Microfluidic biofuel cell based on cholesterol oxidase/laccase enzymes.

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Abstract. In this research two electrodes were developed, a bioanode where the enzyme oxidase was immobilized on Sigracet GDL 39 by means of the covalent binding method and a biocathode with laccase enzyme immobilized on Toray carbon paper by the adsorption method. The evaluation of these electrodes was carried out in a microfluidic fuel cell using cholesterol as fuel and oxygen as oxidant. The electrical conductivity of the electrodes was measured by the kelvin method. Microfluidic fuel cell (mFFC) was constructed using Poly-(methyl methacrylate) (PMMA) for the fuel and oxidant channels; the cathode electrode was incorporated as a wall to obtain the major O₂ quantity for the reduction reaction. The evaluation of the microfluidic cell was carried out using different solutions of cholesterol all in phosphate buffer solutions at pH 7.5, where it was possible to obtain 1.38 mW/cm² of maximum power density and 0.75V of open circuit potential using 500mg/dL of cholesterol.

1. Introduction

Biofuel cells will benefit from the development of microfluidic cell technologies since mass transport convection is improved, allowing an enzymatic rotation rate to reach its full capacity associated with transport by diffusion; there is a greater use of the surface area ratio of the fuel cell, inherent in microstructured devices to promote electrochemical reactions catalyzed by immobilized enzymes [1]. The fuel and oxidant also contain supporting electrolyte that facilitates ionic transport within the streams, thereby eliminating the need for a separate electrolyte. Cholesterol is presented as one of the attractive fuels to be used in this technology, mainly in implantable applications or biomedical devices. Cholesterol is a fatty alcohol and an essential component of almost all tissues in humans and animals. Is the precursor of a large number of biologically important compounds as steroid hormones, vitamin D and bile acids. Cholesterol imbalance leads to an increase of its presence in the bloodstream; a high level correlates with various cardiovascular diseases, cerebral thrombosis, and atherosclerosis, which currently are the leading causes of death worldwide [2].

Cholesterol oxidases are bacterial flavo-oxidoreductases, which act on cholesterol and other substrates having the cholesterol moiety, such as 7α/7β- hydroxy-cholesterol, 7-ketocholesterol, ergosterol, β-sitosterol, pregnenolone and cholestenol [3]. Cholesterol oxidase is a flavin adenin dinucleotide
(FAD)-dependent enzyme that catalyses the oxidation of cholesterol (cholest-5-en-3β-ol) using oxygen as an electron acceptor to form cholest-4-en-3-one and hydrogen peroxide as products [4]. The laccases are polyphenol oxidases enzymes, which belong to the family of multi-copper blue oxidases. These enzymes catalysed the oxidation of an electron related to the reduction of four electrons of molecular oxygen to water. The mechanism for the ORR by laccases involves the transfer of four electrons and occurs in an active site formed by 4 copper atoms distributed over three sites (i.e., one copper atom is located in each of the T1 and T2 sites while two are located in the T3 centre) [5, 6]. The immobilization of these enzymes for application in microfluidic fuel cells can be very important for the development of this technology since it could allow making use of more complex fuels as is the case of the cholesterol that can be obtained from different biological fluids as is the case of human blood, being of great interest for future applications in biomedical devices.

2. Experimental

2.1 Bioanode.

A catalytic ink was prepared using 1mg cholesterol oxidase (EC 1.1.3.6 lyophilized powder, ≥20 units/mg protein), 10 μL of Nafion solution (Sigma Aldrich), Vulcan carbon, 1mL of 0.1M of buffer phosphate pH= 7.5 and 10 mg of tetrabutylammonium bromide (Sigma Aldrich). The catalytic ink was deposited by dipping on carbon Sigracet GDL 39 whose dimensions were of 2.5x30 mm.

2.2 Biocathode construction.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)) di-ammonium salt (Sigma-Aldrich) was used for the preparation of the ABTS/Carbon (ABTS/C) composite by adsorbing 800 μL of 5 mM ABTS solution in a phosphate buffer solution (PBS) at pH 5 and drying at room temperature for 24 h. After that, 800 μL of 1 U mL$^{-1}$ laccase solution (Sigma) was adsorbed on the ABTS/C [6].

2.3 Microfluidic fuel cell evaluation.

The microfluidic device used was previously reported for application in enzymatic microfluidic fuel cells using urease enzyme immobilized at the bioanode [7].

![Figure 1. Schematic representation of bioelectrodes and microfluidic device.](image)

This device (Figure 1) consisted of two supporting plates made of Poly-(methyl methacrylate) (PMMA) and patterned using a computer numerical control CNC, a home-made silicone elastomer film (Silastic®, Dow Corning, prepared using an Elcometer® Film Applicator with a final thickness of 200 μm) used as both the gasket and cell channel structure [8]. The device was evaluated at different cholesterol concentration in Triton X 100 with 0.1M buffer phosphate pH 7.4 (4 U.P.D. Praxair) as...
fuel and oxygen taken from the air as the oxidant, respectively with 70 μL min⁻¹ of flow rate for both streams.

3. Results and discussion

The electrical conductivity of the electrodes was measured by the kelvin method (four-terminal sensing), the results showed that the resistivity dropped from 7.34 mΩ cm to 6.56 mΩ cm improving the electrical conductivity when the cholesterol oxidase was immobilized over the toray paper (Figure 2).

![Figure 2](image2.png)

**Figure 2.** Tests of electrical conductivity of a) naked carbon paper and b) with the immobilized cholesterol oxidase.

![Figure 3](image3.png)

**Figure 3.** Scanning electrochemical microscopy images a) bioanode in the absence and b) in the presence of 300 ng/mL cholesterol in buffer phosphate pH=7.5; ET=0.7V, v=20 μm.

The enzymatic activity of oxidase immobilized was investigated via scanning electrochemical microscopy (SECM) using a Biological scanning system instrument model ac-SECM / SECM470, employing a four electrode cell consisting of an platinum ultramicroelectrode (UME) of 25 μm diameter, Ag | AgCl | 3 M KCl as reference, where it was possible to observe the formation of
hydrogen peroxide caused by the enzymatic reaction (Figure 3). The effect of cholesterol as fuel to produce energy was clearly observed at the polarization curves obtained 1.38 mW cm\(^{-2}\) of maximum power density (Figure 4). When 100 mg dL\(^{-1}\) was aggregated, the cholesterol oxidation took place and the power density increased near to 0 until 0.4 mW cm\(^{-2}\), at the same time, the performance increased by the concentration effect.

![Figure 4](image)

Figure 4. Representative polarization and power density curves for microfluidic fuel cell evaluated at different cholesterol concentrations as fuel in 0.1M buffer phosphates pH 7.4 and 0.3M KOH saturated with O\(_2\) as oxidant, at 10 mV s\(^{-1}\).

The current density was improved from 2.7 to 4.6 mA cm\(^{-2}\) due the cholesterol molecules available over the electrode surface increased, furthermore, the potential enhanced close to 100 mV (maximum open circuit potential 0.75V) as dictated by the Nernst equation when the concentration increase. On the other hand, at high concentration of cholesterol (500 mg dL\(^{-1}\)) the diffusion effect begins to appear in the polarization curve to potentials below 0.45 V (slope change), because, the available oxygen is insufficient for cholesterol that is being oxidize.

4. Conclusions
This research work evidenced the possibility to use the cholesterol as alternative fuel for energy production employing a microfluidic biofuel cell. The covalent binding method for immobilized the cholesterol oxidase enzyme was the key to cholesterol oxidation reaction and achieve a 1.38 mW cm\(^{-2}\) of power density. As a perspective, the developed anode in this work and the cholesterol present in blood could be used to produce energy or some medical application.

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