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Deactivation of bilirubin oxidase by a product of the reaction of urate and O_2

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Abstract

The "wired" bilirubin oxidase (BOD) bioelectrocatalyst is superior to pure platinum as an electrocatalyst of the four-electron electroreduction of O_2 to water. Not only is its overpotential for O_2 reduction lower, but unlike platinum, it is not affected by organic compounds like glucose. The "wired" BOD-coated carbon cathode operates for >1 week at 37 °C in a glucose-containing physiological buffer solution. One of its key applications would be in a glucose–O_2 biofuel cell, which would operate in living tissues. The cathode is, however, short-lived in serum, losing its electrocatalytic activity in a few hours. Here we show that the damaging serum component is a product of the reaction of urate and dissolved oxygen. Exclusion of urate, by application of Nafion™ film on the cathode, improves the stability in serum.

Keywords: Bilirubin oxidase; Urate; O_2

1. Introduction

A simple biofuel cell, consisting merely of two "wired" enzyme-coated carbon electrodes, could react with glucose and O_2 available in living tissues [1–3]. On the anode, glucose would be electrooxidized to gluconolactone at a reducing potential; on the cathode, oxygen would be electroreduced to water at an oxidizing potential. Such a cell already operates at 37 °C in a pH 7.2 physiological buffer solution and in a living plant, a grape. In the buffer, it loses only about 5% of its power per day. The cell is, however, unstable in serum, where it loses most of its output in a few hours.

Blue copper-containing oxidases such as laccase, bilirubin oxidase (BOD), and ascorbate oxidase, catalyze the reduction of O_2 to water [4–6], and are components of cathodic bioelectrocatalysts of biofuel cells [7–15]. Some of the reported cells are very simple, consisting merely of two bioelectrocatalyst-coated carbon fibers. They do not have a membrane or a case. This is of essence if the cells are to be implanted in the body, drawing on the glucose and O_2 of its organs. The membraneless and caseless cells require that their anodic and cathodic enzymes, as well as their electron-shuttling redox mediators, be immobilized. This is feasible only when the enzymes are immobilized in and are electrically connected ("wired") by electron-conducting hydrogels.

Several O_2 cathodes have been built by "wiring" blue copper enzymes. In one, the reaction centers of laccase from *Coriolus hirsutus* were "wired" to a hydrophilic carbon cloth, composed of 10-μm-diameter fibers, by the redox polymer, PVI-Os(tpy)(dme-bpy) [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)]. At 37.5 °C, in pH 5 citrate buffer, in the absence of chloride, O_2 was electroreduced at a current density of 5 mA cm^{-2} at −0.13 V vs. the reversible potential of the O_2/H_2O electrode in the same buffer [10,11]. In a

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second, BOD from *Myrothecium verrucaria* (*Mv*) was "wired" to the carbon cloth by the copolymer of polyacrylamide and poly(*N*-vinylimidazole) complexed with [Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl]$^{+/2+}$ [14]. O_2 was electroreduced, now under physiological conditions (pH 7.4, 0.15 M NaCl, 37.5 °C), at a current density of 5 mA cm^{-2} at −0.18 V vs. the reversible potential of the O_2/H_2O electrode in the same buffer. On a third cathode, made by similarly "wiring" BOD from *Trachyderma tsunodae* (*Tt*), O_2 was electroreduced to water under physiological conditions (pH 7.4, 0.15 M NaCl, 37.5 °C), at a current density of 3 mA cm^{-2} at −0.14 V vs. the reversible potential of the O_2/H_2O electrode in the same buffer, a much smaller overpotential than for smooth platinum [15,16]. A miniature biofuel cell, formed by combining the "wired" *Tt*-BOD cathode with a "wired" glucose oxidase anode, operated in a living plant, a grape [1,2].

In serum, unlike in the physiological buffer solution, the "wired" *Tt*-BOD electrocatalyst lost its activity in a few hours. Because O_2 cathodes of fuel cells operate necessarily at oxidizing potentials, damage by an oxidation product of a serum constituent was suspected [17–19]. The initial experiments pointed at urate [14,15], shown to oxidatively electropolymerize and precipitate in the electrocatalytic films of glucose electrooxidizing "wired" glucose oxidase anodes [20]. In these anodes, the electrooxidatively formed polyanionic polymer precipitated as an electrostatic adduct with the polycationic "wire" of glucose oxidase [20]. Upon precipitation, the formerly mobile segments of the "wire" lost their mobility. This reduced the diffusivity of electrons, which depends on electron-transferring collisions between the redox functions carried by the segments of the "wire." Thus, the urate damaged the anodic "wired" glucose oxidase film by making it resistive, not by damaging its enzyme. As will be shown in this article, urate reacts with oxygen in a reaction catalyzed both by BOD and its "wire" to form a product that irreversibly deactivates the BOD of the film.

2. Experimental

2.1. Chemicals and materials

BOD from *Tt* was purchased from Amano (Lombard, IL). Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) was purchased from Polysciences (Warrington, PA). The DEAE-Sephacel anion exchanger and the Superdex 75 HR 10/30 gel filtration column were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Uric acid and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma (St. Louis, MO) and Nafion™ (5 wt.% in a mixture of lower aliphatic alcohols and water) from Aldrich (Milwaukee, WI). All were used as received. The BOD was purified by anion exchange chromatography on the DEAE-Sephacel anion exchanger and gel filtration chromatography on Superdex 75 HR 10/30 [6]. The purification increased the specific activity threefold [21,22]. When the purified BOD was refrigerated, its specific activity decayed in 1 month to the activity of the unpurified enzyme. For this reason, the enzyme was used within 2 weeks after its purification. Purified enzyme was used in the preparation of the cathodes. For all other purposes, the enzyme was used as received. The pH-7.4 20 mM phosphate, and the pH-7.4 physiological 20 mM phosphate, 0.15 M NaCl (phosphate-buffered saline, or PBS) buffers were prepared using deionized water. The electrochemical measurements were performed in PBS and the pH-7.4 20 mM phosphate, without NaCl, was used for dissolving the enzyme and the redox polymer. The 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. Carbon cloth (Toray TGPH-030) was received from E-TEK (Somerset, NJ). Glassy carbon electrodes of 3 mm diameter, mounted in Teflon sleeves, were used for electrochemical measurements and for mounting the carbon cloth.

The BOD "wiring" copolymer of polyacrylamide and poly(*N*-vinylimidazole) complexed with [Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl]$^{+/2+}$ (PAA-PVI-[Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl]$^{+/2+}$) was synthesized as described [14].

2.2. Electrodes

Carbon cloth electrodes (BOD cathodes, area of 0.107 cm^2) were made by the reported three-step procedure [11,14]. The glassy carbon electrode was initially sanded with 600 and 1200 SiC papers, then polished with a 0.3-μm alumina slurry and sonicated in deionized water. The cleaned glassy carbon electrodes showed featureless voltammograms. The catalytic films were formed of a mixture of 14.4 μl of 3 mg ml^{-1} redox polymer in water, 1.8 μl of buffer, 3.4 μl of 9 mg ml^{-1} BOD in buffer, and 2.8 μl of 2.1 mg ml^{-1} PEGDGE in deionized water. A 9-μl aliquot of the mixed solution was pipetted onto the hydrophilic carbon cloth mounted on the glassy carbon disc, 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. Rotation of the electrodes was controlled by 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 [21,22] 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^{-1}; the specific activity is the number of units per mg (U mg^{-1}).

3. Results and discussion

3.1. Urate destabilizes the "wired" BOD O_2-electroreducing cathode

As seen in Fig. 1A, addition of urate to the oxygenated buffer solution in which the cathode operates results in the prompt loss of about one-third of the O_2 electroreduction current. The prompt loss, which is observed when O_2 is electroreduced at any potential in the 0.1–0.3 V vs. Ag/AgCl range, increases with the potential at which the "wired" BOD cathode is poised. (Fig. 1B). While at 0.1 V vs. Ag/AgCl about one-third of the current was promptly lost, about half of it was lost at 0.25 V vs. Ag/AgCl and nearly two-thirds at 0.3 V vs. Ag/AgCl. We note that urate is electrooxidized at >0.20 V vs. Ag/AgCl [23–25].As a result, the apparent O_2 electroreduction current is expected to decrease, simply because of the additivity of the opposing currents of urate electrooxidation and O_2 electroreduction, the threshold of which is ~0.5 V vs. Ag/AgCl at pH 7.4. Thus, the loss increment when the potential is raised from 0.1 V vs. Ag/AgCl, where urate is not electrooxidized, to 0.25 V vs. Ag/AgCl, where it is, is attributed to the flow of the opposing current, which increases at 0.3 V vs. Ag/AgCl. When the urate electrooxidation current is measured in the absence of O_2 and the current is subtracted from the prompt current loss of Fig. 1B, b or c, then the prompt loss at any of the three potentials is the same, about one-third of the current.

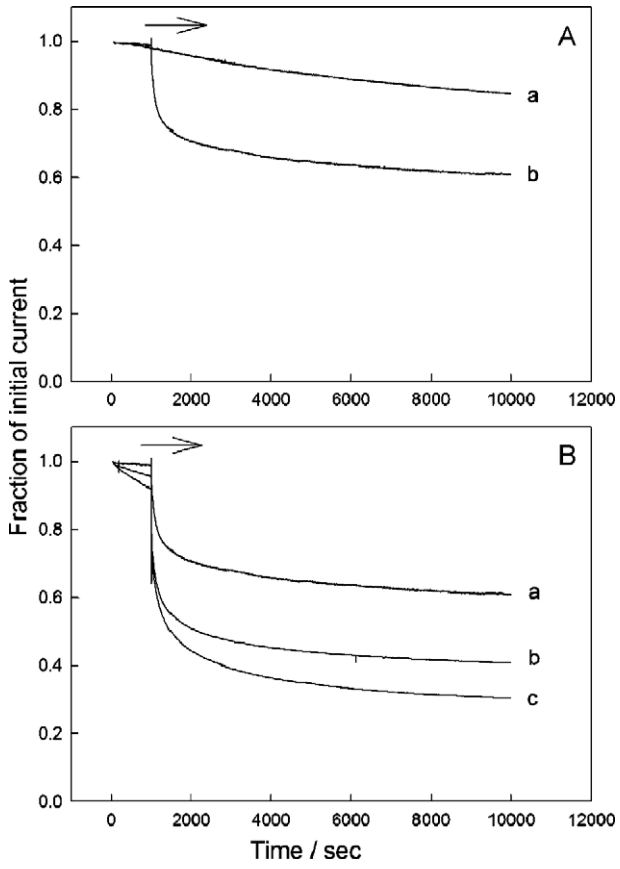


Fig. 1. Time dependence of the fraction of the residual O_2 electroreduction current. (Aa) Without urate; (Ab) urate added at t=1000 s to produce a 0.5 mM solution. Solution equilibrated with 1 atm O_2; electrode poised at 0.1 V vs. Ag/AgCl and rotating at 200 rpm in PBS (37.5 °C). (B) As in (Ab), with the electrode poised at 0.10 V (a), 0.25 V (b), and 0.30 V (c).

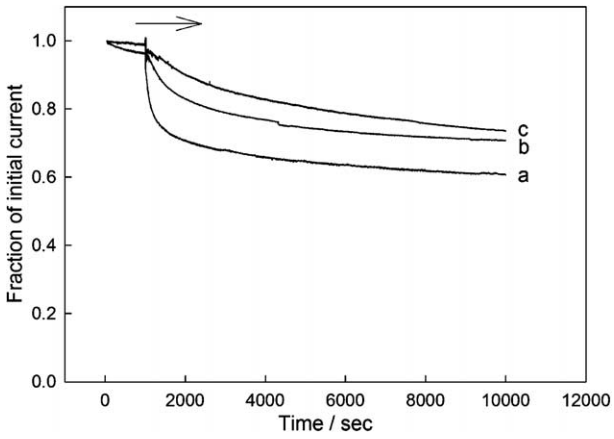


Fig. 2. Time dependence of the fraction of the residual O_2 electroreduction current. Conditions as in Fig. 1Ab, except that the Cl^- concentration was varied: 0.15 M Cl^- (a); 0.65 M Cl^- (b); and 1 M Cl^- (c).

Because chloride competes with urate for the cationic sites of the "wire", which is a crosslinked anion exchanger, it was expected that the urate-caused damage would be reduced at high chloride concentrations. Indeed, the damage was reduced as the Cl^- concentration was increased (Fig. 2). At 1 M Cl^-, the prompt loss of O_2 electroreduction current upon adding urate was barely noticeable. At 0.65 M Cl^-, it was about one-fifth of that in PBS.

3.2. Destabilization by urate requires O_2

The dependence of the O_2 electroreduction current on the potential of the "wired" BOD O_2 cathodes with different histories is shown in Fig. 3. Voltammograms of fresh cathodes are seen in curves 3Aa, 3Ba, and 3Ca. They show a wave which in the composite of two electroreduction waves, one of O_2 and the other of the PAA-PVI-complexed $Os(4,4'\text{-dichloro-}2,2'\text{-bipyridine})_2Cl]^{2+}$ redox centers, the formal potential of which is 0.31 V vs. Ag/AgCl. Curve 3Ab shows a cathode after it was disconnected and slowly rotated, for 1 h, in O_2-saturated PBS buffer, which did not

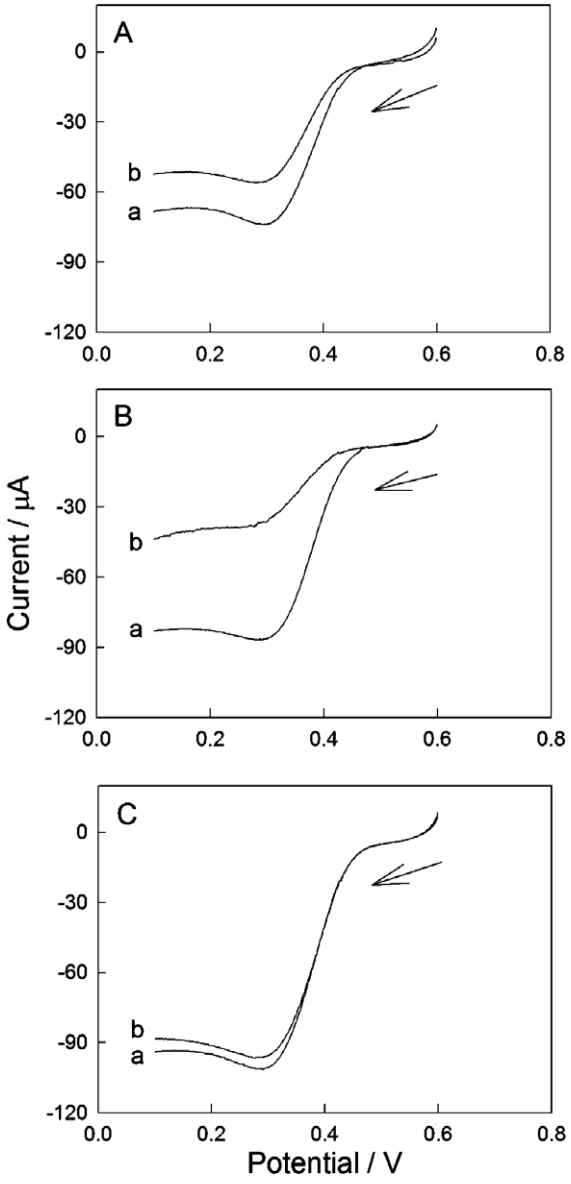


Fig. 3. Dependence of the O_2 electroreduction current on the applied potential for electrodes of different histories. The voltammograms *a* and *b* were obtained, respectively before and after the electrode were immersed in a phosphate buffer solution, (A) O_2 bubbled, buffer only; (B) O_2 bubbled, 0.5 mM urate; (C) 0.5 mM urate, solution under Ar. Immersion: 1 h, no potential applied, with the electrode rotating at 100 rpm. Measurement: solution equilibrated with 1 atm O_2, 500 rpm, scan rate 2 mV s^{-1}. Other conditions as in Fig 1Ab.

contain urate. The height of the wave was reduced by about 25%. Curve 3Bb, obtained after the electrode was similarly rotated in O_2-saturated PBS with 0.5 mM urate, shows drastic reduction of the wave. Curve 3Cb was obtained after the electrode was rotated in PBS with 0.5 mM urate, but without O_2. There was little change showing that, in the absence of O_2, urate did not damage the catalyst. Because the electrode was disconnected in the experiment of Fig. 3B, the damage by the combination of urate and O_2 was chemical, not electrochemical. The massive loss was not reversed when the electrode was placed in a solution without urate. Although the loss was large, the damaged electrode was not totally inactive: some O_2 electroreduction persisted, although only at more reducing potentials.

3.3. BOD catalyzes the oxidation of urate

That BOD catalyzes the oxidation of urate is shown in the experiments of Fig. 4. The figure shows the voltammograms of urate electrooxidation. In the absence of BOD, the concentration of urate is unaffected by bubbling O_2 for 80 min (Fig. 4A). Urate is, however, oxidized by O_2 in the presence of dissolved BOD (Fig. 4B).

3.4. The "*wire*" also catalyzes the oxidation of urate

Not only BOD, but also the Os^{3+} centers of the "wire" oxidize urate. This was observed upon mixing a drop of the 0.5 mM urate solution and the brown solution of the oxidized "wire." The color changed to wine red—the color of the Os^{2+} complex. The two oxidation processes of urate are shown in Scheme 1.

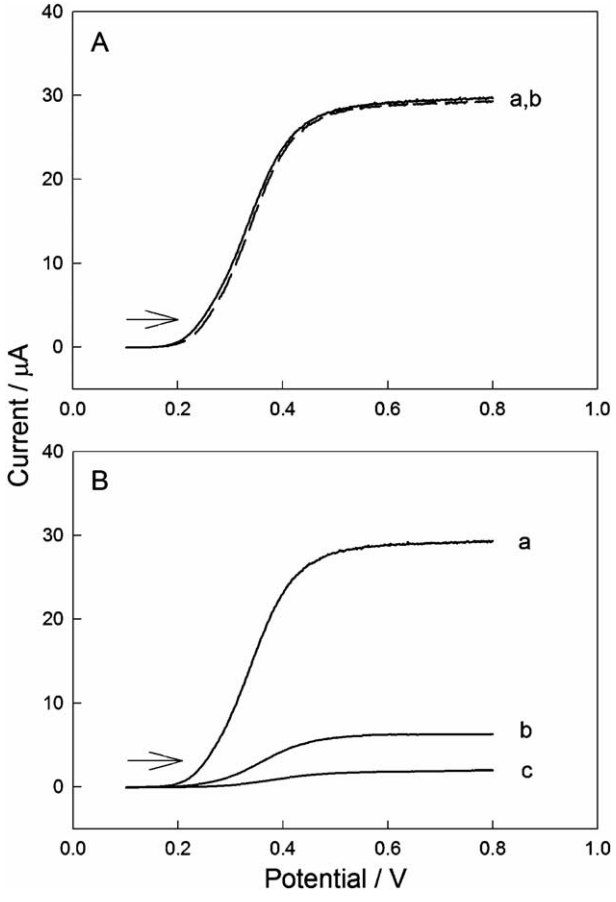
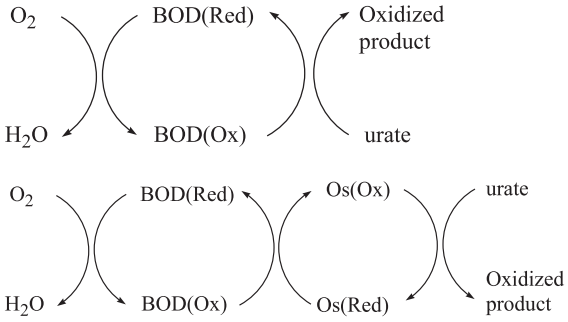


Fig. 4. Voltammograms of uncoated vitreous carbon electrodes in 0.5 mM urate in PBS. (A) Without BOD, before (a, solid line) and after (b, dashed line) bubbling O_2 for 80 min. (B) Without BOD, before bubbling O_2 (a); with 0.5 mg ml^{-1} BOD, after bubbling O_2 for 40 min (b); after bubbling O_2 for 80 min (c). 500 rpm, scan rate, 2 mV s^{-1}. Conditions as in Fig. 1Ab.



Scheme 1. Urate oxidation catalyzed by the BOD enzyme or the BOD+Os complex system.

3.5. A reaction product of urate and O_2 coordinates and precipitates Cu^{2+} of its tetraimidazole complex

In the oxygen-binding cluster of the copper ions, the most frequent ligand is histidine [6,26]. In order to assess the likelihood that an oxidation product of urate could deactivate the BOD by altering the coordination sphere of its copper ions, we probed for change in the coordination of the tetraimidazole complex of copper $Cu(im)_4^{2+}$ upon adding urate or its oxidation products [23–25], allantoin and allantoic acid, in the presence and absence of O_2. Allantoin and allantoic acid did not react with $Cu(im)_4^{2+}$ in the absence of O_2. In the presence of O_2, a pale green precipitate formed when urate was added. Thus, it appears likely that the oxidation product of urate extracts copper from the enzyme.

3.6. A reaction product of urate and O_2 deactivates BOD

The BOD-catalyzed ABTS oxidation rates were compared for solutions containing no additive, urate, allantoin, and allantoic acid. The four solutions contained 0.42 mg ml^{-1} BOD and none or one of the three additives at 0.50 mM concentration. The spectral change was measured after a 10-μl aliquot was added to 2 ml of 1 mM ABTS. The time dependence of the specific activities is seen in Fig. 5. Only urate affected the activity, reducing it from 40 to 26 U mg^{-1} in 2 h. The results obtained in homogeneous solutions parallel those obtained with the immobilized bioelectrocatalyst (Fig. 3). All of the three sets of experiments suggest that a reaction product of urate with O_2 causes the damage, most likely by coordinating one or more of the copper ions of BOD. They also show that the damaging molecule is neither allantoin nor allantoic acid.

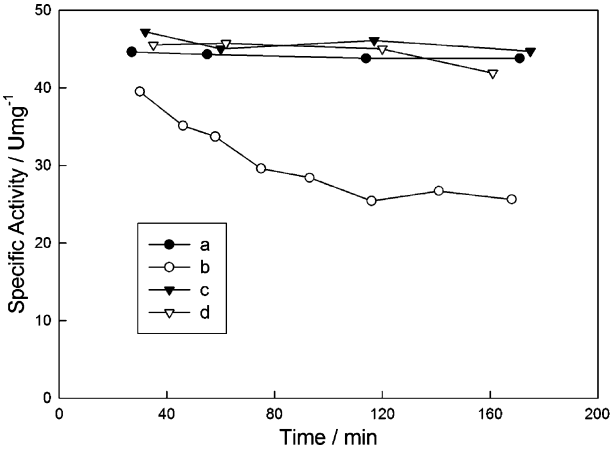


Fig. 5. Time dependence of the activity of a 0.42 mg ml^{-1} solution of *Tt*-BOD. Without any additive (a); with 0.50 mM urate (b); with 0.50 mM allantoin (c); and with 0.50 mM allantoic acid (d).

In a previous study, the damage to a "wired" glucose oxidase anode was attributed to the electrooxidative polymerization product of urate, which reduced the conductivity of the bioelectrocatalyst, not the activity of its enzyme [20]. In the present system, the main cause of the loss is BOD deactivation by coordination of its Cu^{2+} by a urate oxidation product [6,26].

Because Cl^- effectively prevented damage to the activity of "wired" BOD by competing for the urate-occupied sites of the anion-exchanging (Fig. 2), we applied a film of cation-exchanging Nafion™ polymer to reduce the flux of oxidizable urate to the catalyst film. An aliquot of 3 μl of 0.25% Nafion™ was applied to the "wired" BOD film on the carbon cloth and was allowed to dry for 1 h. As seen in Fig. 6A, the

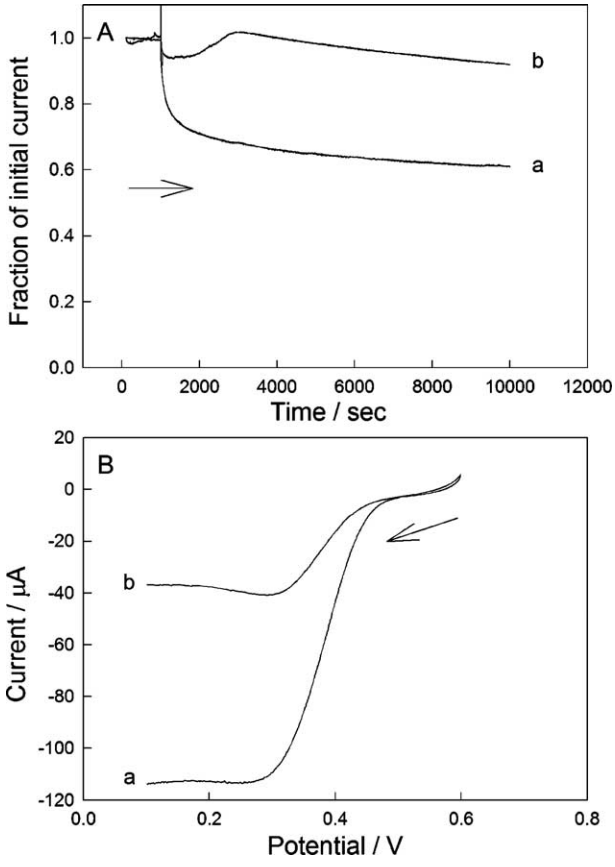


Fig. 6. (A) Time dependence of the fraction of the residual O_2 electroreduction current. Uncoated "wired" BOD cathode (a); Nafion™-coated "wired" BOD cathode (b). Conditions as in Fig. 1Ab. (B) Voltammogram of the uncoated "wired" BOD cathode (a); of the Nafion™-coated BOD cathode (b). Equilibrated with 1 atm O_2, 500 rpm, scan rate 2 mV s^{-1}. Other conditions as in Fig. 1.

Nafion™ stabilized the O_2 electroreduction current. Without Nafion™, the current declined by 39% in the 9000 s following the addition of the urate (Fig. 6Aa). With the Nafion™, the decline was only 8%. (Fig. 6Ab). Application of the Nafion™ reduced, however, the activity of the "wired" BOD electrocatalyst. (Fig. 6B).

4. Conclusions

The current associated with the electrocatalytic reduction of O_2 to water on "wired" BOD is reduced when urate is added. The reversible part of the current loss results in the electrooxidation of urate. Its dominant irreversible part results in damage to the BOD of the electrocatalyst, attributed to coordination of one or more of the four Cu^{2+} ions of BOD by a product of the BOD and redox polymer-catalyzed reaction of urate with dissolved O_2. Exclusion of urate from the electrocatalytic film, either by adding chloride, which competes for the cationic sites of the cation-exchanging "wire", or by a Nafion™ film applied on the bioelectrocatalyst, reduces the damage.

Acknowledgements

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