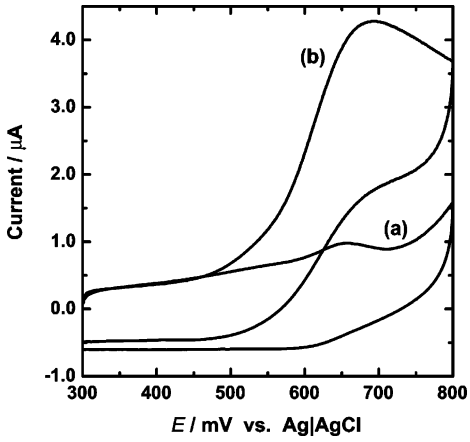

Figure 4. Cyclic voltammograms of AET and thiol-terminated PT-PA co-immobilized Au electrode in 0.05 mol dm^{-3} sodium acetate buffer solution (pH 5.1) containing 0.05 mol dm^{-3} D-glucose at a scan rate of 10 mV s^{-1} in the absence (a) and presence (b) of 4.46 μmol dm^{-3} GOx.

and the FAD center of GOx in a buffer, although the catalytic current was smaller for the PT-PEO1000-modified Au electrode than for the PT-PA-modified electrode. The fact that there is no clear difference between the PT-PA- and PT-PEO1000-immobilized systems in the amount of immobilized PT groups and the sweep rate dependence of the anodic peak current indicates that the oxidation process of PT groups is not responsible for the difference in the catalytic current.

Kinetic Analysis. To obtain the detailed kinetic information, we derived theoretical equations for the steady-state catalytic current of mediated bioelectrocatalysis (for the oxidation of substrate), where enzyme (E) and substrate (S) molecules are dissolved homogeneously in a quiescent buffer, and mediators (M) are immobilized on the electrode surface. The procedure for the derivation is similar to that for the steady-state catalytic current of meditated bioelectrocatalysis where E, S, and M molecules are dissolved in a quiescent solution,[44−47] although the adjustment of dimension between the reactions at the electrode surface and those in a buffer is necessary in the present work.

The reaction of mediators immobilized on the electrode surface consists of two steps: the generation of the oxidized state of mediator, M_{ox}, through the ET to the electrode (eq 1) and the enzyme-catalyzed production of the reduced state of mediator, M_{red} (eq 2).

$$M_{red} \underset{k_{red}}{\overset{k_{ox}}{\rightleftharpoons}} M_{ox} + n_M e^- \tag{1}$$

$$S + (n_S/n_M)M_{ox} \xrightarrow{v} P + (n_S/n_M)M_{red} \tag{2}$$

where n_S and n_M are the stoichiometric numbers of electrons involved in the redox reactions of S and M, k_{ox} and k_{red} are the rate constants for oxidation and reduction of the immobilized mediator (unit: s^{-1}), respectively; v is the rate of M_{red} production; and P is product. Both of the reactions occur in the restricted space at the electrode/electrolyte interface. The current for the electrode reaction of M is given by

$$I = n_M FA(k_{ox}\Gamma_{red} - k_{red}\Gamma_{ox}) \tag{3}$$

where F is the Faraday constant, A is the area of electrode surface and Γ_{red} and Γ_{ox} are the amounts of M_{red} and M_{ox} adsorbed on an electrode (unit: mol cm^{-2}), respectively. In the steady state, $d\Gamma_{OX}/dt$ is set equal to zero, as shown below.

$$\frac{d\Gamma_{ox}}{dt} = 0 = k_{ox}\Gamma_{red} - k_{red}\Gamma_{ox} - (n_S/n_M)v_{SS}$$

$$= \frac{I_{SS}}{n_M FA} - (n_S/n_M)v_{SS} \tag{4}$$

The rate of the M_{red} production via the reaction of M_{ox} with a reduced enzyme under the steady state, v_{SS}, is described with the steady-state current, I_{SS}, by

$$v_{SS} = \frac{I_{SS}}{n_S FA} \tag{5}$$

It should be noted that the unit of v_{SS} is mol cm^{-2} s^{-1}, because the mediators are immobilized on the electrode surface.

The enzyme-catalyzed production of M_{red} (eq 2) is expressed by the ping−pong bi−bi mechanism consisting of two Michaelis−Menten-type reactions

$$M_{ox} + E_{red} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EM \xrightarrow{k_4} M_{red} + E_{ox} \tag{6}$$

$$S + E_{ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E_{red} \tag{7}$$

where EM and ES are the enzyme−mediator and enzyme−substrate complexes, and E_{red} and E_{ox} are the reduced and oxidized states of the enzyme, respectively. Applying the steady-state approximation to eqs 6 and 7, the following three equations are obtained,

$$\frac{d\Gamma_{EM}}{dt} = k_3\Gamma_{ox}[E_{red}] - (k_{-3} + k_4)\Gamma_{EM} = 0 \tag{8}$$

$$\frac{dE_{red}}{dt} = k_2[ES] + k_{-3}\Gamma_{EM}/L - k_3[E_{red}]\Gamma_{ox}/L = 0 \tag{9}$$

$$\frac{dE_{ox}}{dt} = k_{-1}[ES] - k_1[S][E_{ox}] + k_4\Gamma_{EM}/L = 0 \tag{10}$$

where Γ_{EM} is the amount of EM formed on the electrode surface. Although the mediators are immobilized on the electrode surface, we assume that they are able to move in the restricted space due to the spacer chain, especially for the PT-PEO1000 mediator. If we define the value of L as the thickness of the mediator−mobile space, the Γ/L value corresponds to the concentration of M or EM in this interfacial space (unit: mol cm^{-3}). Although the Γ

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values are almost the same, the L value is greater for PT-PEO1000 than for PT-PA due to the long and flexible PEO chain. The magnitude of the Γ/L value is, thus, PT-PA > PT-PEO1000. From eqs 8−10, eqs 11−13 are obtained.

$$[E_{ox}] = \frac{\Gamma_{EM} k_4 (k_{-1} + k_2)}{L[S] k_1 k_2} \quad (11)$$

$$[E_{red}] = \frac{\Gamma_{EM} (k_{-3} + k_4)}{\Gamma_{ox} k_3} \quad (12)$$

$$[ES] = \frac{\Gamma_{EM} k_4}{L k_2} \quad (13)$$

Since the total amount of enzyme in the mediator−mobile space, $[E_{total}]LA$, is described by eq 14, the rate of the M_{red} production under the steady state, v_{SS} (= $k_4\Gamma_{EM}$), is given in eq 15 by substituting eqs 11−13 into eq 14,

$$[E_{total}]LA = \{[E_{ox}] + [E_{red}] + [ES]L + \Gamma_{EM}\}A \quad (14)$$

$$v_{SS} A = \left(\frac{k_{cat}[E_{total}]}{1 + K_M L/\Gamma_{ox} + K_S/[S]}\right)LA \quad (15)$$

where k_{cat} (= $k_2k_4/(k_2 + k_4)$) is the catalytic constant, and K_M (= $(k_{-3} + k_4)k_2/(k_2 + k_4)k_3$) and K_S (= $(k_{-1} + k_2)k_4/(k_2 + k_4)k_1$) are the Michaelis constants of M_{ox} and S, respectively. Equation 15 describes the rate of the M_{red} production in the mediator−mobile space rather than at the electrode surface. Considering the fact that the rate of v_{SS} in eq 5 is equal to that in eq 15, the catalytic current at the steady state, I_{SS}, in the present system can be expressed by a Michaelis−Menten-type formula,

$$I_{SS} = \frac{n_S FALk_{cat}[E_{total}]}{1 + K_M L/\Gamma_{ox} + K_S/[S]} \quad (16)$$

where $n_s = 2$ in the present case. Since I_{SS} was measured at 690 mV, which is sufficiently more positive than the oxidation potential of PT, under a substrate-saturated condition, it can be assumed that [S] is sufficiently larger than K_S and that all mediators are in the oxidized state ($K_S/[S] \approx 0$, $\Gamma_{ox} \approx \Gamma_{med}$). The following two cases are considered, depending on the relative magnitude between K_ML and Γ_{med} values.

$$\text{Case 1 } (K_M L \ll \Gamma_{med}):\ I_{SS} = 2FALk_{cat}[E_{total}] \quad (17)$$

$$\text{Case 2 } (K_M L \gg \Gamma_{med}):\ I_{SS} = 2FAk_{obs}[E_{total}]\Gamma_{med} \quad (18)$$

$$k_{obs} = (k_{cat}/K_M) = k_3k_4/(k_{-3} + k_4)$$

The k_{obs} value reflects the ET rate from FADH/$FADH_2$ to PT^+.

Estimation of the ET Rate Constants from $FADH_2$/FADH to PT^+ Groups. The catalytic current was extensively measured as the GOx concentration was varied in the range from 0.1 to 5 μmol dm^{-3} under a substrate-saturated concentration of D-glucose (0.05 mol dm^{-3}). The Γ_{med} value is experimentally determined to be of the order of 10^{-11} mol cm^{-2}, but the relative magnitude between K_ML and Γ_{med} values is unknown. Case 1 is ruled out by the fact that the steady-state current depends on Γ_{med}. Figure 5 depicts the plots of I_{SS} vs $[E_{total}]\Gamma_{med}$ where I_{SS} is the CV current at 690 mV. Although the CVs in the presence of GOx exhibit hysteresis, as shown in Figure 4, the anodic current at 690 mV was taken as I_{SS}. The linear relationship between I_{SS} and the value of $[E_{total}]\Gamma_{med}$ indicates the validity of the equation derived for case 2. The values of k_{obs} calculated from the slope of the plots according to eq 18 are 1.1×10^5 dm^3 mol^{-1} s^{-1} for the PT-PA mediator and 1.4×10^4 dm^3 mol^{-1} s^{-1} for the PT-PEO1000 mediator.

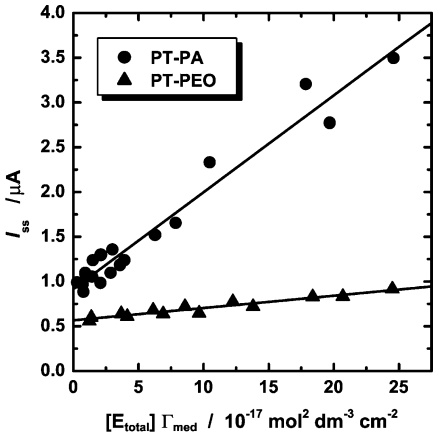

Figure 5. I_{SS} vs $[E_{total}]\Gamma_{med}$ plots measured for AET/thiol-terminated PT-PA (●)- and AET/thiol-terminated PT-PEO1000 (▲)-immobilized Au electrodes.

Anicet et al. investigated the ET of GOx monolayer-modified electrodes with freely moving or attached Fc-terminated PEO mediators.[31] The ET rate constants ranging from 2.2×10^5 to 6.0×10^5 dm^3 mol^{-1} s^{-1} were obtained for the freely moving Fc-PEO systems and the ET rate from 1.0×10^4 to 1.0×10^5 dm^3 mol^{-1} s^{-1} for the attached Fc-PEO systems. We observed the rate constant of 5.2×10^5 dm^3 mol^{-1} s^{-1} for the intermolecular ET between GOx and PT-PEO1000, both of which were dissolved in the buffer solution.[16] The ET rate constants of the present system are comparable to those of the enzyme-confined systems and are slightly smaller than those of the freely diffusing systems. This indicates that the surface-immobilized systems where both the enzyme and mediator or either of them is attached to the electrode at the monolayer level are not necessarily unfavorable in terms of the ET rate.

In the GOx hybrid systems where the mediators are covalently immobilized on the enzyme surface, the long and flexible PEO spacers enable PT mediators to approach close to the cofactor[15,16] and provide an order of magnitude larger ET rate to the hybrids modified with PT-PEO1000 than those modified with PT-PA. In contrast, the k_{obs} value at the present mediator-attached electrode surface is 1 order of magnitude greater for the PT-PA-modified system than for the PT-PEO1000-attached system. This means that a long PEO spacer does not effectively promote the ET between the FAD group in GOx and PT^+ immobilized on the electrode. For the ET of a GOx monolayer modified on electrodes with the freely moving or attached Fc-terminated PEO mediators, Anicet et al. concluded that the ET rate is controlled by the efficiency of the formation of an EM complex prior to the ET.[31] The lower efficiency of the EM complex formation due to the

smaller apparent concentration of PT groups in the mediator-mobile space on the electrode surface (Γ_{med}/L) is one possible explanation for the smaller ET rate of PT-PEO1000 system in this work.

The area occupied by one PT-PEO1000 molecule on the electrode surface is estimated to be 1.28 nm^2 from the typical Γ_{med} value, which is smaller than the cross section of the molecule of 3.14 nm^2 calculated from the hydrodynamic radius determined electrochemically by assuming that PT-PEO1000 has a spherical shape.[16] This implies that PT-PEO1000 molecules immobilized on the electrode form the "interacting mushroom"-like structure[48] and cover the whole electrode surface, although the surface roughness of the Au electrode is not taken into account. It is probable that high-molecular-weight GOx molecules are not able to penetrate into the inside of the polymer mushroom, and this accelerates the effect of the smaller apparent concentration. In addition, it seems that the accessibility of some part of the PT groups to GOx molecules is restricted in the PT-PEO1000 monolayer with the "interacting mushroom"-like structure. Those could be other reasons for the smaller ET rate of the PT-PEO1000 system.

CONCLUSION

We found that the PT-PA and PT-PEO1000 covalently bonded to AET SAM-modified gold electrodes act as effective mediators for the ET between the electrode and the FAD center of freely diffusing GOx in solution. The ET rate constant estimated from the catalytic current using the newly derived equation was an order of magnitude larger for the PT-PA-modified system than for the PT-PEO1000 system, which highly contrasts with the GOx hybrid systems previously investigated.[15,16] The lower mediation ability of the long PEO spacer for the ET between the FAD group and PT^+ immobilized on the electrode could be explained by the smaller apparent PT concentration at the electrode interface due to the high mobility of the PEO chain. Inhibition of the penetration of GOx molecules into the monolayer and of the access of some part of PT groups to GOx molecules could also be reasons for the lower mediation ability of PT-PEO1000 thickly modified on the electrode.

(48) Vermette, P.; Meagher, L. *Colloids Surf. B Biointerfaces* **2003**, *28*, 153−198.

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