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Surface Display of Redox Enzymes in Microbial Fuel Cells

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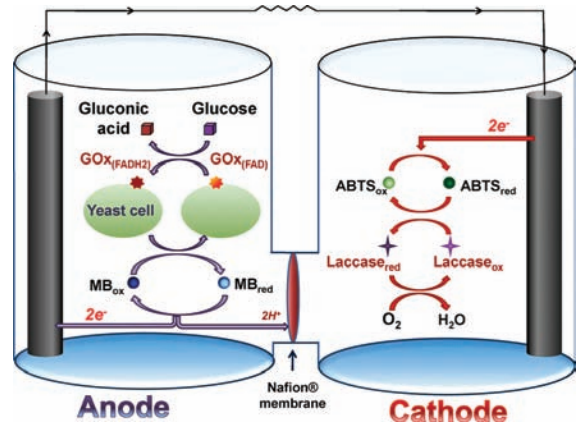
Enzyme-based biofuel cells utilize purified enzymes as catalysts for the oxidation/reduction of metabolites at the anode or cathode, respectively, at room temperature.[1] Microbial-based fuel cells (MFCs), however, exploit the metabolism of an entire organism for catalyzing the oxidation of fuels,[2] ranging from glucose and ethanol to starch and cellulose.[3] *Saccharomyces cerevisiae* (*S. cerevisiae*) was used in MFCs in several earlier examples and was proven to be an efficient catalyst in such devices (with or without the use of external mediators).[4] A biofuel cell containing bacteria at the anode and laccase as the reducing enzyme for oxygen reduction at the cathode was recently demonstrated.[5]

Enzyme-based biofuel cells suffer from a very prominent disadvantage for long-term operation, due to loss in enzyme activity; hence, a system in which the enzyme could be regenerated will be very beneficial. The display of enzymes on the surface of microorganisms suggests a solution to this caveat. Surface display techniques were developed during the 1990s, when these systems were developed both for *Escherichia coli* (*E. coli*)[6] and for yeast.[7] Among their many other uses, these systems were designed to serve as factories for the production of enzymes, eliminating the need for laborious and time-consuming enzyme purification.[8] Yeast surface display systems for the screening of combinatorial polypeptide libraries have demonstrated robust display capabilities. Not only is it easier to express eukaryotic proteins using yeast, but such systems have also successfully displayed larger enzymes than those displayed by the *E. coli* based systems,[9] and with much higher copy numbers. Moreover, a good method to control given reaction conditions, pH, use of cofactors, and different salt concentrations as well as a good accessibility for fuel opens up a whole new array of possibilities for MFCs. Fuels that otherwise could not be used by a given organism could be thus introduced to an MFC, by the display of a non-self-enzyme on the surface of a given organism, also eliminating the need for transport or diffusion of the fuel across the cell membrane.

Herein, we present a novel concept whereby microorganisms at the anode surface display redox enzymes that are used as catalysts for the oxidation of glucose. We have successfully displayed glucose oxidase (GOx), a highly efficient redox enzyme, on the surface of *S. cerevisiae*. GOx, a homodimeric flavo-enzyme, has an apparent molecular weight of 150 kDa, which includes two FAD cofactors.[10] GOx converts β-D-glucose in the presence of oxygen to gluconо-δ-lactone and hydrogen peroxide.[11] In contrast to purified enzyme based systems, microorganism display allows for a self-regenerating system, due to continuous growth and expression of enzymes on the surface. Our MFC, depicted in Scheme 1, contains in the anode compartment yeast cells displaying GOx on their outer surface, methylene blue (MB) as a redox mediator, and glucose as fuel (under anaerobic conditions). The cathode compartment consists of acetate buffer solution containing the oxygen-reducing enzyme, laccase, from *Trametes versicolor* and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a redox mediator.

Displaying GOx was achieved by cloning the amplified *gox* gene lacking its secretion signal peptide[12] from *Aspergillus niger* into the plasmid pCTCON (Supporting Information) to result in the plasmid pC-GOx(-). The displayed enzyme was fused with a myc tag on its c-termini that was detected using an antimyc-FITC labeled antibody, followed by flow cytometry analysis. Measured signals were compared with negative control signals, generated by cells not containing the display system, and positive controls, i.e., cells expressing a well-displayed protein. Strong signals for the GOx display system indicated high expression levels of GOx on the surface of the yeast cells. Flow cytometry results are shown in Figure S1 of the Supporting Information.

Scheme 1. Principle of Operation of the YSD-GOx Fuel Cell



Next, we wanted to test whether the displayed enzyme is functional, as was tested both biochemically and electrochemically. The biochemical assay used is a colorimetric assay detecting hydrogen peroxide production by functional GOx upon the oxidation of glucose. Using horseradish peroxidase (HRP) and *ortho*-phenylenediamine (OPD) we followed the oxidation of OPD by HRP in the presence of hydrogen peroxide produced by GOx. We then constructed a calibration curve and estimated the number of functional GOx per cell unit to be ca. 40 000 copies. The electrochemical assay employed was based on the biocatalytic oxidation of glucose by GOx in the presence of an electron transfer mediator, ferrocene dicarboxylic acid (FDA). Figure 1A shows the cyclic voltammograms (CVs) of FDA (a), the bioelectrocatalytic current resulting from the oxidation of glucose in the presence of FDA and GOx-displaying yeast (b), and purified GOx (concentration is 1000 times higher than GOx displayed on yeast; lower concentrations of purified GOx did not result in a detectable electrobiocatalytic current) (c). The CV for yeast not displaying GOx, in

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the presence of mediator and glucose, is similar to that of the mediator alone (a). It can be clearly seen that a biocatalytic current appeared only in the presence of GOx (and not with unmodified yeast) in the presence of glucose, indicating specific oxidation of glucose on the cell surface. If oxidation of glucose had occurred within the cells, it would not be observed by this method.

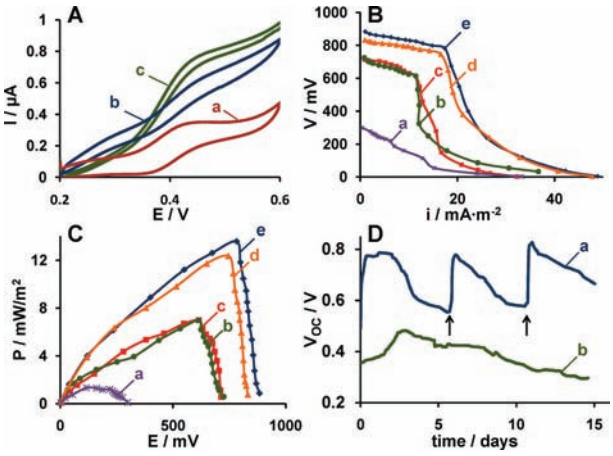

Figure 1. A. CVs of the bioelectrocatalytic current generated upon the oxidation of glucose by GOx in the presence of FDA. (a) FDA (similar CV appears for added yeast that do not display GOx); (b) FDA in the presence of GOx displayed on the cell surface and glucose; (c) FDA in the presence of purified GOx and glucose. Scan rate is 1 mV/s. The reference electrode is a saturated calomel electrode (SCE). B. Current densities and C. Power densities of fuel cell (in phosphate buffer solution) constructed with (a) background currents; (b) purified GOx; (c) unmodified yeast; (d) purified GOx in the presence of unmodified yeast. (e) GOx-displaying yeast. All measurements were conducted under variable external loads. Purified GOx concentration was kept equivalent to displayed GOx concentrations. D. V_{oc} over 15 days of biofuel cell operation in growth media. (a) GOx expressing yeast; (b) Purified GOx. Arrows indicate induction of enzyme expression.

The next step was to assemble the biofuel cell with modified yeast expressing GOx and test its performance, in comparison with biofuel cells based upon unmodified yeast cells or with purified GOx. We have conducted electrochemical and Faradaic impedance measurements for anode and cathode compartments separately and concluded that our specific fuel cell design is cathode limited. Figure 1B shows the polarization curves of the fuel cell upon application of variable external loads. It can be seen from these curves that the maximal achieved potential is highest for the modified yeast (curve e), where the open circuit potential (V_{oc}) is 884 mV, as compared with purified GOx (curve b) or with unmodified yeast (curve c), systems that reached a similar V_{oc} value of only 725 mV. This difference is an indication of an additive effect of having both the GOx on the surface and using the entire metabolism of the yeast cells for power production, reflecting the truly hybrid effect of using the enzyme display system. To test this notion we have also constructed a fuel cell that consisted of unmodified yeast and purified GOx as the catalysts (curve d) which resulted in similar values of power output and V_{oc} as the GOx displaying yeast. Figure 1C shows the power outputs of five MFCs constructed with either our engineered yeast cells or purified GOx or the combination of both. We can clearly see that power densities obtained from the GOx surface-displaying yeast cells (e) are much higher (13.6 $mW \cdot m^{-2}$) than those obtained using purified GOx (b) or unmodified yeast (c) (ca. 7 $mW \cdot m^{-2}$).

To test the long-term stability of current production of our engineered MFC and to demonstrate its self-regenerating abilities, we have compared the performance of two different fuel cells: GOx displaying yeast with that of purified GOx for 15 days. Figure 1D shows the V_{oc} of the two different fuel cells. We could observe that the activity of purified GOx (b) has deteriorated already after 4 days as opposed to the GOx displaying yeast for which, upon further induction of GOx expression after 5 days, full activity of the fuel cell has been restored. The measured V_{oc} for the purified GOx after 15 days was identical to that of the background V_{oc} (ca. 300 mV), an indication of a loss in enzyme activity which was estimated biochemically to be 30% of the initial enzyme activity. This result is further evidence that, in addition to the GOx displaying yeast being higher in activity comparing to unmodified yeast cells, its high activity as a catalyst can be maintained by the production of consecutive active generations of yeast cells displaying GOx.

In conclusion, we were able to demonstrate that surface display of a redox enzyme can be successfully used in an MFC. This is a first example of a new class of biofuel cells that serves as a general approach for the display of any redox enzyme on the surface of microorganisms. This approach allows in principle for a large variety of fuels to be potentially used in such a fuel cell, depending on the choice of the enzyme displayed. It can be further expanded for the use of unnatural fuels by the use of enzymes evolved by directed evolution to utilize these potential fuels.

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Supporting Information Available: Full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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