

## **Direct electron transfer of bilirubin oxidase (*Myrothecium verrucaria*) at an unmodified nanoporous gold biocathode**

Urszula Salaj-Kosla<sup>a,b</sup>, Sascha Pöller<sup>a</sup>, Yvonne Beyl<sup>a</sup>, Michéal D. Scanlon<sup>b</sup>, Sergey Beloshapkin<sup>b</sup>, Sergey Shleev<sup>c</sup>, Wolfgang Schuhmann<sup>a\*</sup>, Edmond Magner<sup>b\*</sup>

<sup>a</sup> Analytische Chemie – Elektroanalytik & Sensorik, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany

<sup>b</sup> Department of Chemical and Environmental Sciences & Materials and Surface Science Institute, University of Limerick, Limerick, Ireland

<sup>c</sup> Biomedical Laboratory Science, Faculty of Health and Society, Malmö University, Södra Förstadsgatan 101, SE-20506 Malmö, Sweden

\*Corresponding authors:

Email address: edmond.magner@ul.ie; tel.: +353 61 202628; fax: +353 61 213529

Email address: wolfgang.schuhmann@rub.de; tel.: +49 234 3226200; fax: +49 234 3214683

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### **Abstract**

Well defined mediatorless bioelectrocatalytic reduction of oxygen with high current densities of 0.8 mA cm<sup>-2</sup> were obtained on nanoporous gold electrodes modified with *Myrothecium verrucaria* bilirubin oxidase. A stable faradaic response was observed when the enzyme

modified electrode was coated with a specifically designed electrodeposition polymer layer. The response of the enzyme electrode was only slightly inhibited by the addition of F<sup>-</sup>.

## 1. Introduction

Bilirubin oxidase (BOD) and laccase have been used extensively in the cathodes of biofuel cells. Both enzymes are 'blue copper' proteins that reduce oxygen to water. Their redox potentials depend on the source of the enzyme. Values of 190 and 460 mV (vs Ag/AgCl, 3 M KCl at pH 7) were reported for the T2/T3 and T1 sites, respectively, for BOD from *Myrothecium verrucaria* (*MvBOD*) [1-3]. *MvBOD* is a monomeric enzyme with a molecular mass of 66 kDa and an isoelectric point of 4.2 [4]. In comparison to laccase, *MvBOD* possesses high catalytic activity at neutral pH while utilising relatively low overpotentials for the reduction of O<sub>2</sub>. The activities of laccase and BOD are inhibited by Cl<sup>-</sup>, with the effect on BOD being far less pronounced [5-8].

Direct electron transfer (DET) between immobilized blue multicopper oxidases, including BOD, on modified gold electrodes has been intensively studied [1-2, 9]. However, in comparison with carbon based surfaces, there are few examples of the use of metallic films in the preparation of biocathodes. DET on bare gold electrodes is difficult with unstable bioelectrocatalytic responses for the few systems that have been examined [9]. Modification of the gold electrode surface with thiol monolayers enables orientation of *MvBOD* on the electrode in a manner which promotes efficient DET. The current density depends on the nature of the terminal groups of the monolayers used to modify the gold electrodes. Carboxyl terminated monolayers enable higher electrocatalytic currents for the reduction of oxygen in comparison to -NH<sub>2</sub>, -OH and -CH<sub>3</sub> terminated monolayers [10]. Covalent binding of *MvBOD* to a gold surface modified with 3-mercaptopropionic acid and stabilised with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) also showed a catalytic response [2].

Improvements in DET on bare gold electrodes can be achieved by changing the morphology of the electrode, with current densities of  $0.5 \text{ mA/cm}^2$  reported for MvBOD immobilised on 15 nm diameter gold nanoparticles in  $\text{O}_2$  saturated solution at pH 7. Further increases in the current density were achieved when a highly porous carbon paper was used as a support for gold nanoparticles. Maximal current densities of  $\sim 4.9 \text{ mA/cm}^2$  and power densities of  $0.87 \text{ mW/cm}^2$  at 300 mV were obtained for a biofuel cell comprised of such a biocathode in combination with a fructose dehydrogenase bioanode (pH 6.0, stirred at 1000 rpm [10]).

Nanoporous gold is an attractive material due to its excellent stability over a wide pH range, high conductivity and biocompatibility [11-12]. Nanoporous gold is mechanically stable and easy to make. It has a high surface-to-volume ratio with tunable pore sizes that allow increased loadings of immobilised proteins. In this study, DET-based bioelectrocatalytic reduction of oxygen by MvBOD immobilized on nanoporous gold electrodes was observed with high current densities of *ca.*  $0.8 \text{ mA/cm}^2$  under aerobic conditions. The inhibitory effect of  $\text{F}^-$  on MvBOD that is usually observed was substantially decreased for the immobilized enzyme.

## **2. Experimental section**

### **2.1. Materials and Methods**

Sulfuric acid, citric acid and disodium phosphate were obtained from J. T. Baker. Sodium fluoride was purchased from Sigma-Aldrich. All chemicals were of analytical grade and used as received without further purification. Buffers were prepared with water ( $0.055 \text{ }\mu\text{S/cm}$ ) purified with Ultra Clean UV (SG). Aerobic and anaerobic conditions were established by bubbling oxygen and argon (Air Liquide), respectively through the solutions for 20 min.

Electrochemical measurements were performed with an AutoLab potentiostat (PGSTAT12, Eco Chemie) using a three-electrode cell with Pt wire, Ag/AgCl (3M KCl) and nanoporous

gold as the counter, reference and working electrodes, respectively. The supporting electrolyte consisted of 0.1 M citrate-phosphate buffer solution at pH 7.0. Current densities were estimated using the geometric surface area of the working electrode. All potentials are reported vs. Ag/AgCl (3 M KCl).

## **2.2. Enzyme**

Partially purified preparations of *Mv*BOD were kindly provided by Amano Enzyme Inc. (Nagoya, Japan). The enzyme was purified to homogeneity using a published procedure [13].

## **2.3. Synthesis of copolymer P017-epoxy**

Polymerisation was carried out by reacting polyethylene glycol methacrylate (395 mg, 0.75 mmol), allyl methacrylate (631 mg, 5.0 mmol), and butyl acrylate (545 mg, 4.25 mmol) in methanol. Excess monomer was removed by precipitation from water. The solid copolymer was re-dissolved in MeOH to give the final polymer suspension (P017; 4.7 % w/w). The synthesis of dimethyldioxirane (DMDO) and epoxidation of P017 was performed according to a published procedure [14]. The dry polymer obtained was re-dissolved in MeOH (1.0 mL). Water was added drop-wise, until the suspension turned milky, to give the final polymer suspension (P017-epoxy).

## **2.4. Modification of nanoporous gold electrodes**

Nanoporous gold electrodes with uniform distribution of Ag were prepared as described previously [15]. The electrodes were cleaned by scanning (10 scans) from 0 to 1.5 V at 200 mV/s in 0.5 M H<sub>2</sub>SO<sub>4</sub>, followed by scanning at 50 mV/s between 0 and 0.8 V in 0.1 M citrate-phosphate buffer at pH 7.0. The electrodes were then placed in a vacuum chamber at 10<sup>-2</sup> mbar for 10 min.

20 µL of a *Mv*BOD solution (40 µg/ml) was placed on a clean, dry nanoporous gold electrode and allowed to adsorb in the vacuum chamber under pressure (10<sup>-2</sup> mbar) for ~3 min. The electrode was removed from the vacuum chamber, covered with 10 µL of P017-epoxy

solution and allowed to dry in air at room temperature for 15 min. As a control experiment, an electrode was modified by drop-casting 20  $\mu\text{L}$  of BOD solution (40  $\mu\text{g/ml}$ ) which was then kept at 4°C for 2 hours prior to use.

### 3. Results and discussion

Bioelectrocatalytic current densities of *ca.* 0.04  $\text{mA/cm}^2$  were obtained at *Mv*BOD modified electrodes prepared by placing a drop of enzyme solution onto the surface of an electrode that had been first dried under vacuum (Fig 1A). On changing the deposition conditions by first drying the electrodes in vacuum, followed by deposition of the enzyme solution in vacuum, significantly higher current densities of *ca.* 0.3  $\text{mA/cm}^2$  (Fig. 1B) were obtained. More importantly, these high bioelectrocatalytic current densities were observed in DET mode for *Mv*BOD immobilised on unmodified, bare gold electrodes. The high catalytic responses observed arise from the high surface areas of the electrodes (roughness factor of 26 (inset Fig. 1B) [15]) combined with the high loadings of enzyme which were achieved by using vacuum to dry the electrode and then adsorb the enzyme. The use of heating to evaporate the solution in the pores was avoided in order to prevent further morphological changes in the electrode arising from thermal stresses [16].

On repeated potential scans the response of the electrode was observed to be unstable with a 50% decrease in the current (Fig. 1B). On modifying the electrode with P017-epoxy, current densities of 0.8  $\text{mA/cm}^2$  were observed which were stable on repeated potential scans (Fig. 1C). This indicates that BOD is relatively loosely bound and easily removed from the surface of the electrode. The role of P017-epoxy is to stabilize the adsorbed *Mv*BOD by creating covalent bonds with nucleophilic groups such as amine, thiol and hydroxyl groups on the surface of the enzyme [17]. Effectively, a cap-like structure was formed on the electrode which prevents leakage of the enzyme (Fig. 2.) and results in very stable currents (Fig. 1C).

In an oxygen saturated solution the onset potential for the biocatalytic reduction of oxygen at *Mv*BOD-modified nanoporous gold electrodes was *ca.* 500 mV (Fig. 1.), in good agreement with the redox potential of 460 mV for the T1 site [1-2, 18]. The theoretical limiting current density of *ca.* 0.6 mA/cm<sup>2</sup> (calculated from the Randles-Sevcik equation and utilising values of  $2.6 \times 10^{-5}$  cm<sup>2</sup>/s and 420 μM for the diffusion coefficient and concentration of oxygen, respectively [19] and a roughness factor of 2.1 for the reduction of dioxygen [14]), indicates that the observed current density of 0.8 mA/cm<sup>2</sup> is controlled by diffusion of O<sub>2</sub> from the bulk solution. At high currents, the response was quite noisy over the potential range 0.35 – 0.1 V, which may have arisen from the highly porous and uneven nature of the electrode. Variations in the amount of oxygen (as well as the amount of adsorbed enzyme) may result in localised variations in the concentration of oxygen, disrupting the response observed. As expected both electrodes did not demonstrate a catalytic current in argon saturated electrolyte. A nanoporous gold electrode coated with P017-epoxy in the absence of enzyme did not show any oxygen reduction current (data not shown).

It is well established that F<sup>-</sup> inhibits *Mv*BOD activity by binding to the T2/T3 copper site, blocking the transfer of electrons from the T1 site to the T2/T3 cluster [20-21]. Surprisingly, the addition of F<sup>-</sup> had little influence on the biocatalytic current of BOD immobilized at nanoporous gold electrodes under aerobic conditions. The maximal loss of activity of a biocathode stabilized with P017-epoxy was *ca.* 3.5%. Such a low inhibitory effect indicates that the normal deleterious effect of F<sup>-</sup> on the response was not observed. The significantly reduced inhibitory effect may arise from exclusion of F<sup>-</sup> from the pores by the polymer and from confinement of the enzyme in the pores of the electrode in a manner which precludes binding of F<sup>-</sup> to the T2/T3 site.

The high catalytic current density of *ca.* 0.8 mA/cm<sup>2</sup> observed on *Mv*BOD modified nanoporous electrodes is in contrast to that at a planar gold electrode where no catalytic

response was observed in this and previous reports [1-2, 9]. It is feasible that enzymes within the 3D porous structure are in an environment where they are in intimate contact with the gold walls of the pores and able to undergo direct electron transfer in an efficient manner (Fig. 2). The use of these electrodes as supports for other redox enzymes in the development of biofuel cells is currently under investigation.

#### **4. Conclusions**

MvBOD immobilised within the pores of nanoporous gold electrodes can undergo efficient DET without surface modification of the electrode. Significantly higher stability was observed when the BOD-nanoporous gold electrode was coated with a P017-epoxy cap. High bioelectrocatalytic current densities of up to 0.8 mA/cm<sup>2</sup>, with minimal inhibition of the response by F<sup>-</sup>, were obtained.

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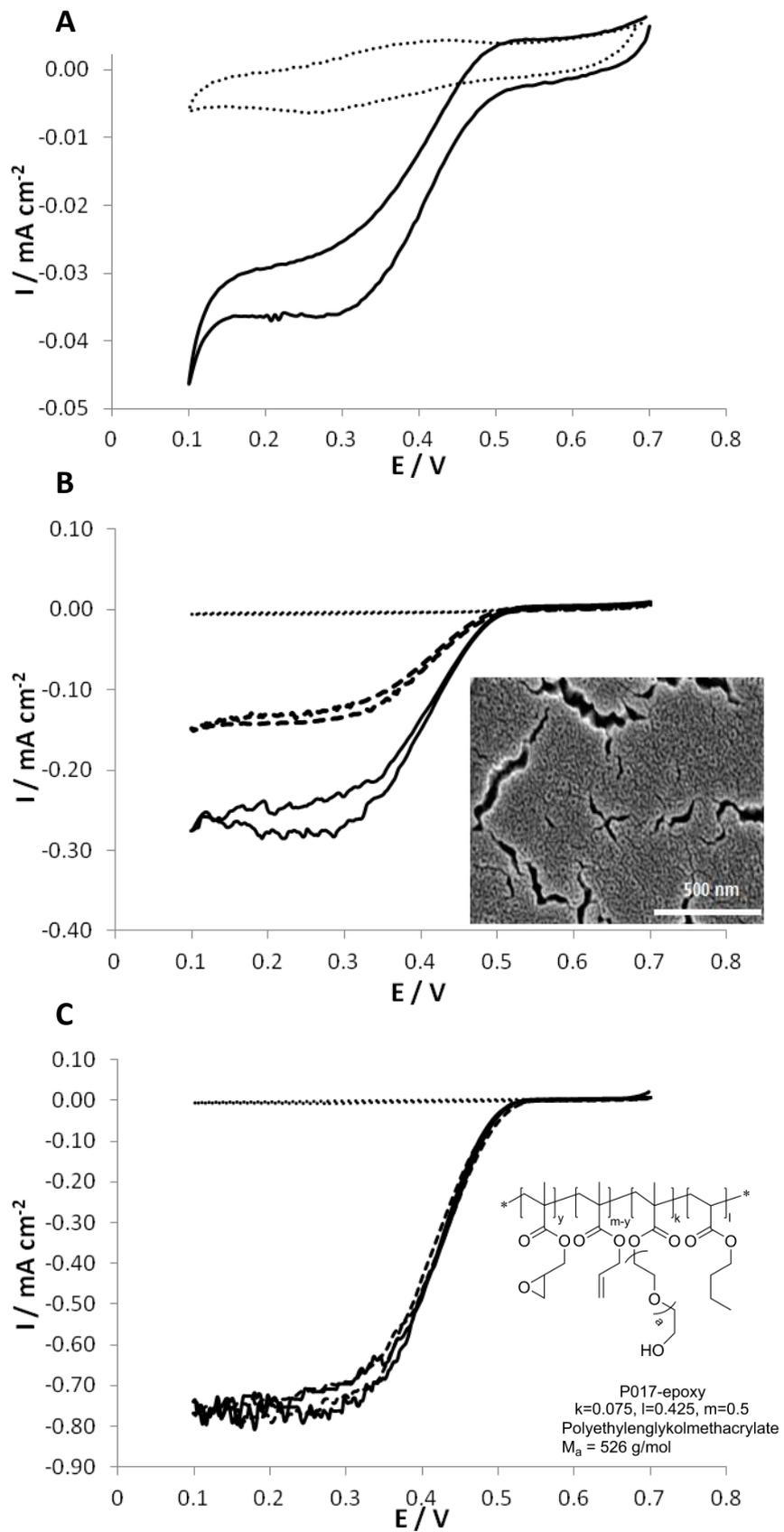
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**Figure captions:**

**Figure 1:** Cyclic voltammograms of *Mv*BOD modified nanoporous gold electrodes (A) prepared by drop-casting in the presence of O<sub>2</sub> (full line) and Ar (dotted line). (B) prepared under vacuum without stabilizer in the presence of O<sub>2</sub> (first scan, full line), (second scan, dashed line) and in the presence of Ar (dotted line); inset: SEM image of the surface of a nanoporous gold electrode. (C) prepared under vacuum with P017-epoxy as stabilizer in the presence of O<sub>2</sub> (first scan, full line), (second scan, dashed line) and in the presence of Ar (dotted line); inset: the proposed structure of P017-epoxy. Conditions: 0.1 M citrate-phosphate buffer, pH 7.0, scan rate of 5 mV s<sup>-1</sup>.

**Figure 2:** Schematic diagram of the cross-section of a nanoporous gold electrode with adsorbed *Mv*BOD without P017-epoxy cap (A) and with P017-epoxy cap (B).

**Figure 1:**



**Figure 2:**

