Significance of Nano-Structures of Carbon Materials for Direct-Electron-Transfer-type Bioelectrocatalysis of Bilirubin Oxidase

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ABSTRACT

Three types of widely used carbon materials were examined as scaffolds for the direct-electron-transfer (DET)-type bioelectrocatalysis of bilirubin oxidase (BOD) as an electrocatalyst for a 4-electron reduction of oxygen (O2). The carbon materials used were: Ketjen Black EC300 (KB) with a primary particle size (ϕp) of ca. 40 nm and a hollow structure, Vulcan XC-72R (Vulcan) with ϕp of 37 nm and a filled structure, and high purity graphite SP series (JSP) with ϕp of 10 μm and well-developed micropore structures. For the three carbon materials, the rotating disk steady-state limiting catalytic current density of the O2-reduction (jlim,OX) increased with the non-Faradaic current (jfl) at small [i]b values and was saturated at large [i]b values. The jlim,jfl ratio in low [i]b range was in the following order: JSP > Vulcan > KB. Electrochemical and microscopic data suggested that microporous structures of JSP are highly effective for the DET-type reaction of BOD. Gaps between several primary particles in the KB and Vulcan aggregates play important roles as scaffolds for BOD. The inner surface of partially broken KB particles is electrochemically active to give large [i]b but not effective as BOD scaffolds.
In this work, three types of widely used carbon materials were examined and compared with each other as scaffolds for DET-type bioelectrocatalysis of BOD: Ketjen Black EC300J (KB) with a size of the primary particle of 30–40 nm and a hollow structure, Vulcan XC-72R (Vulcan) with a size of 30–40 nm and a filled structure, and high purity exfoliated graphite J-SP (JSP) with a size of 10 µm and well-developed micropore structures. In order to characterize the porous electrodes, we also focused on the electrochemically effective surface area by using a charging current as a measure. The effect of carbon nanostructure on DET-type bioelectrocatalysis was considered on the basis of the electrochemical effective area. In addition, the structure of the carbon material-modified electrodes visualized by scanning electron microscopy and laser microscopy.

2. Experimental

2.1 Materials

Bilirubin oxidase (BOD) (EC 1.3.3.5) from Myrothecium verrucaria was purchased from Amano Enzyme Inc. (Japan) and used without further purification. KB, Vulcan, and JSP were purchased from Lion Co. (Japan), Cabot Corporation (U.S.A.), and Nippon Graphite Co., Ltd. (Japan), respectively. Poly(1,1,2,2-tetrafluoroethylene) fine powder 6-J (PTFE) was purchased from DuPont-Mitsui Fluorochemicals Co., Ltd. (Japan). Unless otherwise specified, all other chemicals were analytical grade and purchased from Wako Pure Chemical Industries, Ltd. (Japan). All solutions were prepared with ion-exchanged water.

2.2 Electrode preparation

Glassy carbon (GC) electrodes with a diameter of 3.0 mm (BAS, Japan) were polished with 0.05 µm alumina slurry followed by sonication and washing with ion-exchanged water. Forty mg of each carbon material and 10 mg of PTFE were mixed with 3.5 mL (L = dm³) of 2-propanol, and the mixture was homogenized with an ultrasonic homogenizer for 10 min. Thereafter, 6.5 mL of ion-exchanged water was added into the slurry, and the mixture was homogenized for 5 min with the ultrasonic homogenizer.

An appropriate amount of the carbon material mixtures was dropped on the surface of the polished GC electrode and dried at room temperature to evaporate the solvent. BOD (0.05 g) was dissolved in 250 µL of a 0.1 M (M = mol dm⁻³) phosphate buffer of pH 7.0, and 10 µL of the BOD solution was spread on the carbon material-modified GC electrodes. The electrodes were left to stand in a water-saturated atmosphere for 1 h at 4 °C. The enzyme-adsorbed carbon material-modified electrodes were washed with the phosphate buffer solution (pH 7.0) before electrochemical measurements.

2.3 Electrochemical measurements

All electrochemical measurements were conducted by an electrochemical analyzer (CompactStat, Ivium Technologies, Netherlands). Steady-state voltammetric measurements were carried out with rotating disk electrodes (RDEs) (RDE-1, BAS, Japan) at a rotating speed (ω) of 4000 rpm and a scan rate (v) of 10 mV s⁻¹ in an O₂-saturated 0.1 M phosphate buffer solution (pH 7.0). A platinum wire and an Ag/AgCl sat. KCl electrode were used as the counter and reference electrodes, respectively. All potentials in this work are reported with respect to the reference electrode.

2.4 Characterization of the carbon materials

The surfaces of the carbon material-modified electrodes were observed by a field emission scanning electron microscope (SEM) (TM4000, Hitachi High-Technologies Co., Japan) and a laser microscope (Lasertec Co., Japan). SEM measurements were done at an acceleration voltage of 15.0 kV. The specific surface areas of the carbon materials were evaluated from adsorption and desorption isotherms of N₂ at −196 °C recorded by an adsorption apparatus (BELSORP-mini II, BEL Japan Inc.).

3. Results and Discussion

3.1 DET-type bioelectrocatalysis of BOD at various carbon material-modified GC electrodes

Figure 1 shows rotating-disk cyclic voltammograms (RDCVs) of O₂ reduction catalyzed in DET-type mode by BOD adsorbed on various amounts of (A) KB-, (B) Vulcan-, and (C) JSP-modified GC electrodes. Clear sigmoidal current-potential curves were observed at all the modified electrodes; BOD worked well as a DET-type bioelectrocatalyst for a 4-electron reduction of O₂ at the carbon material-modified electrodes. In this work, the current density (j) is defined as the current per projective unit area of the electrodes.

![Figure 1. RDCVs of bioelectrocatalytic O₂ reduction at BOD-adsorbed electrodes and KB-modified GC electrodes. The carbon materials used were (A) KB at W = a) 4, b) 8, c) 12, d) 16, e) 24, f) 80, and g) 160 mg, (B) Vulcan at W = a) 4, b) 8, c) 12, d) 16, e) 24, f) 80, and g) 160 mg, and (C) JSP at W = a) 4, b) 12, c) 16, d) 24, e) 80, f) 160, g) 320, and g) 640 mg. The RDCVs were recorded in an O₂-saturated 0.1 M phosphate buffer (pH 7.0) at ω = 4000 rpm, v = 10 mV s⁻¹, and room temperature.](image334to522x580)
The catalytic (Faradaic) and non-Faradaic currents increased with an increase in the weight of the applied carbon materials (W). The KB-modified electrodes provided rather clear limiting values in the catalytic waves, while the Vulcan- and JSP-modified electrodes showed so-called residual slope; the reductive catalytic currents increased almost linearly with a decrease in the electrode potential after the sigmoidal increase. The residual slope is ascribed to the random orientation of the enzyme on the electrodes.\textsuperscript{13,29} In this work, the background-subtracted catalytic current density measured at 0 V was defined as the limiting one (j\textsubscript{c,lim}), though definitive limiting currents were not obtained in some cases with residual slopes. Considering the mass-transfer and electro-catalytic reaction kinetics, the j\textsubscript{c,lim} value can be given by a Koutecký-Levich-type equation:

\[
\frac{1}{j_{c,lim}} = \frac{1}{j_{dif}} + \frac{1}{j_{cat}}
\]

where \(j_{dif}\) and \(j_{cat}\) are, respectively, the limiting current densities controlled by the mass transfer of O\(_2\) at an RDE and by the electro-catalytic reaction kinetics. The \(j_{dif}\) value of a 4-electron reduction of O\(_2\) in an O\(_2\)-saturated aqueous solution can be estimated by Levich equation to be 8.4 mA cm\(^{-2}\) at \(\omega = 4000\) rpm and 25 °C.\textsuperscript{30,31} The maximum value of the experimentally obtained \(j_{c,lim}\) was smaller than the estimated value of \(j_{dif}\). Therefore, we concluded that under our experimental conditions, \(j_{c,lim}\) was predominantly determined by \(j_{cat}\) given by:

\[
j_{cat} = -n_F F k_{cat} \Gamma
\]

where \(n_F\), \(F\), \(k_{cat}\), and \(\Gamma\) are the number of electrons of the enzyme, the Faraday constant, the catalytic constant (in one catalytic turnover divided by \(n_F\)), and the surface concentration of the enzyme adsorbed on the electrode, respectively.

The value of W is often used as an important parameter for the optimization of the electrode modification.\textsuperscript{15,32,34} Figure 2 shows the W value dependence of \(j_{c,lim}\). The value of \(j_{c,lim}\) increased linearly with W at low W range and showed saturation behavior at increased W range at every carbon material-modified electrode. The saturation behavior obtained at the KB-modified electrodes was similar to that at the Vulcan-modified electrodes, and those \(j_{c,lim}\) values were much larger than the values at the JSP-modified electrodes. The radii of the primary particles of KB and Vulcan are similar to each other (about 30 nm), but KB and Vulcan have hollow and filled structures, respectively.\textsuperscript{31} Therefore, the specific surface area of KB is much larger than that of Vulcan. Actually, the non-Faradaic currents at the KB-modified electrodes were much larger than that of the Vulcan-modified electrodes (Fig. 1). Therefore, Vulcan seems to provide the best characteristics as scaffolds for the DET-type reaction of BOD among them.

3.2 The relationship between \(j_{c,lim}\) and \(|j_b|\)

Since the effective electrode surface area is an important parameter, in the following we used the width of the background RDCVs at 0.55 V (|\(j_b|\)) and at \(\nu = 10\) mV s\(^{-1}\) without BOD (that is, the difference in non-Faradaic (or capacitive) current densities of the anodic and cathodic scans of RDCVs) as a measure of the electrochemically active surface area. Since the Debye length in the buffer solution is smaller than the primary particle sizes of the carbon materials examined, the value of |\(j_b|\) is appropriately considered to correspond to the electrochemically active surface area. Figure 3 shows the relationship between |\(j_{c,lim}|\) and |\(j_b|\) for the three kinds of carbon material-modified electrodes. KB provided large values of |\(j_{c,lim}|\) and |\(j_b|\). Vulcan provided large values of |\(j_{c,lim}|\), while |\(j_b|\) values were rather small. JSP gave small values of |\(j_{c,lim}|\) and |\(j_b|\).

![Figure 2](image1.png)

**Figure 2.** Relationships between the absolute values of the catalytic current density and the weight of applied carbon materials; (●) KB, (▲) Vulcan, and (■) JSP.

![Figure 3](image2.png)

**Figure 3.** The relation between the absolute value of the catalytic current density and the background current density at the three-carbon material-modified electrodes. (A) KB, (B) Vulcan, and (C) JSP. The inset in panel C shows a magnified view around the origin. Broken lines indicate the slopes at the intersection at the x-axes.
The $[\mathcal{J}_{\lim}]$ values of the three carbon materials increased rapidly with $[\mathcal{J}_{\lim}]$ at low $[\mathcal{J}_{\lim}]$ range and tended to be saturated at increased $[\mathcal{J}_{\lim}]$ range. The estimated slopes at the intersection of the $x$-axes for KB, Vulcan, and JSP are 10, 38, and 133, respectively. As shown in Fig. 1, the $[\mathcal{J}_{\lim}]$ values at the KB-modified electrodes were extremely large compared with those at the Vulcan-modified electrodes, despite that the radii of the primary particles of KB and Vulcan are similar to each other. The primary particle of KB has a hollow structure with a radius of about 10 nm. On the other hand, the primary particles of Vulcan has a filled structure. The primary particles of KB are partially broken and will have holes connected to the inner pore. The electrolyte solution can penetrate into the hole of KB, and then KB gave large $[\mathcal{J}_{\lim}]$ compared with Vulcan. However, it seems to be difficult for BOD to penetrate the hole of KB. Therefore, the inner surface of the hole of KB would not be utilized as scaffolds for BOD adsorption.

These results also show the importance of the outer surface of the primary particles of KB and Vulcan as scaffolds for BOD. Considering very low bioelectrocatalytic activity of BOD at planar GC electrodes, the outer surface of the primary particle with a radius of ca. 30 nm does not seem to be suitable scaffolds for BOD. Therefore, the aggregation of KB and Vulcan seems to be essential for DET-type communication of BOD with the carbon materials as in the case of gold nanoparticles. The gaps among several primary particles in the aggregate seem to play important roles as scaffolds for BOD thanks to curvature effects of the gap-type mesoporous structures.

On the other hand, the situation for JSP is completely different. The size of the primary particle of JSP is very large (10 µm) compared to BOD (5 nm). Therefore, the size of the gap among the particles in the aggregate of JSP is too large for the DET-type communication of BOD. However, the slope of the $[\mathcal{J}_{\lim}]$ vs $[\mathcal{J}_{\lim}]$ curve of JSP at low $[\mathcal{J}_{\lim}]$ is the largest among the carbon materials examined. JSP is characterized to develop relatively higher degrees of microporous structures (Fig. S1). On the other hand, the increased electric field at the edge of microstructures is considered to enhance the interfacial electron transfer kinetics. Therefore, we can conclude that the microporous structures at the surface of JSP are very effective for the DET-type reaction of BOD. However, the $[\mathcal{J}_{\lim}]$ values at low $[\mathcal{J}_{\lim}]$ region was much lower than those of KB and Vulcan. The aggregate of JSP with a large $\phi$ does not seem to be suitable for the DET reaction of BOD. Most probably, the penetration of BOD into a stacked JSP layer was difficult. Some hindrances in the mass transfer of O$_2$ into a stacked JSP layer may also occur.

### 3.3 Microscopic observation of aggregated carbon materials

In the case of gold nanoparticles, initial straight relations in $[\mathcal{J}_{\lim}]$ vs $[\mathcal{J}_{\lim}]$ curves often give negative intercepts. This is also ascribed to the significant contribution of gaps constructed among gold nanoparticles; such gaps are not formed at very low surface coverage due to the rather homogeneous distribution of gold nanoparticles. In the case of JSP, the negative intercept of the initial straight line is observed. However, in the cases of KB and Vulcan, the straight lines in Fig. 3 go through the origin. This seems to be ascribed to the inhomogeneous distribution of the carbon particles and formation of aggregations on the electrode surface even at low surface coverages of KB and Vulcan. In order to justify the prediction, microscopic observation of the carbon materials on the electrode surfaces was carried out.

Figure 4 shows SEM and laser microscopy images of the three kinds of the carbon materials examined on the GC electrodes at very low $W$ values in the range of the straight relation between $[\mathcal{J}_{\lim}]$ and $W$ (Fig. 2). The distribution patterns of KB and Vulcan were similar to each other, while those of JSP were somewhat different from those of KB and Vulcan. The difference in the distribution patterns seems to be ascribed to the difference in the radii of the primary particles of the carbon materials. In any events, the microscopic observation well supported our prediction; KB and Vulcan were not uniformly dispersed on the GC surface and aggregates were formed when low amounts of the carbon materials were dispersed. The laser microscopy of the JSP modified electrode shows the distribution of JSP at the GC surface is relatively homogeneous. The aggregation seems to occur due to the hydrophobic interaction of the carbon particles in the drying process. Therefore, gap-type mesoporous structures effective for the DET-reaction of BOD can be formed among the carbon particles even at low surface coverages of the carbon materials. This seems to be the reason that the $[\mathcal{J}_{\lim}]$ vs $[\mathcal{J}_{\lim}]$ curves go through the origin. The situation is different from that of gold nanoparticles.

When the amounts of the carbon materials were increased, the aggregates macroscopically appeared to homogeneously cover the electrode surface (Figs. S2–S4). However, the surface of the aggregates of KB and Vulcan had deep cracks. These cracks are also formed during the drying process. Such large cracks themselves do not seem to play important roles in DET-type reactions of BOD, as judged from the results in Fig. 2.

In the case of JSP, the particle size is comparable to the thickness of the diffusion layer at the RDE at 4000 rpm (mFDc/8.4 mA cm$^{-2} = 10$ µm). The number of paths of the substrate transfer from the bulk solution into the aggregates of JSP seems to be very limited and the path length would be rather long compared with the diffusion layer thickness. Since the structure of densely packed JSP showed disadvantages for the substrate transfer, the saturated value of $[\mathcal{J}_{\lim}]$ for JSP is smaller than that for KB or Vulcan. One of
the advantages of JSP maybe that cracks were not observed at increased W. This property may be utilized as a base of scaffold for enzyme adsorption.

Since the electrolyte solution may be difficult to penetrate (hydrophobic) microporous structures of carbon materials, the electrochemically effective area does not necessarily coincide with the specific surface area determined, for example, by gas adsorption. The estimated BET surface area was 720, 230, and 11 m² g⁻¹ for KB, Vulcan, and JSP, respectively. The values of |j|₀ linearly increased with increasing W on the electrode surface, as shown in Fig. 5. The slopes for KB, Vulcan, and JSP are 0.015 ± 0.002, 0.0022 ± 0.0002, and 0.00004 ± 0.00001 mA cm⁻²µg⁻¹, respectively. The ratio of the BET surface area and the slope for KB agrees with that for JSP. On the other hand, as mentioned above, these ratios disagree with that for Vulcan. On the other hand, as mentioned above, these ratios disagree with that for JSP, since the penetration of the enzyme into micropore is difficult. The |j|₀,lim curve shows characteristics of the saturation, since the penetration of the enzyme into mesopores in the stacked JSP is also difficult. In addition, the mass transfer of O₂ seems to be inhibited in the densely packed layer of JSP, though JSP particles provide a suitable scaffold for the DET-type reaction of BOD at low surface coverages.

4. Conclusions

KB, Vulcan, and JSP have been investigated by electrochemically and microscopically as scaffolds for the DET-type bioelectrocatalysis of BOD in view of pore size, distribution of porous structures, and three-dimensional structures of the aggregated carbon materials. The comparison between KB and Vulcan shows that gap-type mesopores constructed three-dimensionally by the aggregation of KB or Vulcan particles with almost the same φp are effective as scaffolds for BOD thanks to curvature effects of the mesopores. Inside faces of partially broken KB particles are electrochemically active to give large |j|₀. However, the inner holes of KB are not effective as BOD scaffolds, since it is difficult for BOD to penetrate the hole. On the other hand, the surface structure of JSP is suitable for the DET-type bioelectrocatalysis of BOD most probably due to well-developed microstructures, on which fast electron transfer would proceed. However, densely packed JSP was not suitable for the DET-reaction of BOD, most probably due to the hindrance in the penetration of BOD as well as mass-transfer of O₂.

The optimization of carbon material-modified electrodes for the DET-type bioelectrocatalysis requires to balance the ratio of macro-, meso, and micropores to construct the three-dimensional structure effective for the mass transport of electrolyte solution and substrates, as well as the penetration of enzymes into the nano-structures, and to enhance the interfacial electron transfer kinetics of macromolecular redox enzymes. The discussion based on the electrochemically active surface area as proposed in this work is important in the optimization of carbon materials for DET-type bioelectrocatalysis of redox enzymes.

Supporting Information

The Supporting Information is available on the website at DOI: https://doi.org/10.5796/electrochemistry.20-64063.

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