Bilirubin Oxidase-Based Nanobiocathode Working in Serum-Mimic Buffer for Implantable Biofuel Cell

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Abstract

Low-density graphite electrodes have been covalently modified with gold nanoparticles and used as scaffold for the oriented immobilization of *Myrothecium verrucaria* bilirubin oxidase. The developed nanobioelectrodes have been tested as a biocathode in a human-serum surrogate buffer that mimics physiological conditions. The nanostructured bioelectrode offered a $-140 \mu\text{A}\cdot\text{cm}^{-2}$ current density under serum-mimic conditions at 0.2 V, and was operational during at least 6 days.

Keywords: bilirubin oxidase, biofuel cell, oxygen cathode, gold nanoparticles

Enzymatic electrodes have many advantages for their use as biosensors or biofuel cells. In particular, biofuel cells can find an application field in powering small devices using biofluids, *i.e.* implantable biomedical devices [1]. In this case a miniaturized biofuel cell must enter in contact with the living body and use the naturally existing biochemical substances as fuel (e.g. glucose in blood or in lachrymal liquid) [2]. Biosensors and biofuel cells may also act together by using the biofuel cell to power an *in vivo* electric device able to read a signal from a biosensor. Bilirubin oxidase (BOx) is a suitable enzyme for this kind of application because it can work as the biocatalyst of an O_2 -cathode at neutral pH and high chloride concentration, unlike other multicopper oxidases [3]. BOx, as other multicopper oxidases, contains four Cu ions that are responsible of its catalytic activity. One Cu ion is located in the T1 site, where the substrate oxidation takes place. The other three Cu ions are located in the interconnected T2/T3 sites, where O_2 is reduced to water using four electrons transferred from the T1 site [4]. In a BOx-electrode the electrons for the catalyzed reaction come from the electrode instead of from the substrate, either by direct electron transfer (DET) [3a-c,5] or mediated electrode transfer [3d,6]. Despite all the research performed on BOx-modified electrodes, there are few works reporting their study in physiological fluids [7].

In the present work we have studied the performance of a BOx cathode when immersed in a serum-like buffer. For this purpose we have used a strategy designed for fabricating robust high area electrodes that allows efficient DET of immobilized BOx aided by covalently bound gold nanoparticles (AuNPs).

The use of low-density graphite (LDG) electrodes, which have a high surface area, covalently modified with AuNPs and BOx was intended for obtaining an O_2 biocathode with high catalytic current densities and was stable in the serum-like buffer. AuNPs ($5 \pm 3 \text{ nm}$) were covalently attached to the *in situ* diazonium-functionalized LDG electrode during 3 days, following the strategy reported by us in a recent work [8]. The presence of the AuNPs was demonstrated by cyclic voltammetry in H_2SO_4 0.1M (Fig. 1). No oxidation or reduction peaks are observed with the non-modified LDG electrode, whereas the AuNP-LDG electrode gave the typical gold oxidation-reduction peaks. The gold oxide reduction signal was integrated to estimate the surface coverage of the covalently attached AuNPs, obtaining a charge of $180 \mu\text{C}$, which corresponds to 1.39 cm^2 using the conversion factor for gold of $482 \mu\text{C}\cdot\text{cm}^{-2}$ [9].

Fig. 1. Cyclic voltammograms of LDG electrode in 0.5 M H_2SO_4 at $100 \text{ mV} \cdot \text{s}^{-1}$ scan rate: (a) prior to immobilization of AuNP; (b) after covalent binding of AuNP_s.

Later, the AuNP-LDG electrode was first modified by electrochemical reduction of the diazonium salt derived from the precursor 6-amino-2-naftoic acid; it has been shown previously that this surface modification favors DET of BOx [3c]. After the 6-naftoxylic acid layer formation, a self-assembled monolayer (SAM) of mercapto propionic acid was formed to block the possible gold-naked areas

remaining on the AuNPs attached to the electrode. Finally, BOx was covalently immobilized by establishing amide bonds between its lysine residues and the carboxylic groups of the electrode surface. Fig. 2. Cyclic voltammograms of a BOx-AuNP-LDG electrode in phosphate buffer 100 mM, pH 7.4 at $10 \text{ mV} \cdot \text{s}^{-1}$ scan rate and 500 rpm: under air (dotted line); 1 atm of O_2 (dashed line); background signal after inhibition with 30 mM NaF (solid line).

The BOx-AuNP-LDG electrode was tested as a cathode for potentially implantable biofuel cells. The electrodes were characterized by cyclic voltammetry in either phosphate or plasma-mimic buffer. Fig. 2 shows the resulting cyclic voltammograms run in phosphate buffer. The measurement with the solution equilibrated with air shows a reductive catalytic wave starting at 490 mV, which is near to the redox potential of BOx T1 site [10]. A catalytic plateau current of approximately $-175 \mu\text{A} \cdot \text{cm}^{-2}$ is reached, considering the electrode geometric area of 0.07 cm^2 and subtracting the capacitive current measured

after the inhibition of the immobilized BOx with fluoride [11]. O₂-saturation of the atmosphere yielded a higher reductive electrocatalytic current of $-375 \mu\text{A}\cdot\text{cm}^{-2}$ (Fig. 2).

Some cross-linking of the immobilized BOx molecules may take place during the EDC coupling step. However, we estimate that it happens to a low extent because in all tested electrodes the addition of redox mediator ABTS to the cell solution only gave a slight increase of the catalytic plateau current of around 5% (Fig. 1 of Supporting Information). This effect can be explained by the high affinity of BOx

molecules to the naftoic acid groups of the electrode surface [3c], favoring immobilization of a BOx monolayer but not multilayers.

Fig. 3. Cyclic voltammograms of a BOx-AuNP-LDG electrode in serum mimic buffer at $10 \text{ mV}\cdot\text{s}^{-1}$ scan rate and 500 rpm electrode rotation: under air (dotted line); 1 atm of O₂ (dashed line); background signal after inhibition with 30 mM NaF (solid line).

The BOx-AuNP-LDG electrode provided also an electrocatalytic response of O₂ reduction when taken into serum-mimic buffer (Fig. 3). The catalytic plateau current was approximately $-140 \mu\text{A}\cdot\text{cm}^{-2}$. However, oxidative processes at 0.3 and 0.47 V are also detected, which are attributed to the presence of ascorbic acid and uric acid in the medium, as they are clearly present in the background current measured after inhibition of BOx with NaF. The presence of many additives in the serum-mimic buffer affected the electrocatalytic measurements, particularly for the case of O₂-saturated solution, as the electrocatalytic current did not increase relative to the air-saturated measurement. This can be explained by the BOx-denaturing effect of the combination of uric acid and O₂ present in the serum-mimic buffer, which has been reported by Heller and coworkers for a wired BOx cathode tested in serum [12]. It is expected that this inhibitory effect will be enhanced when the medium is saturated with O₂, thus cancelling the positive effect of the higher substrate concentration on electrocatalysis. Nevertheless, this result may not be a problem in regard to its *in vivo* applications, as the O₂ concentration will not be above the air-saturated value.

The catalytic current plateau measured in the serum-buffer with our BOx-cathode is 6-7 times lower than that reported recently by Edembe et al. for another BOx-cathode operating in serum [7b]. However, this later biocathode is not based on DET of the enzyme but on mediated electron transfer using an immobilized polycationic redox polymer. On the other hand, higher DET-based catalytic current is obtained (nearly 3-fold) in our biocathode compared to that reported by Wang et al. in human plasma for BOx immobilized on a three dimensional electrode [7c].

Fig. 4. Chronoamperometric response of a BOx-AuNP-LDG electrode measured at 0.2 V and 500 rpm electrode rotation. The arrow placed in day 6 indicates the addition of 35 mM NaF to the solution.

The operational stability of the BOx-AuNP-LDG in the serum-mimic buffer was studied by chronoamperometry at 0.2 V and 500 rpm electrode rotation. A large decrease of the catalytic current happened after the first day of continuous operation of the biocathode, being only 19% of the initial value. However, after this strong initial decrease the biocathode proved to be quite stable in the following 6 days (Fig. 4). The high initial loss of electroactivity of the biocathode can be explained by the deleterious effect of uric acid on BOx activity mentioned before [12]. Two recent articles report a higher operational stability of BOx-cathodes in serum or plasma during the first 3-8 hours, although data at longer times was not presented [7b,c]. Nevertheless, the long term operational stability after the first 24 hours of the BOx-NA/MP-AuNP-LDG cathode developed in the present work can be attributed to its robust configuration, in which both AuNPs and enzyme molecules are covalently bound to the electrode [8]. The enzymatic origin of the cathodic current is demonstrated after 6 days of continuous measurement by the addition of 30 mM fluoride, which reduces the current to the background level.

The bioelectronic system developed in this work by modifying a high-surface porous graphite to serve as scaffold for AuNPs covalent linkage and further BOx immobilization has led to high density currents of O₂ reduction. This nanostructured biocathode is suited for its application as O₂ biocathode in biofuel cells operating in physiological fluids. In this biocathode, 19% of the initial electroactivity was retained after 1 week of continuous operation in serum-mimic buffer. Future work regards its study for its application in a membrane-less biofuel cell developed to obtain power from biofluids.

Experimental

Enzyme

Purified preparations of *Myrothecium verrucaria* BOx, expressed recombinantly in *Aspergillus oryzae*, have been kindly provided by Novozymes A/S.

Chemicals

All reagents were purchased on ACS purity grade. 6-amino-2-naftoic acid, 3-mercaptopropionic acid, NaNO₂, HAuCl₄, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-morpholinoethanesulfonate (MES) hydrate 99.5% and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma. Tetrakis(hydroxymethyl)-

phosphonium chloride (THPC), 80%, was obtained from Fluka. Water Milli-Q (18.2 M Ω ·cm) was used for all the experiments.

Gold nanoparticles synthesis

AuNPs for graphite electrodes modification were freshly prepared following a known procedure [13], which consists on reducing 1 mM HAuCl₄ in 60 mM NaOH by addition of 1 mM THPC.

Modification of graphite electrodes with AuNPs and further attachment of BOx

LDG rods of 3 mm diameter (Sigma) were modified with AuNPs according to a recently developed method [8]. The AuNP-LDG electrodes were taken into an electrochemical cell filled with an ice-cooled solution, prepared immediately before its use by mixing 5 mL of 20 mM 6-amino-2-naftoic acid in acetonitrile and 5 mL of 2 mM NaNO₂ in 2 M HCl aqueous solution. Two reductive cyclic voltammograms from 0.6 V to -0.3 V at 200 mV·s⁻¹ scan rate were recorded. The electrode was immersed overnight in a aqueous solution of 1 mM mercapto propionic acid. After rinsing the electrode with water, 20 μ L of 0.83 mg·mL⁻¹ BOx in 10 mM MES buffer pH 6 were deposited on the electrode surface and covered with a lid to avoid evaporation. 30 min after, covalent immobilization was performed by adding 5 μ L of 36 mM EDC and 5 μ L of 17 mM NHS, both of them dissolved in 10 mM MES buffer pH 6, during 90 min.

Serum-mimic buffer preparation

150 mM NaCl, 18 mM NaHCO₃, 5.55 mM glucose, 4.3 mM urea, 0.4 mM fructose, 0.4 mM uric acid, 0.055 mM ascorbic acid, 0.1 mM L-cysteine and 0.87 mM MgSO₄ were added to 100 mM phosphate buffer pH 7.4.

Electrochemical measurements

Experiments were performed with an Autolab PGSTAT30 analyzer controlled by GPES 4.9 software (Eco Chemie). Experiments were run in a three-electrode glass cell, thermostated at 27°C by a water jacket, using a BAS Ag/AgCl, 3 M KCl reference electrode (204 mV vs. NHE) and a platinum wire counter electrode. All redox potentials mentioned in the text and figures are given vs. the Ag/AgCl reference electrode. The LDG electrodes were connected to a MSR electrode rotator from Pine Instruments

Supporting Information

Fig. S1: Comparison of the electrocatalytic current of a BOx-AuNP-LDG electrode in absence and in presence of ABTS as redox mediator. This material is available free of charge via the internet.

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References

- [1] S. C. Barton, J. Gallaway, P. Atanasov, *Chem. Rev.* **2004**, *104*, 4867-4886.
- [2] a) A. Heller, *Anal. Bioanal. Chem.* **2006**, *385*, 469-473. b) F. Tasca, L. Gorton, W. Harreither, D. Haltrich, R. Ludwig, G. Noll, *J. Phys. Chem. C* **2008**, *112*, 9956-9961. c) F. Tasca, L. Gorton, M. Kujawa, I. Patel, W. Harreither, C. K. Peterbauer, R. Ludwig, G. Noll, *Biosens. Bioelectron.* **2005**, *25*, 1710-1716.
- [3] a) S. Shleev, A. E. Kasmi, T. Ruzgas, L. Gorton, *Electrochem. Commun.* **2004**, *6*, 934-939. b) S. Tsujimura, Y. Kamitaka, K. Kano, *Fuel Cells* **2007**, *7*, 463-469. c) L. dos Santos, V. Climent, C. F. Blanford, F. A. Armstrong, *Phys. Chem. Chem. Phys.* **2010**, *12*, 13962-13974. d) F. Durand, C.H. Kjaergaard, E. Suraniti, S. Gounel, R. G. Hadt, E. I. Solomon, N. Mano, *Biosens. Bioelectron.* **2012**, *35*, 140-146.

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- [4] S. Shleev, J. Tkac, A. Christenson, T. Ruzgas, A. I. Yaropolov, W. Whittaker, L. Gorton, *Biosens. Bioelectron.* **2005**, *20*, 2517-2554.
- [5] a) S. Shleev, V. Andoralov, M. Falk, C. T. Reimann, T. Ruzgas, M. Srnec, U. Ryde, L. Rulisek, *Electroanalysis* **2012**, *24*, 1524-1540. b) A. Ciaccafava, A. De Poulpiquet, V. Techer, M. T. Giudici-Orticoni, S. Tingry, C. Innocent, E. Lojou, *Electrochem. Commun.* **2012**, *23*, 25-28.
- [6] M. D. Scanlon, U. Salaj-Kosla, S. Belochapkine, D. MacAodha, D. Leech, Y. Ding, E. Magner, *Langmuir* **2012**, *28*, 2251-2261.
- [7] a) C. Kang, H. Shin, A. Heller, *Bioelectrochemistry* **2006**, *68*, 22-26. b) L. Edembe, S. Gounel, M. Cadet, F. Durand, N. Mano, *Electrochem. Commun.* **2012**, *23*, 80-82. c) X. Wang, M. Falk, R. Ortiz, H. Matsumura, J. Bobacka, R. Ludwig, M. Bergelin, L. Gorton, S. Shleev, **2012** *Biosens. Bioelectron.* *31*, 219-225.
- [8] C. Gutiérrez-Sánchez, M. Pita, C. Vaz-Dominguez, S. Shleev, A. L. De Lacey, *J. Am. Chem. Soc.* **2012**, *134*, 17212-17220.
- [9] U. Oesch, J. Janata, *Electrochim. Acta* **1983**, *28*, 1237-1246.
- [10] A. Christenson, S. Shleev, N. Mano, A. Heller, L. Gorton, *Biochim. Biophys. Acta.* **2006**, *1757*, 1634-1641.
- [11] a) A. Naki, S. D. Varfolomeev, *Biochemistry-Moscow* **1981**, *46*, 1344-1350. b) F. Xu, R. M. Berka, J. A. Wahleithner, B. A. Nelson, J. R. Shuster, S. H. Brown, A. E. Palmer, E. I. Solomon, *Biochem. J.* **1998**, *334*, 63-70.
- [12] C. Kang, H. Shin, Y. Zhang, A. Heller, *Bioelectrochemistry* **2004**, *65*, 83-88.
- [13] M. Pita, J. M. Abad, C. Vaz-Dominguez, C. Briones, E. Mateo-Martí, J. A. Martín-Gago, M. P. Morales, V. M. Fernández, *J. Coll. Interf. Sci.* **2008**, *321*, 484-492.