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Short communication

Development of glycerol/O_2 biofuel cell

Robert L. Arechederra, Becky L. Treu, Shelley D. Minteer *

Department of Chemistry, Saint Louis University, 3501 Laclede Avenue, St. Louis, MO 63103, United States

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Abstract

Glycerol is an attractive fuel for a fuel cell, because it is non-toxic, non-volatile, non-flammable, has high energy density, and is abundant due to the fact that it is a byproduct of biodiesel production. However, it has not been an effective fuel for low temperature, precious metal catalyzed fuel cells. In this paper, we describe the use of glycerol as a fuel in an enzymatic biofuel cell. An alcohol dehydrogenase and aldehyde dehydrogenase-based bioanode has been developed that oxidizes glycerol, a safe high energy density fuel. Glycerol/O_2 biofuel cells employing these bioanodes have yielded power densities of up to 1.21 mW cm^{-2}, and have the ability to operate at 98.9% fuel concentrations. Previous biofuel cells could not operate effectively at high fuel concentrations due to the nature of the solid fuel such as sugar or the solvent characteristics of fuels such as lower aliphatic alcohols. The glycerol/O_2 biofuel cell provides improved power densities compared to ethanol biofuel cells due to ability to more completely oxidize the fuel.
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1. Introduction

One of the important characteristics of a fuel for a fuel cell is high energy density. However, in low temperature fuel cells, high theoretical energy density is weighed with the degree of oxidation of the fuel that can occur at the precious metal anodes. For this reason, researchers have examined the electrochemistry of a number of complex, high energy density fuels, like glycerol [1–3] and ethylene glycol [3], but have not developed PEM fuel cells for these fuels, because the degree of oxidation is typically minimal. Precious metal-based catalysts can only oxidize the first 4 electrons of a total 14 electrons for glycerol at precious metal surfaces [2] leading to a decrease in energy density of the fuel cell. However, biofuel cells have the ability to increase the degree of oxidation, because there are natural enzymes and enzymatic pathways to completely oxidize these fuels.

A biofuel cell is similar to a traditional polymer electrolyte membrane (PEM) fuel cell in that it consists of a cathode and anode separated by a polymer electrolyte membrane that allows for proton transport from the anode to the cathode. Biofuel cells differ from a traditional fuel cell by the catalyst used to catalyze the electrochemical reaction. Rather than using precious metals as catalysts, biofuel cells rely on biological molecules such as enzymes to carry out the reaction. Although early biofuel cell technology employed metabolic pathways of whole microorganisms, the problems associated with this approach include low volumetric catalytic activity of the whole organism and low power density outputs [4]. Enzyme isolation techniques spurred advancement in biofuel cell applications by increasing volumetric activity and catalytic capacity [4]. Isolated enzyme biofuel cells yield increased power density output by overcoming interferences associated with cellular membrane impedance with electron transfer and lack of fuel consuming microbial growth [5].

Most enzyme-based biofuel cells to date rely on traditional biofuels such as simple primary alcohols, sugars, and other small molecule fermentation products (i.e. lactate). However, in this work glycerol is shown as a new fuel for biofuel cells that allows for fuel concentrations up to 98.9% to be used without swelling the Nafion, which traditionally has been problematic in most alcohol-based fuel cells. Glycerol, which is a byproduct of the production of biodiesel, has a higher energy density (6.260 kWh L^{-1} pure liquid) compared to ethanol (5.442 kWh L^{-1} pure liquid), methanol (4.047 kWh L^{-1} pure liquid), or glucose (4.125 kWh L^{-1} saturated solution) making it energetically, a very attractive fuel. Glycerol is non-toxic,

* Corresponding author. Tel.: +1 314 977 3624; fax: +1 314 977 2521.
E-mail address: minteers@slu.edu (S.D. Minteer).

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non-flammable, and non-volatile, which make it ideal for a wide variety of power applications.

Previous research has utilized NAD^+-dependent alcohol dehydrogenase (NAD^+-ADH) to carry out the oxidation of an alcohol to an aldehyde [6,7]. The range of commercially available ADH enzymes is limited to those, which are NAD^+ or $NADP^+$-dependent. NAD^+-ADH when used as a fuel cell catalyst has shown to be problematic due to the fact that it requires additional species to help mediate the exchange of electrons between the enzyme and the electrode, which adds complexity to the system and decreases the current density of the electrode. During the last decade, a series of membrane-bound dehydrogenases involved in the oxidation of selected carbohydrates and alcohols have been identified and characterized for numerous acetic acid bacteria [8]. Pyrroloquinoline quinine-dependent alcohol dehydrogenase (PQQ-ADH) will oxidize a wide range of alcohols and can be isolated from *Gluconobacter* species [9]. The enzyme contains the prosthetic group PQQ along with multiple heme *c*-containing subunits to facilitate electron transport between the enzyme and electrode surface [10,11]. PQQ-dependent aldehyde dehydrogenase (PQQ-AldDH) is also present in *Gluconobacter* species and can be isolated in conjunction with PQQ-ADH due to similar molecular weights (145 kDa for PQQ-ADH [9] and 140 kDa for PQQ-AldDH [12]) and theoretical pI's (6.1 for PQQ-ADH and 5.3 for PQQ-AldDH). Researchers have shown that PQQ-dependent dehydrogenases can undergo direct electron transfer at a variety of carbon surfaces [10,13–15]. In this research, PQQ-ADH and PQQ-AldDH are the enzymes used to oxidize glycerol at the anode surface of a biofuel cell. Studies of degree of oxidation, fuel concentration, and electrochemical performance were completed on the functioning biofuel cell.

2. Experimental

2.1. Reagents

Glycerol (Sigma), potassium hydrogen phosphate (Sigma), sodium dihydrogen phosphate (Sigma), D-mannitol (Sigma), $(NH_4)HPO_4$ (Acros), $MgSO_4 \cdot 7H_2O$ (Sigma), sodium chloride (Sigma), sodium hydroxide (Sigma), $CaCl_2$ (Baker Adamson), sodium deoxycholate (Sigma), sucrose (Mallinckrodt), Triton X-100 (Sigma), $^{13}C_3$-labeled glycerol (Isotec), Toray carbon paper (E-Tek), trimethyloctylammonium bromide (Sigma), Nafion 1100EW suspension (Aldrich) and Nafion 112 (Alfa Aesar) were used as received.

2.2. Alcohol and aldehyde dehydrogenase isolation and purification

In order to obtain PQQ-ADH and PQQ-AldDH, *Gluconobacter* sp. 33 was purchased from DSMZ and was cultivated aerobically in a basal media containing yeast extract, D-mannitol, $(NH_4)HPO_4$, and $MgSO_4 \cdot 7H_2O$ at 30 °C for 20 h. The cell paste was centrifuged at 5000 × *g* for 5 min then twice washed with 0.9% NaCl and stored at −20 °C until use. The thawed cell paste was suspended in 0.2 M phosphate buffer pH 7.0 containing 1 mM $CaCl_2$, 10% sodium deoxycholate (the final concentration 0.5%), and 1 mL of lysozyme (10 mg lysozyme in 1 mL 0.3 M potassium phosphate buffer pH 7.2). The solution was incubated at 4 °C with gentle stirring for 1 h, sonicated at a pulsed frequency for 1 min, and followed by centrifugation for 1 h at 12,000 × *g* to remove insoluble materials. A 10% $CaCl_2$ solution is added to the supernatant via vortexing (0.5% final concentration) to form a calcium phosphate gel and allowed to equilibrate at 4 °C for 45 min. The resultant gel was collected by centrifugation for 20 min at 6000 × *g*, resuspended in 0.3 M potassium phosphate buffer pH 7.2, and stirred gently for 10–20 min to elute bound enzyme. An insoluble material was discarded after centrifugation for 30 min. A buffer exchange in 20 mM Tris–HCl buffer pH 7.2 containing 1 mM $CaCl_2$ and 1% sucrose was performed via dialysis overnight at 4 °C.

After removing an insoluble precipitate by centrifugation, the enzyme extract was applied to a DEAE Toyo-Pearl 650 M® column, which was equilibrated with the dialysis buffer. The column was washed by passing two bed volumes of the same buffer and two bed volumes of 75 mM Tris–HCl buffer pH 7.2 containing 1% sucrose and 1 mM $CaCl_2$. PQQ-ADH and PQQ-AldDH are eluted with the same buffer containing 0.2% of Triton X-100. Fractions with PQQ-ADH and PQQ-AldDH activity are collected. A buffer exchange was performed overnight via dialysis at 4 °C in 5 mM potassium phosphate buffer pH 7.2 containing 1 mM $CaCl_2$. The dialyzed enzyme solutions were concentrated (to 1 mL) using a Centriprep®. The concentrated enzyme solutions were applied to CM-Toyo-Pearl® column equilibrated with 5 mM potassium phosphate buffer pH 7.2 containing 1 mM $CaCl_2$ and 1% sucrose. Fractions containing PQQ-ADH and PQQ-AldDH activity were collected and concentrated via a Centriprep®.

2.3. Electrode preparation

The bioanode consists of 1 cm × 1 cm square Toray paper modified with PQQ-ADH/PQQ-AldDH immobilized in a trimethyloctylammonium bromide (TMOA)-modified Nafion. Casting solutions for making the mixture-cast membranes of Nafion and quaternary ammonium bromides were prepared as discussed in Ref. [16]. One milliliter of the mixture-casting solution was placed in a weighing boat and allowed to dry. Previous studies have shown that all of the bromide ions that were introduced into a membrane were ejected from the membrane upon soaking that membrane in water [17]. Therefore, 7.0 mL of 18 MΩ water were added to the weighing boats and allowed to soak overnight. The water was removed and the films were rinsed thoroughly with 18 MΩ water and dried. The salt-extracted films were then resuspended in 1.0 mL of lower aliphatic alcohols.

Enzyme/TMOA-modified Nafion casting solutions with a ratio of 1:1 (100 μL of 1.0 mg mL^{-1} PQQ-ADH/PQQ-AldDH in phosphate buffer: 100 μL TMOA-modified Nafion suspension) were vortexed in preparation for coating on electrode. Fifty microliters of the solution was pipeted onto the electrode, allowed to soak into the Toray paper electrode and dried in a low humidity environment for 12 h.

2.4. Physical cell apparatus

The physical test cell consisted of custom fabricated “U” shaped cylindrical glass tubing with 2.6 cm diameter, 14.8 cm height, and 7.6 cm length. Approximately 50 mL of solution was contained on both sides of a Nafion 112 membrane (Alfa Aesar). The cathodic compartment of the test cell contained pH 7.15 phosphate buffer solution. The cathode material consisted of a gas permeable ELAT electrode with 20% Pt on Vulcan XC-72 (E-Tek). The anodic compartment of the test cell is filled with pH 7.15 buffer with 1 M NaCl electrolyte ranging from 10 mM to 12.61 M glycerol fuel. Extra electrolyte had to be dissolved into the glycerol to maintain ionic strength when the concentration of glycerol exceeded 8 M. The PQQ-ADH/PQQ-AldDH-modified electrode served as the anode. The complete cell was allowed to equilibrate for a minimum of 2 h before data collection. All data was collected and analyzed for the test cell with a CH Instruments 650 potentiostat interfaced to a PC computer. The biofuel cells were tested in triplicate and all reported uncertainties correspond to 1 standard deviation.

3. Results and discussion

When investigating low-temperature fuel cells employing liquid fuels, there are two main types of fuel cells being developed (the direct methanol fuel cell (DMFC) and the direct ethanol fuel cell (DEFC)). Both fuel cells employ platinum alloy catalysts to oxidize the fuel. More research and commercial investigations are focused on DMFCs, because although the fuel is more toxic than ethanol, it can undergo near complete oxidation to carbon dioxide under optimal temperature, fuel mixture, and partial pressure of oxygen [18,19]. In theory, ethanol is an attractive alternative to methanol, because it is a renewable fuel and is less toxic than methanol, but DEFCs have had problems due to the difficulty in Pt-based catalysts breaking C–C bonds [20]. Oxidation of ethanol at platinum-based electrodes typically results in the formation of the partial oxidation byproducts acetaldehyde and acetic acid [21], which greatly minimizes the energy density of the fuel cell. Therefore, more complex fuels like glycerol have not been used in low-temperature fuel cells, because the triol oxidation is even more complex at precious metal surfaces [2]. In acidic media, platinum has been shown to exhibit catalytic activity [22,23], but platinum, palladium, and gold have been shown to exhibit catalytic activity in alkaline media [1,2,23]. Strong adsorption of glycerol is an issue for all of these metallic systems [2], which has limited its possible use in precious metal-catalyzed fuel cells at room temperature, but the main issue with glycerol catalysis at precious metal electrodes is partial oxidation. The main product is glycerate [2], which only results in 28.6% oxidation of the fuel limiting the energy that can be derived from the fuel. However, when compared to other alcohol fuels, glycerol is an ideal fuel choice, because it is inexpensive, non-toxic, non-flammable and widely available. Also, glycerol is a renewable energy source that is produced in large quantities as a byproduct of biodiesel. Using enzymatic catalysts, PQQ-ADH and PQQ-AldDH isolated from *Gluconobacter* sp. 33 have shown catalytic activity for the multi-step oxidation of glycerol, with power outputs better than ethanol biofuel cells employing the same enzymes. This is due to the fact that glycerol is a triol having more alcohol and subsequently aldehyde oxidation sites than ethanol.

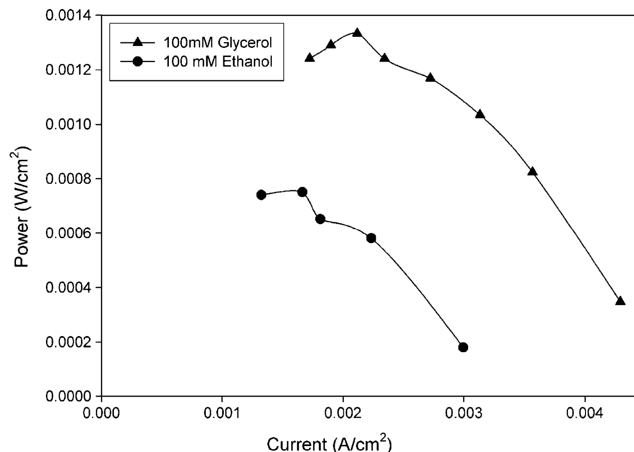

Fig. 1. Comparison of the power curves for a single biofuel cell with two different analyte fuels (100 mM ethanol and 100 mM glycerol) at room temperature.

Bioanodes employing PQQ-ADH and PQQ-AldDH immobilized in a TMOA-modified Nafion membrane have shown open circuit potentials ranging from 0.55 V to 0.73 V at 20 °C and a buffer of pH 7.15 for both bioanodes in glycerol fuel solutions and ethanol fuel solutions. The average maximum power density for the glycerol-based biofuel cell is $1.13 \pm 0.15\ \mathrm{mW\,cm^{-2}}$. A representative power curve for the glycerol/O_2 fuel cell after 24 h of operation is shown in Fig. 1. The maximum power increased approximately 10% over the first 45 days of operation. The same bioanode in a 100 mM ethanol fuel solution shows a decreased performance as shown in Fig. 1. The bioanode when oxidizing glycerol as fuel showed a 62.5% increase in power output compared to oxidizing ethanol, which is indicative of a different oxidation mechanism than ethanol. This is expected, since there are three available alcohol oxidation sites in glycerol compared to ethanol, which has only one alcohol oxidation site.

In order to determine the oxidation mechanism for glycerol in the biofuel cell, ^{13}C NMR was utilized to study reaction products from the fuel cell. A glycerol bioanode containing immobilized PQQ-ADH and PQQ-AldDH was placed into the test cell containing 1% ^{13}C-labeled glycerol in pH 7.15 buffer with 20% D_2O. A load was put on the cell to hold a cell potential of 0.05 V for 16 h. A control consisted of 1% ^{13}C-labeled glycerol in D_2O. ^{13}C NMR of the control showed one doublet at 62.5 ppm and one triplet at 72.0 ppm, corresponding to the primary and the secondary carbons, respectively. ^{13}C NMR was run on the sample that contained the reaction products after the PQQ-ADH and PQQ-AldDH bioanode had been functioning in the glycerol fuel for 16 h with an applied load. The ^{13}C NMR spectrum showed a complex mixture of intermediate oxidation products after 16 h. Peaks at 216 ppm and 215 ppm corresponding to the presence of primary and secondary carbonyls attached to the labeled backbone indicated that dihydroxyacetone, glyceraldehyde, and tartronic aldehyde may have been formed as reaction products. Dihydroxyacetone

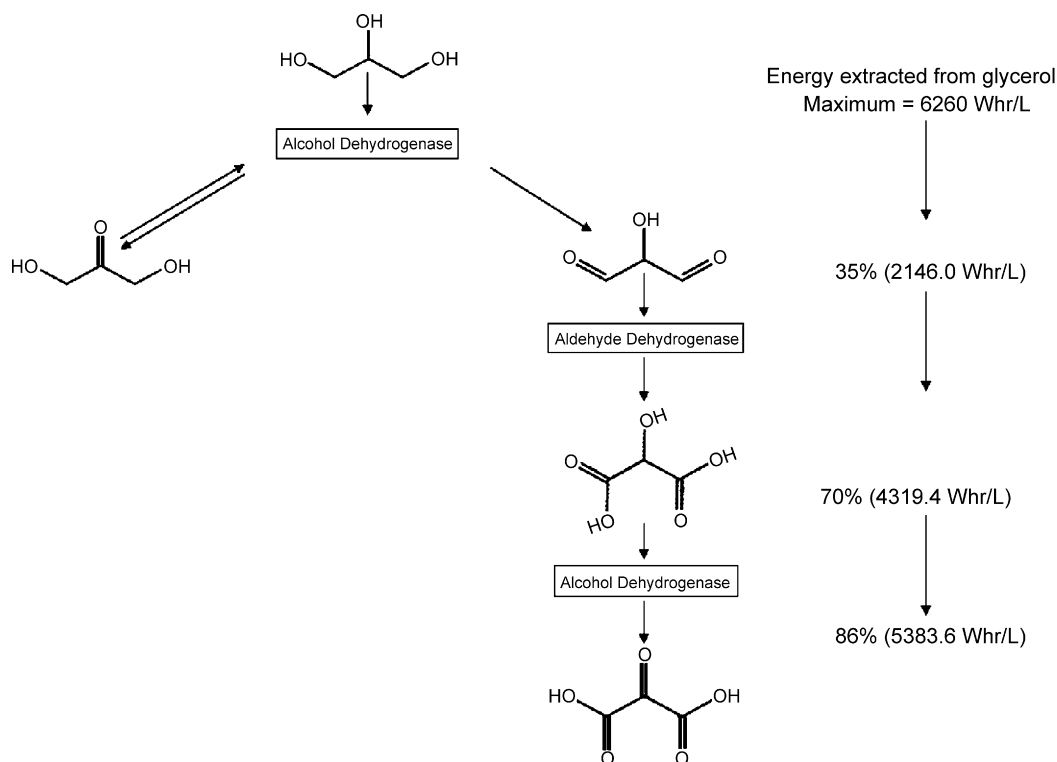

Fig. 2. Oxidation sequence for glycerol at a PQQ-ADH/PQQ-AldDH-modified bioanode.

and glyceraldehyde are actually expected byproducts, because the *Gluconobacter* bacteria that the enzymes are isolated from is wellknown for producing dihydroxyacetone [24–26] and glyceraldehyde [27] upon treatment with glycerol substrate. There is no literature discussion of tartronic aldehyde formation in *Gluconobacter* after glycerol treatment, but it is a reasonable intermediate to the oxidation pathway to mesoxalic acid and is seen in other glycerol-oxidizing bacteria [28], along with chemical oxidation. In addition to these carbonyl peaks, there were peaks at 177 ppm and 176 ppm indicating that there were carboxylic acids attached to the labeled backbone corresponding to the glyceric acid, tartronic acid, mesoxalic acid (often referred to as ketomalonic acid). Other peaks that support these products being formed are 119 ppm for the secondary carbon in mesoxalic acid, and 89 ppm for the secondary carbon in tartronic acid. These are the common byproducts for selective chemical oxidation of glycerol and are to be expected [29]. Fig. 2 shows the generalized oxidation pathway of glycerol in the biofuel cell as supported by the major peaks of the ^{13}C NMR study. This is very different than metallic electrodes which show glycerate as the only detectable oxidation product of glycerol [2] and shows that biofuel cell can allow for deeper oxidation of the glycerol fuel, which increases the efficiency and energy density of the fuel cell.

The use of the TMOA-modified Nafion membrane for enzyme immobilization produces glycerol/O_2 biofuel cells with milliwatt power densities that are comparable to the ethanol biofuel cell. Upon studying the effect of fuel concentration of the glycerol biofuel cell performance, it was shown that the fuel cell continues to perform at fuel concentration up to 98.9 wt.%. The change in maximum power density of the glycerol biofuel cell as a function of fuel concentration is shown in Fig. 3. The fuel cell performance at 98.9 wt.% glycerol is 37.9% of the fuel cell performance at 0.8 wt.% glycerol. This is due to the fact that the viscosity of the fuel solution is increasing with increasing glycerol concentration, thereby, decreasing the mass transport of fuel through the enzyme immobilization membrane. The Stokes–Einstein expression shows that the diffusion coefficient (D) of a molecule is inversely proportional to viscosity (η):

$$D = \frac{k_B T}{6\pi\eta r}$$

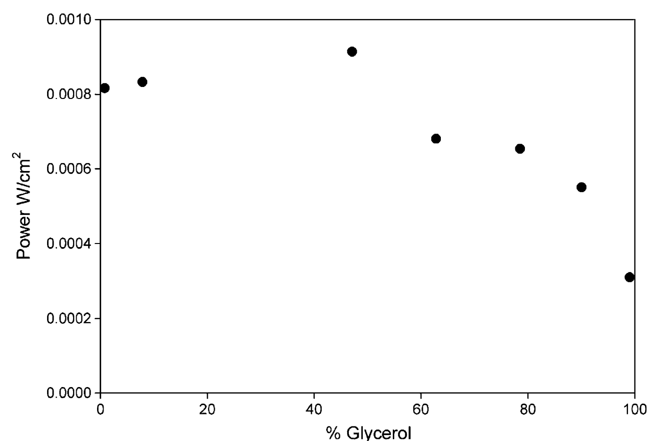

Fig. 3. Maximum power density for a single glycerol/oxygen biofuel cell with varying glycerol concentration in the analyte.

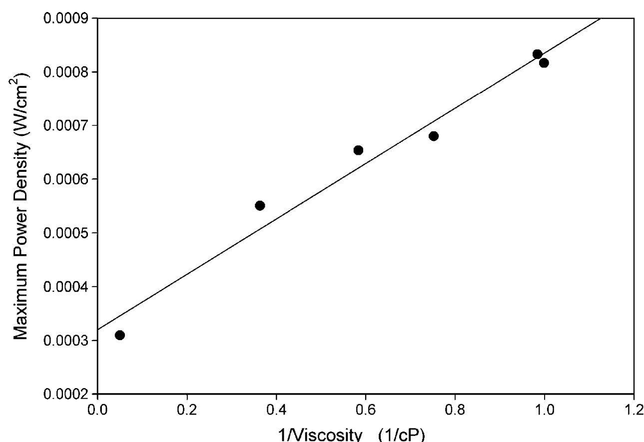

Fig. 4. Maximum power density of a glycerol biofuel cell as a function of 1/viscosity of the fuel solution.

where k_B is the Boltzmann's constant, T the temperature, and r is the radius of the molecule. Fig. 4 is a plot of the power density of the fuel cell as a function of $1/\eta$. The plot shows a linear correlation ($R^2 = 0.97$) between the maximum power density of the fuel cell and the inverse of the viscosity of the fuel solution.

Overall, this paper is the first evidence that glycerol can be employed as a fuel and oxidized in an enzymatic biofuel cell. This paper also shows that deeper oxidation of glycerol (86%) can be accomplished with PQQ-ADH and PQQ-AldDH, because the enzymes can react at multiple sites on the glycerol molecule. This allows for more complete oxidation than ethanol (33%), because the only reactions that occur in the ethanol system are PQQ-ADH-oxidizing ethanol to acetaldehyde and PQQ-AldDH-oxidizing acetaldehyde to acetate. Finally, this paper has shown the first evidence that high fuel concentrations can be used in a biofuel cell. Previous to this work, most biofuel cells had employed ethanol, methanol, or glucose as fuels. Glucose is a solid fuel that can only be dissolved in aqueous solution to 47.6 wt.%, so higher fuel concentrations, which would correspond to higher energy density fuel cells, are not possible. On the other hand, ethanol and methanol swell Nafion 112 and Nafion 117 polymer electrolyte membranes and the enzyme immobilization membranes, so concentration above 30–40% destroy the physical structure of the biofuel cell. Therefore, this study is encouraging, because being able to use high fuel concentrations (up to 100%) is necessary for high energy density to be realized in enzymatic biofuel cells and this is the first evidence that this may be a possibility for some fuels.

4. Conclusions

Over the last two decades, researchers have attempted to use more complex and higher energy density fuels for traditional precious metal-based PEM fuel cells, but these types of fuel cells have been plagued by low oxidation efficiency, many of which result in toxic partial oxidation byproducts, and electrode passivation due to strong adsorption of fuel and fuel byproducts. However, enzymatic systems do not have these limitations, because living organisms have evolved to completely metabolize fuels employing enzymatic cascades (multi-enzyme systems) and do not contain active sites (catalytic sites) that are capable of strongly adsorbing fuels and fuel byproducts. Therefore, we were able to immobilize two oxidoreductase enzymes (PQQ-dependent alcohol dehydrogenase and PQQ-dependent aldehyde dehydrogenase) at the surface of a carbon anode and undergo a multi-step oxidation process to oxidize glycerol to mesoxalic acid; thereby, utilizing 86% of the energy density of the glycerol. These glycerol bioanodes were incorporated into a glycerol/oxygen biofuel cell and resulted in power densities up to 1.21 mW cm^{-2} at room temperature. Due to the fact that glycerol does not swell the PEM (Nafion membrane), we were able to utilize glycerol in a glycerol/oxygen biofuel cell at fuel concentrations up to 98.9 wt.% glycerol.

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