Short Communication

A non-compartmentalized glucose | O₂ biofuel cell by bioengineered electrode surfaces

Eugenii Katz a, Itamar Willner a,*, Alexander B. Kotlyar b

a Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
b Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

Received 12 August 1999; received in revised form 15 October 1999; accepted 15 October 1999

Abstract

A novel glucose | O₂ biofuel cell element was assembled by the engineering of layered bioelectrocatalytic electrodes. The anode consists of a surface reconstituted glucose oxidase monolayer, whereas the cathode is presented by the reconstituted cytochrome c/cytochrome oxidase couple. At the GOx monolayer-functionalized electrode, bioelectrocatalyzed oxidation of glucose to gluconic acid occurs, whereas at the Cyt. c | COx layered electrode, the reduction of O₂ to water takes place. The alignment of the glucose oxidase monolayer on the electrode surface yields an extremely efficient electrical communication, and the electron transfer turnover rate between the redox-center and the conductive support leads to an oxygen insensitive enzyme electrode. This enables the operation of the biofuel cell without the compartmentalization of the anode and the cathode. The system paves the way to tailoring invasive biofuel cells for generating electrical power. The analysis of the interfacial electron transfer processes of the electrodes suggests that by appropriate genetic engineering of the proteins, and appropriate chemical architecturing of the redox-proteins on the electrodes, the extracted power of the biofuel cell element could be further improved. © 1999 Elsevier Science S.A. All rights reserved.

Keywords: Biofuel cell; Cytochrome c; Electrochemistry; Enzyme catalysis; Monolayers

The development of biofuel cells that catalyze the conversion of chemical energy stored in abundant organic raw materials, or biomass, to electrical energy is a continuous challenging effort of bioelectrochemistry [1,2]. Biomass products such as ethanol or glucose may be used as an oxidizable substrate (fuel) at the anode whereas H₂O₂, and, preferably, molecular oxygen can be used as the reducible products at the cathode (oxidizer). Enzymes or whole cells can be used as biocatalysts in biofuel cells in two different routes [3–5]: (i) The biocatalysts may generate the fuel substrate for the cell by biocatalytic transformations or metabolic processes. For example, the microorganism Desulfovibrio desulfuricans catalyzes the metabolic conversion of sulfate to sulfide ions [6]. The latter product acts as the fuel substrate at the anode compartment where it is oxidized to sulfate, and oxygen is reduced at the cathode compartment. (ii) The biocatalysts may participate in the electron transfer chain of the biofuel cell, and enzymes may enhance the oxidation of the substrate at the anode and facilitate the reduction of oxygen at the cathode. Most of the redox enzymes, however, lack direct electrical communication with electrode supports, and various electron mediators were used to contact the biocatalyst electrically with the electrode [7]. For example, N,N-dimethyl-7-amino-1,2-benzophenoxazinium was used as an electron mediator in a glucose dehydrogenase | O₂ fuel cell [8]. The extractable power from the biofuel cell (P = V_{cell}I_{cell}) is controlled by the cell voltage (V_{cell}) and cell current (I_{cell}). Although the ideal cell voltage is the difference in the formal potentials of the fuel substrate and oxidizer, irreversible losses in the cell voltage as a result of kinetic limitations of electron transfer, ohmic resistances and concentration gradients in the cell are observed. Similarly, the cell current is controlled by the

* Corresponding author. Tel.: +972-2-6585272; fax: +972-2-6527715.
E-mail address: willnea@vms.huji.ac.il (I. Willner)
electrode sizes, the ion permeability and transport across the membrane separating the catholyte and analyte compartments of the biofuel cell, and specifically by the electron transfer rates at the respective electrodes. A major limitation of biofuel cell elements is the need to separate the cathode and anode since the oxidizer interferes, and often blocks, the biocatalyzed oxidation of the fuel substrate at the anode.

Here we wish to report on the assembly of a glucose | O2 biofuel cell by the engineering of the anode and cathode with biocatalytic monolayer interfaces that enable the operation of the biofuel cell without compartmentalization of the electrodes. The interfacial bioelectrocatalytic processes at the electrodes are efficient as a result of the structural alignment and orientation of the bioengineered electrodes, thus precluding an interfering reaction between oxygen and the biocatalytic anodic interface. The method could provide a novel approach to tailor invasive biofuel cell elements that extract electrical energy from plants or the living organism. The study suggests that the extractable power from the cell could be improved by further genetic engineering of the redox-proteins, and by additional chemical architecture of the electrodes. Scheme 1 shows the configuration of the biofuel cell element. It consists of two electrodes, where the anode is functionalized by a surface-reconstituted glucose oxidase, GOx, monolayer [9], and the cathode is modified with an integrated biocatalytic assembly composed of cytochrome c (Cyt c), and cytochrome oxidase, COx. At the GOx monolayer-functionalized electrode biocatalyzed oxidation of glucose to gluconic acid occurs, whereas at the Cyt. c | COx layered electrode the reduction of O2 to water takes place. Scheme 2A shows the method to assemble the GOx-functionalized electrode. It involves the surface reconstitution of apo-GOx onto a pyrroloquinoline quinone (PQQ)-FAD monolayer associated with an Au-electrode [10,11]. The cyclic voltammogram of the surface-reconstituted GOx layer in the presence of glucose shows an electrocatalytic anodic current at the redox-potential of the PQQ component (E° = −0.12 V vs. SCE at pH 7.0), implying that PQQ-mediated bioelectrocatalyzed oxidation of glucose by the reconstituted protein occurs. The anodic current transduced by the enzyme monolayer-electrode is unprecedentedly high and implies effective electrical communication between the redox-protein and the electrode support. Previous studies [10,11] indicated that the electron transfer exchange rate between the surface-reconstituted enzyme and the electrode exceeded the electron transfer turnover between the enzyme and its native electron acceptor, molecular oxygen. The turnover rate between GOx and O2 is ca. 600 s−1 at 25°C. Taking into account the footprint dimension of GOx, 58 nm2, a randomly densely packed monolayer, 1.7 × 10−12 mol cm−2, is expected to yield a current density of 200 μA cm−2, provided that the electron transfer exchange rate with the electrode is similar to that of molecular oxygen. The experimental transduced current density at a glucose concentration of 80 mM corresponds to ca. 200 μA cm−2. Thus, the electron transfer turnover between the surface-reconstituted GOx exceeds the electron transfer rate between the biocatalyst and the native acceptor. This extremely effective electrical communication between the redox-center of the enzyme and the electrode support is attributed to the alignment of all of the protein units with respect to the electrode support. The PQQ unit acts as an electron transfer mediator that bridges the electrical contact between the electrode and the redox-center. This result has important implications on the possible use of the surface-reconstituted GOx as the anode in the biofuel cell. The extremely high turnover of electrons between the enzyme redox-center and the electrode suggests that the bioelectrocatalytic activity of the enzyme should not be affected by molecular oxygen. In fact, we have previously reported [10,11] that the bioelectrocatalytic functions of the surface-reconstituted GOx are not affected by typical interferants such as ascorbic acid, uric acid or molecular oxygen.

The assembly of the bioelectrocatalytic interface of the cathode is shown in Scheme 2B. Cytochrome c lacks direct electrical contact with Au electrodes. Previous studies have indicated that surface modification of Au electrodes with molecular promoters, such as 4-pyridinethiol, or the functionalization of the electrode with negatively charged monolayer interfaces, lead to the electrical contact of Cyt. c with the conductive support [12,13]. This electrical communication between Cyt. c and the electrode is a result of the appropriate
alignment of the hemoprotein on the electrode [14]. We have aligned Cyt. c on the electrode by the site-specific coupling of the 102-Cys residue of Cyt. c (from *Saccharomyces cerevisiae*), to a maleimide monolayer [15] assembled on the electrode surface. A cyclic voltammogram of the Cyt. c monolayer assembled on the electrode shows a quasi-reversible redox wave of the hemoprotein, $E^0 = 0.03 \text{ V (vs. SCE)}$, indicating that the covalently-linked Cyt. c exhibits electrical communication between the heme-site and the electrode. This electrical contact between Cyt. c and the electrode is attributed to the alignment of the hemoprotein on the conductive support as a result of the site-specific covalent attachment. Coulometric assay of the reduction (or oxidation) wave of Cyt. c, indicated a surface coverage of the hemoprotein is $8 \times 10^{-12} \text{ mol cm}^{-2}$, that corresponds to a nearly densely packed monolayer (taking into account the diameter of Cyt. c, ca. 45 Å). The transient current decay upon the pulsed reduction of the heme center in a chronoamperometric experiment enables us to calculate the interfacial electron-transfer rate-constant to be $20 \text{ s}^{-1}$. Electron transfer rate constants for cytochromes immobilized on self-assembled monolayers vary from 0.2 to 1000 $\text{ s}^{-1}$ [16]. Thus, the rate constant achieved in our system could be enhanced by the appropriate change of the cytochrome molecule and the mode of its immobilization.

The cytochrome c-mediated electron transfer to cytochrome oxidase results in the four-electron reduction of oxygen to water [17,18]. This mediated electron transfer originates from an interprotein high affinity Cyt. c–COx complex that facilitates electron transfer from the Cyt. c heme to the redox centers of COx. We have shown previously, that cofactor–protein affinity complexes on electrode surfaces can be crosslinked to yield electrically-contacted, integrated, two-dimensional bioelectrocatalytic assays [19–21]. The Cyt. c monolayer assembled on the electrode was interacted with COx to generate the interprotein affinity complex (Scheme 2B). The resulting layered array is sufficiently stable to allow the subsequent crosslinking of the affinity complex with glutaric dialdehyde to yield the integrated and aligned protein layered electrode. Microgravimetric, quartz-crystal-microbalance (QCM), analyses indicate that the surface coverage of COx on the base Cyt. c monolayer is $2 \times 10^{-12} \text{ mol cm}^{-2}$.

At a bare Au-electrode the reduction of O$_2$ occurs at ca. $-0.3 \text{ V versus SCE}$. Many electrocatalytic and bioelectrocatalytic systems have been reported to decrease the overpotential for the reduction of O$_2$ [22–
24]. However, organized layered enzyme systems were never used for this purpose. Modification of the electrode with the Cyt. c monolayer results in a cathodic wave at ca. −0.5 V versus SCE, corresponding to the reduction of oxygen. This shift indicates that the modification of the electrode surface with Cyt. c introduces an electron transfer barrier for the reduction of oxygen, resulting in the observed overpotential. In the presence of the integrated Cyt. c | COx layer an electrocatalytic cathodic current, corresponding to the reduction of O₂, is observed. The reduction of oxygen is positively shifted to ca. 0.0 V versus SCE. That is, even though two proteins are associated with the electrode, the electrochemical reduction of O₂ is facilitated, and the electron transfer barriers are eliminated. Thus, the electron transfer cascade to COx biocatalyzes the reduction of O₂. The COx biocatalyzed reduction is anticipated to occur via a concerted four-electron reduction process to H₂O, without the intermediate formation of H₂O₂. It is important to verify that the biocatalyzed reduction of O₂ by the integrated Cyt. c | COx layered electrode does not yield H₂O₂, as the latter product could interfere as oxidizer in the biofuel cell element. The possible formation of H₂O₂ in the system was assayed by a rotating disc-ring experiment [25]. The Au-disc electrode was modified by the Cyt. c | COx assembly. On the bare Au-ring electrode, the potential +1.14 V was applied as a constant potential for the oxidation of H₂O₂. We found that the ring electrode does not generate any current upon sweeping of the potential on the disc electrode and stimulation of the biocatalyzed reduction of O₂, indicating that no H₂O₂ is formed.

The potential of the reconstituted GOx monolayer-electrode saturates at a glucose concentration of 1 mM. Thus, the biofuel cell performance was examined at a concentration of glucose corresponding to 1 mM, and using an electrolyte solution saturated with air. Fig. 1 shows the current voltage behavior of the biofuel cell at different external loads. The power of the cell at different loads is shown in the Fig. 1(inset). The maximum power is 4 μW at an external load of 0.9 kΩ. The ideal voltage–current relationship for an electrochemical generator is rectangular [26]. The experimental voltage–current plot deviates from the rectangular shape, and the fill factor, \( f = P_{\text{max}}V_{\text{oc}}^{-1} \), corresponds to ca. 40%. The \( I_{\text{cell}} - V_{\text{cell}} \) curve, Fig. 1, includes a region of low resistance, attributed to the ohmic cell-resistance, and a domain of high resistance, observed at high current values, attributed to the interfacial electron transfer resistance at the electrode support [26]. This high resistance may be attributed to the electron transfer at the Cyt. c | COx-modified electrode that revealed low interfacial electron transfer kinetics.

The stability of the biofuel cell element was examined for 48 h, using a flow system that preserves the glucose concentration in the cell to a constant value of 1 mM. We find that the cell potential and current are not altered during this period. It should be noted that the power extracted from the biofuel cell element is relatively low. In order to optimize the performance of the biofuel cell element, the interfacial bioelectrocatalytic transformations at the two electrodes should be optimized. As stated earlier, the bioelectrocatalyzed oxidation of glucose proceeds with a turnover rate of ca. 600 s⁻¹. The kinetics of the reduction of oxygen were examined by RDE experiments. The substantial deviation of the experimental current from the theoretical current calculated according to the Levich equation implies that the bioelectrocatalyzed reduction of oxygen is limited by electron transfer rather than by the diffusion of the substrate or ions. The turnover rate for the bioelectrocatalyzed reduction of O₂ was estimated to be ca. 20 s⁻¹. This value is very close to the interfacial electron transfer rate constant of Cyt. c, determined by chronocoulometry. Thus, we conclude that the rate of the reduction of O₂ by the integrated Cyt. c | COx layered electrode, is controlled by the primary event of electron transfer to Cyt. c. Although we were successful in attaining electron transfer communication between Cyt. c and the electrode by the site-specific coupling and alignment of the hemoprotein on the conductive support, the protein architecture on the surface is probably in a non-optimized configuration. Changing of the hemoprotein configuration by appropriate genetic engineered mutants of specific cysteine residues, could be a method to improve the interfacial electron transfer kinetics. A further contribution to the relatively low extracted power from the cell is the low potential difference existing between the anode and cathode. The bioelectrocatalyzed oxidation of glucose occurs at the

---

Fig. 1. Current–voltage behavior of the biofuel cell at different external loads. (Inset) Electrical power extracted from the biofuel cell at different external loads. The biocatalytic cathode and anode (ca. 0.8 cm² geometrical area, roughness factor ca. 1.3) were assembled in a thin-layer electrochemical cell with the distance between electrodes 5 mm.
redox-potential of the PQQ-electron mediator, $-0.125$ V versus SCE at pH 7.0. This yields a potential difference of only ca. 130 mV between the anode and the cathode. By the application of electron mediators that exhibit more negative potentials, the extractable power from the cell could be enhanced.

Acknowledgements

This research is supported by the Belfer Foundation.

References