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An innovative powerful and mediatorless H_2/O_2 biofuel cell based on an outstanding bioanode

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ABSTRACT

A H_2/O_2 biofuel cell based on a hyperthermophilic O_2-tolerant hydrogenase and bilirubin oxidase was designed by one step covalent immobilization of the enzymes on functionalized carbon nanotubes. Under pure H_2 and O_2 saturated buffer solutions and no redox mediator, the biofuel cell delivers power densities up to 300 μW cm^{-2} at 0.6 V with an open circuit voltage of 1.1 V. These performances, which are demonstrated to be dependent on hydrogenase characteristics at high potentials, are the best ever obtained. Promising stability of the biofuel cell during 24 h of continuous use has been obtained, which allows considering this device as an alternative power supply for small portable applications.

1. Introduction

Enzymatic biofuel cells are promising alternative and renewable power sources. Devices based on enzymatic glucose oxidation and oxygen reduction have been widely studied for implantable and microelectronics applications [1–6]. Biofuel cells based on hydrogen oxidation have received much less attention although meaningful for many portable applications, essentially because of the extreme oxygen sensitivity of hydrogenase, the key enzyme for H_2 oxidation. During the last years, four membrane-bound hydrogenases have been discovered from aerobic or extremophilic organisms (*Ralstonia eutropha* [7], *Escherichia coli* (*E. coli*) [8], *Hydrogenovibrio marinus* [9], *Aquifex aeolicus* (Aa) [10,11]), that belong to the [NiFe]-hydrogenase family and have been demonstrated to oxidize H_2 in the presence of oxygen. The crystallographic structure of three of them has been resolved, showing that an uncommon [4Fe–3S] cluster proximal to the active site prevents deleterious oxygen attack. Due to this outstanding property, development of H_2/O_2 biofuel cells can be now considered.

Extensive studies on the understanding of the catalytic mechanisms for H_2 oxidation by [NiFe] hydrogenases immobilized on graphite and gold electrodes have allowed the definition of the parameters that help direct electron transfer [12]. In our group, original use of a hyperthermophilic hydrogenase from Aa (mbHI) allowed the increase in the catalytic current for H_2 oxidation on a large range of temperature up to 70 °C. Attempts to enhance the number of electrically connected mbHI succeeded by the use of coatings of chemically oxidized single-walled carbon nanotubes (SWCNT) [13]. Otherwise, direct connection on carbon nanotube-modified electrode of blue copper proteins such as bilirubin oxidase for oxygen reduction is widely documented [14]. We thus can envision mbHI coupled to bilirubin oxidase in a H_2/O_2 biofuel cell where both cathode and anode are modified with carbon nanotube coatings.

Very recently, a biofuel cell device based on *E. coli* hydrogenase and fed with an 80/20 hydrogen-air gas mixture has been elaborated in F.A. Armstrong's group using pyrene-carbon nanotube coatings as electrode materials. This fuel cell exhibited around 120 μW cm^{-2} power density at room temperature [15]. Herein we describe an innovative H_2/O_2 biofuel cell based on mbHI able to operate under pure H_2 aqueous solutions at various temperatures in the anodic compartment and pure O_2 in the cathodic one. As far as we know, this biofuel cell displays the highest power density ever reported with H_2 as a fuel. The temperature effect on the power density of this biofuel cell from 25 to 80 °C is investigated. The influence of bioanode characteristics at high potentials on the performance of the device is discussed and allows to determine the regime in which it efficiently operates.

2. Experimental

2.1. Methods and instrumentation

Bilirubin oxidase (BOD) from *Myrothecium verrucaria* was a gift from Amano Enzyme Inc. (Nagoya, Japan) and mbHI was purified as described in [13]. Electrochemical experiments were performed using an Autolab PGSTAT 128N. The pyrolytic graphite electrode (PG)

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from Bio-logic was the working electrode. All current densities are calculated using geometrical area of the PG electrode (A = 0.07 cm^2). The biofuel cell parameters were examined in 50 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (Hepes) buffer, pH 6.8 under 100% H_2 and 100% O_2 for anode and cathode respectively, separated by a Nafion® membrane (Nafion® 117 from DUPONT — USA). Each half-cell was independently thermoregulated. The cell current and voltage were measured by polarization curves, after stabilization of the system. Current densities were measured at −0.25 V or +0.1 V for mbHI and BOD, respectively. Normalized current density j/j_0 was obtained by dividing the current at each time by the current at initial time. Scan rate is 3.33 mV s^{-1}.

2.2. Design of the bioelectrodes

SWCNT (from Sigma) functionalization with carboxylic groups (SWCNT–COOH) was realized as described in Lojou et al. [16] and diluted in Milli-Q water. 10 µL of SWCNT–COOH was dropped onto PG electrode until complete drying. SWCNT–COOH modified PG electrode was dipped in a 40 mM/10 mM *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC)/*N*-Hydroxysuccinimide (NHS) solution, for one hour. Then 8 µL of mbHI 5 µM or BOD 20 µM was deposited on it and left to dry under N_2 stream on the bench for covalent attachment.

3. Results and discussion

3.1. Covalent binding of the enzymes on SWCNT–COOH coated PG electrodes

The advantage of carbon nanotube coating on H_2 oxidation by mbHI was reported before [13]. In this work we took benefit of the carboxylic functions carried by the nanotubes, and of the knowledge that mbHI and BOD can achieve direct electron transfer on negatively charged electrodes. Covalent attachment of the enzymes was thus performed to prevent instability of the system.

Polarization curves of the bioanode at 60 °C under 100% H_2 and biocathode at 25 °C under 100% O_2 were compared with and without SWCNT–COOH coatings (Fig. 1A). In all cases catalytic currents were obtained in the absence of any redox mediator, although very weak in the case of BOD on bare PG. H_2 enzymatic oxidation current exhibits a classical decrease at potentials higher than −200 mV, due to the formation of an inactive but reversible state of the [NiFe] mbHI active site [10]. As expected, great enhancement of anodic and cathodic currents was obtained on SWCNT–COOH coatings, leading to at least 35 and 300 fold increase for mbHI and BOD, respectively. Denaturating SDS-PAGE gels were used to assess the optimal concentration of the EDC/NHS linker from 4/1 mM, 40/10 mM to 400/100 mM. The appropriate EDC/NHS concentration that avoids enzyme aggregation, i.e. 40/10 mM, yields great stabilization of the catalytic currents. The loss drops dramatically from 25% and 60% for BOD and mbHI respectively, to less than 10%.

3.2. Temperature effect

MbHI is a hyperthermophilic hydrogenase with an optimal activity at a temperature of 85 °C. The current density delivered by the biofuel cell at a potential of 0.65 V was followed as a function of the temperature, which was independently controlled in the anodic and cathodic compartments (Fig. 1B). The cell current density follows an expected evolution with temperature up to 60 °C/40 °C values. A decrease in the current density value and in the stability of the current with time is recorded for higher temperatures. MbHI has been shown to be stable at high temperature when immobilized on carbon nanotube layers [13] and able to resist to various temperature stresses. As expected for a mesophilic enzyme, BOD was shown in this work to lose half of its activity above 50 °C, being responsible for the

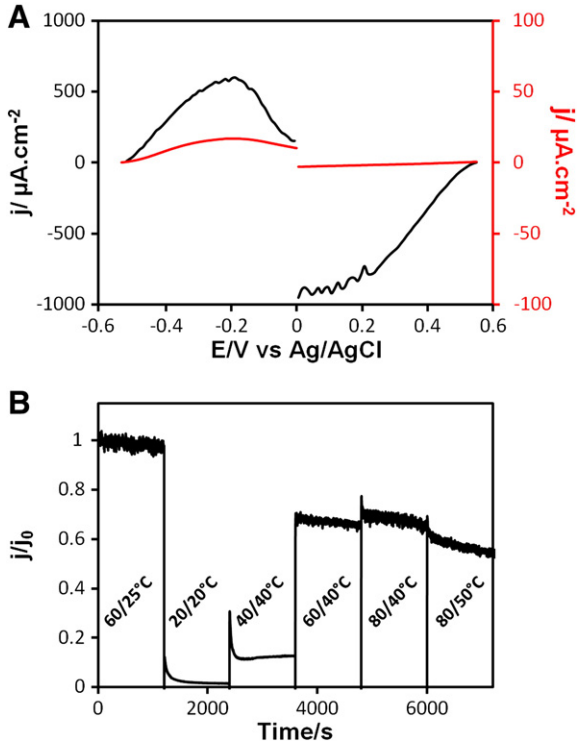

Fig. 1. (A) Polarization curves for SWCNT–COOH modified (black lines) *vs* bare PG (red lines) bioanode and biocathode. (B) Chronoamperometry at 0.65 V of the biofuel cell as a function of temperature in each half cell (T_a/T_c).

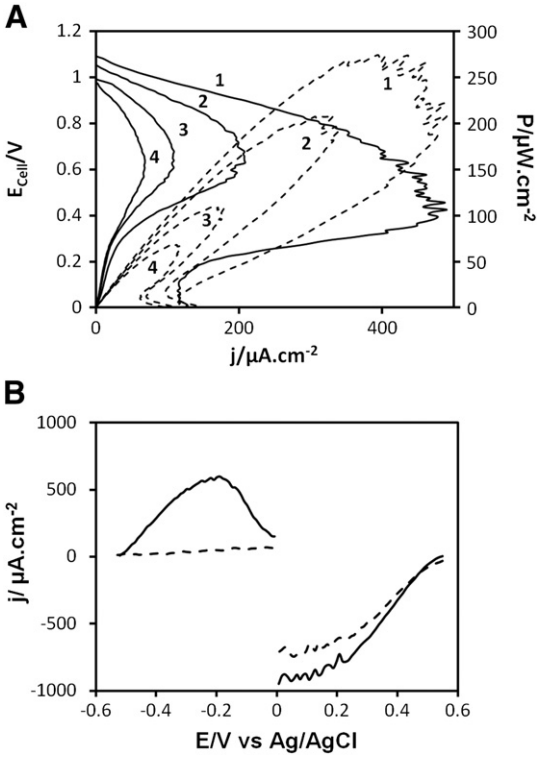

Fig. 2. (A) Evolution of four consecutive cell power measurements (dashed lines) obtained from polarization curves (black lines). (B) Polarization curves for SWCNT–COOH modified PG bioanode and biocathode after the first (black lines) and the fourth (dashed lines) power cell measurement. Temperatures of the anodic and cathodic compartments are 60 and 25 °C, respectively.