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An enzymatic biofuel cell based on electrically wired polyphenol oxidase and glucose oxidase operating under physiological conditions

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Abstract

This paper reports the development and optimisation of a glucose/O_2 biofuel cell. At the anode, the glucose oxidation was performed by glucose oxidase (GOx) co-immobilised with a phenanthrenequinone mediator. At the cathode, the catalysis of O_2 using tetrathiafulvalene and polyphenol oxidase (PPO) has been demonstrated for the first time. The open circuit voltage (OCV) of the optimised biofuel cell reached 0.32 V, and the maximum power density achieved was 40.8 $\mu W\ cm^{-2}$ at 0.2 V. This original new GBFC showed quasi-stability in both OCV and power density for more than 25 days.

Keywords: biofuel cell, glucose oxidase, polyphenol oxidase, tetrathiafulvalene, enzyme wiring

1. Introduction

Throughout the last decade, the development of biofuel cells (BFC) that convert chemical energy into electrochemical energy via biochemical reactions has attracted great research attention [1-6]. The production of electricity is obtained via the oxidation of a fuel at the anode and the reduction of an oxidant at the cathode. For the glucose/O_2 biofuel cell, the oxidisable substrate is glucose and the oxidant is dioxygen. Enzymes are well-adapted to play the role of biocatalysts in biofuel cells. Due to the deep localisation of the redox active site of enzymes, the electron transfer from enzymes to electrodes can be achieved by direct electron transfer communication (DET) by using very specific materials [7-11] such as carbon nanotubes [9]. Another approach to insure the electron transfer is the introduction of artificial redox mediator molecules [12] such as osmium complexes [13, 14] and 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid [15, 16].

Glucose-O_2 biofuel cells are interesting devices because of their in vivo availability, allowing their implantation in living organisms [17]. Since the first example reported about glucose-O_2 biofuel implanted in the abdomen of a rat [17], different strategies regarding the method of implantation have been reported [18-21]. New implantable systems have been tested in cockroaches [20] and molluscs, in snails [18] and clams [19]. Nevertheless, for implantation, the activity of bioelectrodes must be stable in physiological fluids. The vast majority of BFC is based on the combination of glucose oxidase and laccase or bilirubin oxidase (BOD) for oxygen reduction. Although GOx is well-adapted for working at physiological pH, the use of laccase or BOD is compromised in physiological fluids. Laccase operates mainly in acidic conditions and is inhibited in the presence of chloride while BOD is inhibited by urate [22]. An alternative consists of using polyphenol oxidase (PPO) to reduce oxygen, as we firstly reported for a PPO-based BFC implanted in rats. However, PPO was electrically wired by a water soluble redox mediator (quinhydrone) requiring the use of a dialysis bag to prevent in vivo quinhydrone release. The use of water-insoluble compounds should enhance the

electrical connection with the enzyme and pave the way for a wider range of redox mediators for the enzyme wiring. In this context, we report here the original screening of various poorly soluble redox mediators for PPO and for GOx via the use of a carbon cavity microelectrode (CME). It is a rapid and simple way to determine if a mediator is suitable for enzymatic electrocatalysis. It consists of using CME, filling the cavity with both the mediator and the associated enzyme and looking at the catalytic effect by electrochemical methods in presence of substrate.

The optimum mediated enzyme systems were then employed for the fabrication of a BFC by mechanical compression with graphite particles. We further investigated the optimisation of the BFC performance under physiological conditions.

2. Experimental section

2.1. Material and reagents

GOx type VII (Aspergillus niger, $\geq$168,800 U g^{-1} solid), catalase (from Bovine Liver, 1610 U mg^{-1}), PPO (from mushroom 3933 U mg^{-1}), syringaldazine, ferrocene, 9,10-phenanthrenequinone, 1,4-naphtoquinone, duroquinone and graphite flakes (GF) were purchased from Sigma-Aldrich. Tetrathiafluvalene (TTF) was purchased from Alfa Aesar, HEPES from Euromedex and NaCl from Carlo Erba. All chemicals were used as received. Distilled water (18.5 MΩ cm^{-1}) was used for all aqueous solutions.

2.2. Electrode fabrication

The carbon cavity microelectrode consists of a cylindrical cavity (about 45μm in diameter and 25μm in depth) which was filled with different mixtures of enzyme and redox mediator powders (1:1 weight ratio), using the electrode as a pestle [23]. Composite graphite discs containing enzyme and redox mediator were designed by mechanical compression with

graphite particles [17]. Electrical contact was made through a platinum wire stuck by carbon paste glue.

2.3. Electrochemical measurements

All measurements were performed using an Autolab PGSTAT 30. A platinum wire and a SCE were used as counter and reference electrodes, respectively. Electrochemical measurements were performed at 25°C in HEPES buffer (0.025 mol L^{-1}, pH 7.2) containing NaCl (0.15 mol L^{-1}) and glucose (5.5 10^{-3} mol L^{-1}). Solutions of D-glucose were prepared 24 h before experiments. The performance of the BFCs was examined by immersing the two bioelectrodes in a one-compartment cell. The BFC power (P_{cell}) was measured using linear sweep voltammetry at 1 mVs^{-1}.

3. Results and discussion

3.1. Electrocatalytic evidence of O$_2$ reduction at TTF/PPO-based electrodes

With the aim of suppressing the release of redox mediators and hence the use of an additional membrane at the biocathode surface, the electrical wiring of PPO, which has a formal potential of 0.30 V/ SCE [24], by poorly soluble redox mediators as ferrocene, syringaldazine, and tetrathiafulvalene (TTF) was examined. Firstly, the electrochemical behavior of these three mediators was evaluated by cyclic voltammetry with CME filled with redox mediator (Fig. 1A) by scanning the positive region first. As shown on Figure 1, the E$_{1/2}$ values of these mediators are 0.203 V/SCE (ΔEp = 0.153V), 0.208 V/SCE (ΔEp = 0.065 V), and 0.165 V/SCE (ΔEp = 0.273 V) for ferrocene, syringaldazine, and TTF, respectively. The E$_{1/2}$ value of syringaldazine is close to the potential of syringaldazine immobilised onto a hydrophobic porous silicate-carbon heterogeneous structure [25]. These results show the possibility of using poorly soluble mediators to insure the connection of PPO to electrode the

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TTF giving the larger currents even if its half wave potential is slightly lower than that of the other two.

The efficiency of the PPO connection to syringaldine, ferrocene and TTF was then investigated with CME filled with enzyme–redox mediator mixtures. Cyclic voltammograms were recorded with these modified CMEs in the presence and the absence of oxygen. For instance, Fig. 1B displays the electrochemical behaviour of a CME filled with a TTF/PPO mixture (ratio 1:1). Under argon, a peak system composed of an anodic peak at 0.25 V/SCE associated to a cathodic peak at 0.06 V/SCE was attributed to the reversible oxidation of TTF to TTF^+. In O_2-saturated condition, the anodic peak relative to the TTF oxidation decreases while the cathodic peak increases. This catalytic phenomenon was ascribed to the formation of TTF^+ generated during the electro-enzymatic reduction of O_2 highlighting thus the efficient electrical wiring of PPO. The comparison of the different electrochemical behaviours indicates that TTF was the best candidate for establishing a stable electrical communication between PPO and the electrode. The PPO/TTF system was therefore investigated for the fabrication of compressed graphite electrodes.

On the linear sweep voltammogram from 0.15 V/SCE to -0.25 V/SCE for a compressed graphite-TTF-PPO electrode, a cathodic peak appears at -0.12 V/SCE (Fig. 2A). It was attributed to the reduction of TTF^+ generated during the enzymatic reduction of O_2. In addition, the same experiments performed with pressed graphite electrodes containing PPO or TTF show no reduction peak in this potential range, thus corroborating the efficient PPO wiring by TTF inside the graphite disc.

Finally, the operational stability of the bioelectrode was investigated by keeping the biocathode immersed in physiological conditions and determining its performance daily, via the bioelectrode polarisation. It appears that the open circuit potential (OCP) is stable for 28 days (141 ± 3 mV/SCE) while the I_{lim} value (recorded at -0.1 V/SCE) remains stable for 22 days and then decreases to 57% of its initial value after 28 days (Fig. 2B).

3.2. Electrocatalytic glucose oxidation at PQ/GOx-Catalase-based electrodes

As direct transfer between GOx and an underlying electrode is difficult or not possible [26,27], in a similar way, the GOx wiring by 9,10-phenanthrenequinone, 1,4-naphtoquinone, and duroquinone was examined by cyclic voltammetry with CME. As described before for cathodic mediators, the anodic mediators have been characterised by cyclic voltammetry (figure 3A) with CME filled with respectively 9,10-phenanthrenequinone (PQ) (curve a) 1,4-naphtoquinone (curve b), and finally duroquinone (curve c) by cycling the negative potentials first. The $E_{1/2}$ measured are respectively -0.208 V/SCE ($\Delta Ep = 0.164$ V), -0.22 V/SCE ($\Delta Ep = 0.160$ V), and -0.045 V/SCE ($\Delta Ep = 1.150$ V). Currents for the three mediators are all of the same order of magnitude, PQ being the mediator with the lowest $E_{1/2}$. To evaluate the electrocatalytic effect of GOx, Fig. 3B shows for example the cyclic voltammograms recorded at a CME filled with PQ/GOx mixture in absence and presence of glucose. Without substrate, an ill-defined cathodic peak appears at -0.290 V/SCE attributed to the reduction of PQ, combined with an anodic peak at -0.164 V/SCE. In the presence of glucose (0.03 mol L^{-1}), the efficient wiring of GOx by PQ is illustrated by the simultaneous decrease of the cathodic charge and the increase of the anodic one. The following resulting mechanism is suggested :

Glucose + GOx (FAD) $\rightarrow$ gluconolactone + GOx (FADH$_2$)

PQ + GOx (FADH$_2$) $\rightarrow$ PQH$_2$ + GOx (FAD)
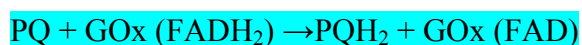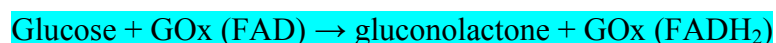
In the presence of glucose (5.5×10^{-3} mol L^{-1}), the polarisation curve for a graphite-PQ-GOx disc shows an OCP of -0.175 V/SCE (figure 4A) with a maximum anodic current intensity (202 μA) at 0.076 V/SCE. In contrast, OCP values for graphite, graphite-GOx and graphite-PQ were in the positive region, confirming the GOx wiring by PQ (Fig. 4A).

The operational stability of the bioanode was also examined by soaking the bioanode continuously in a 5.5×10^{-3} mol L^{-1} glucose solution. After an initial stabilisation period, OCP and I$_{lim}$ (recorded at +0.1 V/SCE) values remain remarkably stable for 120 days (Fig. 4B).

3.3. Optimisation of biocathode and bioanode

In order to design the optimum BFC configuration, the influence of graphite/mediator ratio or enzyme/mediator ratio on the bioelectrode performance was examined. Table 1 summarises the OCP and I$_{lim}$ values recorded for the different bioelectrode configurations. It appears that O$_2$ electro-reduction and glucose electro-oxidation currents were dependent on both ratios. It should be noted that the optimum value for I$_{lim}$ was considered to be more important than the OCP value to determine the optimum configuration. For the bioanode, the importance of the choice of the ratio between carbon graphite and the mediator is also important. The influence of the proportion of PQ was measured for carbon /PQ ratios ranging from 95/5 to 33/66 (for a total mass of 100 mg) and for 3.3 mg of GOx (Table 1). Comparing the anodic polarisation curves of electrodes with several different mass ratios, it appears that the best performance is obtained for a ratio of 75/25 with a current of 44.6 µA at a potential of 66 mV. Once this parameter is set, the increase of the proportion of enzymes indicates that the value of current measured at the catalytic oxidation potential of PQ is proportional to the amount of enzyme immobilised on the electrode. So, 25 mg PQ and 13.2 mg GOx for 75 mg graphite constitute the optimum composition that corresponds to a molecular mediator/enzyme ratio of 600 taking into account the price of enzymes for the production of the electrodes.

As for the cathode, the influence of the proportion of TTF was measured for carbon/TTF ratios ranging from 25/75 to 98/2 (for a total mass of 100 mg) and 5 mg of PPO (Table 1). The catalytic effect of PPO on the TTF was measured for three different concentrations of the enzyme. The increase in this enzyme/mediator ratio induces a small increase (10 % for the

current) which is negligible. Thus, the optimum biocathode configuration was 5 mg TTF and 5 mg PPO for 95 mg graphite that corresponds to a molecular mediator/enzyme ratio of 1500.

3.4. Performance of the BFC under physiological conditions

Fig. 5A shows the dependence of the current and the power as a function of the cell voltage in aqueous solution (pH 7.2) containing 5.5×10^{-3} mol L^{-1} of glucose. OCP reached 0.32 V and the maximum power density, calculated from the polarization curve, was 40.8 µWcm^{-2} at 0.2 V of the cell voltage. It should be noted the good correlation between the BFC performances determined by cyclic voltammetry at 1 mVs^{-1} and calculated from the chronopotentiometric curves (Fig. 3A) as previously reported [28]. The relatively low power output is due to the weak operating voltage of the cell, which is mainly due to the biocathode potential induced by TTF. Although the TTF potential was less positive than those used in other biocathodes [29, 30], its hydrophobic character reinforces the stability of the biocathode. As expected, the BFC stability (25 days) is in good agreement with the stability of each bioelectrode measured separately.

Compared to the previously fabricated biofuel cells [17], by changing the mediators at both electrodes, performances of the biofuel cell have been improved; the open circuit potential shifted from 0.246 V to 0.320 V in association with an increase of the power output by a factor of 13.5.

The operational stability of the BFC was investigated by recording the cell voltage as a function of time for constant current discharges from 10 to 50 µA (Fig. 5B). As expected, the cell voltage decreases with an increase of the discharge current. For a current of 50 µA, the cell voltage drops to 45 % of its initial value over 2 minutes whereas for a 10 µA discharge, only a slow decrease of the voltage (9%) is observed, illustrating the potentialities of this BFC.

Conclusion

Herein, we have reported the original use of cavity microelectrodes for the choice of the redox mediators able to connect enzymes for BFC. The combination of PPO and TTF led to a biocompatible biocathode for oxygen reduction. Its association with a bioanode based on GOx and 9,10-phenanthrenequinone provided a BFC able to operate under physiological conditions and hence be used for implantation in living tissues. It is expected that this easy screening tool and fabrication procedure for enzyme electrodes will facilitate the development of a new generation of biofuel cells.

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Table 1: Electrochemical characteristics of bioelectrodes immersed in aqueous solutions mimicking the physiological fluids and exhibiting various graphite/mediator/enzyme loadings. Mediators were 9,10-phenanthenequinone (PQ) at the anode and tetrathiafulvalene (TTF) at the cathode. Enzymes were GOx and catalase at the anode and PPO at the cathode.

Fig. 1: A- Cyclic voltammograms recorded at 100 mV s^{-1} at a carbon CME filled with (a) ferrocene, (b) syringaldazine, (c) tetrathiafulvalene immersed in a buffered aqueous solution. B- Cyclic voltammograms recorded at 100 mV s^{-1} at a carbon CME filled with a TTF/PPO 1:1 mixture and immersed in a buffered aqueous solution under an argon (line) or O$_2$ (dots) atmosphere.

Fig.2: A- Polarization curves of compressed graphite electrodes: graphite (line), graphite/TTF (dashes), graphite/PPO (dots) and graphite /TTF/PPO (red dots) at 1 mV s^{-1}. B- Stability of OCP (circles) and I$_{lim}$ at -0.1 V/SCE (squares) values obtained for a biocathode. There is an uncertainty of ± 3 mV for E and ± 2μA for I . 0.15 mol L^{-1} NaCl, 0.025 mol L^{-1} HEPES (pH 7.2, 20 °C).

Fig. 3: A- Cyclic voltammograms recorded at 100 mV s^{-1} at a carbon CME filled with (a) 9,10-phenanthenequinone (b) 1,4 naphtoquinone (c) duroquinone immersed in a buffered aqueous solution. B- Cyclic voltammograms recorded at 100 mV s^{-1} at a carbon CME filled with a PQ/GOx 1:1 mixture and immersed in a buffered aqueous solution in presence (dots) or absence (line) of glucose (0.03 mol L^{-1}).

Fig.4: A- Polarization curves of compressed graphite electrodes: graphite (line), graphite/PQ (dashes), graphite/GOx (dots) and graphite/PQ/GOx (blue dots) at 1 mV s^{-1}. B- Stability of OCP (circles) and I$_{lim}$ at +0.1 V/SCE (squares) values obtained for a bioanode. There is an uncertainty of ± 3 mV for E and ± 2μA for I. Buffered aqueous solution (pH 7.2, 20°C) containing 0.15 mol L^{-1} NaCl, 0.025 mol L^{-1} HEPES and glucose (5.5 10^{-3} mol L^{-1}) for polarization curves and stability experiments.

Fig. 5: A- Polarization curve obtained at 1 mVs^{-1} for a BFC (line) based on graphite /TTF/PPO and graphite/PQ/GOx discs and dependence of the power output as a function of the current. Dotted values (triangle and cross) correspond to chronopotentiometric determination. Potential values (cross) measured after 120 seconds of constant current discharges in the range 10-50 μA. B- Evolution of the BFC voltage as a function of time for

constant current discharges from 10 to 50μA. Experimental conditions: aqueous solution (pH 7.2, 20°C) containing NaCl (0.15 mol L^{-1}), HEPES (0.025 mol L^{-1}) and glucose (5.5 × 10^{-3} mol L^{-1}).

Table 1

Electrode	Mediator (mg)	Enzymes (mg)	OCP (V) Value ± 0.003 V	I_{lim} (µA)
bioanode (Mediator: PQ Enzymes: GOx - Catalase)	66	3.3 - 1.6	-0.059	5.0± 0.1
	50	3.3 - 1.6	-0.067	12.7± 0.2
	25	3.3 - 1.6	-0.112	44.6± 0.3
	10	3.3 - 1.6	-0.1390	34.1± 0.2
	5	3.3 - 1.6	-0.080	22.3± 0.2
	0	3.3 - 1.6	0.104	-
	25	6.6 - 3.2	-0.175	95.9± 0.8
	25	**13.2 - 6.4**	**-0.168**	**191± 2**
	25	19.8 - 9.6	-0.163	215± 2
biocathode (Mediator: TTF Enzyme: PPO)	75	5	0.117	-35.9± 0.2
	50	5	0.111	-62.7± 0.6
	25	5	0.111	-81.2± 0.7
	10	5	0.129	-118 ± 1
	5	**5**	**0.130**	**-151± 2**
	2	5	0.149	-118± 1
	0	5	0.090	-4.0± 0.1
	10	10	0.129	-130± 2
	10	2.5	0.124	-109± 2

Fig. 1

Fig. 2

Fig.3

Fig. 4

Fig. 5

A

B