

# Electrochemistry of bilirubin oxidase at carbon nanotubes

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**Abstract** In this paper, we compared the direct electron transfer and electrocatalytic properties of bilirubin oxidase (BOD) immobilized at two kinds of carbon nanotubes (CNTs), bamboo-CNTs and uniform-CNTs. X-ray diffraction and X-ray photoelectron spectroscopy results indicated that the ratio of  $sp^2$  band to  $sp^3$  band and the content of oxygen-containing groups at the surface of uniform-CNTs were higher than that of bamboo-CNTs. Moreover, uniform-CNTs can be well separated at the surface of the electrode. Better electrochemical and electrocatalytic properties of BOD immobilized at uniform-CNTs were shown.

**Keywords** Carbon nanotubes · Bilirubin oxidase · Direct electron transfer · Oxygen reduction · Biofuel cell

## Introduction

Biofuel cell (BFC) is a special kind of fuel cell which uses natural enzyme as a catalyst or directly transforms chemical energy to electrical energy via electrochemical reaction involving biochemical pathways [1–4]. In comparison with inorganic fuel cells, BFCs have many advantages, such as corrosion-free electrolyte and more moderate temperature. The enzymatic BFCs have attracted considerable research attention in recent years [1–20], yet the works still remain at their early stage. The main reason is that the rate of

electron transfer between the redox center of biocatalysts and the underlying electrodes is very slow, leading to the low efficiency and small power output of BFCs. To solve this problem, great efforts have been made to improve the direct or mediated electron transfer reactions of, i.e., laccase and bilirubin oxidase by immobilizing them onto various interfaces, i.e., spectrographic graphite [21, 22], poly-L-lysine/plastic-formed carbon, and highly oriented pyrolytic graphite (PG) [23].

Carbon nanotubes (CNTs) have been proved to be a novel type of nanostructure with unique structural, mechanical, electrical, physical, and chemical properties since their discovery [24]. Attempts over the last decade have revealed that the CNTs constitute a new form of carbon materials that are finding striking applications in many fields, such as energy conversion and storage, electromechanical actuators, and chemical sensing, especially in nano-devices such as quantum wires, ultrahigh-strength engineering fibers and sensors [25–27]. It has been documented in the recent literatures that the CNTs have better electrocatalytic activity and higher surface area compared with other kinds of carbon-based materials, such as glass carbon (GC), PG, and diamond [28]. Our recent interests in the direct electron transfer of electrochemistry of multi-copper oxidase, i.e., laccase and bilirubin oxidase (BOD), onto various CNTs have been primarily focused on the development of CNT-based bioelectronic nanodevices such as biofuel cells [20, 29–31]. In the present study, we further detailed the investigated effect of the direct electron transfer and bioelectrocatalytic activities of BOD at two kinds of CNTs, which may be not only fundamentally helpful for understanding the relationship between the structure of carbon-based materials and the interfacial electron transfer properties of multi-copper oxidases but also practically useful for the development of novel kinds of CNT-based biosensors and biofuel cells.

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## Experimental

### Reagents

Uniform-CNTs, the inner structure of which consisted of uniform tubular (diameters ranging from 10 to 30 nm), were purchased from Shenzhen Nanotech Port Co., Ltd (Shenzhen, China). Bamboo-CNTs, the inner of which consisted of hollow, tapered tube structures (20–40 nm in length), were self-synthesized by thermal chemical vapor deposition (CVD) using Fe as the catalyst. A gas mixture of methane and hydrogen was used in the CVD process with a growth temperature of about 600°C. Both uniform-CNTs and bamboo-CNTs were purified by refluxing in 3.0 M HNO<sub>3</sub> for 5 h prior to use. Bilirubin oxidase (EC 1.3.3.5) from *Myrothecium verrucaria* and bovine serum albumin (BSA) were purchased from Sigma and used without further purification. Other chemicals were of at least analytical grade and used as received. Aqueous solutions were prepared with doubly distilled water.

### Electrode preparation

Glassy carbon (3 mm diameter), before use, was firstly polished with emery paper (# 2000), 0.3 and 0.05 μm granularity alumina slurry on a woolen cloth, and then cleaned under bath sonication for 5 min and finally thoroughly rinsed with distilled water. The bamboo-CNTs and uniform-CNTs were dispersed into *N,N*-dimethylformamide to give a 2 mg mL<sup>-1</sup> homogeneous dispersion, respectively. Four microliters of the bamboo-CNTs dispersion was dip-coated onto the GC electrodes and dried at ambient temperature for at least 1 h. Then, a 2-μL volume of 0.2 U μL<sup>-1</sup> BOD was mixed with 2 μL of 1% (wt.%) BSA to give an enzyme–BSA mixture, which was coated onto the bamboo-CNTs-modified electrodes and then cross-linked with glutaraldehyde by applying 2 μL of 40 mM glutaraldehyde onto the electrodes. The resulting electrodes (denoted as BOD/bamboo-CNTs-modified GC) were dried at ambient temperature and rinsed with distilled water before use or stored at 4°C in a refrigerator while not used. The BOD was cross-linked onto uniform-CNTs-modified GC electrodes to prepare BOD/uniform-CNTs-modified GC electrodes with the same procedures as those of the BOD/bamboo-CNTs-modified electrode.

### Apparatus and electrochemical measurements

The electrochemical measurements were carried out using a computer-controlled electrochemical analyzer (CHI 650C, Chen-Hua, Shanghai) with a two-compartment and three-electrode cell. The modified GC electrodes were used as working electrodes, with a platinum spiral

wire as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode. Phosphate buffer, at 0.10 M (pH 7.0), was used as the supporting electrolyte. For the experiments conducted under anaerobic conditions, the electrolyte was bubbled with pure nitrogen gas for more than 30 min, and nitrogen gas was kept flowing over the solution during the electrochemical measurements. All electrochemical measurements were performed at ambient temperature (18±2°C).

Scanning electron microscope images of CNTs were measured using environmental scanning electron microscopy (AMRAY-1910FE). The crystallinity of the CNT samples were characterized by X-ray diffractometer (XRD) equipped with CuK<sub>α</sub> source and operating at 40 kV and 40 mA. The diffraction patterns were obtained at a scan rate of five per minute. The samples were also analyzed by X-ray photoelectron spectroscopy (XPS) using an AXIS Ultra spectrometer (Kratos, UK) equipped with a focused monochromatic X-ray source (AlK<sub>α</sub>, *h<sub>ν</sub>*=1,486.6 eV) operated at 225 W with a corresponding voltage of 15 kV. The data were converted into VAMAS file format and imported into CasaXPS software package for manipulation and curve-fitting.

## Results and discussion

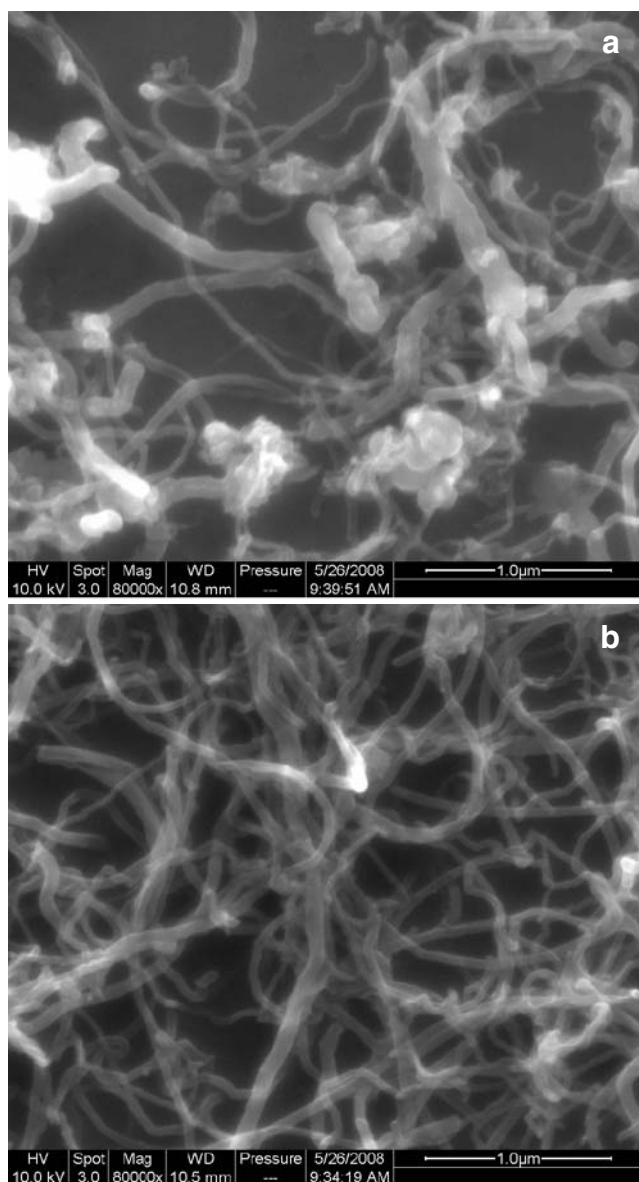
### Characterization of CNTs

Typical SEM images of two kinds of CNTs were shown in Fig. 1a, b. It can be seen that the bamboo-CNTs exhibit aggregated morphology, in comparison with the uniform-CNTs under the same preparation process. Based on the comparison of the SEM images, uniform-CNTs are expected to have significantly higher surface area compared to the bamboo-CNTs. The active surface areas of the bamboo-CNTs- and uniform-CNTs-modified electrodes can be estimated according to the Randles–Sevcik equation for the redox couple [Fe(CN)<sub>6</sub>]<sup>3-</sup>/[Fe(CN)<sub>6</sub>]<sup>4-</sup> [32]:

$$i_p = 0.4463 nFAC (nFvD/RT)^{1/2}$$

Here, *n* is the number of electrons appearing in half-reaction for the redox couple, *v* is the rate at which the potential is swept, *F* is Faraday's constant, *A* is the electrode area, *R* is the universal gas constant, *T* is the absolute temperature, and *D* is the analyte's diffusion coefficient. For 1.0×10<sup>-3</sup> mol L<sup>-1</sup> K<sub>3</sub>Fe(CN)<sub>6</sub> in the 0.10 mol L<sup>-1</sup> KCl electrolyte, *n*=1 and *D*=7.6×10<sup>-6</sup> cm<sup>2</sup> s<sup>-1</sup>. They are 1.39 mm<sup>2</sup> for bamboo-CNTs- and 2.87 mm<sup>2</sup> for uniform-CNTs-modified electrode (average of three measurements), respectively.

The examination of the bamboo-CNTs and uniform-CNTs samples with X-ray diffraction analysis revealed the



**Fig. 1** Typical SEM image of bamboo-CNTs (a) and uniform-CNTs (b) samples

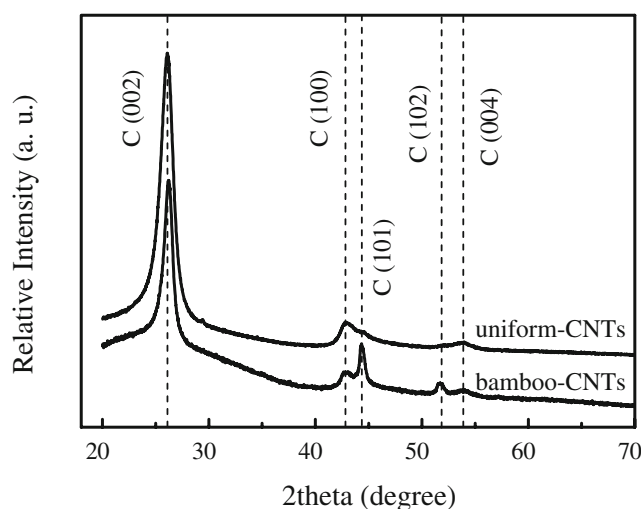
formation of graphite-like lattice structures. As shown in Fig. 2, the diffraction peaks at  $26.0^\circ$ ,  $42.8^\circ$ , and  $53.8^\circ$  observed for the two kinds of CNTs can be attributed to the hexagonal graphite (002), (100), and (004) planes. For the bamboo-CNTs, the observed diffraction peaks at  $44.3^\circ$  and  $51.7^\circ$  were attributed to the hexagonal graphite (101) and (102) planes. Moreover, the intensity of the (002) diffraction peak for the uniform-CNTs was much higher than that for the bamboo-CNTs, which indicated that the uniform-CNTs sample has a much higher graphitization degree, resulting in the higher electron conductivity for electron transfer during electrochemical reactions [33, 34].

XPS, as one of the surface analytical techniques, is capable for readily providing information about chemical

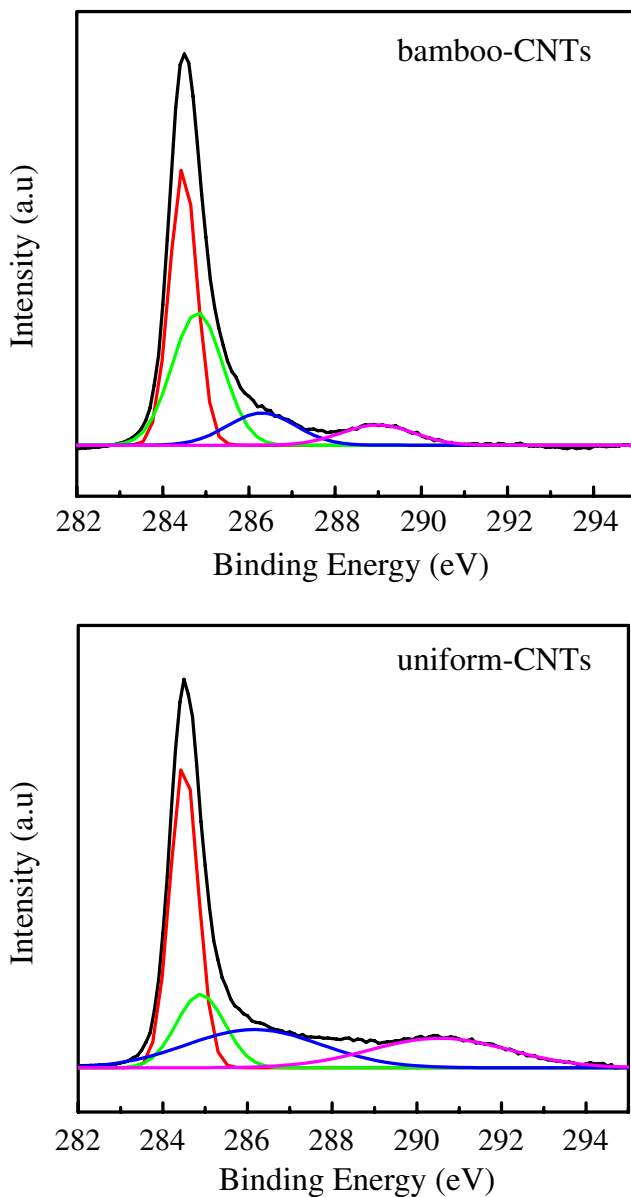
bonding on the surface. XPS was performed on two kinds of CNTs samples to get the elemental information on the surface in the present study. The detailed XPS C 1s and O 1s spectra for uniform-CNTs and bamboo-CNTs samples were shown in Fig. 3. The main peak at 284.5 eV for two kinds of CNTs corresponded to the combination of the  $sp^2$  C=C and  $sp^3$  C-C structure of carbon nanotubes [35]. Compared with uniform-CNTs, firstly, the bamboo-CNTs had a lower ratio of  $sp^2$  band to  $sp^3$  band. The local  $sp^3$  defects were produced by disrupting the graphitic-like  $sp^2$  network of carbon in the preparation and purification process. Secondly, there were fewer oxygen-contained functional groups in bamboo-CNTs, leading to a lower electrochemical activity [36]. Thirdly, the relative oxygen atom percentage of uniform-CNTs was less than that of bamboo-CNTs, 2.9% vs. 5.5%, as calculated from XPS oxygen narrow spectra (not shown here). More defects existing on the surface would facilitate the absorption of oxygen in air and result in the higher oxygen content [37]. It could be conjectured that the production of associated carboxylic acid groups on the surface of uniform-CNTs might lead to their higher activity.

#### Direct electrochemistry and electrocatalysis of BOD at two kinds of CNTs

BOD is a multi-copper oxidase catalyzing the oxidation of bilirubin to biliverdin accompanied with the four-electron reduction of dioxygen to water at a high potential near pH 7.0 and its catalytic activity is not readily affected by chloride [38], which normally exists in biological systems. Therefore, BOD has been utilized in the construction of the dioxygen biocathode operating at neutral pH in BFCs [8–14, 18–20, 39–41]. In this study, we used cyclic voltam-



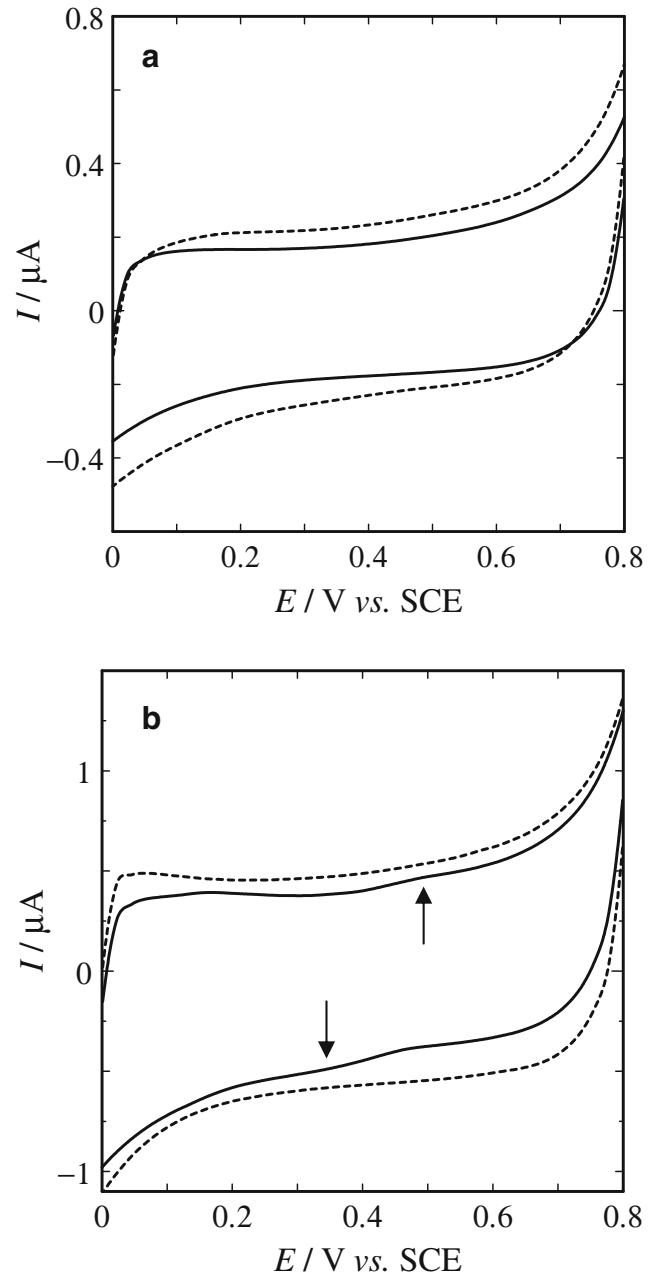
**Fig. 2** X-ray diffraction patterns for bamboo-CNTs and uniform-CNTs samples



**Fig. 3** C 1 s core level XPS spectra of bamboo-CNTs and uniform-CNTs

metry to study the direct electron transfer of BOD at two kinds of CNTs-modified electrodes. As could be seen from Fig. 4a, the cross-linking of BOD at the bamboo-CNTs-modified electrode (solid line) did not produce any observable redox response, which was distinct from that at the bamboo-CNTs-modified electrode (dashed line). This observation essentially suggested that the voltammetric response resulted from the direct electron transfer of BOD was unable to be recorded at the bamboo-CNTs-modified electrode under anaerobic conditions. In contrast, under the same condition, a pair of well-defined voltammetric response was recorded for the BOD at the uniform-CNTs-modified GC electrode with a formal potential  $E^{0'}$  of  $425 \pm 15.9$  mV (vs. SCE), as marked by an arrow in Fig. 4b. The

$E^{0'}$  was very close to the redox potential of T1 site of the BOD (670 mV vs. normal hydrogen electrode, NHE) [42]. The above results indicated that the uniform-CNTs could facilitate the direct electron transfer of the enzyme and enhance the electrochemical signal. It is known that, as a new kind of carbon materials, the CNTs possess unique structural and electronic properties and have been widely used as electrode materials to realize direct electron transfer



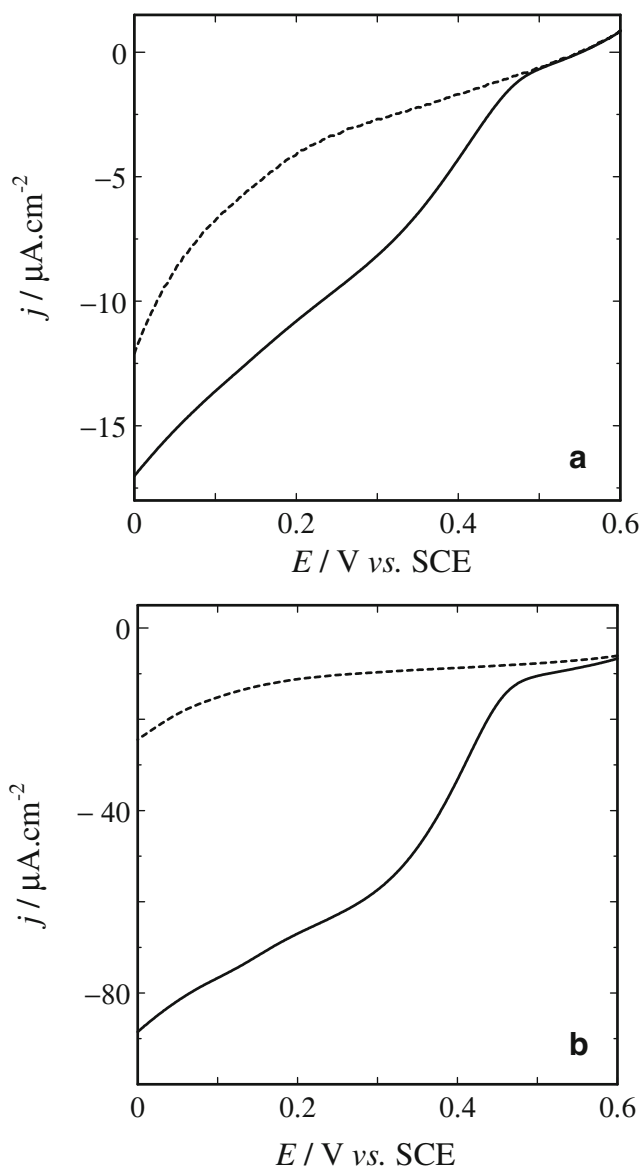
**Fig. 4** Cyclic voltammograms observed at **a** BOD/bamboo-CNTs- (solid line) and bamboo-CNTs-modified (dashed line) GCs and **b** BOD/uniform-CNTs- (solid line) and uniform-CNTs-modified (dashed line) GCs in  $N_2$ -saturated 0.10 M phosphate buffer, pH 7.0. Scan rate,  $10 \text{ mV s}^{-1}$

of biomacromolecules (i.e., proteins and enzymes) [32, 36, 43]. This is mainly because the enzymes and proteins immobilized on CNTs possess relatively complex orientations and structures, making them exposing the electroactive centers and realizing their direct electron transfer [44]. Therefore, the observed difference might result from the direct electron transfer owing to the favorable orientations of BOD immobilized on uniform-CNTs and the higher electroactive area of the uniform-CNTs-modified electrode.

As mentioned above, the direct electron transfer between BOD and the modified electrode was expected to be important for the development of the cathode of BFC, because it could reduce the overpotential of oxygen reduction to water in a near-neutral solution. Thus, the bioelectrocatalytic activity of the BOD immobilized onto the two kinds of CNTs-modified electrodes toward the reduction of oxygen was further studied. Figure 5 showed the polarization curves obtained for the catalytic reduction of oxygen at BOD modified by different CNTs electrodes in 0.10 M phosphate buffer (pH 7.0). As could be seen, the electrocatalytic current of oxygen reduction started at 480 mV (vs. SCE) at the BOD/bamboo-CNTs- (solid line, Fig. 5a) and BOD/uniform-CNTs-modified (solid line, Fig. 5b) GC electrodes. However, the oxygen reduction commencing at 100 mV was observed for both bamboo-CNTs- (dashed line, Fig. 5a) and uniform-CNTs-modified- (dashed line, Fig. 5b) electrodes. This phenomenon revealed that the potential for the  $O_2$  reduction at the BOD modified by two kinds of CNTs electrode positively shifted ca. 380 mV, indicating that the  $O_2$  reduction was catalyzed by BOD. Moreover, the reduction potentials at the BOD-modified bamboo-CNTs electrode and BOD-modified uniform-CNTs electrode were more positive than those obtained using redox mediators and close to the thermodynamic equilibrium potential of the four-electron reduction of oxygen under the present conditions (i.e., 816 mV vs. NHE) [45]. These results indicated that the BOD-catalyzed  $O_2$  reduction could reduce the overpotential of oxygen reduction to water and approve the feasibility of its application in constructing BFCs. In addition, it could be seen from Fig. 5 that the current density of  $O_2$  reduction observed for BOD/uniform-CNTs/GC electrode was higher than that for BOD/bamboo-CNTs/GC electrode. Moreover, these values of the current density were smaller than that in the previous report [23], probably because the favorable orientations and structures of BOD have been affected when immobilizing with uniform-CNTs and bamboo-CNTs.

## Conclusions

In this study, we demonstrated the direct electron transfer properties of BOD at two kinds of CNTs with different



**Fig. 5** Polarization curve observed at **a** BOD/bamboo-CNTs- (solid line) and bamboo-CNT-modified (dashed line) GCs and **b** BOD/uniform-CNTs- (solid line) and uniform-CNT-modified (dashed line) GCs in air-saturated 0.10 M phosphate buffer, pH 7.0. Scan rate, 1 mV s<sup>-1</sup>

structures, which were characterized by SEM, XRD, and XPS. Compared with uniform-CNTs, the bamboo-CNTs had more local  $sp^3$  defects and fewer oxygen-contained functional groups and lower graphitization degree. Moreover, uniform-CNTs can be separated well and form the three-dimensional structure, which led to the smaller electrochemically accessible surface area and faster charge transfer rate at the electrode/electrolyte interface. The direct electron transfer of BOD is largely facilitated onto the uniform-CNTs, which has been illustrated with the excellent bioelectrocatalytic activities of the BOD/uniform-



CNTs-modified electrodes toward the reduction of oxygen. These results given above would be significant for the investigations on the interfacial electron transfer of multicopper oxidases and the development of the CNT-based biosensors and biofuel cells.

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## References

- Barton SC, Gallaway J, Atanassov P (2004) *Chem Rev* 104:4867. doi:10.1021/cr020719k
- Bullen RA, Arnot TC, Lakeman JB, Walsh FC (2006) *Biosens Bioelectron* 21:2015. doi:10.1016/j.bios.2006.01.030
- Davis F, Higson SPJ (2007) *Biosens Bioelectron* 22:1224. doi:10.1016/j.bios.2006.04.029
- Barton SC, Kim HH, Binyamin G, Heller A (2001) *J Am Chem Soc* 123:5802. doi:10.1021/ja010408b
- Habrioux A, Sibert E, Servat K, Vogel W, Kokoh KB, Alonso-Vante N (2007) *J Phys Chem B* 111:10329. doi:10.1021/jp0720183
- Kim H, Mano N, Zhang Y, Heller A (2003) *J Electrochem Soc* 150:209
- Tsujimura S, Kano K, Ikeda T (2002) *Electrochemistry* 70:940
- Tsujimura S, Fujita M, Tatsumi H, Kano K, Ikeda T (2001) *Phys Chem Chem Phys* 3:1331. doi:10.1039/b009539g
- Tsujimura S, Tatsumi B, Ogawa J, Shimizu S, Kano K, Ikeda T (2001) *J Electroanal Chem* 1-2:69. doi:10.1016/S0022-0728(00)00239-4
- Shleev S, Tkac J, Christenson A, Ruzgas T, Yaropolov AI, Whittaker JW, Gorton L (2005) *Biosens Bioelectron* 20:2517. doi:10.1016/j.bios.2004.10.003
- Yuhashi N, Tomiyama M, Okuda J, Igarashi S, Ikebukuro K, Sode K (2005) *Biosens Bioelectron* 20:2145. doi:10.1016/j.bios.2004.08.017
- Kim HH, Zhang YC, Heller A (2004) *Anal Chem* 76:2411. doi:10.1021/ac035487j
- Tominaga M, Otani M, Kishikawa M, Taniguchi I (2006) *Chem Lett* 35:1174. doi:10.1246/cl.2006.1174
- Kang C, Shin H, Heller A (2006) *Bioelectrochemistry* 68:22. doi:10.1016/j.bioelechem.2005.03.002
- Dronov R, Kurth DG, Mohwald H, Scheller FW, Lisdat F (2008) *Angew Chem Int Ed* 47:3000. doi:10.1002/anie.200704049
- Togo M, Takamura A, Asai T, Kaji H, Nishizawa M (2008) *J Power Sources* 178:53. doi:10.1016/j.jpowsour.2007.12.052
- Otsuka K, Sugihara T, Tsujino Y, Osakai T, Tamiya E (2007) *Anal Biochem* 370:98. doi:10.1016/j.ab.2007.06.011
- Lim J, Cirigliano N, Wang J, Dunn B (2007) *Phys Chem Chem Phys* 9:1809. doi:10.1039/b618422g
- Dronov R, Kurth DG, Scheller FW, Lisdat F (2007) *Electroanalysis* 19:1642. doi:10.1002/elan.200703900
- Gao F, Yan Y, Su L, Wang L, Mao L (2007) *Electrochem Commun* 9:989. doi:10.1016/j.elecom.2006.12.008
- Shleev S, Kasmi AE, Ruzgas T, Gorton L (2004) *Electrochem Commun* 6:934. doi:10.1016/j.elecom.2004.07.008
- Tsujimura S, Kawaharada M, Nakagawa T, Kano K, Ikeda T (2003) *Electrochem Commun* 5:138. doi:10.1016/S1388-2481(03)00003-1
- Tsujimura S, Kano K, Ikeda T (2005) *J Electroanal Chem* 576:113. doi:10.1016/j.jelechem.2004.09.031
- Iijima S (1991) *Nature* 354:56. doi:10.1038/354056a0
- Xiao Y, Li CM (2008) *Electroanalysis* 20:648. doi:10.1002/elan.200704125
- Baughman RH, Zakhidov AA, Heer WA (2002) *Science* 297:787. doi:10.1126/science.1060928
- Carpani I, Scavetta E, Tonelli D (2008) *Electroanalysis* 20:84. doi:10.1002/elan.200704054
- Sherigara BS, Kutner W, D'Souza F (2003) *Electroanalysis* 15:753. doi:10.1002/elan.200390094
- Yan Y, Yehezkeli O, Willner I (2007) *Chem Eur J* 13:10168. doi:10.1002/chem.200700806
- Zheng W, Li Q, Su L, Yan Y, Zhang J, Mao L (2006) *Electroanalysis* 18:587. doi:10.1002/elan.200503444
- Zheng W, Zhou HM, Zheng YF, Wang N (2008) *Chem Phys Lett* 457:381. doi:10.1016/j.cplett.2008.04.047
- Gooding JJ (2005) *Electrochim Acta* 50:3049. doi:10.1016/j.electacta.2004.08.052
- Wu G, Xu B (2007) *J Power Sources* 174:148. doi:10.1016/j.jpowsour.2007.08.024
- Park KW, Sung YE, Han S, Yun Y, Hyeon T (2004) *J Phys Chem B* 108:939. doi:10.1021/jp0368031
- Xu T, Yang J, Liu J, Fu Q (2007) *Appl Surf Sci* 253:8945. doi:10.1016/j.apsusc.2007.05.028
- Gong K, Yan Y, Zhang M, Su L, Xiong S, Mao L (2005) *Anal Sci* 21:1383. doi:10.2116/analsci.21.1383
- Xia W, Wang Y, Bergsträßer R, Kundu S, Muhler M (2007) *Appl Surf Sci* 254:247. doi:10.1016/j.apsusc.2007.07.120
- Xu F, Shin W, Brown SH, Wahleithner JA, Sundaram UM, Solomon EI (1996) *Biochim Biophys Acta* 1292:303
- Tsujimura S, Tatsumi H, Ogawa J, Shimizu S, Kano K, Ikeda T (2001) *J Electroanal Chem* 496:69. doi:10.1016/S0022-0728(00)00239-4
- Mao N, Kim H, Zhang Y, Heller A (2002) *J Am Chem Soc* 124:6480. doi:10.1021/ja025874v
- Mao N, Kim H, Heller A (2002) *J Phys Chem B* 106:8842. doi:10.1021/jp025955d
- Christenson A, Shleev S, Mano N, Heller A, Gorton L (2006) *Biochim Biophys Acta* 1757:1634. doi:10.1016/j.bbabioc.2006.08.008
- Wang J (2005) *Electroanalysis* 17:7. doi:10.1002/elan.200403113
- Heller A (1990) *Acc Chem Res* 23:128. doi:10.1021/ar00173a002
- Bard AJ, Faulkner LR (1980) *Electrochemical methods. fundamentals and applications*, Wiley, New York