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A laccase–glucose oxidase biofuel cell prototype operating in a physiological buffer

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Abstract

Here we report on the design and study of a biofuel cell consisting of a glucose oxidase-based anode (*Aspergillus niger*) and a laccase-based cathode (*Trametes versicolor*) using osmium-based redox polymers as mediators of the biocatalysts' electron transfer at graphite electrode surfaces. The graphite electrodes of the device are modified with the deposition and immobilization of the appropriate enzyme and the osmium redox polymer mediator. A redox polymer [Os(4,4′-diamino-2,2′bipyridine)$_2$(poly{*N*-vinylimidazole})-(poly{*N*-vinylimidazole})$_9$Cl]Cl ($E^{0\prime} = -0.110$ V versus Ag/AgCl) of moderately low redox potential is used for the glucose oxidizing anode and a redox polymer [Os(phenanthroline)$_2$(poly{*N*-vinylimidazole})$_2$-(poly{*N*-vinylimidazole})$_8$]Cl$_2$ ($E^{0\prime} = 0.49$ V versus Ag/AgCl) of moderately high redox potential is used at the dioxygen reducing cathode. The enzyme and redox polymer are cross-linked with polyoxyethylene bis(glycidyl ether). The working biofuel cell was studied under air at 37 °C in a 0.1 M phosphate buffer solution of pH range 4.4–7.4, containing 0.1 M sodium chloride and 10 mM glucose. Under physiological conditions (pH 7.4) maximum power density, evaluated from the geometric area of the electrode, reached 16 μW/cm^2 at a cell voltage of 0.25 V. At lower pH values maximum power density was 40 μW/cm^2 at 0.4 V (pH 5.5) and 10 μW/cm^2 at 0.3 V (pH 4.4).

Keywords: Biofuel cell; Biocatalyst; Laccase; Redox mediator; Osmium redox-polymer; Oxygen reduction

1. Introduction

The development of ever more miniaturized and sophisticated implantable biomedical devices provides the driving force for the design of small, reliable and bio-compatible, low power source systems. Biofuel cells are being developed for such applications. Indeed, they are simple in design and easy to miniaturize. Biofuel cells consist of a two electrode set (of any stable and electrically conducting material) modified by biocatalytic enzymes to specifically oxidize/reduce substrates. For implantable biofuel cells these substrates are available in vivo. The electrode reactions should not produce any harmful side products, and hence allow the direct and safe conversion of the chemical energy present within naturally available molecules into usable electrical power. In addition, the specificity of the enzyme reactions implies that no other components are required for a working biofuel cell, contrary to conventional fuel cells that contain at least a case and a membrane between the anode and the cathode. Recent approaches to the development of biofuel cells have been reviewed [1–5]. One approach towards the design of an implantable, membrane-less and bio-compatible biofuel cell consists of catalyzing the oxidation of glucose at the anode using either glucose oxidase or glucose dehydrogenase enzymes, coupled to the reduction of dioxygen at the cathode by a dioxygen-reducing enzyme such as laccase, bilirubin oxidase or cytochrome *c*/cytochrome oxidase [6–16]. Electron transfer to/from the biocatalytic active sites can be mediated by polymer bound or entrapped redox complexes [1–16]. Both water-soluble fuel molecules (glucose and O_2) are found in body fluids with concentrations in blood of respectively ca. 10 and 0.1 mM. Besides, these molecules are converted at the electrodes into naturally occurring degradation molecules, in low concentration (gluconolactone and water). The maximum theoretical electromotive force (emf) allowed by the thermodynamics of glucose oxidation and dioxygen reduction, at physiological pH, is approximately 1 V. Taking advantage of these electrode reac-

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tions requires the immobilization of the enzyme and of a redox mediator at the electrode surface. We recently reported our initial strategy towards development of an implantable biofuel cell, focused on rational optimization in the design of a laccase-based cathode [17]. In this account, we present our first assembly and study of a working biofuel cell under physiological conditions.

2. Experimental

2.1. Materials

4,4′-Diamino-2,2′-bipyridine and [Os(4,4′-diamino-2,2′-bipyridine)$_2$Cl$_2$] were synthesized according to the recently published improved procedure [18]. [Os(1,10-phenanthroline)$_2$Cl$_2$] [19,20] poly(vinylimidazole) [21] and polyacrylamide-polyvinylimidazole co-polymer [22] were synthesized as reported previously. The weight-average molecular weight of the poly(vinylimidazole) as determined by viscometry in ethanol [23] was 10,000 g/mol. Redox polymers were synthesized following procedures described by Forster and Vos, using a molar ratio of 1:10 of redox complex to polymer repeat unit [24]. All other chemicals were purchased from Sigma–Aldrich Chemical Co. (Ireland). All buffers were prepared from solutions of the selected base, then adjusted to the desired pH using solutions of the acid. The laccase isozyme II from *Trametes versicolor* source 52J (ATCC 96186) was produced and purified in our laboratory as described previously [25]. Laccase activity was determined by oxidation of 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS) as described previously [26].

2.2. Apparatus

All voltammetric experiments were performed using either CH Instruments 650 (USA) or EcoChemie Autolab PGSTAT10 (NL) potentiostats, coupled to single compartment electrochemical cells containing a Ag/AgCl reference electrode (Bioanalytical Systems, UK) a platinum wire counter electrode (Goodfellow, UK) and a graphite (Goodfellow) or glassy carbon (BAS) electrode in a phosphate buffer. Assembled fuel cells were studied with the modified graphite disk anode and cathode dipped in a cell containing 200 mL or more of a solution, thermostated at 37 °C, containing glucose at physiological concentration (10 mM) and 0.1 M of sodium chloride. Both electrodes were externally connected through a resistor (1 kΩ to 10 MΩ) and the voltage between the electrodes was measured with a multimeter (Keithley, UK) for each load.

2.3. Methods

Graphite disc electrodes (6 mm diameter), formed by shrouding graphite rods in glass tubes using heat-shrinkable tubing and establishing an electrical connection at the rear, were modified by drop-coating of a solution (typically 100 μL) containing the enzyme (50 μL of a 1600 U/mL laccase or 245 U/mL glucose oxidase solution in water), the redox polymer (25 μL of an 8–10 mg/mL solution/suspension in water) and polyoxyethylene bis(glycidyl ether) (25 μL of a 6–7 mg/mL solution in water) as a cross-linker onto a roughly polished and washed (milliQ water) graphite electrode, followed by at least 24 h drying of the film. All current and power densities are reported with respect to the geometric areas of the graphite electrodes to allow comparison to previously published biofuel cell densities.

3. Results and discussion

In our initial study [17] on the evaluation of a glucose/dioxygen biofuel cell concept and subsequent optimization and study of a laccase-based cathode, we selected [Os(bipyridine)$_2$(poly{*N*-vinyl imidazole})$_2$-(poly{*N*-vinyl imidazole})$_8$]Cl$_2$ (polymer I), as a good compromise for mediation of electron transfer to laccase at the cathode. Indeed its redox potential ($E^{0\prime}$ = +0.40 V versus Ag/AgCl) is high enough to obtain an appreciable voltage output in a putative assembled biofuel cell, where the anode would function at a low enough potential (ca. $-0.3\,\text{V} \leq E^{0\prime} \leq 0\,\text{V}$ versus Ag/AgCl since the glucose oxidase $E^{0\prime}$ is ca. −0.320 V versus Ag/AgCl for *Aspergillus niger* [27]). However, the redox potential of polymer I remains negative (by ca. 185 mV) of the laccase enzyme $E^{0\prime}$ of +0.585 V versus Ag/AgCl for *T. versicolor* [28]. Hence, the catalytic reduction of dioxygen ($E^{0\prime}$ = +0.593 V versus Ag/AgCl at pH 7) by the laccase enzyme (+0.585 V versus Ag/AgCl) mediated by polymer I ($E^{0\prime}$ = +0.40 V versus Ag/AgCl) is sufficiently thermodynamically driven downhill.

We found that our laccase cathodes, modified with polymer I, were efficient electrocatalysts of dioxygen reduction, yielding current densities of ∼240 μA/cm^2 at pH 5.0. However, consistent with the known pH dependence of this laccase activity in homogeneous solution [29–32], the laccase-modified cathode yielded only 7% of the catalytic current observed at pH 5.0, at a pH of 7.4. This low current density at physiological pH seems to preclude the in vivo use of this redox polymer-modified electrode. Nevertheless, we have further optimized our laccase-based cathode and connected it to a glucose oxidase-based anode in order to design a working biofuel cell. The design of our first assembled membrane-less biofuel cell is reported here together with the discussion of its performance in physiological conditions and its response to pH change.

First, following on the optimization strategy developed previously [17] we have synthesized two new redox polymers as candidates for improved mediation of the reduction of O_2 by laccase at the cathode. Polymer IIa and IIb, Fig. 1, consist of an osmium bis(1,10-phenanthroline) center coordinated via two imidazole moieties to the polymer backbone. Polymer IIb is a homopolymer of poly(vinylimidazole) whilst the IIa polymer backbone is a copolymer of poly(vinylimidazole) and polyacrylamide. Polyacrylamide may be introduced as a copolymer as it has been shown not to bind strongly or irreversibly to proteins [22]. Cyclic voltammograms of films of these polymers on glassy carbon electrodes, Fig. 2, demonstrate that they possess indistinguishable redox potentials of $E^{0\prime}$ = +0.49 (±0.01) V versus Ag/AgCl, within the precision of cyclic voltammetry. Films of both of these metallopolymers on the carbon electrodes exhibit linear increases in peak cur-

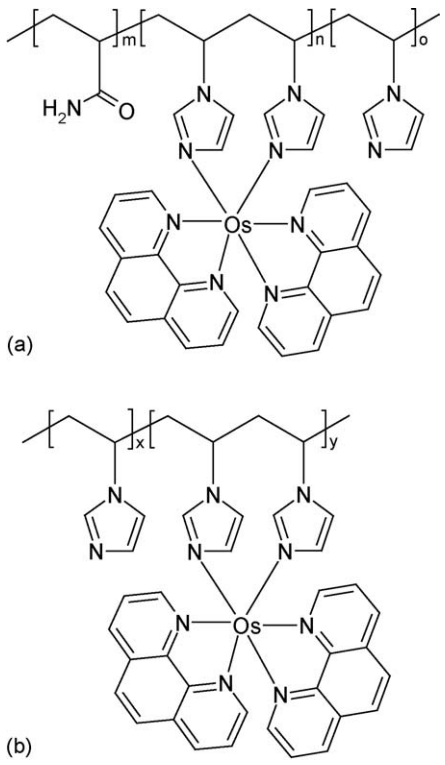

Fig. 1. Proposed structure of polymers IIa and IIb ($E^{0\prime} = 0.49$ V vs. Ag/AgCl) designed for the dioxygen reducing cathode, where m is 17.5, n is 1, o is 0.5, x is 8 and y is 2, assuming complete reaction of starting material.

rent as a function of scan rates for scan rates up to 50 mV/s, indicative of surface behaviour. At scan rates above 50 mV/s the peak currents scale with square root of scan rates, indicative of semi-infinite diffusion. The synthetic strategy for the preparation of these metallopolymers involves the successive substitution of the chloride ligands of an Os(1,10-phenanthroline)$_2$Cl$_2$ complex with the imidazolium nitrogens of the polymer backbone. The reported metal loading of the polymers here assumes complete reaction of the metal complex and polymer starting materials. Complete substitution, using the 1:10 polymer monomeric unit:metal complex ratio is not achieved. This is evident from the small peaks centred at $E^{0\prime} \sim +0.25$ V, in the cyclic voltammograms in Fig. 2, due to the presence of a minor component of the [Os(1,10-phenanthroline)$_2$(Pol)$_9$Cl]$^+$ intermediate, with Pol representing the polymer backbone, and where only one of the chloride ligands of the Os(1,10-phenanthroline)$_2$Cl$_2$ starting material is substituted. The surface coverages of the two forms of osmium complex present in these metallopolymers may be estimated by integration of the charge under the slow-scan cyclic voltammograms peaks. Surface coverage of osmium as [Os(phenanthroline)$_2$(poly{*N*-vinylimidazole})$_2$-(Pol)$_8$]Cl$_2$ is 5×10^{-10} mol cm^{-2} for both polymer IIa and IIb. It may be estimated that approximately 10% of the osmium in the metallopolymer films remains as the [Os(1,10-phenanthroline)$_2$(Pol)$_9$Cl]$^+$ intermediate. The polymer II $E^{0\prime}$ values remain ~0.1 V negative of the $E^{0\prime}$ of the laccase enzyme and allows a potential gain of 0.1 V in cell emf with respect to polymer I used previously. Polymer IIa however, showed low solubility in water, which precluded its co-deposition from aqueous solution with the enzyme and cross-linker on the electrode.

Two other redox polymers were synthesized for the anode. The redox active center in these polymers was an osmium bis(4,4′-diamino-2,2′-bipyridine)chloride coordination complex linked to the polymer backbone via one imidazole moiety of respectively, poly(vinylimidazole) (IIIb) and poly(vinylimidazole)/polyacrylamide copolymer (IIIa), Fig. 3.

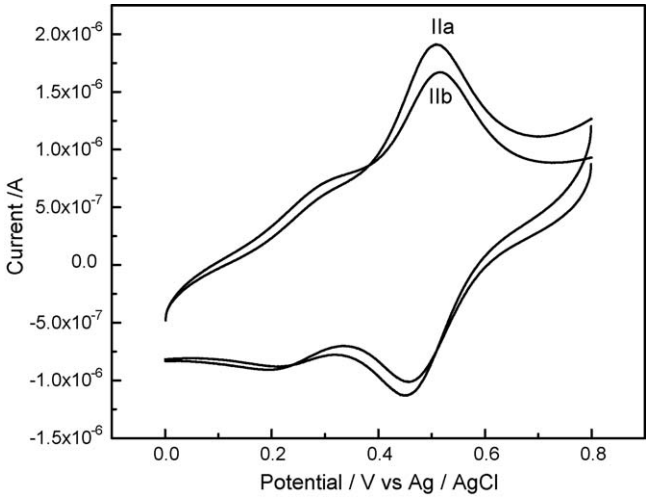

Fig. 2. Cyclic voltammetry of films of polymer IIa and IIb deposited onto 3 mm diameter glassy carbon electrodes. Scan rate 50 mV/s in 0.05 M phosphate buffer pH 7.4, 25 °C.

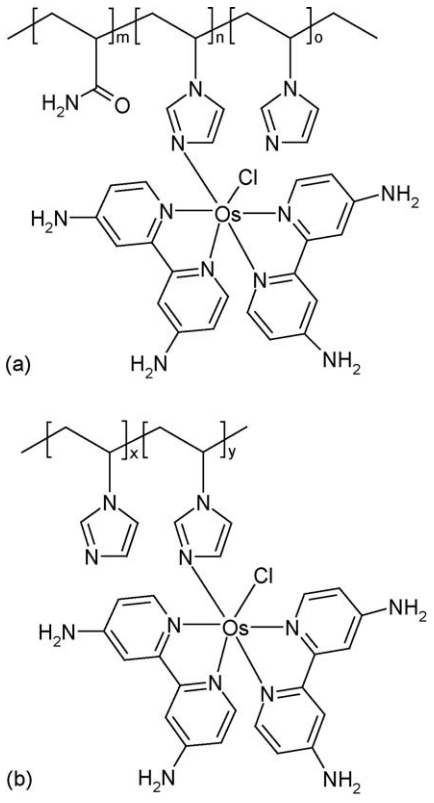

Fig. 3. Proposed structure of polymers IIIa and IIIb designed for the glucose oxidizing anode, where m is 8.75, n is 1, o is 0.25, x is 9 and y is 1, assuming complete reaction of starting material.

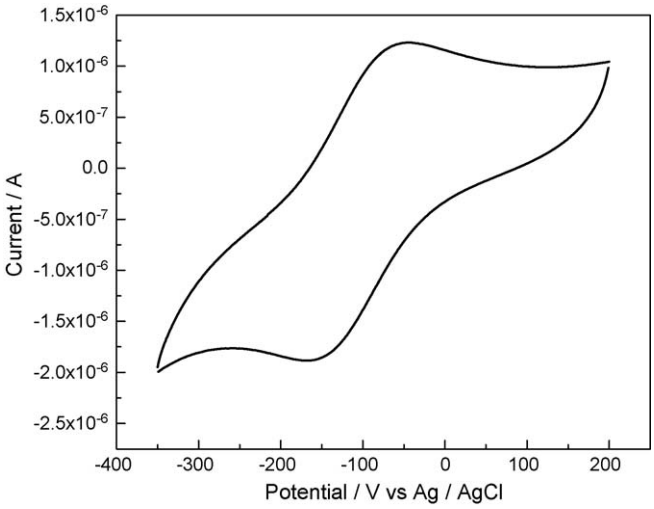

Fig. 4. Cyclic voltammetry of a film of polymer IIIb deposited onto 3 mm diameter glassy carbon electrodes. Scan rate 50 mV/s in 0.05 M phosphate buffer pH 7.4, 25 °C.

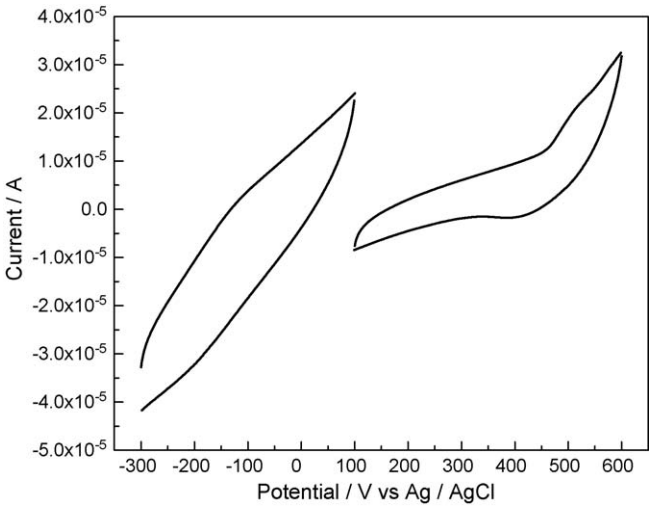

Fig. 6. Cyclic voltammetry at graphite electrodes modified with polymer IIb/laccase (cathode) and polymer IIIb/glucose oxidase (anode) in a single compartment 200 mL cell containing 0.05 M phosphate buffer, pH 7.4, 0.1 M NaCl, 10 mM glucose at 37 °C. Scan rate 1 mV/s.

Both polymers IIIa and IIIb have indistinguishable redox potentials of $E^{0\prime} = -0.11$ (±0.01) V versus Ag/AgCl, within the precision of cyclic voltammetry, Fig. 4. Once again, films of both of these metallopolymers on the carbon electrodes exhibit surface behaviour up to 50 mV/s scan rates and semi-infinite diffusion behaviour at higher scan rates. Polymer IIb (0.49 V) and IIIb (−0.11 V) were selected for further investigations because of their superior solubility in water.

Slow scan cyclic voltammograms of films of polymer IIb co-immobilized with laccase and polymer IIIb co-immobilized with glucose oxidase on graphite electrodes (6 mm diameter) are shown in Fig. 5 (pH 4.7) and 6 (pH 7.4) under conditions approximating physiological conditions (10 mM glucose and 0.1 M NaCl in phosphate buffer at 37 °C). At pH 4.7 (Fig. 5), biocatalytic oxidation of glucose occurs at the anode, mediated by redox polymer IIIb, attaining maximum catalytic currents at ca. −0.1 V, whilst biocatalytic reduction of oxygen occurs at the cathode, mediated by redox polymer IIb, attaining maximum catalytic currents at ca. 0.3 V, indicating that an emf cell output of at least 0.4 V is possible with a biofuel cell under these conditions. The maximum current density is ca. 80 μA/cm^2 at the anode and ca. 280 μA/cm^2 at the cathode, under these conditions. When the electrolyte is altered to pH 7.4 (Fig. 6) the potential observed for maximum current is similar to that at pH 4.7 for the anode, whilst maximum current is attained at 0.4 V at the cathode, an increase in 0.1 V compared to at pH 4.7, to give a possible emf of 0.5 V for a biofuel cell operating under these conditions. However, the maximum current density under these conditions has dropped to ca. 65 μA/cm^2 for the anode and ca. 20 μA/cm^2 for the cathode.

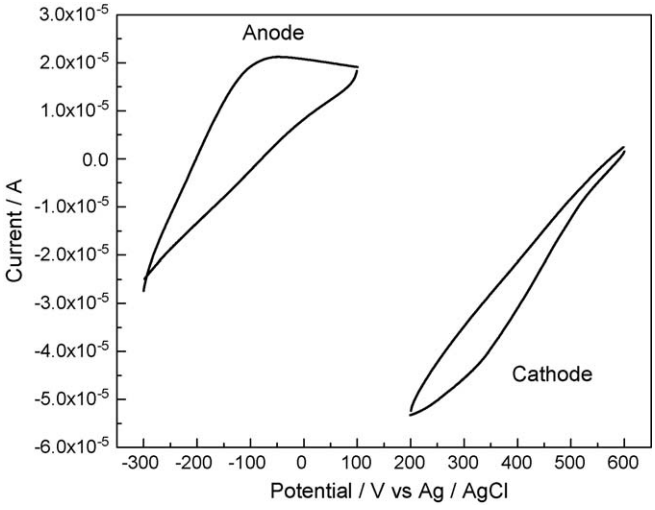

Fig. 5. Cyclic voltammetry at graphite electrodes modified with polymer IIb/laccase (cathode) and polymer IIIb/glucose oxidase (anode) in a single compartment 200 mL cell containing 0.05 M phosphate buffer, pH 4.7, 0.1 M NaCl, 10 mM glucose at 37 °C. Scan rate 1 mV/s.

Biofuel cells were assembled using films of these redox polymers and biocatalysts at graphite electrodes, by inserting the modified graphite disc electrodes into a phosphate buffered solution containing 10 mM glucose, 0.1 M NaCl and thermostated at 37 °C, as to model physiological conditions, under varying loads. We obtained the maximum performance for our cell at pH 5.5, Fig. 7, with a maximum power density of 40 μW/cm^2 at an operating voltage of 0.4 V, consistent with the optimum pH of laccase [29–32] and within the operating pH of glucose oxidase (5.5 with a broad range of high activity: 4–7) [33,34]. At the lower pH of 4.4, the lower maximum power density of 10 μW/cm^2 at 0.3 V can be explained by the remoteness of the optimum pH for glucose oxidase enzyme, with the anode thus limiting the power density, as expected from Fig. 5.

At physiological pH (7.4) the maximum power density was 16 μW/cm^2 at an output of 0.25 V. The lower output can be explained by examination of the pH activity profile of the *T. versicolor* laccase [29–32]. This enzyme is an active oxidant of inorganic redox complexes with an operating pH of 3–6 [21,32], but its activity for these substrates drops dramatically between pH 6 and 7. The power density for the cell is thus limited in this

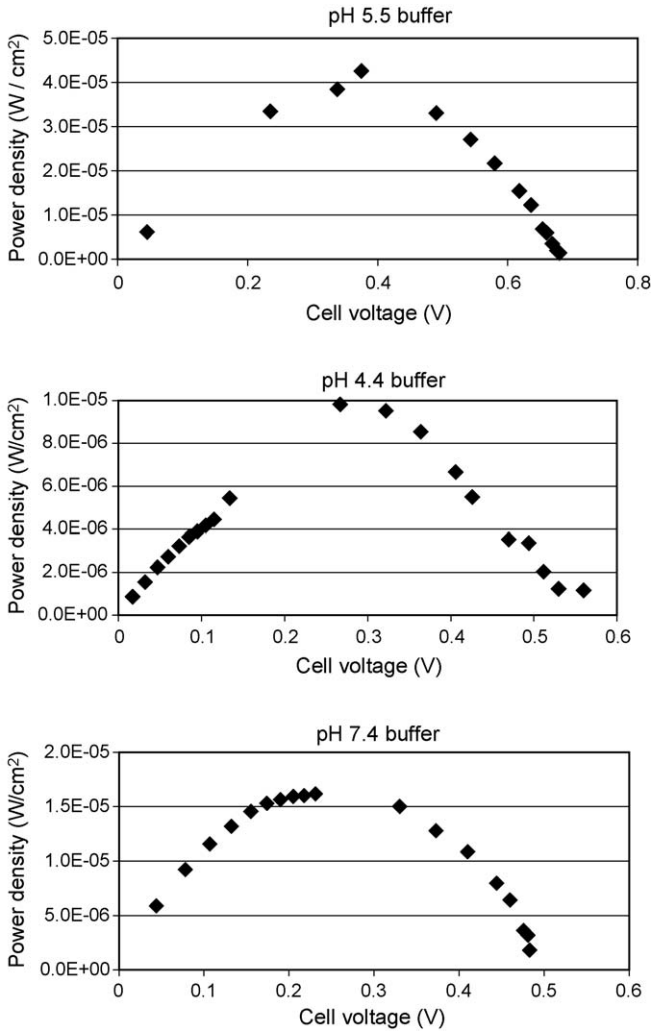

Fig. 7. Power density (W/cm^2) vs. cell voltage (V) curves at different pH (5.5, 4.4 and 7.4) for a biofuel cell consisting of graphite electrodes modified with polymer IIb/laccase (cathode) and polymer IIIb/glucose oxidase (anode) in a single compartment 200 mL cell containing 0.05 M phosphate buffer, 0.1 M NaCl, 10 mM glucose at 37 °C.

case by the cathode. This output compares favourably with an output of 5 μW/cm^2 at 0.06 V, observed by Katz et al. [6] for their glucose–O_2 biofuel operating in physiological solutions, based on a glucose oxidase anode and a cytochrome *c*/cytochrome *c* oxidase cathode. It should be noted also that in homogeneous solution laccases are weakly inhibited by chloride ions. In our hands, unlike that reported by others for laccases immobilized in redox polymer films [15,16,35] the catalytic reduction of dioxygen by *Tramates versicolor* laccase, and indeed by tyrosinase, co-immobilized in the osmium redox polymers does not diminish upon addition of physiological levels (up to 150 mM) of NaCl to the cell [32]. This may be due to a Donnan-type potential effect operating within these redox polymer films, given the high concentration of the positively charged osmium redox sites (~1 M) and corresponding counter-anions present in the films.

Thus, even though laccase is sensitive to chloride in homogeneous solution, and less active at neutral pH, our results show that this enzyme can nevertheless be used in physiological conditions and yield a significant power output at an appreciable operating voltage at electrodes of low microporosity. We are in the process of improving our deposition procedure as well as the surface functionalization, since the cell output was not stable (below 1 day) to yield improved biofuel cells. To improve the efficiency of the laccase/glucose oxidase biofuel cell working in physiological conditions, genetic engineering or selection of mutated strains might prove valuable to confer the enzymes specific properties to improve their activity under defined conditions and/or conferring the enzymes with properties adapted to different deposition or functionalization procedures [36–38]. In addition, improved power densities may be obtained by miniaturisation of the biofuel, and by increasing the micro/nano-porosity of the electrode surfaces.

An obvious alternative approach to avoid disadvantages of the pH activity profile of fungal laccases is the use of other enzymes that are active in physiological conditions, such as the oxygen reducing enzyme bilirubin oxidase, as demonstrated by Tsujimura et al. [9]. This group achieved a power density of 50 μW/cm^2 at 0.19 V, for a glucose–O_2 biofuel cell operating in physiological solutions, based on glassy carbon electrodes modified with osmium redox polymers co-immobilized with pyrroloquinoline quinone-dependent soluble glucose dehydrogenase at the anode and bilirubin oxidase at the cathode. The Heller research group have refined their glucose–O_2 biofuel cell, which initially used laccases at the cathode [7,11], to one which uses osmium redox polymers co-immobilized with glucose oxidase at the anode and bilirubin oxidases at the cathode [8,10,39], to yield a miniature biofuel cell capable of yielding a power density of 480 μW/cm^2 at 0.36 V. In these cases, however, the redox potential of the bilirubin oxidases is over 300 mV more negative than laccase from *T. versicolor*, which reduces the maximum possible emf of a glucose–O_2 biofuel-cell. We intend to improve on our laccase-based biofuel cells using a combination of the approaches identified above.

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