# An Oxygen Cathode Operating in a Physiological Solution

**ARTICLE** *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · JULY 2002

Impact Factor: 12.11 · DOI: 10.1021/ja025874v · Source: PubMed



**4 AUTHORS**, INCLUDING:

Nicolas Mano
Centre de Recherche Paul Pascal
**119** PUBLICATIONS **3,666** CITATIONS
SEE PROFILE

Hyug-Han Kim
Dankook University
**34** PUBLICATIONS **1,628** CITATIONS
SEE PROFILE

Yongchao Zhang
Morgan State University
**22** PUBLICATIONS **1,508** CITATIONS
SEE PROFILE

J|A|C|S
ARTICLES
Published on Web 05/10/2002

# An Oxygen Cathode Operating in a Physiological Solution

Nicolas Mano, Hyug-Han Kim, Yongchao Zhang, and Adam Heller*

*Contribution from the Department of Chemical Engineering and the Texas Materials Institute, The University of Texas, Austin, Texas 78712*

Received February 8, 2002

***Abstract:*** We report the electroreduction of $O_2$ to water under physiological conditions (pH 7.4, 0.15 M NaCl, 37.5 °C) at a current density of 5 mA $cm^{-2}$ and at a potential only 0.18 V reducing versus that of the reversible $O_2/H_2O$ electrode at pH 7.4. The immobilized electrocatalyst enabling the reduction is the electrostatic adduct of bilirubin oxidase from *Myrothecium verrucaria*, a polyanion at pH >4.1, and the polycationic redox copolymer of polyacrylamide and poly (*N*-vinylimidazole) complexed with [Os (4,4′-dichloro-2,2′-bipyridine)$_2$Cl]$^{+/2+}$, cross-linked on carbon cloth. The current density of the rotating electrodes was $O_2$ transport limited up to 8.8 mA $cm^{-2}$; their kinetic limit was reached at 9.1 mA $cm^{-2}$. The operational life of the electrodes depended on their angular velocity, which defined not only the current density but also the mechanical shear stress stripping the electrocatalyst. When the electrodes were rotated at 300 rpm and were poised at −256 mV versus the potential of the reversible $O_2/H_2O$ electrode, their 2.4 mA $cm^{-2}$ initial current density decreased to 1.3 mA $cm^{-2}$ after 6 days of continuous operation at 37.5 °C.

## Introduction

The four-electron electroreduction of $O_2$ to water under physiological conditions is of interest because miniature biofuel cells[1] with $O_2$ electroreducing cathodes and glucose electrooxidizing anodes[2,3] may power in the future sensors and actuators implanted in the body. They are likely to be smaller than batteries, because unlike batteries they do not require a case and a seal.

The blue, copper-containing oxidases, examples of which include laccases, ascorbate oxidase, and bilirubin oxidase, catalyze the four-electron reduction of $O_2$ to water.[4−8] Among these enzymes, the laccases were the most studied.[9−14] Tarasevich et al. reported that monolayers of laccases on vitreous carbon catalyze the four-electron reduction of $O_2$. The reported electrode operated at pH 5 at a current density of 175 $\mu$A $cm^{-2}$ when poised near the thermodynamic potential of the reversible $O_2/H_2O$ electrode.[15] Tarasevich et al. also reported operation of an electrode made of a composite of high surface area porous carbon and an unidentified laccase at pH 3.5 at −0.2 V relative to the thermodynamic potential at a current density approaching 10 mA $cm^{-2}$. They did not provide, however, enough detail to allow others to reproduce their experiment.[16] Two recent studies[17,18] describe the electroreduction of $O_2$ to water, in the absence of chloride, in a pH 5 citrate buffer at a current density of 5 mA $cm^{-2}$ at −0.13 V versus the reversible potential of the $O_2/H_2O$ electrode at 37.5 °C. The electrocatalyst of these electrodes was a composite of laccase from *Coriolus hirsutus* cross-linked with a redox polymer on a hydrophilic cloth of 10-$\mu$m-diameter carbon fibers. The redox polymer, PVI−Os(tpy)(dme-bpy)$^{2+/3+}$, [poly-*N*-vinylimidazole with one-fifth of the imidazoles complexed with [Os(tpy)(dme-bpy)]$^{2+/3+}$ (tpy = terpyridine; dme-bpy = 4,4′-dimethyl-2,2′-bipyridine)], electrically connected ("wired") the laccase reaction centers to the fibers. A glucose/$O_2$ biofuel cell built with the "wired" *C. hirsutus* laccase cathode had a 5-fold higher power density than earlier glucose/$O_2$ cells.[1]

However, the current density of the "wired" *C. hirsutus* laccase−carbon cloth cathode was very small at the physiological pH of 7.4 and at the physiological chloride concentration of 0.14 M, because the *C. hirsutus* laccase is practically inactive at neutral pH and is inhibited by $Cl^-$. The current density of a

* Author to whom correspondence should be addressed. E-mail: heller@che.utexas.edu.

(1) Chen, T.; Barton, S. C.; Binyamin, G.; Gao, Z.; Zhang, Y.; Kim, H.-H.; Heller, A. *J. Am. Chem. Soc.* **2001**, *123*, 8630−8631.
(2) Katz, E.; Willner, I.; Kotlyar, A. B. *J. Electroanal. Chem.* **1999**, *479*, 64−68.
(3) Katz, E.; Bückmann, A. F.; Willner, I. *J. Am. Chem. Soc.* **2001**, *123*, 10752−10753.
(4) Messerschmidt, A. *Multi-Copper Oxidases*; World Scientific: Singapore, 1997.
(5) Spiro, T. G. *Copper Proteins*; John Wiley and Sons: New York, 1981; Vol. 3.
(6) Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. *Chem. Rev.* **1996**, *96*, 2563−2605.
(7) Solomon, E. I.; Lowery, M. D. *Science* **1993**, *259*, 1575−1581.
(8) Palmore, G. T.; Whitesides, G. M. *ACS Symp. Ser.* **1994**, *No. 566*, 271−290.
(9) Palmore, G. T.; Kim, H.-H. *J. Electroanal. Chem.* **1999**, *464*, 110−117.
(10) Yaropolov, A. I.; Kharybin, A. N.; Emmeus, J.; Marko-Varga, G.; Gorton, L. *Bioelectrochem. Bioenerg.* **1996**, *40*, 49−57.
(11) Santucci, R.; Ferri, T.; Morpurgo, L.; Savini, I.; Avigliano, L. *Biochem. J.* **1998**, *332*, 611−615.
(12) Trudeau, F.; Daigle, F.; Leech, D. *Anal. Chem.* **1997**, *69*, 882−886.
(13) Thuesen, M. H.; Farver, O.; Reinhammar, B.; Ulstrup, J. *Acta Chem. Scand.* **1998**, *52*, 555−562.
(14) Gelo-Pujic, M.; Kim, H.-H.; Butlin, N. G.; Palmore, G. T. *Appl. Environ. Microbiol.* **1999**, *65*, 5515−5521.
(15) Tarasevich, M. R.; Yaropolov, A. I.; Bogdanovskaya, V. A.; Varfolomeev, S. D. *Bioelectrochem. Bioenerg.* **1979**, *6*, 393−403.
(16) Tarasevich, M. R.; Bogdanovskaya, V. A.; Gavrilova, E. F.; Orlov, S. B. J. *J. Electroanal. Chem. Interfacial Electrochem.* **1986**, *206*, 217−227.
(17) Barton, S. C.; Kim, H.-H.; Binyamin, G.; Zhang, Y.; Heller, A. *J. Phys. Chem.* **2001**, *105*, 11917−11921.
(18) Barton, S. C.; Kim, H.-H.; Binyamin, G.; Zhang, Y.; Heller, A. *J. Am. Chem. Soc.* **2001**, *123*, 5802−5803.


**10.1021/ja025874v CCC: $22.00** 

smooth vitreous carbon electrode, made by "wiring" the laccase from *Pleurotus ostreatus*, nevertheless reached ~100 $\mu$A·cm$^{-2}$ at a potential 0.19 V reducing relative to the reversible potential at pH 7 in phosphate-buffered 0.15 M NaCl.[19] The projected current density for this electrode, were it made as a carbon cloth composite, would have been ~0.3 mA cm$^{-2}$.

Tsujimura et al. recently described[29] a carbon felt cathode coated with bilirubin oxidase from *Myrothecium verrucaria*, an enzyme used in the clinical assay[20−23] of serum bilirubin. The enzyme catalyzes the oxidation of bilirubin to biliverdin (eq 1)

$$\text{bilirubin} + {}^1/_2O_2 \rightarrow \text{biliverdin} + H_2O \qquad (1)$$

and then to a yet unidentified purple pigment.[24−28] The electrode poised at −0.17 V versus the reversible potential of the $O_2$/$H_2O$ electrode operated at 0.5 mA cm$^{-2}$ only for 2 h in a pH 7 phosphate-buffered 0.1 M KCl solution.[29]

Bilirubin oxidase (BOD) is a monomeric protein with a molecular mass of 60 kDa. Parts of its sequence are homologous with those of other multicopper oxidases, such as laccases,[30] ascorbate oxidase,[31] and ceruloplasmine[32] having a copper-binding His−Cys−His[33] triad. The copper ions of these enzymes are classified into three types by their optical and magnetic properties.[4] Type I (blue) copper ions have a characteristic Cys to Cu (II) charge-transfer band near 600 nm. The type I copper center accepts electrons from the electron-donating substrate of the enzyme and relays these to the $O_2$ reduction site. The latter is a trinuclear cluster, consisting of a type II copper ion and a type III pair of cupric ions with a characteristic 330-nm shoulder.[4,5] Shimizu et al.[33,34] has shown that BOD is also a multicopper oxidase, containing one type I, one type II, and two type III copper ions.

Here we describe persistent four-electron electroreduction of $O_2$ to water under physiological conditions at previously unattained current densities and potentials. The reaction is catalyzed by the electrostatic adduct of BOD and a redox polymer tailored to donate protons and mediate the transport of electrons from the electrode to the enzyme.

## Experimental Section

**Chemicals and Materials.** Bilirubin oxidase (EC 1.3.3.5) from *M. verrucaria*, catalase from bovine liver (EC 1.11.1.6), uric acid (sodium salt), L-sodium ascorbate, 4-acetaminophen, $NaIO_4$, NaCl, NaOH, KCNS, KBr, and NaF were purchased from Sigma (St. Louis, MO). Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) was purchased from Polysciences Inc. (Warrington, PA). A fresh solution of BOD in pH 7.4 20 mM phosphate buffer (PB) was prepared daily. The uric acid was dissolved in dilute NaOH and then neutralized with dilute $H_3PO_4$ to yield a 10 mM aqueous solution.[35] The electrochemical measurements were performed in PBS (pH 7.4 20 mM phosphate-buffered 0.15 M NaCl) except in the experiments where the pH and anion dependences of the steady-state electroreduction of $O_2$ were studied. In these, borate, citrate, acetate, phosphate, and Tris buffers were employed. All solutions were made with deionized water that was passed through a purification train (Sybron Chemicals Inc., Pittsburgh, PA). Carbon cloth (Toray TGPH-030) was received, as a sample, from E-TEK (Somerset, NJ). Ultrapure $O_2$ and argon were purchased from Matheson (Austin, TX).

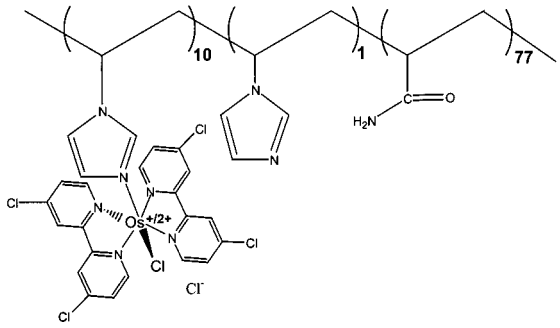


***Figure 1.*** Structure of the bilirubin oxidase "wiring" redox polycation PAA−PVI−[Os(dcl-bpy)$_2$Cl]$^{+/2+}$.

**Synthesis of the Redox Polymer PAA−PVI−[Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl]$^{+/2+}$.** 4,4′-Dinitro-2,2′-bipyridine *N*,*N*′-dioxide was prepared as described.[36,37] 4,4′-dichloro-2,2′-bipyridine (dcl-bpy) was synthesized from 4,4′-dinitro-2,2′-bipyridine *N*,*N*′-dioxide by modifying the procedure of Maerker et al.[36,38] Os(dcl-bpy)$_2$Cl$_2$ was prepared as follows: $(NH_4)_2OsCl_6$ and ″dcl-bpy were dissolved in ethylene glycol in a 1:2 molar ratio and refluxed under argon for 1 h (yield 85%).[39] The Os(dcl-bpy)$_2$Cl$_2$ was then complexed with the 1:7 polyacrylamide−poly(*N*-vinylimidazole) (PAA−PVI) copolymer and purified as described.[39] Figure 1 shows the structure and the stoichiometry of the PAA−PVI−[Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl]$^{+/2+}$ redox polymer.

**Electrodes.** The carbon cloth electrodes were made by the reported three-step procedure.[17] Their substrates were 3-mm-diameter vitreous carbon electrodes mounted in Teflon sleeves. In the first step, the substrates were polished with 0.05-$\mu$m $Al_2O_3$ powder (Buehler, Lake Bluff, IL) rinsed and sonicated for 10 min in ultrapure water. The polishing step was repeated until no voltammetric features beyond water oxidation were observed in a 50 mV s$^{-1}$ scan in PBS through the 0.2- and 1-V range. The electrodes were then dried in an air stream. In the second step, the 350-$\mu$m-thick carbon cloth (nominal 78% void fraction, composed of 10-$\mu$m-diameter fibers) was cut into 4-mm-diameter disks. These were cemented, using conductive carbon paint (SPI, West Chester, PA), to the surface of the substrate electrodes. The substrate-bound cloth was made hydrophilic by exposure to a 1 Torr $O_2$ plasma for 5 min.[40] The rotating ring−disk electrodes (RRDE), with 3-mm-diameter vitreous carbon disks and platinum rings were similarly made. In these, the platinum electrodes were cycled in 0.5 M $H_2SO_4$ until the

(19) Barton, S. C.; Pickard, M.; Vasquez-Duhalf, R.; Heller, A. *Biosens. Bioelectron.* **2002**, *00*, 00−00.
(20) Andreu, Y.; Galban, J.; De Marcos, S.; Castillo, J. R. *Fresenius J. Anal. Chem.* **2000**, *368*, 516−521.
(21) Doumas, B. T.; Wu, T. W.; Poon, K. C. P.; Jendrzejczak, B. *Clin. Chem.* **1985**, *31*, 1677−1682.
(22) Lavin, A.; Sung, C.; Klibanov, A. M.; Langer, R. *Science* **1985**, *230*, 543−545.
(23) Kosaka, A.; Yamamoto, C.; Morisita, C.; Nakane, K. *Clin. Biochem.* **1987**, *20*, 451−458.
(24) Tanaka, N.; Murao, S. *Agric. Biol. Chem.* **1983**, *47*, 1627−1628.
(25) Murao, S.; Tanaka, N. *Agric. Biol. Chem.* **1982**, *46*, 2031−2034.
(26) Tanaka, N.; Murao, S. *Agric. Biol. Chem.* **1982**, *46*, 2499−2503.
(27) Tanaka, N.; Murao, S. *Agric. Biol. Chem.* **1985**, *49*, 843−844.
(28) Koikeda, S.; Ando, K.; Kaji, H.; Inoue, T.; Murao, S.; Takeuchi, K.; Samejima, T. *J. Biol. Chem* **1993**, *268*, 18801−18809.
(29) Tsujimura, S.; Tatsumi, H.; Ogawa, J.; Shimizu, S.; Kano, K.; Ikeda, T. *J. Electroanal. Chem.* **2001**, *496*, 69−75.
(30) Ducos, V.; Brzowski, A. M.; Wilson, K. S.; Brown, S. H.; Ostergaard, P.; Schneider, P.; Yaver, D. S.; Pedersen, A. H.; Davies, G. J. *Nat. Struct. Biol.* **1998**, *5*, 310−316.
(31) Messerschmidt, A.; Ladenstein, R.; Huber, R. *J. Mol. Biol.* **1992**, *224*, 179−205.
(32) Zaitseva, I.; Zaitsev, V.; Card, G.; Moshkov, K.; Bax, B.; Ralph, A.; Lindley, P. *J. Biol. Inorg. Chem.* **1996**, *1*, 15−23.
(33) Shimizu, A.; Kwon, J. H.; Sasaki, T.; Satoh, T.; Sakurai, N.; Sakurai, T.; Yamaguchi, S.; Samejima, T. *Biochemistry* **1999**, *38*, 3034−3042.
(34) Shimizu, A.; Sasaki, T.; Kwon, J. H.; Odaka, A.; Satoh, T.; Sakurai, N.; Sakurai, T.; Yamaguchi, S.; Samejima, T. *J. Biochem.* **1999**, *125*, 662−668.
(35) Binyamin, G.; Chen, T.; Heller, A. *J. Electroanal. Chem.* **2001**, *500*, 604−611.
(36) Anderson, S.; Constable, E. C.; Seddon, K. R.; Turp, E. T.; Baggott, J. E.; Pilling, J. *J. Chem. Soc., Dalton Trans.* **1985**, 2247−2250.
(37) Kenausis, G.; Taylor, C.; Rajagopalan, R.; Heller, A. *J. Chem. Soc., Faraday Trans.* **1996**, *92*, 4131−4135.
(38) Maerker, G.; Case, F. H. *J. Am. Chem. Soc.* **1958**, *80*, 2475−2477.
(39) Zakeeruddin, S. M.; D. M. Fraser, D. M.; Nazeeruddin, M.-K.; Gratzel, M. *J. Electroanal. Chem.* **1992**, *337*, 253−256.
(40) Sayka, A.; Eberhart, J. G. *Solid State Technol.* **1989**, *32*, 69−70.

voltamogramms showed the characteristics of a clean platinum electrode. In the third step, the electrocatalyst was deposited on the carbon cloth. The deposition solution consisted of 10 $\mu$L of 10 mg mL$^{-1}$ aqueous redox polymer solution, 2 $\mu$L of PB, 2 $\mu$L of 46 mg mL$^{-1}$ BOD in PB, and 2 $\mu$L of 7 mg mL$^{-1}$ PEGDGE in water. A 5-$\mu$L aliquot of the mixed solution was pipetted onto the mounted hydrophilic carbon cloth, which was promptly wetted and penetrated by the solution. The electrodes were cured for at least 18 h at room temperature before they were used.

**Instrumentation and Cell.** The measurements were performed using a bipotentiostat (CH-Instruments, electrochemical detector model CHI832) and a dedicated computer. The temperature was controlled with an isothermal circulator (Fisher Scientific, Pittsburgh, PA). The dissolved $O_2$ concentration was monitored with an $O_2$ electrode purchased from BAS (West Lafayette, IN). The electrodes were rotated using a Pine Instruments rotator (Austin, TX). The measurements were carried out in a water-jacketed electrochemical cell at 37.5 °C containing 50 mL of PBS. At the start of the experiments, argon was bubbled through the solution for a least 15 min, followed by oxygen. To maintain a fixed volume of solution in the cell, the bubbled gases were presaturated with water by passage through a bubbler, which also contained PBS. The potentials were measured versus a commercial Ag/AgCl (3 M KCl) reference electrode. The counter electrode was a platinum wire (BAS). In the coulometric measurements, the scan rate was 1 mV s$^{-1}$.

**BOD Assay.** The absorption spectra of the BOD solutions were measured at 25 °C with an Agilent 8453 UV−visible spectrophotometer following the procedure of Hirose.[41] The concentration of BOD was calculated using its reported molar absorption coefficient of 3870 M$^{-1}$ cm$^{-1}$ at 610 nm.[41,42]

## Results

The open circuit potential of a vitreous carbon electrode on which BOD (without redox polymer) was adsorbed was +360 mV versus Ag/AgCl in pH 7.4 PBS under 1 atm $O_2$ at 37.5 °C or −196 mV versus the potential of the reversible $O_2/H_2O$ under the same conditions. The open circuit potential of a similar electrode, but with a thin adsorbed film of the electrostatic adduct of the redox polymer and BOD, was remarkably higher, +530 mV versus Ag/AgCl, only −26 mV versus the potential of the reversible $O_2$ /$H_2O$ electrode. No spectroscopic evidence was found for complex formation between the copper ions of BOD and the polymer: When the water-soluble copolymer of acrylamide and *N*-vinylimidazole was added to the BOD solution in PB, the spectrum of the enzyme did not change.

Figure 2 shows the cyclic voltammogram of the composite "wired" BOD-coated carbon cloth electrode under argon at 50 mV s$^{-1}$ in PBS buffer at 37.5 °C. The coating of the electrode consisted of 44.6 wt % BOD, 48.5 wt % polymer, and 6.9 wt % PEDGE. Its voltammogram is characteristic of a polymer-bound osmium complex with an apparent redox potential of +350 mV versus Ag/AgCl. At 1 mV s$^{-1}$ scan rate, the voltammogram exhibited a symmetrical wave, with only slight separation of the oxidation and reduction peaks ($\Delta E_P = 5$ mV). At 50 mV s$^{-1}$ the separation was substantially increased ($\Delta E_P = 53$ mV). The width of the peaks at half-height, $E_{whm}$, was 90 mV, close to the theoretical width of 90.6 mV for an ideal Nernstian one-electron-transfer reaction. In contrast with wired laccases, where $\Delta E_p$ and $E_{whm}$ increased with the PEDGE weight fraction in the catalyst, $\Delta E_p$ and $E_{whm}$ did not vary with the PEGDGE weight fraction in the "wired" BOD electrodes. As will be shown below, the kinetic limit of their current density depended, however, on the weight fraction of the PEGDGE cross-linker. Upon 4-h cycling of the potential at 50 mV s$^{-1}$ between 0.0 and +0.5 V versus Ag/AgCl, the heights of the voltammetric peaks of the electrodes rotating at 1000 rpm decreased only by less than 5%, at 37.5 °C.

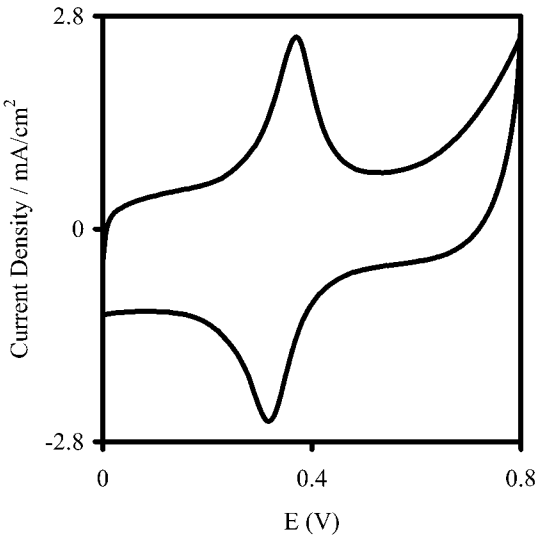


***Figure 2.*** Cyclic voltammogram of the "wired" bilirubin oxidase-coated carbon cloth electrode under argon. PBS (0.15 M NaCl, pH 7.4; 20 mM phosphate) buffer, 37.5 °C, and 50 mV s$^{-1}$ scan rate. Total loading, of the 44.6 wt % BOD, 48.5 wt % redox polymer, 6.9 wt % PEGDGE cross-linker composite, 0.6 mg cm$^{-2}$. Current densities based on the 0.126-cm$^2$ geometrical area of the 4-mm-diameter carbon cloth disk.

The reproducibility of the $O_2$ electroreduction currents of the electrodes rotating at 1000 rpm was ±10% or better both for different batches ($n = 4$) and within batches ($n = 12$). When the electrodes were rotated at 4000 rpm, the reproducibility was only ±28% between the batches and ±20% within the batches.

Because two-electron BOD-catalyzed electroreduction of $O_2$ to $H_2O_2$[44,45] might compete with the desired four-electron reduction, $H_2O_2$ production was tested for using RRDEs having "wired" BOD carbon cloth disks and platinum rings. If $H_2O_2$ were produced on the disk, it would have been detected at the ring poised at +0.750 mV versus Ag/AgCl, the $H_2O_2$ being electrooxidized to $O_2$ on the platinum ring at this potential. (A similar experiment was performed to investigate the formation of $H_2O_2$ during the electroreduction of $O_2$ on disks of RRDE electrodes modified with cytochrome *c* and analogs.[46−49]).

When $O_2$ was electroreduced on the disk at increasing current densities between 0 and 5 mA cm$^{-2}$, the ring current remained unchanged and was negligibly small (Figure 3A), showing that $H_2O_2$ was not produced on the disk. Furthermore, catalase,

(41) Hirose, J.; Inoue, T.; Sakuragi, H.; Kikkawa, M.; Minakami, M.; Morikawa, T.; Iwamoto, H.; Hiromi, K. *Inorg. Chim. Acta* **1998**, *273*, 204−212.
(42) Hiromi, K.; Yamaguchi, S.; Sugiura, Y.; Iwamoto, H.; Hirose, J. *Biosci. Biochem.* **1992**, *56*, 1349−1352.
(43) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods. Fundamentals and Applications*; John Wiley and Sons: New York, 2001.
(44) Shoham, B.; Migron, Y.; Riklin, A.; Willner, I.; Tartakovsky, B. *Biosens. Bioelectron.* **1995**, *10*, 341−352.
(45) Wang, J.; Ozsoz, M. *Electroanalysis* **1990**, *2*, 647−650.
(46) Collman, J. P.; Fu, L.; Herrmann, P. C.; Zhang, X. *Science* **1997**, *275*, 949−951.
(47) Collman, J. P.; Fu, L.; Herrmann, P. C.; Wang, Z.; Rapta, M.; Broring, M.; Schwenninger, R.; Boitrel, B. *Angew. Chem., Int. Ed. Engl.* **1998**, *1998*, 3397−3400.
(48) Collman, J. P.; Rapta, M.; Broring, M.; Raptova, L.; Schwenninger, R.; Boitrel, B.; Fu, L.; L'Her, M. *J. Am. Chem. Soc.* **1999**, *121*, 1387−1388.
(49) Pardo-Yissar, V.; Katz, E.; Willner, I.; Kotlyar, A. B.; Sanders, C.; Lill, H. *Faraday Discuss.* **2000**, *116*, 119−134.

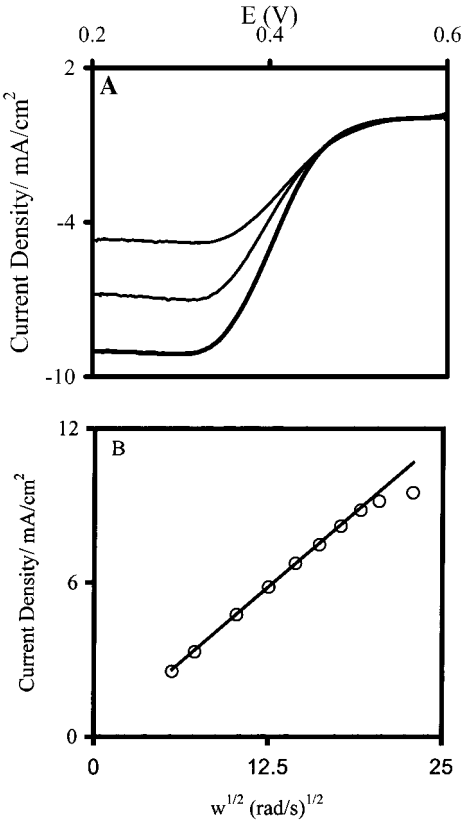


***Figure 3.*** (A) Polarization of the cathode under 1 atm $O_2$, rotating at 1000 (top curve), 2300 (middle curve), and 4000 rpm (bottom curve), in PBS buffer at 37.5 °C. Scan rate, 1 mV $s^{-1}$. Other conditions as in Figure 2. (B) Current density versus the square root of the angular velocity ($\omega^{1/2}$) for the electrode poised at +300 mV vs Ag/AgCl in PBS at 37.5 °C (open circles) and the current density calculated by Levich equation (solid line) (see text). Conditions, other than angular velocities and the 1 atm $O_2$ pressure, as in Figure 2.

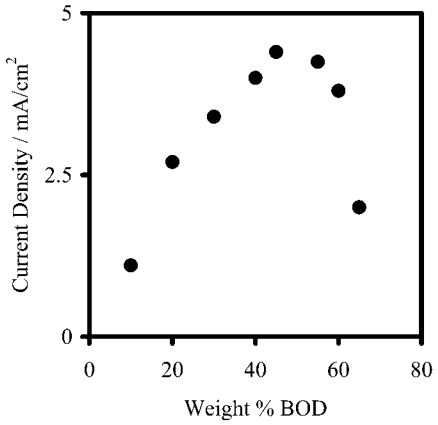


***Figure 4.*** Dependence of the current density on the BOD enzyme weight percentage. Electrode poised at +300 mV vs Ag/AgCl, 1 mV $s^{-1}$ scan rate, 1000 rpm, and 1 atm $O_2$. Other conditions as in Figure 2.

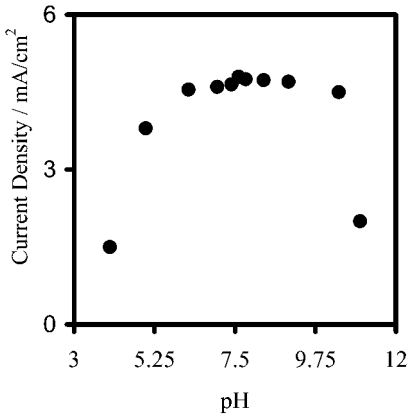


***Figure 5.*** pH dependence of the steady-state current density under 1 atm $O_2$ for the electrode poised at +300 mV vs Ag/AgCl, 1000 rpm. Other conditions as in Figure 2.

which would have decomposed any $H_2O_2$ that might have been produced into $O_2$, had no effect on the ring current, confirming that $H_2O_2$ was not a byproduct of the electroreduction of $O_2$ to water. After 10 h of $O_2$ electroreduction at ~1 mA current, the 50-mL solution of the cell tested negative for $H_2O_2$ in a colorimetric ABTS−horseradish peroxidase test that would have detected 1 $\mu$M $H_2O_2$.

Figure 3B shows the dependence of the current density on the square root of the angular velocity of the rotating electrode, $\omega^{1/2}$. Up to 3500 rpm, where the current density reached 8.8 mA $cm^{-2}$, the current increased linearly with $\omega^{1/2}$ in accordance with the Levich equation, showing that it was $O_2$ transport-limited under 1 atm $O_2$ when the electrode was poised at +300 mV versus Ag/AgCl. Above 3500 rpm, the current continued to increase with $\omega^{1/2}$, but no longer linearly, until it reached the kinetic limit of 9.1 mA $cm^{-2}$.

The optimal composition of the electrocatalyst was determined for electrodes rotating at 1000 rpm and poised at +300 mV versus Ag/AgCl. In the first group of experiments, the cross-linker (PEDGE) was fixed at 6.9 wt %, and the total loading of all film components was fixed at 0.6 mg $cm^{-2}$. Figure 4 shows the dependence of the current density of BOD through the 20−70 wt % range. From 20 to 45 wt %, the current density increased with the weight percentage of BOD, reaching 4.6 mA $cm^{-2}$ at 45.2 wt %. Above 50 wt % BOD, the current density declined rapidly. At 60 wt % BOD, precipitation was observed. The precipitation is attributed to the formation of a charge-neutral electrostatic adduct between the cationic polymer and the anionic enzyme (p$I$ = 4.1). In experiments where the weight percentage of the cross-linker was varied, while the BOD/redox polymer weight ratio was fixed at 1:1, the optimal PEGDGE weight percentage was found to be 6.9. The resulting optimal catalyst was composed of 44.6 wt % BOD, 48.5 wt % polymer, and 6.9 wt % PEDGE at 0.6 mg $cm^{-2}$ total loading. The effect of the total loading on the kinetic limit of the catalytic current was not optimized, because the optimum would have varied with the ratios of the components.

Unlike in the cases of wired glucose oxidase[50−52] and of wired laccases,[19] cross-linking by periodate oxidation of the BOD oligosaccharides did not lead to useful wired BOD electrodes, even though BOD contains 6.3 wt % carbohydrate.[6]

The pH dependence of the steady-state current density of $O_2$ electroreduction was measured with the electrode poised at +300 mV versus Ag/AgCl in 0.15 M NaCl while the electrode rotated at 1000 rpm. Phosphate, borate, citrate, or Tris was added at 20 mM concentration to maintain the desired pH. The dependence of the current on the pH is shown in Figure 5. As seen in the figure, the current density increased with pH until

(50) Binyamin, G.; Heller, A. *J. Electrochem. Soc.* **1999**, *146*, 2965−2967.
(51) Vreeke, M.; Maidan, R.; Heller, A. *Anal. Chem.* **1992**, *64*, 3084−3090.
(52) Binyamin, G.; Cole, J.; Heller, A. *J. Electrochem. Soc.* **2000**, *147*, 2780−2783.

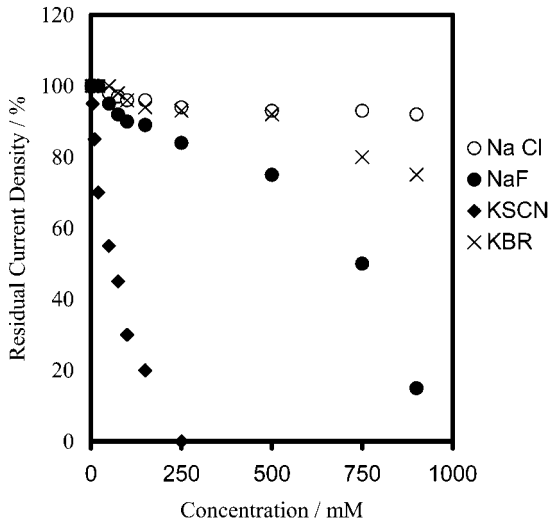


***Figure 6.*** Dependence of the current density on the concentrations of different anions under 1 atm $O_2$ for the electrode poised at +300 mV vs Ag/AgCl, 1000 rpm. Other conditions as in Figure 2.

it reached a plateau at pH 7.5 and then declined above pH 10.5. In the pH 6−10.5 range, the current density was nearly independent of pH, varying by less than ±10%. Up to pH 9, there was no irreversible change in the current characteristics; above pH 10.5, the drop in the current density was irreversible.

Because copper-binding anions, particularly halide anions, inhibit the laccases and because blood contains 0.14 M chloride, the effect of added anions was investigated. In these experiments, the electrode rotated at 1000 rpm and was poised at + 300 mV versus Ag/AgCl in a pH 7.4, 20 mM phosphate buffer solution. The results are summarized in Figure 6. The current density was nearly independent of the concentration of chloride, added as NaCl, through the 0−1 M range. Above 1.5 M NaCl concentrations, a decline in the current density was, nevertheless, observed. When bromide was added, the current density declined monotonically as the KBr concentration was raised from 0 and 1 M. At 1 M KBr, the current density was about 25% lower than in the absence of bromide. A steeper decline was again observed at >1.5 M KBr concentration. Adding of fluoride (as NaF) inhibited the electroreduction of $O_2$, about 30% of the current density being lost when the concentration of fluoride was raised from 0 to 0.5 M and about 90% being lost when the concentration was further raised to 1 M. Thiocyanate, added as KCNS, strongly inhibited the electroreduction, the current density dropping to 0 already at 0.25 M KCNS. The maximum current density was about the same when the solutions were buffered at pH 7 with Tris, acetate, or phosphate.

Figure 7 shows the temperature dependence of the current density of the electrode poised at +300 mV versus Ag/AgCl, rotating at 1000 rpm in PBS under 1 atm $O_2$. The current density increased with temperature up to 60 °C and then declined rapidly. The increase was reversible only up to 50 °C, the enzyme being denatured at higher temperatures. The Arrhenius plot (not shown) of the temperature dependence yielded an activation energy of $E_{act} = 28.2$ kJ mol$^{-1}$. The observed activation energy for the denaturing of the enzyme was 77 kJ mol$^{-1}$.

When rotating at 4000 rpm, where the shear stress on the rim on the fiber cloth was 0.7 N m$^{-2}$, the electrodes were mechanically unstable. For this reason, their stability was tested

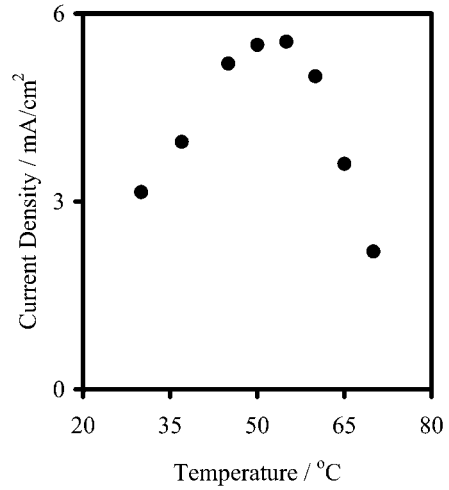


***Figure 7.*** Temperature dependence of the current density. 1 atm $O_2$, electrode poised at +300 mV vs Ag/AgCl, 1000 rpm. Other conditions as in Figure 2.

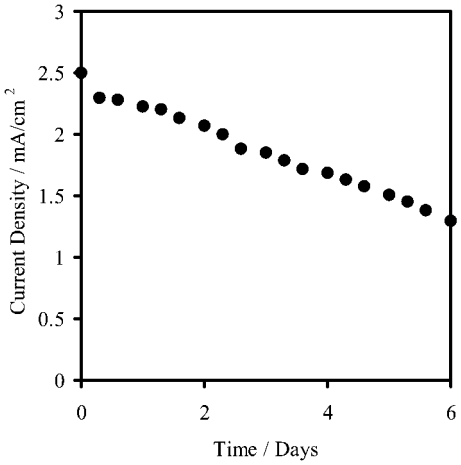


***Figure 8.*** Stability of the 'wired' enzyme carbon cloth electrode poised at +300 mV vs Ag/AgCl under 1 atm $O_2$, 300 rpm. Other conditions as in Figure 2.

at 300 rpm, where the current was $O_2$ transport limited. At 300 rpm, the shear stress acting on the rim of the 4-mm-diameter electrode was $1.4 \times 10^{-2}$ N m$^{-2}$. The time dependence of the current density of an electrode poised at + 300 mV versus Ag/AgCl rotating at 300 rpm in PBS under 1 atm $O_2$ at 37.5 °C is shown in Figure 8. In the first 6 days of operation, the current dropped by less than 10% per day: The initial 2.4 mA cm$^{-2}$ current density dropped to 1.3 mA cm$^{-2}$ after 6 days of continuous operation. After storage of the dry electrodes for 1 month at 4 °C under air, 95 ± 3% of the initial current density was retained. The presence of electrooxidizable blood constituents did not harm the electrodes. A transient 5% increase in the current density was observed when either 0.1 mM ascorbate or 0.1 mM ascorbate and 0.17 mM acetaminophen was added, and a transient 25% increase in current density was observed with 0.48 mM urate in the solution.

## Discussion

**Potentials.** Xu et al. measured spectrophotometrically the redox potential of an aerated pH 5.3 solution of recombinant *M. verrucaria* BOD at 20 °C, reporting +260 mV versus Ag/AgCl or −440 mV relative to the potential of the reversible

$O_2/H_2O$ electrode at the same pH.[53] In pH 7.8 50 mM Tris buffer, Shimizu et al. reported a potential of +373 mV versus Ag/AgCl[34] at ambient temperature, −174 mV versus the reversible $O_2/H_2O$ electrode potential at this pH. The open circuit potential of the $O_2$ electrode made by adsorbing BOD on vitreous carbon was +360 mV versus Ag/AgCl under 1 atm $O_2$ at 37.5 °C and at pH 7.4, consistent with the latter value. This potential was −196 mV versus that of the reversible $O_2/H_2O$ electrode under these conditions. The open circuit potential of the "wired" BOD electrode, made by cross-linking the electrostatic adduct of BOD, a polyanion above pH 4.1, and the redox polycation of Figure 1 on carbon cloth, was +530 mV versus Ag/AgCl, only 26 mV below that of the reversible $O_2/H_2O$ electrode, at pH 7.4 under 1 atm $O_2$ and at 37.5 °C. The cause of the remarkable increase in potential upon wiring the BOD is not known. The redox potential of the PAA−PVI−[Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl]$^{+/2+}$ "wire" (Figure 1) was +350 mV versus Ag/AgCl or −180 mV versus the pH 7.4 open circuit potential of the "wired" BOD electrode under 1 atm $O_2$.

**Voltammetric Characteristics.** The slight separation of the voltammetric peak heights at 1 mV $s^{-1}$ scan rate is indicative of a reversible surface-bound couple. The linear dependence of the peak heights on the scan rate also shows that the redox couples of the polymer are surface-confined.[43] The voltammetric peaks were narrower and less separated than those in the earlier PVI−Os(tpy)(dme-bpy)$^{2+/3}$-based laccase electrode, where $\Delta E_p$ was 120 mV.[17] The difference is attributed to faster charge transport through the "wire" of this study. Repetitive cycling over a 4-h period at 37.5 °C did not change the shape of the voltammograms of the electrodes rotating at 1000 rpm, and their peak currents decreased only by less than 5%.

**Reproducibility.** When the electrodes rotated at 1000 rpm, the apparent batch-to-batch reproducibility, as well as the within-the batch reproducibility, of the $O_2$ electroreduction currents was better than ±10%. This reproducibility does not necessarily imply that the composite catalysts were identical within ±10%, because at 1000 rpm the current was limited by $O_2$ transport, not by the kinetics of the electrocatalyst. When the electrodes were rotated at 4000 rpm, where the currents were already limited by the kinetics of the catalytic films, but the electrodes were already mechanically unstable, the reproducibility was only ±28% between the batches and ± 20% within the batches.

**Composition.** The optimal enzyme-to-polymer weight ratio was near 1:1 (Figure 4). At a lower weight fraction of enzyme, the current was limited by the BOD-catalyzed rate of $O_2$ reduction. When the weight fraction of enzyme was higher, the current was limited by the electronic resistance of the film, the redox polymer being an electron conductor and the enzyme an insulator.[54,55] Above 60 wt % BOD, where the electrostatic adduct precipitated, the increased resistance resulted in increased separation of the voltammetric peaks. At the optimal weight ratio, the upper limits of three currents, of the flow of electrons from the electrode to the redox polymer, from the redox polymer to BOD, and from BOD to $O_2$ are equal. The optimal ratio decreases when the maximum turnover rate of the enzyme is higher and decreases when electrons diffuse more rapidly through the redox polymer. The ratio also depends on the nature of the bond between the wire and the enzyme, which determines the maximum electron current from the redox polymer to BOD. In the absence of bonding, the BOD and the redox polymer would phase-separate, the entropy of mixing of two macromolecules being too low prevent their separation. As discussed above, intimate contact between the electrostatically bound BOD and its wire lowers the kinetic resistance for electron transfer from the wire to BOD.

Cross-linking reduces the segmental mobility on which the electron conduction in the redox polymer films depends. Because electrons diffuse as they are transferred when reduced and oxidized redox centers collide, the highest electron diffusivities are being reached when the film is not cross-linked.[56] However, in absence of cross-linking the films dissolve, and when inadequately cross-linked, they are sheared off the rotating electrodes.[50] With 6.9 wt % PEDGE cross-linker, the films were mechanically stable at 300 rpm where the maximum shear stress at the rims of the electrodes was $1.4 \times 10^{-2}$ N $m^{-2}$. At this angular velocity, the current density was 2.4 mA $cm^{-2}$.

**Polarization.** Electroreduction of $O_2$ started at the open circuit potential of the wired BOD electrode, +530 mV versus Ag/AgCl. When the electrode rotated at 4000 rpm where, as will be shown below, the current was no longer $O_2$ transport limited, the current density was 6.8 mA $cm^{-2}$ at +380 mV versus Ag/AgCl (−176 mV versus the potential of the reversible $O_2/H_2O$ electrode at the same pH) and the current density reached 9.1 mA $cm^{-2}$ at +300 mV versus Ag/AgCl, −276 mV versus the reversible potential. The value of 6.8 mA $cm^{-2}$ at −176 mV versus the potential of the reversible $O_2/H_2O$ electrode represents a greater than 13-fold increase over current density reported by Tsujimara et al.,[29] whose solution was not a physiological buffer, and whose cathodes operated only for a few hours.

**Mass Transport and Kinetics.** The Levich−Koutecky plot[57] for electrode rotating at an angular velocity $\omega$ (Figure 3B) shows that the Levich equation $I = 0.62nFAcD^{2/3}\nu^{-1/6}\omega^{1/2}\omega^{1/2}$ is rigorously obeyed up to 3500 rpm, where the current density is 8.8 mA $cm^{-2}$. The correspondence between the measured (circles) and the predicted currents (solid line) is exact for the $O_2$ diffusivity $D = 2.4 \times 10^{-5}$ $cm^2$ $s^{-1}$, the $O_2$ solubility $c = 1.07 \times 10^{-6}$ mol $cm^{-3}$, and the kinematic viscosity $\nu = 0.091$ $cm^2$ $s^{-1}$ of the 0.15 M NaCl solution at 37.5 °C, when $n$, the number of electrons electroreducing the $O_2$, equals 4 and the area $A$ is 0.126 $cm^2$.[58−60] Above 3500 rpm, the current is controlled by the kinetics of the electrocatalytic reaction. When the electrode rotates at 4000 rpm and the current is kinetically controlled, the measured Tafel slope is −122 mV/decade, the theoretical slope for $O_2$ electroreduction at 37.5 °C.[61]

**Absence of Inhibition in Physiological Solution.** The pH dependence of the current density of the "wired" BOD electrode differed from the pH dependence of the maximal turnover rate $V_{max}$ of the dissolved enzyme. When the enzyme is dissolved,

(53) Xu, F.; Shin, W.; Brown, S. H.; Walhleithner, J. A.; Sundaram, U. M.; Solomon, E. I. *Biochim. Biophys. Acta* **1996**, *1292*, 303−311.
(54) Ohara, T. J.; Rajagopolan, R.; Heller, A. *Anal. Chem.* **1993**, *65*, 3512−3517.
(55) Ohara, T. J.; Rajagopolan, R.; Heller, A. *Anal. Chem.* **1994**, *66*, 2451−2457.
(56) Aoki, A.; Rajagopalan, R.; Heller, A. *J. Phys. Chem.* **1995**, *99*, 5102−5105.
(57) Bard, A. J.; Faulkner, L. R. *Electrochemical methods: Fundamentals and applications*, 2nd ed.; John Wiley and Sons: New York, 2001; p 341.
(58) Battino, R. *Oxygen and Ozone*, Solubility data series (Pergamon, Oxford, 1981), vol. 7.
(59) Lobo, V. M. M. *Handbook of electrolyte solutions* (Elsevier, Amsterdam, 1989).
(60) Weast, R. C. *Handbook of Chemistry and Physics* (CRC Press, Boca Raton, 1986).
(61) Bard, A. J.; Faulkner, L. R. *Electrochemical methods: Fundamentals and applications*, 2nd ed.; John Wiley and Sons: New York, 2001; p 103.

$V_{max}$ was high only through the pH 6−8 range. $V_{max}$ declined rapidly at both higher and lower pH, only one-third of $V_{max}$ being retained at pH 9.[26] In contrast, the "wired" BOD electrode retained its high current density through the pH 6−10 range. Importantly, the variation of the current was negligibly small within the pH 7.35−7.45 range of normal human arterial blood (Figure 5). Above pH 10.5, the electrode was irreversibly damaged because of enzyme denaturation. The observed damage above pH 10.5 is consistent with results of Samejima et al. who found, by tracking the circular dichroism as a function of pH, that the enzyme is denatured upon ionization of its tyrosine residues.[62] The voltammetric characteristics of the redox polymer did not change up to pH 11.

Laccases are strongly inhibited by halide anions,[63−65] including the chloride anion, because these bind with their catalytic type II Cu centers and prevent electron transfer from their type I Cu to their type III Cu clusters.[26,41] The current density of the "wired" laccase electrodes declined at pH 5 by 60% when the chloride concentration was raised from 0 to 0.1 M.[19] In contrast, the current density of the "wired" BOD electrode declined only by 6%. The loss remained small even in 1 M NaCl at pH 7.4 (Figure 6). Other copper-binding anions inhibited the electroreduction of $O_2$, the inhibition declining in the order $CNS^- \gg F^- \gg Br^- > Cl^-$ (Figure 6). This order is only in partial agreement with the order $CNS^- > F^- \gg Cl^- > Br^-$ of solution-phase inhibition of the free enzyme reported by Hirose et al.[41] for BOD and by Xu et al.[53,63] for laccases. Significantly, the inhibition of the "wired" BOD cathode by chloride at its 0.096−0.106 M concentration in normal human blood was so small that physiological variation in chloride concentration will not affect the $O_2$ electroreduction current. The electrode differed in this respect from the dissolved BOD-based cathode of Tsujimura et al.,[29] the current of which depended strongly on the concentration of KCl in the 0−0.1 M range. The observed partial loss of $O_2$ electroreduction current at very high chloride concentration (>1.5 M NaCl) is not attributed to specific binding of chloride to copper centers of BOD. Its likely cause is the screening of the charges of the electrostatically bound polycationic and polyanionic segments of the cross-linked redox polymer−BOD adduct at high ionic strength, causing their dissociation and making the "wiring" of the enzyme ineffective.[54,55,66]

**Temperature Dependence.** The $O_2$ electroreduction current increased with the temperature up to 60 °C when the rate of increase was about 10 °C $h^{-1}$ and declined rapidly when the temperature exceeded 60 °C. The decline is attributed to the denaturation of the enzyme (Figure 7). The apparent stability of the "wired" enzyme electrode was better than that of the dissolved enzyme, for which an optimal temperature of 40 °C was reported.[25,26] The apparent activation energy for $O_2$ electroreduction was $E_{act} = 28.2$ kJ $mol^{-1}$ for the pH 7.4 solution with the electrode rotating at 1000 rpm under 1 atm $O_2$, where the current was $O_2$ transport controlled. Because the current was $O_2$ transport limited at 1000 rpm, the 28.2 kJ $mol^{-1}$ value represents the activation energy for diffusion of oxygen in the solution.[67] The 77 kJ $mol^{-1}$ activation energy for current loss is similar to that for the thermal denaturation of other enzymes.[68]

**Stability.** At high angular velocity (>1000 rpm), the electrode was mechanically unstable, its catalytic film being sheared off. When the electrode, poised at +300 mV versus Ag/AgCl, was rotated at 300 rpm under 1 atm $O_2$ at 37.5 °C, it lost less than 10% of its current per day of continuous operation for 6 days (Figure 8). The "wired" BOD electrode was far more stable than suggested by Tanaka's study of the stability of the dissolved enzyme, showing loss of half the activity after 1 h in phosphate buffer at 37 °C.[26] It was also more stable than that of an immobilized BOD electrode used in the clinical monitoring of the concentration of bilirubin. The half-life of the clinical bilirubin-monitoring electrode was much shorter than that of the cathode of this study, only 17 h at 37 °C[69] and 8 h at 40 °C.[44]

The most readily cathode electooxidized constituents of blood, urate, ascorbate and acetaminophen, at their physiological concentrations,[70] did not damage the cathode poised at +300 mV/Ag/AgCl in PBS buffer at 37.5 °C. When 0.1 mM ascorbate, or 0.1 mM ascorbate and 0.17 mM acetaminophen was added, the current transiently decreased by 5%, and when 0.48 mM urate was added, the decrease was 25%. While Binyamin et al.[35] showed that the electrooxidative polymerization of urate in a redox polymer film with a higher density of cationic sites and thus a higher urate concentration in the film caused degradation of its electrocatalytic properties, no such damage was observed in the present films.

## Conclusion

The study describes the first electrode on which $O_2$ is electroreduced to water physiological conditions at a current density of 9.1 mA $cm^{-2}$ at a potential −0.26 V relative to the reversible potential of the $O_2/H_2O$ electrode. The electrode has no leachable components, making it suitable for use in flow systems, and with a yet to be added thin bioinert film, for use in animals. It could well serve as the cathode of a miniature biofuel cell that might power implanted sensors and actuators for about 1 week.

**Acknowledgment.** The study was supported by the Office of Naval Research (Grant N00014-02-1-0144). The authors thank Ting Chen, Keith A. Friedman, Scott Calabrese Barton, Gary Binyamin, and Vonda Totten for their advice and assistance.

JA025874V

(62) Samejima, T.; Wu, C. S.; Shiboya, K.; Kaji, H.; Koikeda, S.; Ando, K.; Yang, J. T. *J. Protein Chem.* **1994**, *13*, 307−313.
(63) Xu, F. *Biochemistry* **1996**, *35*, 7608−7614.
(64) Xu, F. *J. Biol. Chem.* **1997**, *272*, 924−928.
(65) Xu, F. *Appl. Biochem. Biotechnol.* **2001**, *95*, 125−133.
(66) Heller, A. *J. Phys. Chem.* **1992**, *96*, 3579−3587.
(67) Perry, R. H.; Green, D. W. *Chemical Engineers Handbook*; McGraw-Hill Book Co.: New York, 1984.
(68) Rob, A.; Hernandez, M.; Ball, A. S.; Tuncer, M.; Arias, M. E.; Wilson, M. T. *Appl. Biochem. Biotechnol.* **1997**, *62*, 159−174.
(69) Sung, C.; Lavin, A.; Klibanov, A. M.; Langer, R. *Biotechnol. Bioenerg.* **1986**, *28*, 1531−1539.
(70) Csoregi, E.; Schmidtke, D. W.; Heller, A. *Anal. Chem.* **1995**, *34*, 1240−1244.