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UV–Ozone Treatments Improved Carbon Black Surface for Direct Electron-transfer Reactions with Bilirubin Oxidase under Aerobic Conditions

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Direct electron-transfer reactions of bilirubin oxidase (BOD) adsorbed onto carbon black (CB) were observed. Heterogeneous electron-transfer rate constants (k°) between BOD and CB were evaluated by analyzing steady-state catalytic voltammograms using a rotating electrode system. By the use of CB treated with UV–ozone, improvements in k° values were observed.

Bilirubin oxidase (BOD) contains multi active center copper sites (type 1, 2, and 3) and catalyzes the oxidation of bilirubin to biliverdin (at the type 1 site) with the concomitant four-electron reduction of dioxygen to water (at type 2 and 3 sites).[1–3] BOD electron-transfer reaction investigations at carbon electrodes are attractive studies, not only from the point of view for the basic understanding of multi-copper protein reactions, but also for the application of dioxygen biocathodes for biofuel cells operating near neutral pH solutions.[4] Several studies on the mediated electrochemistry of BOD using monomeric and redox polymer mediators have been reported.[5,6] Recently, the direct electron-transfer reaction of BOD at carbon electrodes under anaerobic and aerobic conditions has been reported.[7,8] In the present study, we investigated electrode reactions of BOD adsorbed on carbon black (CB). CB has an advantage for large surface area-to-volume ratios. Heterogeneous electron-transfer rate constants (k°) between BOD and CB were evaluated by analyzing steady-state catalytic voltammograms under aerobic conditions. We found that k° values improved with the use of UV–ozone treated CB.

Bilirubin oxidase (BOD, EC 1.3.3.5, from *Myrothecium verrucaria*) was purchased from MP Biomedicals Inc., France, and used without further purification. Carbon black (CB, #7400, TOKAI Carbon Co., Ltd., Japan) had a diameter of 18–42 nm as evaluated by TEM, and the specific surface area evaluated by nitrogen adsorption was 85 $m^2\,g^{-1}$.

A UV–ozone treatment system (Model OC-2503, Eye Graphics Co., Japan) was used in this study.[9] UV light had main emissions at 185 and 254 nm. The density at 230–280 nm (sensitivity peak: ca. 255 nm) was evaluated as 11 $mW\,cm^{-2}$ by an ultraviolet ray integration illuminance meter. The concentration of ozone was ca. 100 ppm.

Electrochemical measurements were conducted on a CH-Instruments Electrochemical Analyzer Model 700B combined with a rotating electrode system to obtain steady-state catalytic currents. An Ag/AgCl (saturated KCl) electrode and a Pt plate were used as reference and auxiliary electrodes, respectively. All potentials were reported with respect to an Ag/AgCl (saturated KCl) electrode. Prior to measuring, electrolyte solutions of phosphate buffer (0.05 $mol\,dm^{-3}$, pH 6.8) were purged with oxygen.

A BOD-modified electrode was prepared as follows: a 3 mm-diameter glassy carbon electrode (Bioanalytical Systems) mounted in Teflon sleeves was polished with a No. 2000-grid SiC paper. Then, a 50 μL portion of a 5 $mg\,mL^{-1}$ CB DMF solution was placed on a polished electrode surface, and allowed to air-dry at room temperature. The active geometric electrode surface area was 1.13 cm^2 after the CB modification. CB-modified electrodes (CB-electrode) were treated by UV–ozone radiation. To protect the electrode from contamination, after UV–ozone radiation, the treated electrode was quickly immersed into a pure water solution and kept there until further use. To modify BOD on the electrode (BOD-modified electrode), unless otherwise noted, a 80 μL portion of a 40 $U\,mL^{-1}$ BOD phosphate buffer (0.05 $mol\,dm^{-3}$, pH 6.8) was placed on a UV–ozone treated CB-electrode. The BOD-modified electrode was rinsed with Milli-Q water prior to measurements.

Figure 1 shows typical cyclic voltammograms of BOD-modified and -unmodified CB-electrodes in oxygen-saturated buffer solutions. An O_2 catalytic reduction current with a peak potential of ca. 0.37 V at the BOD-modified CB-electrode was observed, where CB was not treated with UV–ozone. The catalytic current was observed from around 0.55 V, which was close to the formal potential (0.46 V) of a type 1 (T1) BOD Cu site.[7] On the other hand, no catalytic current was observed at a BOD-unmodified CB-electrode. These facts indicate that the direct electron-transfer reaction at the BOD T1 site occur at the CB surface. The aforementioned behavior is similar to results reported by a previous study.[7]

Recently, important approaches to analyze the quantitative interpretation of steady-state O_2 reduction catalytic currents by BOD adsorbed on a carbon electrode with direct electron-transfer reactions were suggested by Kano, Ikeda, and co-workers as follows:[7,10–12]

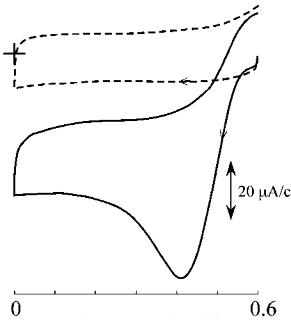

Figure 1. Typical cyclic voltammograms for BOD-modified and -unmodified CB-electrodes in oxygen saturated buffer (0.05 $mol\,dm^{-3}$, pH 6.8) solutions. The potential sweep rate was 5 $mV\,s^{-1}$.

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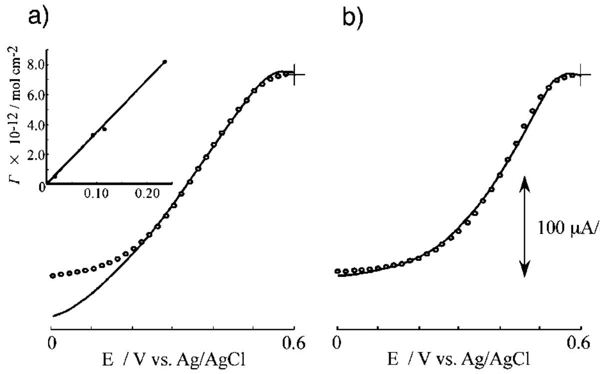

Figure 2. Steady-state linear sweep voltammograms at BOD-modified CB-electrodes without (a) and with (b) UV–ozone treatment for 1 min in a phosphate buffer (0.05 mol dm^{-3}, pH 6.8) purged with oxygen. Inset: The relationship between the observed current density vs the evaluated total surface concentration of BOD. The k° and α values used for analysis were 70 s^{-1} and 0.3, respectively.

$$I = \frac{nFk_c\Gamma}{1 + k_c/k_f + k_b/k_f} \tag{1}$$

$$k_f = k^\circ \exp[-\alpha(nF/RT)(E - E^{\circ\prime})] \tag{2}$$

$$k_b = k^\circ \exp[(1 - \alpha)(nF/RT)(E - E^{\circ\prime})] \tag{3}$$

To evaluate the heterogeneous electron-transfer kinetics between BOD and CB, the steady-state catalytic voltammogram was carried out using a rotating electrode system. Figure 2a shows background current corrected steady-state linear sweep voltammograms at a BOD-modified CB-electrode in a buffer solution. The obtained voltammogram was analyzed by fitting to Equations 1–3 with k°, Γ, and α as adjustable parameters. The values of k°, Γ, and α were determined to be 70 s^{-1}, 8.2×10^{-12} mol cm^{-2} and 0.3, respectively. The relationship between current density and the total surface concentration of electrically active BOD was investigated by changing the quantity of modified BOD. The relationship was linear as shown in the inset of Figure 2a, indicating that the kinetic analysis in the present study was satisfied by Equation 1.

Negatively charged electrode surfaces could be suitable for direct electron reactions with BOD, because it is expected that active center moieties of BOD T1 sites would be positively charged.[7] We tried to improve the surface of CB by treating it with UV–ozone to introduce oxygenated functional groups to its surface.[9] Figure 2b shows the background current corrected steady-state linear sweep voltammogram at a BOD-modified CB-electrode in a buffer solution, where CB was UV–ozone treated for 1 min. From regression curve analyses, values of k° and Γ were determined to be 140 s^{-1}, 8.4×10^{-12} mol cm^{-2}, respectively. The obtained Γ value was almost the same as that obtained when untreated CB was used. On the other hand, k° improved with the use of UV–ozone treated CB. The k° constant was about two times larger than the value obtained from untreated CB. Figure 3 shows changes in k° and Γ values as a function of UV–ozone treatment time. The k° value reached a maximum during UV–ozone exposure for 1–3 min, and reached a value of 70 s^{-1} when the untreated CB electrode was used. The Γ value was almost constant when UV–ozone exposure lasted for ca. 3 min. We do not have a clear reason to explain the behavior of k° and Γ values vs UV–ozone exposure, however, it may have something to do with the change in the molecular orientation of BOD adsorbed onto the UV–ozone treated CB surface.

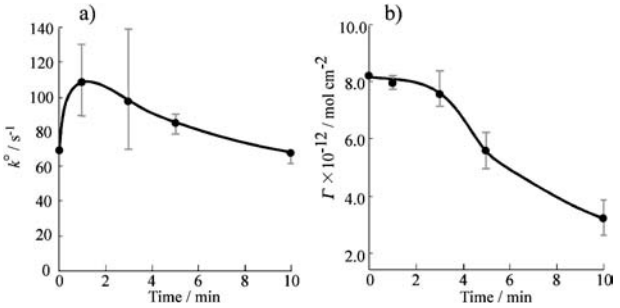

Figure 3. Change in k° and Γ values as a function of UV–ozone treatment time. Steady-state linear sweep voltammograms at a BOD-modified CB-electrode were obtained in a phosphate buffer (0.05 mol dm^{-3}, pH 6.8) purged with oxygen.

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11 In equation 1, n, F, and Γ are the number of electrons ($n = 1$ for type 1 Cu of BOD), the Faraday constant and total surface concentration of the electrically active BOD, respectively. k_c is a function of the intermolecular electron-transfer rate constant from type 1 Cu to type 2 and 3 Cu active centers and the intermolecular electron-transfer rate constant for O_2 reduction at the type 2 and 3 active centers. In this study, the k_c value was used as a maximum catalytic constant for BOD of 250 s^{-1}, in solution according to a previous study.[7]
12 In equation 2 and 3, $E^{\circ\prime}$, k°, and α are the formal potential (0.46 V) for the BOD type 1 Cu site, the heterogeneous electron-transfer rate constant at $E^{\circ\prime}$ and the transfer coefficient, respectively.

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