Electron Transfer Reaction of Glucose Oxidase Hybrids Modified with Phenothiazine *via* Poly(ethylene oxide) Spacer on Acidic Amino Acid Residues

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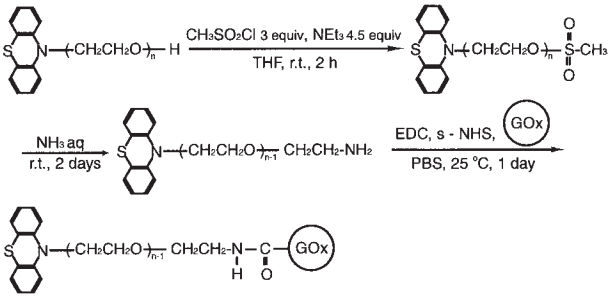
GOx hybrids [GOx-(PT-PEO-NH_2)] are prepared by covalently bonding phenothiazine(PT)-labeled poly(ethylene oxide) (PEO) oligomers having an amino end group, PT-PEO-NH_2, to acidic amino acid residues on the enzyme surface. The rate constant for the mediated FADH/$FADH_2$ oxidation calculated from the catalytic current under substrate-saturated conditions ranges from 1.7 to 388 s^{-1}, and the largest value is obtained for GOx hybrids with PT-PEO of molecular weight 3000. Surprisingly effective electron transfer from FADH/$FADH_2$ to PT^+ is achieved in the GOx-(PT-PEO-NH_2) hybrids due to the PT modification to aspartic or glutamic acid residues, many of which are located close to the FAD center.

Glucose oxidase (GOx) is a redox enzyme that catalyzes the oxidation of glucose to gluconolactone accompanying the reduction of FAD to $FADH_2$. The $FADH_2$ is reoxidized to FAD by molecular oxygen in nature. Instead of oxygen, mediators which shuttle electrons between the prosthetic FAD group and electrodes are necessary to electrochemically observe the enzymatic reaction of GOx since the direct electron transfer (ET) between them is very slow.[1–4] The modification of ET mediators to the FAD group[5] or on the enzyme surface[6–14] is an effective way to provide the electrochemical activity to enzymes. We have studied the electrochemical properties of GOx hybrids, in which PEO having electroactive PT group at one end (PT-PEO) was modified to lysine residues of GOx surface.[13,14] PT groups bounded to surface lysine residues *via* PEO chain with the optimum length effectively mediate the fast ET between electrodes and FAD due to the fast local motion of the hydrophilic PEO chain. The presence of the optimum PEO chain length in terms of the ET from FADH/$FADH_2$ to PT^+ implies that the location of PT-PEO modification with respect to the FAD center is important to achieve the fast mediation reaction. We here report electrochemical properties of new GOx hybrids with PT-PEO groups covalently bonded to surface aspartic or glutamic acid residues. While all 15 lysine residues per GOx monomer unit are located more than 23 Å away from the FAD center, the through-space distance from the FAD center is less than 16 Å for ten of 66 aspartic or glutamic acid residues.[15] We expect that the modification of PT-PEO groups to the latter residues on GOx surface realize the faster mediated $FADH_2$ oxidation compared with the modification to lysine residues.

PT-PEO-NH_2 oligomers with different molecular weights, which have an amino end group for the covalent immobilization to acidic amino acid residues, were synthesized and covalently bonded to surface aspartic or glutamic acid residues [GOx-(PT-PEO-NH_2) hybrids]. (Scheme 1) The relative enzymatic activity of GOx-(PT-PEO-NH_2) hybrids to native GOx decreased with increasing the number of attached PT-PEO-NH_2 groups, but didn't depend on the molecular weight of PT-PEO-NH_2. Carboxyl groups of aspartic or glutamic acid residues on GOx surface were activated by *N*-hydroxysulfo-succinimide (s-NHS) and 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) for amide bond formation between these residues and PT-PEO-NH_2. The GOx activation by s-NHS and EDC might be a reason for the lower relative enzymatic activity of GOx-(PT-PEO-NH_2) hybrids compared with the previous GOx-(PT-PEO) hybrids,[13,14] in which COOH groups of PT-PEO groups were activated. However, more than 40% of relative enzymatic activity was retained in the present hybrids. Compared with the modification to lysine in the previous work,[13,14] the higher number of modification was possible due to the greater number of surface acidic amino acid residues. The modified number of PT groups was determined by UV-Vis spectroscopy. Purified GOx hybrids in 0.05 mol dm^{-3} sodium acetate buffer (pH 5.1) was immediately transferred to an electrochemical cell and deoxygenated by N_2 bubbling. Cyclic voltammograms (CVs) were recorded using a glassy carbon working electrode, a Ag|AgCl| saturated KCl reference electrode, and a Pt counter electrode.



Scheme 1. Synthetic pathway of GOx-(PT-PEO-NH_2) hybrids.

While only a small peak assigned to PT appeared around 0.55 V in the absence of glucose (Figure 1a), a large catalytic oxidation current, i_{cat}, was obtained at a potential more positive than the redox potential of PT group after the addition of glucose (Figure 1b). The i_{cat} measured at 0.62 V for the hybrids increased with the glucose concentration and leveled off above 30 mmol dm^{-3} glucose (Figure 1, inset). These results indicate that PT groups bound to glutamate or aspartate residues on GOx surface through PEO chains function as electron mediators between electrodes and FAD.

Figure 2 shows a relation between the i_{cat} at 0.62 V and the number of modified PT-PEO-NH_2 for GOx-(PT-PEO-NH_2) hybrids with different molecular weights of PT-PEO-NH_2 under a glucose-saturated and diffusion-limited condition. The i_{cat} increased with the number of modified PT-PEO-NH_2, showing that plural PT groups are responsible for the ET reaction. The manner of the current increase depends on the molecular weight of modified PT-PEO-NH_2. Saturation of the i_{cat} increase was observed for GOx hybrids with PT-PEO-NH_2 of molecular weight 1000, 2000, and 3000. For GOx hybrids with PT-PEO-NH_2 of molecular weight 4200 and 8000, a maximum appeared for the i_{cat} in Figure 2. The modification number at the i_{cat} maximum became smaller for the

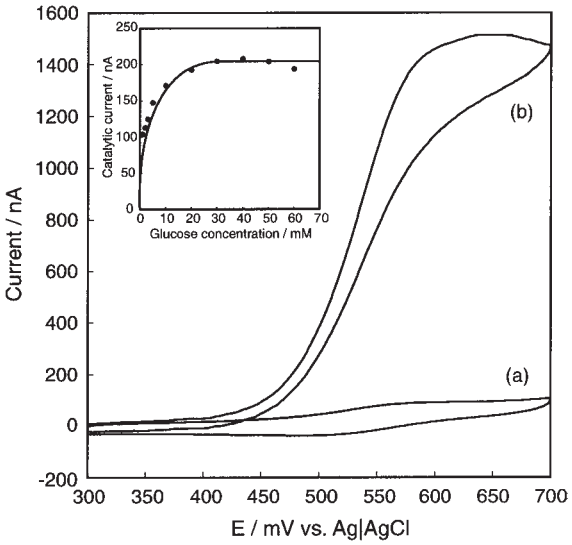


Figure 1. CVs of GOx-(PT-PEO-NH_2 2000)$_{5.7}$ hybrid (9.0 μmol dm^{-3}) measurd at 10 mV s^{-1} at a glassy carbon electrode in 0.5 mol dm^{-3} sodium acetate buffer (pH 5.1) in the absence (a) and presence (b) of 0.05 mol dm^{-3} glucose. Inset shows the dependence of the i_{cat} at 0.62 V on the glucose concentration for GOx-(PT-PEO-NH_2 2000)$_{2.0}$ (9.0 μmol dm^{-3}).

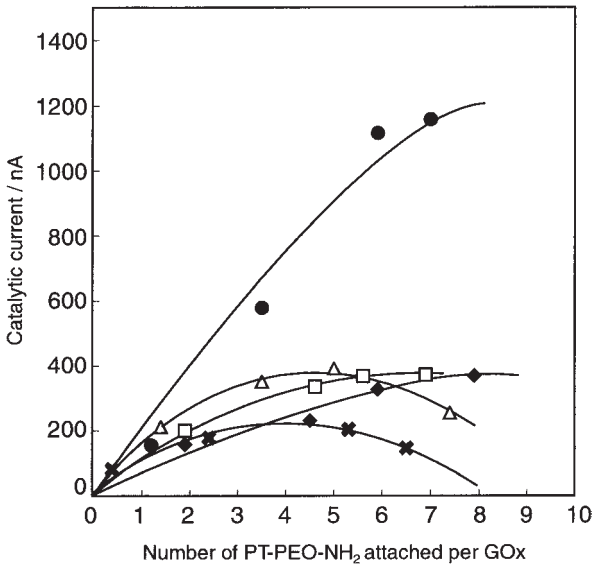


Figure 2. Relationship between the number of PT-PEO-NH_2 attached per GOx hybrid molecule and the i_{cat} at 0.62 V of hybrids (9 μmol dm^{-3}) at a glassy carbon electrode in 0.05 mol dm^{-3} sodium actate buffer (pH 5.1) containing 0.05 mol dm^{-3} glucose. The molecular weights of modified PT-PEO-NH_2 are 1000(◆), 2000(□), 3000(●), 4200(△), and 8000(✖).

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^+. The optimum PEO chain length was also observed in the previous lysine modification[13,14] and attributable to 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 GOx surface in the case of too much long PEO chain. The overlap of the accessible area of PT groups on GOx surface seems to occur in the present GOx hybrids with a large number of and/or high molecular weight PT-PEO-NH_2 groups, since several aspartic and glutamic acid residues locate closer to the FAD center than lysines. This resulted in saturation of the i_{cat} increase and the i_{cat} maximum as shown in Figure 2, which were not observed for the previous lysine modification.

The rate constant of the ET from FADH/$FADH_2$ to PT^+, k_{obs}, was estimated from the i_{cat} using Equation 1.[9]

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

where D_{hybrid} is the diffusion coefficient of the hybrid[16] and C_{hybrid} is the concentration of the hybrid. The obtained k_{obs} values range from 1.7 to 388 s^{-1}, that are greater than the reported values for GOx with Ferrocene-labeled long spacer chains attached to sugar or acidic amino acid residues on its surface.[7,8] Comparing the k_{obs} values for GOx-(PT-PEO-NH_2) hybrids with those for the lysine-modified GOx-(PT-PEO) hybrids[13,14] at a similar number of modified PT groups, a difference was observed in the case of shorter PEO chains. Greater values were obtained for the former hybrids, in which the effective ET between PT^+ and $FADH_2$/FADH was attained due to the PT modification to acidic amino acid residues, many of which are located closer to FAD than lysine residues. The higher number of modification in the present hybrids than in the previous hybrids is also responsible for the greater k_{obs} values.

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16 The D value for native GOx (4.1×10^{-7} cm^2 s^{-1} at 25 °C in the reference 6) was used for all GOx hybrids in this work.