Evaluation of alcohol dehydrogenase and aldehyde dehydrogenase enzymes as bi-enzymatic anodes in a membraneless ethanol microfluidic fuel cell

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Abstract. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AldH) enzymes were immobilized by covalent binding and used as the anode in a bi-enzymatic membraneless ethanol hybrid microfluidic fuel cell. The purpose of using both enzymes was to optimize the ethanol electro-oxidation reaction (EOR) by using ADH toward its direct oxidation and AldH for the oxidation of aldehydes as by-products of the EOR. For this reason, three enzymatic bioanode configurations were evaluated according with the location of enzymes: combined, vertical and horizontally separated. In the combined configuration, a current density of 16.3 mA cm⁻², a voltage of 1.14 V and a power density of 7.02 mW cm⁻² were obtained. When enzymes were separately placed in a horizontal and vertical position the ocp drops to 0.94 V and to 0.68 V, respectively. The current density also falls to values of 13.63 and 5.05 mA cm⁻². The decrease of cell performance of bioanodes with separated enzymes compared with the combined bioanode was of 31.7% and 86.87% for the horizontal and the vertical array.

1. Introduction

The use of immobilized enzymes onto the electrode surfaces in fuel cells has been of great interest, the major issues to overcome in membraneless microfluidic fuel cells are the increase of power density of such devices and find strategies for the enzymatic immobilization [1-3]. Ethanol has been recognized as the most promising alcohol for use in the energy conversion area due to its physicochemical properties, including high energy density (8.0 kWh⁻¹ kg⁻¹) and the multiple pathways to obtain it [4-5]. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are the principal enzymes responsible for metabolism of ethanol in humans, catalyzing the conversion of ethanol to acetaldehyde and then to acetate, respectively [6-8]. The ADH system can be regarded as major detoxifying machinery for alcohols and aldehydes. The conversion of ethanol to acetaldehyde coupled with the
reduction of NAD$^+$ to NADH results in an altered NAD$^+$/NADH ratio and is responsible for many metabolic effects of ethanol [9]:

$$\text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ = \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+$$

Immobilization is the key factor to enhance the activity of enzymes, especially if two enzymes are immobilized in the same electrode. In this way, with a good immobilization, it is possible to improve almost all the catalytic properties of enzymes: stability, activity, specificity, selectivity, and the inhibition reduction [10]. In this work, alcohol dehydrogenase and aldehyde dehydrogenase were used as catalysts for the ethanol oxidation and the acetaldehyde oxidation (as byproduct of ethanol reaction) respectively, in order to improve the obtaining electrical energy. For this reason, three enzymatic arrangement on Toray paper electrodes were tested: a physical mixture dispersed on the electrode, and the enzymes horizontal and vertically separated. The effect of the electrode array was analyzed on the cell performance of an ethanol membraneless microfluidic fuel cell.

2. Experimental

2.1 Electrodes preparation

Three catalytic inks were prepared, one for alcohol dehydrogenase (EC 1.1.1.1 initial activity $\geq 300$ units/mg protein from *Saccharomyces cerevisiae*), other for aldehyde dehydrogenase (EC 1.2.1.5 initial activity $\geq 2$ mg protein from *Saccharomyces cerevisiae*) and also a third, which involve the mixture of both enzymes. The catalytic ink was prepared using $5 \mu$L of Nafion (Sigma Aldrich), $10 \mu$L of glutaraldehyde, $1000 \mu$L of deionized water, $2 \text{mg}$ β-nicotinamide adenine dinucleotide sodium salt (NAD$^+$, Sigma Aldrich) and $20 \text{mg}$ of tetrabutylammonium bromide (Sigma Aldrich) per milligram of the first two enzymes individually and per milligram of the mixture of enzymes (half of alcohol dehydrogenase and half aldehyde dehydrogenase).

The bioanodes consisted of two $2.5 \times 30 \text{ mm}$ (wide and long) Toray porous paper electrodes (EC-TP1-060T for combined immobilization of enzymes across the electrode and the vertical configuration) and two $1.25 \times 30 \text{ mm}$ Toray porous paper electrodes (EC-TP1-060T for horizontal configuration). These carbon paper slides were covered with $100 \mu$L of 5:100 v/v % Nafion-water and dried with hot air. After, theses slides were employed for the methylene blue electro-polymerisation using a Biologic VSP Potentiostat/Galvanostat, by cyclic voltammetry from -1 to 1 V vs. SCE for 15 cycles at 0.05 V s$^{-1}$ scan rate. The electrolytic bath consisted of 0.05 M methylene blue. The inks were then sonicated for 20 min and mixed by vortex for other 15 min. Three configurations for the immobilization were performed: a) the first corresponded to the use of the combined enzymatic mixture. This was done placing 100 μL of the catalytic ink over the entire surface of the electrode. b) In the second, two smaller electrodes were used and 50 μL of the alcohol dehydrogenase ink was depositing onto the surface of one them and 50μL of the aldehyde dehydrogenase ink on the other electrode. c) A slide was vertically divided in two sections. In the superior section were deposited 50 μL of alcohol dehydrogenase ink and 50 μL of ink enzyme aldehyde dehydrogenase in the bottom section (Figure 1). All the electrodes with deposited ink were dried at room temperature.

The Pt-based abiotic cathode was prepared using commercial Pt/C (30% E-TEK) as the catalyst and carbon nanofoam (2.5×30 mm, Markotech®) as the electrode. Briefly, 120 μL of isopropyl alcohol and 14 μL of Nafion were added per milligram of catalyst and mixed for 30 minutes. The catalytic ink was deposited on the carbon nanofoam surface using the spray technique until the electrode weight increase 1 mg.

2.2 Hybrid microfluidic fuel cell evaluation
The design of the microfluidic fuel cell [11] consists of three main sections, which were constructed using non-sophisticated techniques. The first section consisted in two supporting plates made of Poly(methyl methacrylate) (PMMA). These were patterned using a computer numerical control (CNC). The second section consists in a homemade silicone elastomer film (Silastic®, Dow Corning, prepared using an Elcometer® Film Applicator with a final thickness of 200 µm) used as both, gasketing and as cell channel structure. The third section consisted in the core of the device, which is the bioanodes and cathode electrodes. The hybrid microfluidic fuel cell tests were performed by injecting 0.1 M ethanol N2-saturated (4 U. P. D. Praxair) as fuel and oxygen from air as oxidant within two aqueous streams that contained phosphate buffer (pH 8.86) and 0.3 M KOH on the anodic and the cathodic side respectively and used as supporting electrolytes. The flow rate was 50 µL min\(^{-1}\) for both streams. Figure 1 shows the configurations of the bioanodes used in microfluidic fuel cells: a) which have the two enzymes immobilized along the electrode, b) having two thin electrodes where the first from right to left is immobilized enzyme alcohol dehydrogenase and the second with aldehyde dehydrogenase enzyme, and c) where the electrode is divided into two, which are immobilized on top alcohol dehydrogenase and bottom aldehyde dehydrogenase.

![Figure 1](image-url)

**Figure 1.** Schematic representation of the configuration of enzymatic bioanodes: a) combined, b) horizontally separated and c) vertically separated.

### 3. Results and discussion

#### 3.1 Microfluidic fuel cell performance

The performance of the bi-enzymatic bioanodes toward the EOR in a membraneless ethanol hybrid microfluidic fuel cell is illustrated in Figure 2 and the obtained results for the three hybrid microfluidic fuel cells are presented in Table 1. The hybrid MFC with the mixture of enzymes exhibited a cell voltage of 1.141 V and a maximum current density of 16.3331 mA cm\(^{-2}\) (Fig. 2). In contrast, the
hybrid MFC with horizontal bioanode configuration exhibited an open circuit voltage of 0.6795V and a current density of 5.0735 mA cm\(^{-2}\). The power density for the hybrid MFC with the vertical configuration was 13.6176 and 4.8158 mW cm\(^{-2}\), respectively. The drop in performance exhibited by the vertical electrode array can be related –according with the behavior of the curve– to a deficiency in both, the electron transference between enzymes and a poor mass transport due to the electrode configuration. In the case of the vertical array, the lower performance compared with the homogeneous enzyme mixture are related to ohmic issues, such as problems with electron transference. The stability tests were performed by chronoamperometry (Fig. 3). The electrode configuration related to a horizontal array showed the poorest stability, the current density fell over the entire period of time that the experiment was carried out. The combined enzyme mixture and the vertical enzymatic array showed good stability over time. The vertical array exhibited a constant current density of 5 mA cm\(^{-2}\) and the combined array of 7.5 mA cm\(^{-2}\).

\textbf{Figure 2.} Polarization and performance curves of the device using three enzymatic bioanodes arrays: combined (blue), horizontally separated (green) and vertically separated (black).

\textbf{Figure 3.} Chronoamperometry using 0.02 V as potential at maximum current density.

\textbf{Table 1.} Performance of microfluidic fuel cells with bi-enzymatic bioanodes: Alcohol dehydrogenase and aldehyde dehydrogenase.

| Bioanode configuration         | Current density (mA/cm\(^2\)) | Open circuit potential (V) | Power density (mW/cm\(^2\)) |
|-------------------------------|--------------------------------|---------------------------|-----------------------------|
| Enzyme combined               | 16.3331                        | 1.141                     | 7.0289                      |
| Enzyme horizontally separated | 5.0735                         | 0.6795                    | 0.9186                      |
| Enzyme vertically separated   | 13.6176                        | 0.9466                    | 4.8158                      |

3.2 Bioanode SEM analysis

Scanning electron microscope analysis of the anode corresponding to the combined enzymatic array immobilized on the Toray paper surface was made (Fig. 4). This electrode was selected because of its high performance in the microfluidic fuel cell. SEM images (Fig. 4a and 4b) were acquired to show the successful use of a covalent binding method to immobilize the alcohol dehydrogenase and aldehyde dehydrogenase enzymes. The micrograph of naked Toray electrode is shown in Fig. 4a, left. Meanwhile, the Toray electrode with the bioanode array (Nafion, methylene blue, glutaraldehyde, TBAB, NAD\(^+\), Nafion) is shown in Fig. 4b right. It is clearly observed from micrograph at the right position that, some material is deposited over the entire surface of the electrode which could indicate that, through electro-polymerisation, ADH and AldH enzymes can be adhered to the carbon-based surface.
Conclusion

The immobilization method as well as the enzymatic arrangement in multi-enzymatic electrodes have a significant effect on the performance of microfluidic fuel cells. In this work, three electrode configurations were proposed in which the enzymes were immobilized on the electrode surface. The homogeneous mixture between both enzymes showed the highest power density (7.0289 mW cm⁻²) herein reported. This performance, compared with the other electrode configuration, was related to an enhancement in the electron transference probably because of the short distance between them which allowed a better conduction of charge from both electro-oxidation reactions. Also, the order of magnitude (milliwatts) of the cell performance was achieved with the use of three-dimensional electrodes as flow-through electrodes. In addition, a covalent binding immobilisation method based on Nafion, poly-(methylene blue), glutaraldehyde, TBAB and NAD⁺ was used to enhance the durability of the MFC.

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