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On the stability of the "wired" bilirubin oxidase oxygen cathode in serum

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Abstract

Oxygen is electroreduced to water on the "wired" bilirubin oxidase (w-BOD) catalyst at a considerably lesser potential than on pure platinum. The w-BOD catalyst could be of value in an implantable glucose–O_2 biofuel cell, operating living tissue, if it were stable in serum. We found, however, that w-BOD loses its activity in a few hours in the combined presence of the urate and O_2, both of which are normal serum constituents (*Bioelectrochemistry*, 2004, 65, 83–88). Here we report a second major instability: When the disconnected w-BOD cathode is allowed, in the absence of urate, to poise itself at the potential of the O_2/H_2O half cell at pH 7.2, it loses its activity rapidly. Unlike the urate/O_2 caused loss, this loss can be avoided either by applying a potential that is reducing relative to the O_2/H_2O half-cell potential, or by excluding O_2 and adding a mildly reducing reagent, such as urate. The w-BOD cathode can be stored, therefore, in deoxygenated serum, which contains urate.

Keywords: w-BOD cathode; Serum; Urate

1. Introduction

Glucose–O_2 biofuel cells, consisting of two "wired" bioelectrocatalyst-coated electrodes, have been recently reported [1–6]. Because the two reactants are present in most tissues, and because in the saline physiological pH 7.2 environment the cell does not require a membrane or a case, a cell consisting merely of two bioelectrocatalyst-coated carbon fibers could function in-vivo. The w-BOD electrocatalyst consists of the blue copper oxidase bilirubin oxidase (BOD) and its "wiring" redox polymer [7–11]. When BOD from *Myrothecium verrucaria* (*Mv*) is "wired" to a carbon cloth by the copolymer of polyacrylamide and poly(*N*-vinylimidazole) complexed with $[Os(4,4'\text{-dichloro-}2,2'\text{-bipyridine})_2Cl]^{+/2+}$ [10], O_2 is electroreduced to water, under physiological conditions (pH 7.4, 0.15 M NaCl, 37.5 °C) at a current density as high as 5 mA cm^{-2} at −0.18 V vs. the reversible potential of the O_2/H_2O electrode in the same buffer. When BOD from *Trachyderma tsunodae* (*Tt*) is "wired," O_2 is electroreduced at a current density of 3 mA cm^{-2} at −0.14 V vs. the reversible potential of the O_2/H_2O electrode [11]. The overpotentials of smooth carbon fibers or disks coated with these bioelectrocatalysts are much smaller than those of smooth platinum fibers or disks [11,12]. Miniature biofuel cells, formed by combining the w-BOD cathode with a "wired" glucose oxidase anode, operate both in a physiological buffer solution at 37 °C for a week and in a living plant, a grape, for a day [2,3].

In serum the w-BOD electrocatalyst loses, however, its activity in a few hours. Among the serum components [13,14], urate is particularly rapidly electrooxidized and was found to damage glucose anodes when operated at oxidizing potentials, near those at which the w-BOD cathodes operate in biofuel cells [15]. The combination of urate and dissolved O_2 damages also the w-BOD cathode [16]. The oxidation of urate by O_2 is catalyzed both by BOD and by its "wire", and a urate oxidation product irreversibly deactivates the BOD. When urate is added to a physiological buffer solution it causes, at its typical serum concentration, a loss of 1/3rd of the O_2 electroreduction current in 1 h, and of

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40% of the current in 3 h in the operating, or connected, cathode. Here we show that when the w-BOD cathode is disconnected and its potential is allowed to "float", poising itself under air near the O_2/H_2O potential, the loss of current is exceptionally rapid, greatly exceeding the urate/O_2 caused loss. We also show that this loss is avoided by poising the w-BOD cathode at its optimal operating potential in biofuel cells, and that the cathode is stable when stored in de-oxygenated serum.

2. Experimental section

2.1. Chemicals and materials

Bilirubin oxidase (BOD) from *Trachyderma tsunodae* (*Tt*) was purchased from Amano, Lombard, IL. Poly (ethylene glycol) (400) diglycidyl ether (PEGDGE) was purchased from Polysciences Inc. (Warrington, PA). Uric acid, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma, St. Louis, MO. The frozen calf serum (Sigma cat. # C8056, from formula-fed bovine calves, iron-supplemented, cell culture test) was melted before use. A fresh solution of BOD was prepared for each experiment. The pH 7.4 20 mM phosphate, and the pH 7.4 physiological 20 mM phosphate, 0.15 M NaCl (PBS) buffers were prepared with de-ionized water. The electrochemical measurements were performed in PBS and pH 7.4 20 mM phosphate, without NaCl, was used for dissolving the enzyme and the redox polymer. Uric acid was dissolved in 1 M KOH, then the pH was brought to 7.4 using KH_2PO_4, to yield a 10 mM urate solution.

The BOD "wiring" copolymer of polyacryamide and poly(*N*-vinylimidazole) complexed with $[Os(4,4'\text{-dichloro-}2,2'\text{-bipyridine})_2Cl]^{+/2+}$ (PAA-PVI-$[Os(4,4'\text{-dichloro-}2,2'\text{-bipyridine})_2Cl]^{+/2+}$) was synthesized as described [10].

2.2. The BOD cathode

Carbon cloth cathodes (0.107 cm^2) were made by the reported three-step procedure, using Toray TGPH-030 carbon cloth from E-TEK, Somerset, NJ [10,17]. The 3 mm diameter glassy carbon electrodes were initially sanded with 600 and 1200 SiC paper, then polished with a 0.3 μm alumina slurry and sonicated in de-ionized water. The cleaned glassy carbon electrodes showed featureless voltammograms. The catalytic films were formed of a mixture of 17.3 μL of 4.5 mg/mL redox polymer in water, 1.44 μL of buffer, 4.8 μL of 15 mg/mL BOD in buffer, and 3.36 μL of 3.2 mg/mL PEGDGE in de-ionized water. A 9 μL aliquot of the mixed solution was pipetted onto the hydrophilic carbon cloth mounted on the glassy carbon disk, which was promptly wetted and penetrated by the solution. The films were cured for at least 18 h at room temperature before use.

2.3. Instrumentation and cell

The measurements were performed using a Model CHI832 potentiostat (CH-Instruments, Austin, TX) controlled through a personal computer. The rotation of the electrode was controlled using a Pine Instrument rotator (Grove, PA). Spectra were measured using an HP 8452A UV–visible spectrophotometer. The three-electrode cell used had a commercial Ag/AgCl (3 M NaCl) reference electrode and an auxiliary platinum wire electrode. The temperature of the cell was controlled with an isothermal circulator (Fisher Scientific, Pittsburg, PA).

2.4. BOD activity assay

BOD was assayed by measuring the time-dependence of the ABTS absorbance at 405 nm [18,19] after adding the assayed BOD aliquot, using the reported 35 mM^{-1} cm^{-1} molar absorption coefficient at 22 °C. A unit of enzyme activity (U) is that generating 1 μmol of oxidized ABTS/min; the specific activity is the number of units per mg (U/mg).

3. Results and discussion

The stabilities of the w-BOD cathodes were determined by comparing their O_2 electroreduction-associated voltammograms, before and after 2 h long immersion and rotation, at 100 rpm, in physiological buffer solutions, with or without 0.5 mM urate, under 1 atm O_2 or under argon, and while disconnected or connected and poised at 0.1 V vs. Ag/AgCl. The voltammograms are shown in Fig. 1A–H and the results are summarized in Table 1. With 0.5 mM urate, under 1 atm O_2 and with the electrode disconnected from the potentiostat, 73% of the O_2 electroreduction current was lost. Poising the electrode at 0.1 V vs. Ag/AgCl, at which both the "wiring" polymer's *N*-vinylimidazole-function bound $[Os(4,4'\text{-dichloro-}2,2'\text{-bipyridine})_2Cl]^{+/2+}$ centers and the Cu atoms of BOD are in the reduced state, reduced the loss to 26%. (Fig. 1A and B). When the 0.1 V vs. Ag/AgCl potential was applied, and oxygen and urate were both present, the loss was 26% (Fig. 1B), but no loss was seen in the absence of either urate or O_2 (Fig. 1D and F), confirming earlier results [16]. In the absence of O_2, but in the presence of urate, the cathode was stable, regardless of whether or not a potential was applied (Fig. 1E and F).

Without urate (Fig. 1C and D) 78% of the current was lost when the electrode was disconnected, but there was no current loss when a 0.1 V vs. Ag/AgCl potential was applied. In the presence of urate, the disconnected electrode under 1 atm O_2 lost 73% of its current (Fig. 1A and E), but there was no loss in the de-oxygenated solution. The disconnected electrode was unstable under 1 atm O_2, where the osmium centers of the "wire", as well as the copper centers of BOD, were in their oxidized states (Os^{3+} and

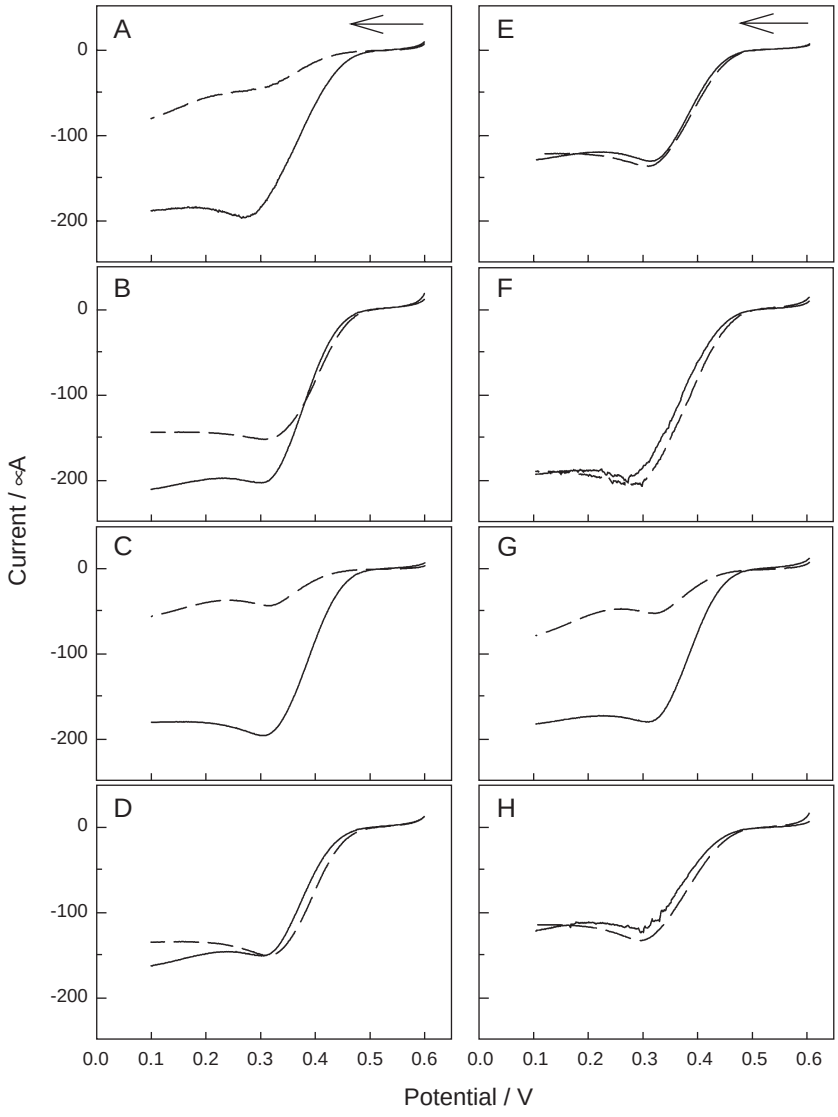

Fig. 1. Voltammograms of "wired" bilirubin oxidase before (solid line) and after (dashed line) 2 h rotation at 100 rpm in PBS (0.15 M NaCl, 0.02 M phosphate). The voltammograms were measured under 1 atm O_2 in PBS at 37.5 °C, with the electrode rotating at 500 rpm, at 1 mV s^{-1} scan rate. The variables, detailed in Table 1, are the atmosphere (1 atm O_2 or argon); the presence or absence of 0.5 mM urate; and the potential applied.

Cu^{2+}). The cathode was, however, stable when a 0.1 V vs. Ag/AgCl potential was applied and the centers were in their reduced (Os^{2+} and Cu^{+}) states. The cause of the 78% loss upon storage of the rotating cathode under 1 atm O_2 was not poor mechanical integrity. Mechanical strengthening, through increasing the weight % of the cross-linker, PEGDGE from 6.7% to 17.7% resulted only in marginal improvement of the stability, the loss being reduced from 78% to 67%.

Table 1
Loss of O_2 electroreduction current at 0.1 V vs. Ag/AgCl in PBS

Fig. 1	Atmosphere	Urate (mM)	Applied potential	% Loss (±5%)
A	1 atm O_2	0.5	None	73
B	1 atm O_2	0.5	0.1 V (Ag/AgCl)	26
C	1 atm O_2	0.0	None	78
D	1 atm O_2	0.0	0.1 V (Ag/AgCl)	0
E	Ar purged	0.5	None	0
F	Ar purged	0.5	0.1 V (Ag/AgCl)	0
G	Ar purged	0.0	None	72
H	Ar purged	0.0	0.1 V (Ag/AgCl)	0

The results show that O_2 by itself, as well as O_2 in combination with urate, destabilize the w-BOD cathode. The O_2-caused damage, avoided by applying 0.1 V vs. Ag/AgCl, is more severe than the damage by O_2 in combination with urate. The cathode does not lose any of its catalytic activity when stored in a deoxygenated urate-free solution, with the 0.1 V vs. Ag/AgCl potential applied (Fig. 1G and H). When the potential was not applied, the cathode was unstable in buffer, but was stable in the urate—containing deoxygenated buffer (Fig. 1E and G), as the urate maintained the w-BOD Os and Cu redox centers in their reduced states. Because the voltammetric peak heights associated with the electrooxidation/reduction of the redox

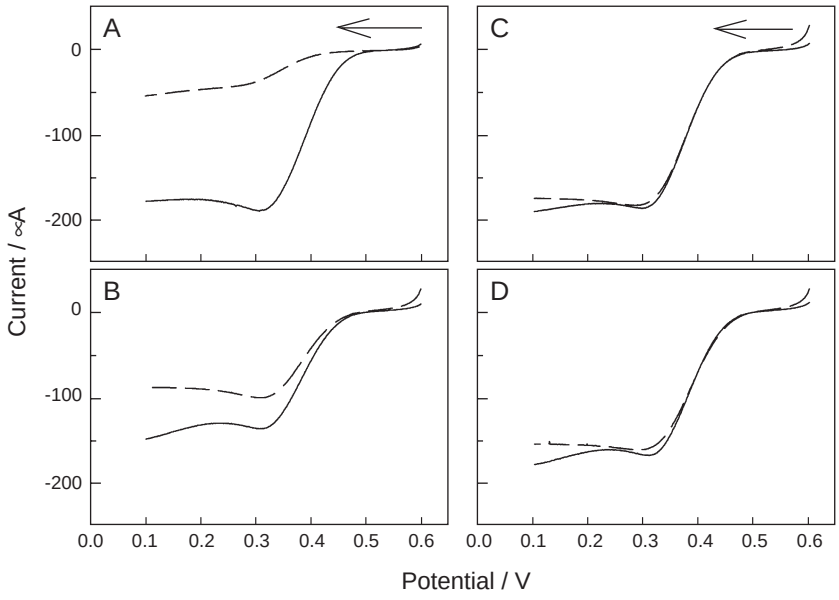

Fig. 2. Voltammograms of "wired" bilirubin oxidase before (solid line) and after (dashed line) 2 h rotation at 100 rpm in calf serum. The voltammograms were measured under 1 atm O_2 in PBS at 37.5 °C, with the electrode rotating at 500 rpm, at 1 mV s^{-1} scan rate. The variables, detailed in Table 2, are the atmosphere (air or argon) and the potential applied.

polymer did not change substantially in any of the 2 h immersions, we conclude that it is the maintenance of the BOD Cu centers in the Cu^{2+} state that causes the dominant instability. Disconnection of the electrode sufficed to cause the large loss; even under argon the loss was large. Correspondingly, dissolved BOD (0.84 mg/mL) lost in 24 h 60±5% of its activity under argon and 55±5% of its activity under air. Urate (1 mM) reduced the 24 h loss under argon to 34±5%.

The results obtained in calf serum are shown in Fig. 2A–D and in Table 2. The tests were performed by rotating the electrodes in calf serum at 100 rpm for 2 h. In serum exposed to air, the disconnected electrodes also lost 72% of their O_2-electroreduction current, but only 29% when connected with the 0.1 V vs. Ag/AgCl potential applied (Fig. 2A and B). When the serum, which contained urate, was de-oxygenated, the cathode was highly stable, irrespective of whether it was disconnected or connected with the 0.1 V vs. Ag/AgCl potential applied (Fig. 2C and D), paralleling the results obtained in urate-containing buffer (Fig. 1E and F). Thus, even though serum has many constituents [13,14], it is the serum urate that controls the stability of the w-BOD cathode. In the presence of O_2 it is the precursor of a BOD-damaging species [16] and in absence of O_2 it stabilizes the BOD by reducing its Cu-centers.

Table 2
Loss of O_2 electroreduction current at 0.1 V vs. Ag/AgCl in serum

Fig. 2	Atmosphere	Applied potential	% Loss (±5%)
A	Air	None	72
B	Air	0.1 V (Ag/AgCl)	29
C	Ar purged	None	0
D	Ar purged	0.1 V (Ag/AgCl)	0

4. Conclusions

In serum, there are two processes damaging the "wired" BOD, catalyzing the electroreduction of O_2 to H_2O. In both processes the damaged catalyst component is BOD, not its "wire". In the first, earlier reported process, a product of the BOD-catalyzed O_2-oxidation of urate deactivates the enzyme [16]. The second process involves the irreversible de-activation of BOD Cu-centers in their oxidized state, and is actually alleviated by urate, which maintains the BOD Cu centers in their reduced state in the absence of O_2. While the disconnected O_2 electroreducing cathodes are drastically and rapidly damaged by the second process, the operating cathodes are not, because they are poised at potentials where the Cu centers are in the reduced state. Furthermore, in the absence of O_2, the disconnected electrodes can be stored in serum without losing their activity, because serum urate maintains the damage-prone Cu-centers in the reduced state.

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