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Direct electron transfer of bilirubin oxidase at a carbon flow-through electrode

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ABSTRACT

The kinetics and isotherms of adsorption of *Myrothecium verrucaria* bilirubin oxidase (*Mv* BOD) on nanoporous carbons (CNP) and carbon nanofibers (CNF) were studied by the solution depletion method. The kinetics of adsorption of *Mv* BOD on both carbons are very fast, reaching equilibrium within 10 min and 30 min for CNP and CNF, respectively. The adsorption isotherms reveal a strong affinity between *Mv* BOD and both carbons. An original flow-through device based on electrodes filled with CNP and CNF connected to an UV–vis spectrophotometer was used to correlate the dioxygen catalytic current reduction to the amount of *Mv* BOD adsorbed on the carbons. It was shown that although the amount of BOD adsorbed on CNP is much lower than the amount adsorbed on CNF, the currents are comparable, suggesting that the *Mv* BOD orientation is more favourable for direct electron transfer in the case of CNP. Chronoamperometry experiments showed that the catalytic current is stable for a few days (70 h). It was emphasized by stop and flow experiments that the current is limited by mass transport inside the column.

1. Introduction

Hydrogen has emerged as a very promising energy carrier to tackle the issues of reserve depletion and pollution that come with the use of fossil sources. Because of its good performances in terms of power density and sustainability, proton exchange membrane fuel cell operating at low temperature is largely studied as a power source for transportation, portable or specific stationary applications. However, its spread on the market is still limited by the use of platinum based electrocatalysts and the proton conducting membrane for cost and availability reasons [1,2]. A new type of fuel cell based on efficient and specific noble metal-free redox enzymes was alternatively proposed. At the anode of these H_2/O_2 enzymatic fuel cells (EFC) enzymes such as hydrogenases are used as biocatalysts for oxidation of hydrogen [3–5]. On the cathodic side, multicopper oxidases (MCO) are generally used to reduce O_2 into water. Most popular are laccase and bilirubin oxidase (BOD) [6].

Myrothecium verrucaria BOD (*Mv* BOD) is one of the most studied oxidase as a biocatalyst in EFC. It has been identified in a filamentous fungus and works in neutral conditions at room temperature. The active site of this enzyme consists of four copper ions: one T1 type, one T2 type and a pair of T3 type. It has been shown that the T1 site is the primary electron acceptor from the physiological substrate. Electrons are transferred via internal pathways to the trinuclear T2/T3 cluster where the copper-cluster-bound O_2 is reduced which leads to the conversion of dioxygen into water in a four-electron reaction. Here, the direct electron transfer (DET) pathway that can be established by connecting the enzyme to the electrode with the T1 Cu (the entry site of electrons), facing the electrode, is considered. This preferred orientation of the enzyme can be achieved either by strong noncovalent binding, such as electrostatic interactions [7,8] or through some adsorbed compounds [9].

To date a few H_2/O_2 EFCs using BOD and hydrogenase have been built delivering power in the mW cm^{-2} range. However, bio-power technology based on enzymatic electron transfer suffers from several limitations coming mainly from the design of the electrodes. Indeed, signal output is dependent on the nature of the

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enzyme, the efficiency of the connection between the enzyme and the electrode surface, the kind of electron transfer, the stability and reproducibility of the layer, and the electrically active surface area [10]. Three dimensional electrode architectures are promising since they would greatly increase the reactive surface area and therefore the enzyme loading, allowing for current enhancement [11–14]. Carbon nanopowders are very interesting as enzyme support because of high specific surface areas, tailorable surface chemistry and textural properties (porosity), good electronic conductivity, low density, availability and low prices for some of them. Immobilization of enzymes has thus been studied on various carbon materials such as carbon cloth, carbon blacks, carbon nanoparticles, graphite particles, pyrolytic graphite, carbon nanotubes (CNT), carbon nanofibers (CNF) or graphene [15,16].

However, enzymes are nanometer size objects, with metal active site embedded in an insulating protein moiety. Hence, depending on the orientation of the enzyme on the carbon surface, there will be a distribution of distances between the carbon substrate and the active site of the enzyme and thus, a distribution in the electron transfer rates [17]. It is well accepted that to obtain an effective direct electron transfer using MCO, the T1 copper site situated at 7–8 Å from the enzyme surface must be at a tunneling distance from the electrode surface. So far, the issue of enzyme orientation has been mostly addressed through engineering of the enzyme or modification of electrode surface chemistry [10]. The porous structure of the carbon electrode may furthermore influence the enzyme orientation, which essentially depends on the relative pore size versus enzyme size [18]. However, when larger surfaces are used by increasing the mass of carbon for example, the efficiency of the enzymes induces fast depletion of the enzyme substrate at the material interface [3,12]. To overcome this issue, gas-diffusion-layer electrodes were proposed [19] but the three-phase-boundaries in such systems are deleterious for the enzymes. There is thus a need for new electrode geometries.

In this work an original study of dioxygen reduction by *Mv* BOD immobilised in a fixed-bed carbon electrode is reported. The electrolyte is able to flow through the carbon bed in order to force mass transport inside the electrode. As the carbon powder is unsupported, the quantity of carbon can vary and the measurements are not influenced by any powder support or binder. Thanks to its UV–vis signature [16,20], BOD adsorption on carbon could be quantified by the solution depletion method. Therefore, by coupling chronoamperometric measurements to *ex situ* and *in situ* UV spectroscopy the correlation between the electrocatalytic activity and the adsorption properties have been studied for two different carbon powders.

2. Experimental

2.1. Materials

A commercial graphitized carbon used without further purification was purchased from Sigma-Aldrich (CAS number 1333-86-4). According to the manufacturer, the carbon consists in 35 nm particles presenting a mesoporosity of 13.7 nm and that further forms 175 nm–400 nm agglomerates. It will be referred to as CNP (Carbon Nanoparticles). The carbon nanofibers used in this study are described in details in Ref. [12]. Briefly, the CNFs were prepared by a conventional chemical vapor deposition (CVD) process using a Ni catalyst on a graphite support. The CNF carbon was then treated under hydrogen at 600 °C in order to obtain a hydrophobic surface. *Mv* BOD from Sigma-Aldrich (CAS number 80619-01-8, 15-65 units/mg protein) was diluted in 0.1 M phosphate buffer (pH 6.9). All reagents were of analytical grade. All the experiments were carried out at 298 K, in an air-conditioned room.

2.2. Characterization of carbons

CNP was characterized by gas adsorption and mercury intrusion porosimetry. Nitrogen sorption isotherms at 77 K were determined with an ASAP2010 apparatus from Micromeritics. The carbon was outgassed overnight before analysis at a temperature of 120 °C and a pressure below 1 Pa. The Brunauer–Emmett–Teller (BET) equation was applied to determine the surface area. The porosity and pore size distribution of CNP were obtained by mercury porosimetry using the Poremaster apparatus from Quantachrome. Intrusion and extrusion were carried out after the sample was evacuated. The cumulative intruded volume was measured as a function of intrusion pressure. This latter was transformed in pore size by applying the Laplace Washburn equation with a contact angle of 130°. The characterization of CNF is described in details in Ref. [12].

2.3. BOD adsorption from solution

The adsorption isotherms at 298 K were determined by the solution depletion method. Different amounts of a stock solution of *Mv* BOD (1 mg cm^{-3}) were introduced into glass tubes containing 4 mg of carbon in a 1 cm^3 of phosphate buffer (0.1 M, pH 6.9). The time necessary to reach equilibrium was experimentally determined from preliminary kinetic measurements. For the adsorption isotherm the tubes were stirred during 24 h, which was longer than the time necessary to reach equilibrium. The suspensions were then centrifuged and the equilibrium concentration of *Mv* BOD was determined at a wavelength of 280 nm using an Agilent UV–vis spectrophotometer. The amount adsorbed on the carbon is given by:

$$Q_{ads} = (C_i - C_e)\frac{V}{m_s} in\ mg\ g^{-1} \quad (1)$$

where C_i and C_e are the initial and equilibrium concentrations of BOD (mg dm^{-3}), V the volume of solution (dm^3) and m_s the mass of the solid sample (g). The reproducibility of these measurements was better than 5% within the range of studied concentrations.

Adsorption kinetics study of *Mv* BOD (presented for $C_i = 0.18$ mg cm^{-3}) were performed by analyzing the adsorption capacity at different time intervals at 298 K until the adsorption equilibrium was reached.

2.4. Design of the packed bed carbon electrode and electrochemical measurements set up

A schematic representation of the setup is given in Fig. 1. Enzymatic dioxygen reduction experiments were performed in a three electrode electrochemical cell containing 5 cm^3 of phosphate buffer at 0.1 M as the electrolyte. An Ag/AgCl/3 M KCl electrode was used as the reference electrode and a platinum wire as the counter electrode. The working electrode consisted of a stainless steel column (length 8 mm, diameter 6.35 mm) filled with the carbon. In the column, the carbon powder was introduced between stainless steel filters to ensure a homogeneous distribution and electrical contact. The entire device including the stainless steel column and the filters was electrically connected to the potentiostat. The total mass of carbon can be varied between 3 and 30 mg, and the number of filters added was adapted to maintain the mechanical stability of the carbon bed. The electrolyte was flowed through the carbon bed electrode with an HPLC pump. The solution flew from the electrochemical cell connected to a potentiostat/galvanostat