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Kinetic and transport analysis of immobilized oxidoreductases that oxidize glycerol and its oxidation products

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ABSTRACT

Glycerol has drawn increasing attention as a possible fuel, because it has many desirable qualities and is abundant due to the fact that it is a byproduct of biodiesel production. Previous research has shown that non-natural enzyme cascades can be used to create a bioanode that can stepwise oxidize glycerol to carbon dioxide. Two of these enzymes are pyrroloquinoline quinone (PQQ) dependant alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AldDH) derived from Gluconobacter. The third enzyme, which is responsible for carbon bond cleavage, is oxalate oxidase (OxOx) derived from barley. Previous research has shown that all three enzymes have demonstrated the ability to undergo direct electron transfer to a carbon electrode which allows for a simple and efficient bioanode that completely oxidizes glycerol. In this study, each enzyme was individually immobilized within modified Nafion® on a glassy carbon rotating disc electrode (GC-RDE) and voltammetric analysis was performed employing different rotation rates in a solution containing each enzyme's respective substrate. This substrate was glycerol for alcohol dehydrogenase, glyceraldehyde for aldehyde dehydrogenase, and mesoxalic acid for oxalate oxidase. From the voltammograms, Levich plots were produced and the solution diffusion coefficient (D_{soln}), the membrane diffusion coefficient (D_{film}), k_{CAT}, K_M, and V_{MAX} were determined.

1. Introduction

Enzymatic biofuel cells which use enzymes as catalysts for electrode reactions have shown great potential as an alternative to traditional precious metal catalyzed fuel cells [1]. Since there are numerous oxidoreductase enzymes that have the ability to oxidize a wide variety of substrates, the limitations of fuel choices that are typically imposed by precious metal catalysts are more easily overcome [2–14]. For example, room temperature precious metal catalysts and their alloys are typically limited to methanol, methane, or hydrogen if complete oxidation is to occur [15–18]. Any fuel molecule that is more complex such as ethanol, propanol, or glycerol can cause catalyst passivation and is incompletely oxidized meaning that the oxidation products are typically the aldehyde or carboxylic acid of the parent molecule [19–21]. The fact that more complex and more energy dense fuels cannot be efficiently utilized with precious metals and their alloys limits the usefulness of such catalysts in applications where low temperature and high energy density are major issues [22–25].

In previous work, we have developed a three-enzyme cascade on an electrode that stepwise oxidizes glycerol to carbon dioxide [4,5]. The motivation for developing this three-enzyme system for a bioanode was because glycerol is an abundant fuel source due to it being the major byproduct of the large quantities of biodiesel that are produced, it has a high energy density, and it can be used at high concentrations, because it does not have the solvent properties of methanol and ethanol. This three-enzyme cascade is not the natural metabolic process for glycerol oxidation in living cells, which involves the glycolytic pathway and the Kreb's cycle, but instead is a simpler enzyme cascade that employs the ability of PQQ-dependent dehydrogenases and oxalate oxidase to oxidize non-natural substrates.

In order to better understand these enzyme-modified electrodes and the effect of non-natural substrates on the electrochemistry, rotating disk electrochemistry was performed with each immobilized enzyme in the modified Nafion® membranes that have been shown to have the highest activity with oxidoreductase enzymes [26]. These two polymers are tetrabutylammonium bromide modified Nafion® (TBAB) and triethylhexylammonium bromide modified Nafion® (TEHA). This allowed for the evaluation of mass transport and kinetics in these enzymatic bioanodes and the comparison to the literature information.

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Table 1
Comparison of the moles of loaded enzyme onto the electrode versus the moles of the loaded enzyme that are electrochemical accessible to the carbon electrode.

Enzyme	Polymer	Cast (moles of enzyme)	Accessible (moles of enzyme)	% Accessible enzyme
PQQ-ADH	TBAB	5.43×10^{-13}	$2.97 \pm 0.38 \times 10^{-13}$	54.69
PQQ-ADH	TEHA	5.43×10^{-13}	$2.18 \pm 0.14 \times 10^{-13}$	40.14
PQQ-AldDH	TBAB	3.33×10^{-12}	$4.11 \pm 0.22 \times 10^{-14}$	1.23
PQQ-AldDH	TEHA	3.33×10^{-12}	$3.07 \pm 1.01 \times 10^{-14}$	0.92
OxOx	TBAB	2.35×10^{-10}	$1.11 \pm 0.03 \times 10^{-13}$	0.05
OxOx	TEHA	2.35×10^{-10}	$1.28 \pm 0.12 \times 10^{-13}$	0.05

2. Experimental

2.1. Materials

Glycerol (Sigma), oxalate oxidase (0.71 U/mg from barley, Sigma), mesoxalic acid (Sigma), sodium phosphate monobasic (Sigma), sodium nitrate (Sigma), sodium hydroxide (Sigma), tetrabutylammonium bromide (Sigma), triethylhexylammonium bromide (Sigma), Nafion® 1100EW suspension (Aldrich), ethanol (Sigma), and glyceraldehyde (Sigma) were used as received. PQQ-ADH and AldDH were obtained from Gluconobacter sp.33 which was grown, extracted, and purified according to the protocol previously established [5,27]. Modified Nafion® was also prepared by a procedure that has been previously established [28].

2.2. Electrode fabrication

Enzyme-modified rotating disk electrodes were fabricated by mixing 1.0 mg of one particular enzyme with 100 μl of 18 MΩ water in a microcentrifuge tube. In a separate microcentrifuge tube, 100 μl of either TBAB or TEHA modified Nafion® was added, followed by the addition of 50 μl of the enzyme solution. The polymer/enzyme solution was mixed for 30 s with a vortex mixer on the highest setting. Then, 20 μl of the polymer enzyme solution was cast onto each glassy carbon rotating disk electrode (5 mm diameter, Pine Instruments) and spread evenly across the surface of the glassy carbon to ensure a complete coating. Once the solution was cast, the rotating disk electrodes were allowed to dry for 3 h at room temperature. Then, the electrodes were soaked overnight in pH 7.15 10 mM phosphate buffer with 100 mM sodium nitrate electrolyte to fully equilibrate the electrodes. The membranes on the rotating disk electrodes were measured with a profilometer to determine their thickness.

2.3. Cyclic voltammetry

After equilibration, cyclic voltammetry was performed on each electrode in the soaking buffer solution with a platinum mesh counter electrode and a saturated calomel reference electrode to determine the amount of enzyme that was electrochemically accessible. For PQQ-ADH and PQQ-AldDH modified electrodes, the scan rate was 0.5 V s^{-1} and the potential window was −0.8 V to +0.6 V. For OxOx modified electrodes, the scan rate was 0.5 V s^{-1} and the potential window was 0.0 V to +0.8 V. Electrochemical measurements were taken with a CH Instruments model 620A potentiostat interfaced to a PC. All experiments were performed in triplicate and the uncertainties reported correspond to one standard deviation.

2.4. Rotating disk electrochemistry

Rotating disc voltammetry was performed on each electrode in 100 μM substrate solution that contained 100 mM $NaNO_3$ electrolyte and pH 7.15 10 mM phosphate buffer. The substrates were selected based on the oxidation of glycerol and its products that have been determined previously by carbon-13 NMR intermediate and product analysis [4]. The substrate for PQQ-ADH was glycerol, the substrate for PQQ-AldDH was glyceraldehyde, and the substrate for OxOx was mesoxalic acid. The electrodes were allowed to soak in their respective substrate solutions for at least 1 h prior to being rotated to ensure that they were equilibrated with the substrate solution. Each electrode was tested with a platinum mesh counter and a saturated calomel reference electrode. The rotator was a Pine Instruments model AFM-SRX. Rotation rates were varied and cyclic voltammetry was performed at each rotation rate. All experiments were performed in at least triplicate and the uncertainties reported correspond to one standard deviation.

3. Results and discussion

Previous research on glycerol bioanodes for biofuel cells employed multi-enzyme cascades where multiple reactions were occurring. However, in order to be able to separate the effects of each enzyme on the system, in this study, we studied the oxidation of one susbstrate at an electrode containing one immobilized enzyme. The information determined in this study should provide information about which enzyme/substrate system is most limiting in the glycerol bioanode.

The amount of electrochemically accessible enzyme was determined from the cyclic voltammogram for each respective enzyme immobilized on the glassy carbon electrode in pH 7.15 phosphate buffer with no added substrate. Both PQQ-dependent enzymes undergo a 6e-process from the hemes and the PQQ when there is no substrate present [29]. Oxalate oxidase undergoes a 2e-process when substrate is not present [30]. The amount of enzyme cast and the amount of enzyme electrochemically accessible for each is shown in Table 1. As can be seen in Table 1, PQQ-ADH has the highest percentage of immobilized protein that is electrochemically accessible and OxOx has the lowest percentage of immobilized protein that is electrochemical accessible. This will be important in further optimizations of glycerol bioanodes to ensure optimal metabolic flux of glycerol oxidation and minimize

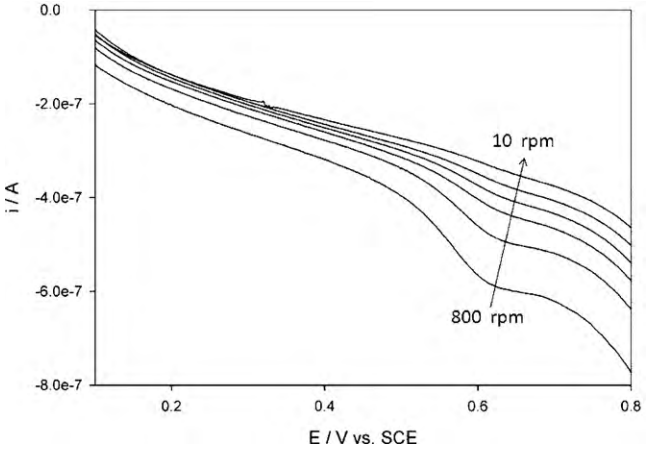

Fig. 1. Representative rotating disk voltammograms for oxalate oxidase enzyme immobilized in TEHA modified Nafion® at a glassy carbon rotating disk electrode in 0.1 mM mesoxalic acid for a variety of rotation rates.

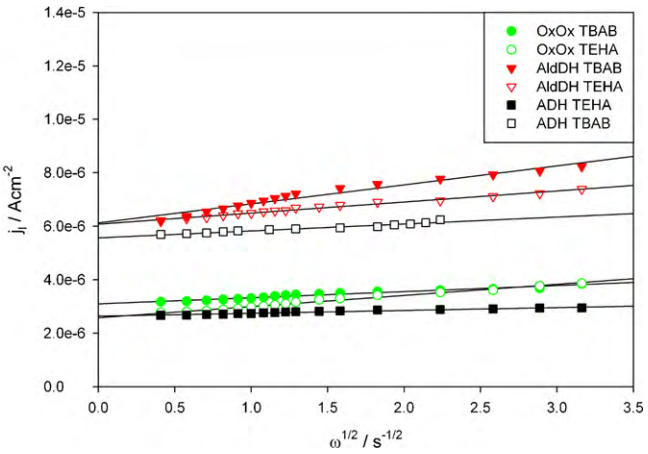

Fig. 2. Levich plots of limiting current of substrate at each enzyme immobilized in each polymer combination.

Table 2
Levich slopes and intercepts for each enzyme/polymer combination in 0.1 mM substrate.

Enzyme	Polymer	Levich slope ($A\,cm^{-2}\,s^{1/2}$)	Levich intercept ($A\,cm^{-2}$)
PQQ-ADH	TBAB	$2.59 \pm 0.16 \times 10^{-7}$	$5.57 \pm 0.34 \times 10^{-6}$
PQQ-ADH	TEHA	$1.51 \pm 0.10 \times 10^{-7}$	$2.60 \pm 0.17 \times 10^{-6}$
PQQ-AldDH	TBAB	$4.89 \pm 4.58 \times 10^{-8}$	$7.52 \pm 6.99 \times 10^{-7}$
PQQ-AldDH	TEHA	$3.93 \pm 0.20 \times 10^{-7}$	$6.04 \pm 0.31 \times 10^{-6}$
OxOx	TBAB	$4.81 \pm 0.67 \times 10^{-8}$	$6.05 \pm 0.85 \times 10^{-7}$
OxOx	TEHA	$8.21 \pm 1.07 \times 10^{-8}$	$5.071 \pm 0.66 \times 10^{-7}$

the enzymatic bottlenecks. The differences in electrochemically accessible enzyme for PQQ-ADH, PQQ-AldDH, and OxOx are due to a number of factors including chemical effects (charge and hydrophobicity) that result in orientation differences on carbon electrode surfaces as well as structural differences between the enzymes which affect their ability to undergo direct electron transfer with the electrode. It is also important to note that although TBAB modified Nafion® membranes provide higher percentage of available enzyme that is electrochemical accessible for both of the dehydrogenase enzymes, TEHA modified Nafion® membranes provided higher percentage of electrochemical accessible enzyme for oxalate oxidase.

Limiting currents were determined for each of the rotating disc experiments at each rotation rate. *Note*: rotation rates were kept low to ensure stability of the modification layer, but this increases the uncertainty in these measurements. Representative voltammograms are shown in Fig. 1 for several rotation rates. Both Koutecky–Levich and Levich plots were graphed for each enzyme and polymer combination. Koutecky–Levich plots were not linear. However, the Levich plots were linear with non-zero intercepts. The Levich plot which is the limiting current versus the square root of the rotation rate (w) is shown in Fig. 2. From this data, the slope and the intercept for the Levich plot was determined for each enzyme/polymer combination, which is shown in Table 2. To calculate the solution diffusion coefficient (D_{soln}), the slope of the Levich plot and the Levich equation was used:

$$\text{slope} = -\frac{0.62nFAD_{soln}^{2/3}\omega^{1/2}C^0}{\nu^{1/6}}$$

where n is the number of electron transfer, F is Faraday's constant, A is the area of the electrode, ν is the kinematic viscosity, and C^0 is the concentration of substrate in solution. The diffusion coefficient in the film (D_{film}) was calculated from the intercept of the Levich plot with:

$$\text{intercept} = nFA\left(\frac{D_{film}\kappa c_A^0}{\varphi}\right)$$

where k is the partition coefficient, which is assumed to be 1, and the film thickness (φ) was determined with profilometry to be 10.0 ± 0.2 μm. The diffusion coefficients in the film and in solution are shown in Table 3. Although diffusion coefficients in aqueous solutions are not known for glyceraldehyde and mesoxalic acid, they are known for glycerol (1.1×10^{-5} $cm^2\,s^{-1}$) [31]. The diffusion of the substrate molecules in solution determined from the RDE experiments is in general agreement with the literature values [31] for similar compounds at infinite dilution which is a good indication that the experiments were successful and the values determined for other constants are accurate. The diffusion of the substrates through the modified Nafion® membranes was approximately two orders of magnitude slower than in solution. Similar phenomena is observed with the diffusion of ethanol or water in solution verses their diffusion through a Nafion® membrane [32].

Kinetic evaluation of the bioelectrodes was also performed. K_M, k_{CAT}, and V_{MAX} were determined by fitting a line to the plot of current versus rotation rate and then extrapolating to the value where it approached its maximum [33,34]. This maximum current is defined as the catalytic current (i_{CAT}) which can be directly converted into k_{CAT} and V_{MAX}, if the amount of electrochemically accessible enzyme is known and the mass of the enzyme. Also, K_M can be calculated from this plot by determining the current at ½V_{MAX} and then solving the Levich equation for the effective concentration at that current, which is K_M. The values determined from the RDE experiments for the enzyme kinetics are shown in Table 4.

Comparing the electrochemical K_M, k_{CAT}, and V_{MAX} to the literature values for free enzyme in solution is more difficult, since the literature values are for the natural substrate and, in this system, we were studying non-natural substrates for each of the enzymes. PQQ-dependent enzymes are known for their broad substrate lack of specificity, so it is not surprising that the V_{MAX} values

Table 4
Kinetic values for each enzyme in each polymer determined from RDE experiments.

Enzyme	Polymer	V_{MAX} ($\mu M\,min^{-1}\,mg^{-1}$)	k_{CAT} (s^{-1})	K_M (mM)
PQQ-ADH	TBAB	44.8 ± 0.6	105 ± 1	0.025 ± 0.002
PQQ-ADH	TEHA	30.1 ± 0.4	70 ± 1	0.011 ± 0.001
PQQ-AldDH	TBAB	438 ± 10	1023 ± 2	0.068 ± 0.063
PQQ-AldDH	TEHA	558 ± 30	1302 ± 68	0.028 ± 0.001
OxOx	TBAB	15.8 ± 0.2	34 ± 1	0.010 ± 0.001
OxOx	TEHA	14.5 ± 0.5	31 ± 1	0.003 ± 0.001

Table 3
Diffusion coefficients determined for solution (D_{soln}) and membrane (D_{film}) for each substrate/polymer/enzyme combination.

Enzyme/substrate	Polymer	D_{film} ($cm^2\,s^{-1}$)	D_{soln} ($cm^2\,s^{-1}$)
PQQ-ADH/glycerol	TBAB	$2.9 \pm 0.2 \times 10^{-7}$	$2.3 \pm 0.2 \times 10^{-5}$
PQQ-ADH/glycerol	TEHA	$1.4 \pm 0.1 \times 10^{-7}$	$1.0 \pm 0.1 \times 10^{-5}$
PQQ-AldDH/glyceraldehyde	TBAB	$3.9 \pm 3.6 \times 10^{-8}$	$1.9 \pm 2.6 \times 10^{-6}$
PQQ-AldDH/glyceraldehyde	TEHA	$3.1 \pm 0.2 \times 10^{-7}$	$4.2 \pm 0.3 \times 10^{-5}$
OxOx/mesoxalic acid	TBAB	$3.1 \pm 0.4 \times 10^{-8}$	$1.8 \pm 0.4 \times 10^{-6}$
OxOx/mesoxalic acid	TEHA	$2.6 \pm 0.3 \times 10^{-8}$	$4.0 \pm 0.8 \times 10^{-6}$

determined for glycerol and glyceraldehyde are consistent with the literature values for ethanol at PQQ-ADH (52 μmole/min/mg) [35] and acetaldehyde at PQQ-AldDH (430 μmole/min/mg) [36]. Oxalate oxidase shows a V_{MAX} for oxalate oxidase in TBAB and TEHA modified Nafion® membranes of 15.8 μmole/min/mg and 14.2 μmole/min/mg, respectively, which is lower than the V_{MAX} for oxalate at oxalate oxidase in solution (34 μmole/min/mg) [30]. This shows that the kinetics of oxalate oxidase are different for non-natural substrates. K_M values were determined for each of the enzymes in both polymers. The literature value for K_M for the natural substrate (ethanol) and PQQ-ADH enzyme is 34 mM [35]. The K_M value for acetaldehyde substrate and PQQ-AldDH enzyme has been shown to be 3.3 mM in the literature [36]. The literature value for K_M for the natural substrate oxalic acid and oxalate oxidase enzyme is 0.78 mM [30]. The K_M values determined for all three immobilized enzyme with their non-natural substrates were lower than the values reported in the literature for free enzyme in solution indicating that the K_M for the non-natural substrate is different for these enzymes immobilized on the electrodes. This is expected due to the fact that the substrate binding for a non-natural substrate is expected to be different than the natural substrate.

4. Conclusions

Overall, the data determined from these electrochemical experiments will be useful for further development of the glycerol biofuel cell anodes and yield insight into other immobilized biocatalytic systems. The PQQ-dependant enzymes when in direct electrical communication with the carbon electrode exhibit activities that are similar to enzyme in solution with the natural substrate indicating the enzyme kinetics are mostly unchanged. However, the enzyme kinetics of oxalate oxidase are different when in direct electrical communication with a carbon electrode and employing a non-natural substrate (mesoxalic acid) than for free enzyme in solution with the natural substrate oxalic acid. The main limiting factor for this system of immobilization is transport through the modified Nafion® membrane which is two orders of magnitude lower than that in solution. In order to enhance current density, future work will focus on improving mass transport within the immobilization layer while also engineering the enzymes to handle non-natural substrates more efficiently.

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