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Citric acid cycle biomimic on a carbon electrode

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ABSTRACT

The citric acid cycle is one of the main metabolic pathways living cells utilize to completely oxidize biofuels to carbon dioxide and water. The overall goal of this research is to mimic the citric acid cycle at the carbon surface of an electrode in order to achieve complete oxidation of ethanol at a bioanode to increase biofuel cell energy density. In order to mimic this process, dehydrogenase enzymes (known to be the electron or energy producing enzymes of the citric acid cycle) are immobilized in cascades at an electrode surface along with non-energy producing enzymes necessary for the cycle to progress. Six enzymatic schemes were investigated each containing an additional dehydrogenase enzyme involved in the complete oxidation of ethanol. An increase in current density is observed along with an increase in power density with each additional dehydrogenase immobilized on an electrode, reflecting increased electron production at the bioanode with deeper oxidation of the ethanol biofuel. By mimicking the complete citric acid cycle on a carbon electrode, power density was increased 8.71-fold compared to a single enzyme (alcohol dehydrogenase)-based ethanol/air biofuel cell.

1. Introduction

Enzymatic biofuel cells are a type of fuel cell where chemical energy is converted to electrical energy by employing enzymes as the electrocatalysts. Most enzymatic biofuel cells in the literature employ a single enzyme to do a partial oxidation of a specific fuel (i.e. glucose, lactate, pyruvate, ethanol). In contrast, living systems undergo metabolic processes such as the citric acid cycle through which they are able to completely oxidize biofuels to carbon dioxide and water. The citric acid cycle processes two carbon units from carbohydrates, amino acids, and fatty acids in the form of acetyl-CoA to generate reducing equivalents NADH and $FADH_2$ for ATP production by the electron transport chain. One of the key issues in developing effective and efficient enzymatic biofuel cells is the successful immobilization of multi-enzyme systems that can completely oxidize the fuel to carbon dioxide in order to increase the overall efficiency of the fuel cell. By doing so, the overall performance of the biofuel cell is increased as well. The first biofuel-based multistep oxidation of alcohols was demonstrated by Palmore et al. (1998) who employed alcohol dehydrogenase (ADH), aldehyde dehydrogenase (AldDH) and formate dehydrogenase (FDH) to completely oxidize methanol to carbon dioxide and water. Since these dehydrogenases are dependent upon NAD^+ reduction, a fourth enzyme diaphorase was introduced to regenerate the NAD^+ by reducing the mediator, benzyl viologen. This dehydrogenase catalyzed methanol/dioxygen biofuel cell produced an open circuit potential of 0.8 V and power density of 0.68 mW/cm^2. Akers et al. studied the two-step oxidation of ethanol to acetate using ADH and AldDH in a novel membrane assembly (MEA) configuration (Akers and Minteer, 2003; Akers et al., 2005). Since dehydrogenase enzymes are NAD^+-dependent, a polymer-based electrocatalyst (poly(methylene green)) was used to regenerate NAD^+ and to shuttle electrons from NADH to the electrode (Thomas et al., 2003). Bioanodes undergoing one-step oxidation were compared to bioanodes undergoing two-step oxidation. The ethanol/O_2 biofuel cell undergoing only one-step oxidation with ADH immobilized in a tetrabutylammonium bromide (TBAB) modified Nafion® membrane has shown open circuit potentials ranging from 0.60 to 0.62 V and the average maximum power density of 1.16 ± 0.05 mW/cm^2. In contrast, the open circuit potential and the maximum power density for the ethanol/O_2 biofuel cell employing a mixture of both ADH and AldDH immobilized in a TBAB modified Nafion® membrane were 0.82 V and 2.04 mW/cm^2, respectively. These bioanodes were able to function for more than 30 days and after 30 days of continuous operation, the bioanode showed an 18.1% decrease in power output. Although the results were successful, the system permitted only a 33% oxidation of the ethanol fuel which represents a low fuel utilization and therefore low energy density (Akers et al., 2005).

In the last few decades, harvesting energy from renewable resources has become an important focus in order to eliminate our dependency on oil and other non-renewable resources necessary as primary power sources (Davis and Higson, 2007; Kjeang et al.,

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2006; Winter and Brodd, 2004). In an attempt to do so, biofuel cell systems have been studied extensively, because of their ability to convert energy derived from biofuels to electrical energy by means of the catalytic activity of microorganisms and/or their enzymes. When building a fuel cell, maximizing both energy density (percentage of total available electrons gained during the oxidation process times the energy density of the fuel (typically measured in W h/L)) and power density (the operating voltage times the current per unit electrode area (typically measured in cm^2)) is of crucial importance. Microbial biofuel cells reported in the literature have been successful in attaining high energy densities due to the capability of completely (100%) oxidizing complex biofuels (i.e. glucose); however, their power densities have been low due to slow mass transport of the fuel across the cell wall (Karube et al., 1981; Lovely, 2006; Matsunaga and Suzuki, 1980; Suzuki and Karube, 1983). On the other hand, enzymatic biofuel cells have eliminated the fuel transport problems that caused low power densities in microbial fuel cells; however, their energy densities have been low due to incomplete oxidation of fuel (Austin, 1967; Nagy, 2003). In order to build an optimal biofuel cell, both energy density and power density need to be addressed and optimized. This work focuses on maximizing both the power and energy density through the use of enzymatic cascades; thereby, maintaining the high power densities of enzymatic biofuel cells while increasing the energy density of the biofuel cell through the ability to completely oxidize the biofuel ethanol.

Most living organisms acquire energy through metabolic enzyme pathways such as the citric acid cycle in order to completely oxidize most biological substrates. The citric acid cycle takes place in the mitochondria of eukaryotic cells where it oxidizes acetyl-CoA, a key metabolic junction, to carbon dioxide and water. Several substrates can be fed into the citric acid cycle via this key metabolic junction and each substrate will require different enzyme cascades. One of these substrates is glucose which can be oxidized through another metabolic pathway called glycolysis to pyruvate, which then gets oxidized to acetyl-CoA in presence of pyruvate dehydrogenase. Besides, glucose and pyruvate, lactate can also be fed into the citric acid cycle by incorporating lactate dehydrogenase and oxidizing it to the key metabolic junction, acetyl-CoA. In addition, ethanol can also be the substrate of choice after incorporating ADH, AldDH, and *S*-acetyl CoA synthetase and oxidizing ethanol to acetyl CoA, which has been done in the research presented in this paper. Nevertheless, the citric acid cycle uses acetyl-CoA as the substrate and undergoes eight enzymatic reactions out of which four are electron producing dehydrogenases. The electron producing enzymes of the citric acid cycle are NAD-dependent dehydrogenases except for succinate dehydrogenase (SDH), which is a FAD-dependent dehydrogenase. The redox couples NAD^+/NADH and $FAD/FADH_2$ are two electron electrochemical processes and their regeneration can be catalyzed by poly(methylene green). In this citric acid cycle biomimic, which uses ethanol as the starting point, expanding from a single enzyme system to a multi-enzyme system allows for additional electrons to be generated which contributes to the overall current and power density of the biofuel cell.

In this research paper, in order to mimic the citric acid cycle at the carbon surface of an electrode, all the enzymes employed for this biomimic were immobilized in a quaternary ammonium bromide salt modified Nafion® membrane layer. Dehydrogenase enzymes along with non-electron producing enzymes and cofactors were immobilized in cascades for the cycle to progress. The starting point for this biomimic was ethanol. ADH, AldDH, and *S*-acetyl-CoA synthetase were incorporated along with all the enzymes and cofactors of the citric acid cycle. The entire ethanol metabolic path can be seen in Fig. 1.

1.1. Modified Nafion® membrane

Enzymes are extremely efficient biocatalysts when immobilized at the electrode surface. When immobilizing an enzyme on an electrode surface, it is important to choose a method of attachment that will prevent loss of enzyme's activity, but not change the chemical nature or reactive groups in the binding site of that enzyme. The immobilization technique employed for this biomimic involves entrapping the respective enzyme(s) in a hydrophobically modified Nafion® polymer. Nafion® is a micellar cation exchange polymer with transport channels that deliver substrate or fuel to the immobilized enzyme. Unmodified Nafion® is hydrophilic, acidic, and has a micellar pore size of 4 nm, which is too small to effectively immobilize the enzymes of the citric acid cycle. Quaternary ammonium bromide salt modified Nafion® membranes modify the micro-environment of the pore to a near neutral pH that resists a decrease in pH, because quaternary ammonium cations have a much higher affinity for the sulfonic acid site than protons due to the hydrophobicity of the quaternary ammonium cation (Thomas et al., 2003). Previous research in our group has shown quaternary ammonium bromide modified Nafion® membranes have enlarged micellar pores and lower proton exchange capacity. This modification creates micellar pores that can accommodate the chemical and biochemical needs of an enzyme while retaining the electrical properties of Nafion®.

1.2. Poly(methylene green)

Most of the enzymes employed in this study are NAD^+-dependent dehydrogenase enzymes. During the oxidation of fuel, this coenzyme (NAD^+) is reduced to NADH. NAD^+ is regenerated by the oxidation of NADH, however, NADH oxidation and regeneration on platinum and carbon electrodes occurs at large overpotential and typically passivates bare electrode surfaces (Blaedel and Jenkins, 1975). Therefore, a polymer-based electrocatalyst, poly(methylene green), was used to regenerate NAD^+ and to shuttle electrons from the NADH to the electrode. Cyclic voltammetric studies of poly(methylene green)-coated glassy carbon electrodes have shown that poly(methylene green) is an electrocatalyst for NADH (Moore et al., 2004; Zhou et al., 1996).

2. Experimental

2.1. Materials

Methylene green (Sigma), sodium nitrate (Fisher), sodium borate (Fisher), sodium phosphate (Sigma), sodium hydroxide (Sigma), Nafion® 1100 EW suspension (Aldrich), ethanol (Sigma) and tetrabutylammonium bromide (TBAB) (Sigma) were purchased and used as received. In addition, Tris–HCl, NaCl, DL-dithiothreitol, EDTA, BSA, and cysteine were purchased from Sigma. Russet potatoes were purchased from a grocery store. Enzymes employed include: alcohol dehydrogenase (ADH) (E.C.1.1.1.1; Sigma), aldehyde dehydrogenase (AldDH) (E.C.1.2.1.5; Roche), *S*-acetyl-coenzyme A synthetase (E.C.6.2.1.1; Sigma), citrate synthase (CS)-(E.C.4.1.3.7; Sigma), aconitase (Aco.) (E.C.4.2.1.3; Sigma), isocitric dehydrogenase (IDH) (E.C.1.1.1.42; Sigma), α-ketoglutarate dehydrogenase (KDH) (E.C.1.2.4.2; Sigma), succinyl CoA synthetase (E.C.6.2.1.4; Bioacatalytics), fumarase (E.C.4.2.1.2; Sigma), and malic dehydrogenase (MDH) (E.C.1.1.1.37; Sigma). All enzymes purchased were stored at −20 °C except AldDH, citrate synthase, fumarase, and MDH which were stored at +2 to +8 °C. The necessary cofactors used were all purchased from Sigma and they include β-nicotinamide adenine dinucleotide hydrate (NAD^+), flavin adenine disodium salt