Membraneless ethanol,O2 enzymatic biofuel cell based on laccase and ADH/NAD+ bioelectrodes

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ABSTRACT: This work describes EtOH,O2 membraneless enzymatic biofuel cells (EtOH,O2 MlessEBFCs) that employ laccase-based biocathodes and ADH/NAD+ bioanode. Laccase biocathodes were prepared by immobilizing a polypyrrole film containing different redox mediators (ruthenium and osmium complexes). The bioanode for EtOH,O2 MlessEBFCs was fabricated by immobilizing multiwalled carbon nanotubes, NAD+-dependent alcohol dehydrogenase enzyme (ADH), poly-methylene green, and poly(amidoamine) (PAMAM) dendrimer onto a carbon cloth platform. Maximum power density and current density were 21.0 ± 0.2 μW cm^{-2} and 0.15 ± 0.07 mA cm^{-2}, respectively, in PBS (pH 6.5). Lifetime tests conducted for EtOH,O2 MlessEBFCs showed promising perspectives for their future application in miniaturized devices.
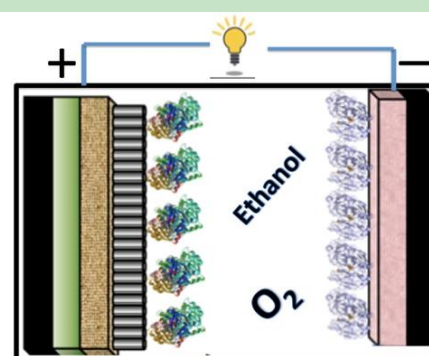
C/MG/Polypyrole/MWCNT/ADH,ethanol,O2/Laccase/Os/C
EtOH,O2 MlessEBFC generated 21 W cm^{-2} and considerable lifetime stability

1. Introduction

Biofuel cells (BFCs) employ enzymes or microorganisms as catalysts to convert chemical energy into electric energy. BFCs can operate under milder temperatures (20-40 °C) and physiological pH, so they could be a strategy to replace traditional batteries that need large amount of hazardous metallic catalysts in small devices. Moreover, BFCs could be employed to produce energy from various fuel sources because enzymes can selectively catalyze different fuels[1,2]. Enzymes have high specific selectivity, which could dismiss the need for a membrane[3]. The first report of a membraneless biofuel cell (MlessBFC) dates from 1997, when a single compartment cell was used to oxidize organic compounds (sugars or alcohols) while simultaneously reducing molecular oxygen (O2) at the biocathode[4].

To prepare MlessBFCs successfully, enzyme immobilization is a key step to obtain a stable long-lasting device, improving electron transfer kinetics, and increasing power densities (PDs). In this context, researchers have sought to enhance enzymatic system robustness and activity—an enzymatic system must be able to survive pH, temperature, and reaction medium changes. This is not a simple task when one deals with biomolecules, but growing interest in this area has advanced knowledge in the field. A 20-day lifetime has been reported for a membraneless ethanol, oxygen enzymatic biofuel cell (EtOH,O2 MlessEBFC) based on alcohol dehydrogenase (ADH) and bilirubin oxidase (BOD) as bioelectrodes[5]. Over the years, numerous architecture designs for MlessEBFCs have been developed in order to achieve higher PD values[6,7]. For instance, Deng and co-workers[8] produced

energy through oxidation of EtOH present in beverages by employing an EtOH,O_2 M_{less}EBFC. The bioanode MB/AuNPs/PSSG-CHI-ADH was constructed with Meldola's Blue (MB), ADH enzyme, gold nanoparticles (AuNPs), partially sulfonated (3-mercaptopropyl-)trimethoxysilane sol-gel (PSSG), and chitosan (CHI) and an AuNPs/PSSG-CHI–laccase biocathode. The authors described PD of 1.78 mW cm^{-2} at 680 mV when they used wine as fuel, which pointed out that this device deserves attention for alternative energy production.

To increase PD, carbon-based materials, such as multiwalled carbon nanotubes (MWCNTs), have been successfully investigated[9]. With respect to electrochemical performance, MWCNTs are claimed to be more efficient than single-walled carbon nanotubes (SWCNTs). Indeed, MWCNTs have greater surface area and wider potential range, provide many active sites for biomolecule immobilization (which promotes faster electron transfer reactions along the tube axis), and display prominent charge transortation features[10,11].

Immobilization aiming at protein microencapsulation has currently gained researchers' attention. In this immobilization mode, intrinsically conductive polymers and dendrimers are employed as imprisonment arrays so that enzymes are physically entrapped in membrane pores or anchored onto the electrode surface. Intrinsically conductive polymers are compounds that can carry electric current without incorporating conductive charges. Also known as conjugated polymers, their electrical, optical, magnetic, and electronic properties resemble the properties of metals and/or semiconductors. Here, we highlight the use of polypyrrole (polyPYR), which is highly chemically and environmentally stable, biocompatible, and biodegradable. This porous polymer has been widely applied in batteries, sensors, and anti-corrosion protective agents, among others. Several methodologies can be employed to obtain polyPYR layers, and use of this polymer, modified or not, has been often reported[12-14]. Our research group prepared enzymatic biocathode and bioanode for biofuel cells[15,16], and an example of M_{less}EBFCs application can be found elsewhere[17]. PolyPYR and MWCNT matrixes have been employed to prepare glucose,O_2 EBFCs based on glucose oxidase (GOx) and pyrroloquinoline quinone (PQQ) redox mediator absorbed on MWCNTs and polyPYR as a MWCNTs-GOx-PQQ-polyPYR

bioanode[17]. PD of 1.1 μW mm^{-2} was achieved at non-compartmentalized BFCs at a cell voltage of 0.167 V in PBS (pH 7.4) for 10 mM glucose (as fuel), and PD of 0.69 μW mm^{-2} was obtained at cell voltage of 0.151 V in human serum containing 5 mmol L^{-1} glucose (37 °C)[17].

PAMAM dendrimer is another promising polymer belonging to the class of branched monodisperse polymers[18,19]. PAMAM has widely uniform structure, low molecular weight, highly functionalized surface and high degree of porosity[19].

We report the construction of a single-chamber EtOH,O_2 biofuel cell to harvest energy from ethanol. Strategies to enhance ET between enzymes and electroactive surfaces include orientation and immobilization of the enzymes and electron mediation. For this laccase-based biocathode metallic redox complexes (Os and Ru) was entrapped in a polyPYR film as redox mediators and the ADH/NAD$^+$ bioanode employed polymethylene green layer as mediator. We also investigate the activity of the membraneless biofuel for a long period (11 months) in other to show their stability.

2. Experimental

2.1. Chemicals

Enzyme ADH (E.C. 1.1.1.1), from *Saccharomyces cerevisiae* lyophilized powder (331 Units mg^{-1}); enzyme Laccase (E.C. 1.10.3.2.), from *Trametes Versicolor* lyophilized powder (21 Units mg^{-1}); coenzyme nicotinamide adenine dinucleotide hydrate (NAD$^+$); 2,2′-azinobis(3-ethyl-2,3-dihydrobenzothazole-6-sulfonic acid (ABTS); pyrrole (PYR); and polyamidoamine generation 4 dendrimer (PAMAM) were purchased from Sigma-Aldrich in reactant grade. All reagents and enzymes were used without purification. Enzyme solutions were freshly prepared and rapidly immobilized on the carbon platform.

Multiwalled carbon nanotubes (MWCNTs) were acquired from Cheap Tubes Inc. (diameter of 8.0 nm, length of 10 to 30 μm, and > 95% purity). All solutions were prepared with high-purity water from a Millipore Milli-Q system. Solution pH was measured with a pH electrode coupled to a Qualxtron model 8010 pHmeter.

2.2. Instrumentation

Electrochemical investigations were conducted on a Potentiostat/Galvanostat AUTOLAB PGSTAT 30 (EcoChemie, Netherlands) at room temperature, (25 ± 1) °C. M_{less}EBFC experiments were carried out in a single-compartment cell (10 mL) by using 200 mmol L^{-1} phosphate buffer solution (PBS) or acetate buffer solution (ABS) at pH 6.5, 1.9 mmol L^{-1} NAD^+, and 100 mmol L^{-1} EtOH as electrolyte.

2.3. Bioelectrode preparation

ADH bioanodes were prepared on carbon cloth platform (HT 1400 w, Elat CDL-Basf), to which MWCNTs and PAMAM were added. Further preparation details can be obtained elsewhere[20].To this end, polymethylene green (poly-MG) was used as immobilized electrocatalyst and was obtained by performing twelve successive voltammetric cycles (−0.3 to 1.3 V vs. Ag/AgCl). After that, a PAMAM anchoring matrix was directly deposited onto the electrode surface, obtained by pippeting 50 μL of 0.025 mol L^{-1} commercial PAMAM solution. Then, 50 μL of MWCNT ink (1 mg of commercial MWCNTs dissolved in EtOH (400 μL) and 100 mmol L^{-1} PBS (600 μL; pH 7.4) and sonicated for at least 4 h) was deposited. After drying, 50 μL of a freshly prepared enzyme solution containing ADH (300 μL; 3.37 mg L^{-1}) + NAD^+ (100 μL; 7.50 mg L^{-1}) + PAMAM (100 μL; 0.025 mol L^{-1}) in PBS (100 μL; 100 mmol L^{-1}; pH 7.4) was cast on the electrode surface. The casting sequence carbon cloth/poly-MG/PAMAM/MWCNTs/ADH was named MWCNTs-ADH configuration.

The osmium $[Os(bpy)_2Cl_2]$ and ruthenium complex $[Ru(bpy)_2Cl_2]$ catalysts for laccase biocathode was synthesized according to the literature[22,23]. $[Os(bpy)_2Cl_2]$ or $[Ru(bpy)_2Cl_2]$ entrapment in polyPYR films (1.5% v/v PYR, 8 mg mL^{-1} $[Os(bpy)_2Cl_2]$ or $[Ru(bpy)_2Cl_2]$ mediator in 100 mmol L^{-1} $NaNO_3$) was accomplished by chronoamperometry at a fixed potential (0.7 V vs. SCE; 10 min). The film containing polyPYR and $[Os(bpy)_2Cl_2]$ or $[Ru(bpy)_2Cl_2]$ mediator was named polyPYR-Os and polyPYR-Ru, respectively.

Laccase biocathodes, which employed $[Os(bpy)_2Cl_2]$ or $[Ru(bpy)_2Cl_2]$ as nanocatalyst and PAMAM dendrimer as enzymatic anchoring matrix, was prepared according to Cardoso and co-workers[15]. To this end, 50 μL of a stock solution, prepared by mixing laccase (300 μL; 3.32 mg mL^{-1}), PAMAM dendrimer (100 μL; 0.025 mol L^{-1}), and acetate buffer solution (ABS; 200 μL; 100 mmol L^{-1}; pH 4), was deposited on the previously modified carbon platform.

Figure 1 displays the representative structures of the bioelectrodes prepared on carbon cloth platforms.
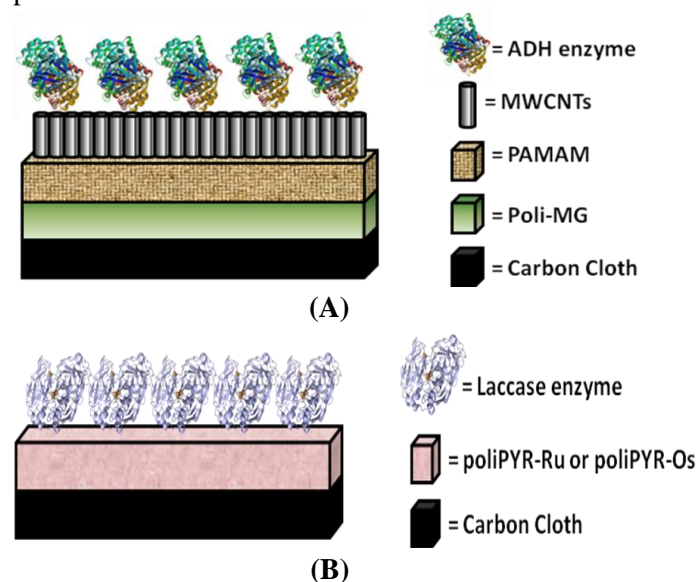

Figure 1. Bioelectrode side schematic representations: A) MWCNTs-ADH Bioanode; B) polyPYR-Os-laccase or polyPYR-Ru-laccase Biocathode.

Bioelectrodes were kept in their appropriate buffer solution (PBS (pH 7.4) for MWCNTs-ADH bioanode or ABS (pH 4.5) for polyPYR-Os-laccase or polyPYR-Os-laccase biocathodes) for at least 24 h before use. After electrochemical experiments, the respective bioelectrodes were kept refrigerated in buffer solution at 4 °C for at least 24 h before a further test was conducted.

pH influence on enzymatic kinetics was determined by assaying laccase and ADH activities at various pH values ranging between 3.5 and 10. To this end, the following 0.1 mol L^{-1} buffer solutions were employed: acetate buffer (NaAc/HAc) for pH 3.5-5, phosphate buffer (NaH_2PO_4/Na_2HPO_4) for pH 6-7, and tris(hydroxymethyl)aminomethane–HCl (Tris+) buffer for pH 8–9. Reaction was initiated by adding substrate to the immobilized protein, depending on the study that was being performed.

2.4. Biofuel cell tests

Power density measurements were accomplished in an EtOH,O_2 M_{less}EBFC described in the Instrumentation Section. First, the EtOH,O_2 M_{less}EBFC open circuit voltage (OCV) was measured at least 1 h before the cell test. After that, polarization curves at a scan rate of 1 mV s^{-1} were registered in triplicate. PD values for all M_{less}EBFCs were obtained by multiplying cell voltage (E_{cell}) by current density (J_{cell}) (PD = E_{cell} × J_{cell}).

3. Results and discussion

3.1. pH effect on semi- M_{less}EBFC

To obtain maximum M_{less}EBFC performance, it is important to investigate bioelectrode enzymatic behavior as a function of pH because hydrogen ion concentration affects enzymatic activity: enzyme spatial conformation depends on pH values and on the presence of protonated/deprotonated groups in the enzyme catalytic site, which can modify the enzyme tertiary structure. pH also influences intrinsic/extrinsic electron transfer reaction strongly. The individual pH behavior of the immobilized enzymes employed here has previously been investigated in detail[15,20]. The optimum pH range for ADH/NAD$^+$ bioanode is between 7.0 and 8.0, achieved by employing PBS as buffer[24]. Laccase works best in more acidic medium (pH 4.5), in ABS buffer solution[15]. Therefore, besides operating in different pH ranges, bioanode and biocathode also use distinct buffer solutions.

Direct correlation between enzymatic kinetics and pH is important to obtain maximum bioelectrode performance. Nevertheless, in a M_{less}EBFC both bioelectrodes seldom operate at their optimum pH. To find the best pH for M_{less}EBFC operation, individual pH curves of each enzyme were plotted together (Fig. 2). On the basis of Fig. 2, pH curves intersect at pH 6.5, which was further employed with M_{less}EBFCs. Even though this pH value is close to physiological conditions, other complications may arise and diminish EtOH,O_2 M_{less}EBFC PD and OCV values as compared to separated biofuel cells. Other factors may also be associated with this behavior such as problems with the enzyme-mediator-electrode electron transfer enzyme, which hinders the redox process underlying EtOH oxidation by ADH and

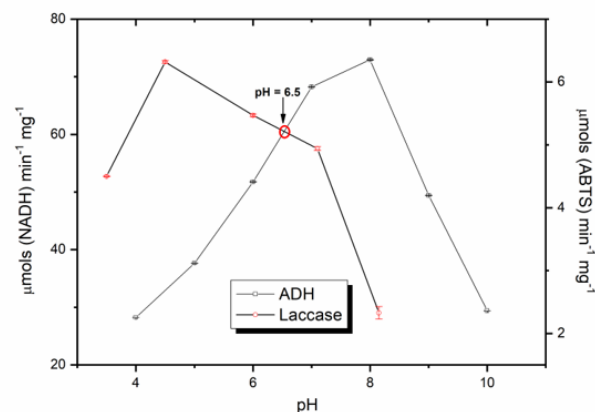O_2 reduction to H_2O by multicopper oxidase enzymes (laccase).

Figure 2. pH influence on the enzymatic activity of bioelectrodes containing immobilized ADH (−■−) in PBS and immobilized laccase (−●−) in ABS.

3.2. EtOH,O_2 membraneless biofuel cell: reaction medium influence (ABS or PBS).

Eliminating proton exchange membrane (PEM) has several advantages. During the reaction process, PEM is subjected to membrane channel obstruction by ions present in the supporting electrolyte, which dries or floods membrane parts, and fuel crossover. To minimize the aforementioned problems, one strategy is to remove the membrane when biocatalyst specificity can be maintained at M_{less}EBFC. However, each bioelectrode must be evaluated for its electron transfer activity and enzymatic selectivity. EtOH,O_2 M_{less}EBFC performance was assessed by analyzing OCV and power density curves obtained from polarization curves (results not shown). To investigate how PBS and ABS buffers influenced M_{less}EBFC activity, EtOH,O_2 M_{less}EBFC performance was measured at pH 6.5 in both buffers (ABS and PBS; buffer concentration = 200 mmol L^{-1}). This "precaution" was necessary because PEM removal resulted in each enzyme facing a medium that was different from its ideal operation condition (ABS (pH 4.5) for laccase and PBS (pH 7.4) for ADH).

Figure 3 shows the results for M_{less}EBFCs containing a MWCNTs-ADH anode and one of the following cathode configurations: polyPYR-Os-laccase or polyPYR-Ru-laccase; either ABS or PBS at pH 6.5 was employed. According to Fig. 3 M_{less}EBFC in PBS buffer operated at higher power density (PD) and current ($J_{cell(max)}$) as compared to M_{less}EBFC in ABS buffer. In PBS buffer (Fig. 3A),

PD and $J_{cell(max)}$ respectively were 21.0 ± 0.2 µW cm^{-2} and 0.15 ± 0.07 mA cm^{-2} for MWCNTs-ADH, polyPYR-Os-laccase, and 13.5 ± 0.2 µW cm^{-2} and 0.11 ± 0.05 mA cm^{-2} for MWCNTs-ADH,polyPYR-Ru-laccase. When these systems were switched to ABS buffer, both PD and $J_{cell(max)}$ decreased significantly (Fig. 3B). For this reason, PBS was selected as buffer for further EtOH,O$_2$ M$_{less}$EBFC investigations.
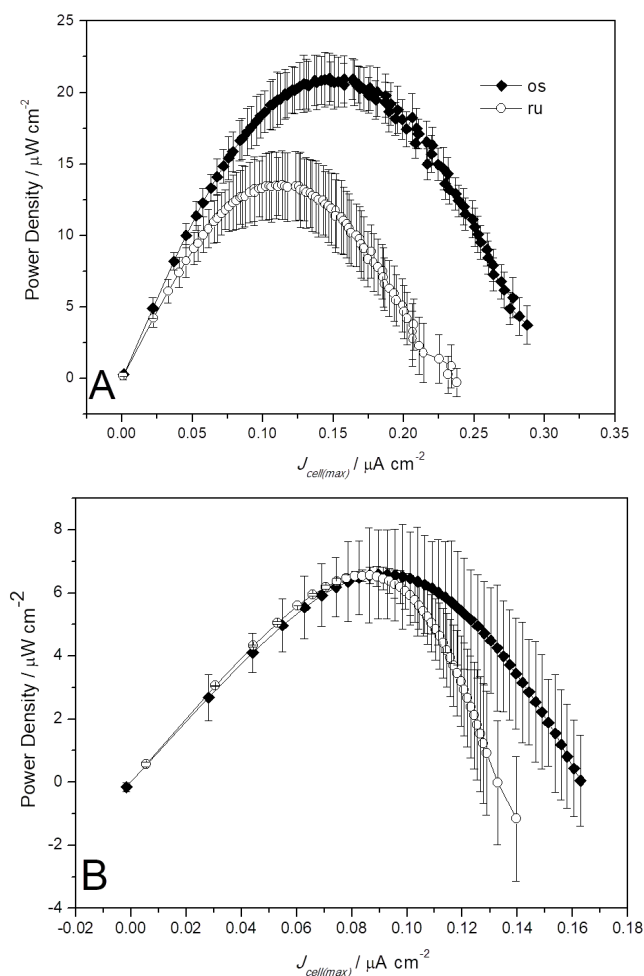

Figure 3. Power density curves for EtOH,O$_2$ M$_{less}$EBFCs employing MWCNTs-ADH as bioanode and ($-♦-$) polyPYR-Os-laccase or polyPYR-Ru-laccase ($-○-$) as biocathode in (A) PBS and (B) ABS medium (200 mmol L^{-1}; pH 6.5, 1.9 mmol L^{-1} NAD$^+$, 100 mmol L^{-1} EtOH, n = 3).

3.3. ABTS influence on EtOH,O$_2$ M$_{less}$BFCs

ABTS is one of the most common oxygen reduction mediators when laccase is employed in EBFCs. Figure 4 illustrates how ABTS influences EtOH,O$_2$ M$_{less}$EBFC performance in PBS medium (pH 6.5) for both anode configurations investigated

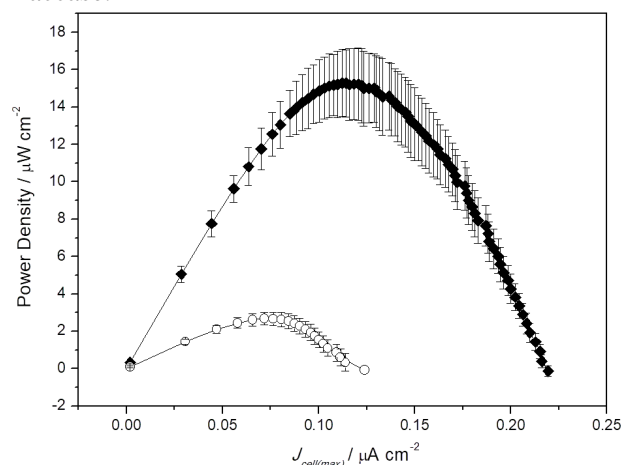here: polyPYR-Os-laccase and polyPYR-Ru-laccase.

Figure 4. Power density curves for MWCNTs-ADH,polyPYR-Os-laccase ($-♦-$) and MWCNTs-ADH,polyPYR-Ru-laccase ($-○-$) in the presence of 1 mmol L^{-1} ABTS (200 mmol L^{-1} PBS (pH 6.5), 1.9 mmol L^{-1} NAD$^+$, and 100 mmol L^{-1} EtOH; n = 3).

Homogeneous ABTS introduction diminishes Ru-mediated cathode cell PD by over 80% as compared to Os-mediated cathode cell. Indeed, PD decreased from 15.3 ± 0.2 µW cm^{-2} to 2.7 ± 0.3 µW cm^{-2} just by changing the Os complex to the Ru complex. Also, when results obtained in the absence (Fig. 3A) and in the presence (Fig. 4) of ABTS are compared for Os-complex in PBS, PD and $J_{cell(max)}$ was approximately 27.5 and 23.8%, respectively. This decrease could be explained by competition between ABTS and mediators incorporated into the polyPYR matrix and the enzymatic redox sites. The best results were achieved for EtOH,O$_2$ M$_{less}$EBFCs based on the MWCNT-ADH,polyPYR-Os-laccase system. These results agreed with literature data[25] claiming that [Os(bpy)$_2$Cl$_2$] can bind to laccase copper hydrophobic T1 active site and establish a strong electrostatic interaction, which entraps the Os-complex in polyPYR, shifts the polyPYR-Os oxidation potential (E$_{oxi}$), and facilitates electron transfer. Our results showed that polyPYR-Ru-laccase did not interact in the same way as the Os-mediator.

Table 1 summarizes all experimental parameters obtained for EtOH,O$_2$ M$_{less}$EBFCs as a function of the different redox mediators entrapped in polyPYR, in the absence or presence of 1 mmol L^{-1} ABTS.

Table 1. Parameters obtained for different EtOH/O$_2$ M$_{less}$EBFCs.

MlessEBFCs	OCV/V	E$_{MAX}$/V	$J_{cell(max)}$/mA cm^{-2}	PD*/μW cm^{-2}
MWCNTs-ADH,polyPYR-Os-laccase (without ABTS)	0.226 ± 0.008	0.14 ± 0.01	0.15 ± 0.07	21.0 ± 0.2
MWCNTs-ADH,polyPYR-Os-laccase (with ABTS)	0.15 ± 0.06	0.11 ± 0.04	0.11 ± 0.05	15.3 ± 0.2
MWCNTs-ADH,polyPYR-Ru-laccase (without ABTS)	0.17 ± 0.02	0.115 ± 0.006	0.11 ± 0.05	13.5 ± 0.2
MWCNTs-ADH,polyPYR-Ru-laccase (with ABTS)	0.048 ± 0.007	0.037 ± 0.008	0.072 ± 0.001	2.7± 0.3

*Average and standard deviation for combinatorial analysis, in triplicate, for a set of three biocathodes and four bioanodes.

On the basis of the results above (Table 1), the best EtOH,O$_2$ M$_{less}$EBFCs was MWCNTs-ADH,polyPYR-Os-laccase in 200 mmol L^{-1} PBS (pH 6.5) in the absence of ABTS. Figure 5 illustrates the selected operation system.
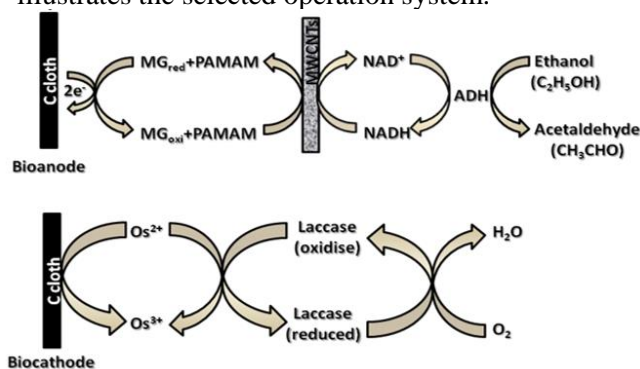

Figure 5. Schematic diagram of mediated electron transfer in EtOH,O$_2$ M$_{less}$EBFCs based on MWCNTs-ADH,polyPYR-Os-laccase.

We have investigated and reported half-cell data for these electrodes configuration before[15,21]. For the biocathode half-cell[15] a gas diffusion membrane (ELAT) consisting of 40% metal in C (Pt0.66Ru0.34, E-TEK commercial mixture) hot pressed in a Nafion NRE-212 membrane was employed as the anode. This configuration furnished at least five times higher power densities values than the value reported for the membraneless fuel cell. For the half-bioanode[21] Pt was used as cathode, also separated by a Nafion membrane. This configuration furnished power density values as high as 0.25 mW cm^{-2}. Table 1 shows that the results for EtOH,O$_2$ M$_{less}$EBFCs are much lower than the separated compartment cell indicating that besides pH effect must be a mutual influence of the fuel and O$_2$ in the performance of the enzymatic systems. Nevertheless, despite differences with respect to enzyme immobilization method, the values measured herein are in the same order of magnitude (μW cm^{-2}) with some data reported in the literature data[5,8,27,28]. Considering the high efficiency in ethanol/acetaldehyde conversion and concomitant O$_2$ reduction to H$_2$O demonstrated by enzymatic systems future application of biofuel cells in miniaturized systems must be solved preparing microfluidic devices that operate with streams of liquid electrolytes[29]. In this configuration it is possible to operate with different electrolytes for anodic and cathodic compartments without any problem. This is our future goal to increase the power density.

Table 2 lists the MWCNTs-ADH,polyPYR-Os-laccase cell storage lifetime under optimum conditions (200 mmol L^{-1} PBS (pH 6.5), 1.9 mmol L^{-1} NAD$^+$, and 100 mmol L^{-1} EtOH).

Table 2. EtOH,O$_2$ M$_{less}$EBFCs storage lifetime for polyPYR-Os-Laccase and MWCNTs-ADH bioelectrodes.

M$_{less}$EBFC	OCV/V	$J_{cell(max)}$/mA cm^{-2}	PD*/μW cm^{-2}
Fresly produced	0.226 ± 0.008	0.15 ± 0.07	21.0 ± 0.2
After 5 months	0.198 ± 0.003	0.09 ± 0.02	13 ± 4
After 11 months	0.140 ± 0.02	0.08 ± 0.02	8 ± 2

*Average and standard deviation analysis, in triplicate, for a set of three biocathodes and four bioanodes.

After five months, PD and $J_{cell(max)}$ decreased by approximately 38% (13 ± 4) μW cm^{-2} and 0.09 ± 0.02 mA cm^{-2}, respectively) as compared to freshly prepared electrodes. These values dropped slowly and reached 62% and 48% (8 ± 2 μW cm^{-2} and 0.09 ± 0.02 mA cm^{-2}, respectively) of the initial values. These results attested that immobilization of the enzymes employed here provided a relatively stable medium for long-term storage tests. This result may be important to apply these devices in new types of nanofluid cells to enhance the power harvest from the ethanol molecule[30].

4. Conclusions

Bioelectrodes containing the enzymes ADH and laccase and different redox mediators (Os or Ru) entrapped in polyPYR films or PAMAM dendrimer were tested. MWCNTs-ADH,polyPYR-Os-laccase employing PBS (pH 6.5) in the absence of ABTS performed the best. PD and $J_{cell(max)}$ remained around 21.0 ± 0.2 μW cm^{-2} and 0.15 ± 0.07 mA cm^{-2} for freshly prepared electrodes. Electrodes retain 38% of their activity after storage for five months storage in a refrigerator. The prepared EtOH,O$_2$ M$_{less}$EBFC generated power densities values comparable with literature data as well as considerable lifetime stability. Therefore, the results presented here for EtOH,O$_2$ M$_{less}$EBFCs are promising and may be employed in microfluidic devises to enhance the activity of the system.

5. Acknowledgments

6. References

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