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Enzyme Cascade for Catalyzing Sucrose Oxidation in a Biofuel Cell

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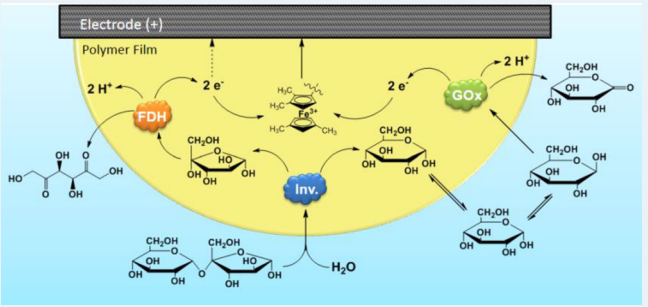
Supporting Information

ABSTRACT: Biofuel cells provide a safe and renewable means of powering small electronic devices. In this work, we demonstrate a bioanode that is capable of extracting four electrons from a single molecule of sucrose by way of a three-enzyme cascade. Invertase, fructose dehydrogenase and glucose oxidase are immobilized in a ferrocene-modified linear poly(ethylenimine) (LPEI) hydrogel onto the surface of a carbon electrode. Fuel sources are generated in the polymer film by (1) hydrolyzing sucrose into fructose and glucose and then (2) electroenzymatically oxidizing fructose and glucose to produce a current response. A previously unreported synergistic effect is observed between glucose oxidase and fructose dehydrogenase that results in a current response that is considerably higher than expected. The newly described enzyme cascade generated 302 ± 57 μA/cm^2 at 25 °C and 602 ± 62 μA/cm^2 at 37 °C and when poised against an air breathing platinum cathode in a biofuel cell, the multienzyme-containing film generated 42 ± 15 μW/cm^2 at 172 mV with a maximum current density of 344 ± 25 μA/cm^2 in 100 mmol/L sucrose at 25 °C. This is the first example of an enzymatic biofuel cell that utilizes both fructose and glucose as oxidation fuel sources.



KEYWORDS: *glucose oxidase, fructose dehydrogenase, invertase, ferrocene, poly(ethylenimine), redox polymer*

INTRODUCTION

Since the late 1980s, enzymatic biofuel cells have been studied as potential power sources for small electronic devices.[1−4] Instead of traditional metal catalysts, enzymatic biofuel cells utilize enzymes as biocatalysts at one or both electrodes. Enzymes provide several advantages over their transition metal counterparts: they have a very high per-molecule activity, the ability to operate near neutral pH, and high substrate specificity. High specificity allows for mixing of the anode and cathode substrates without the possibility of fuel crossover and therefore eliminates the need for an ion-exchange separator membrane (i.e., Nafion), which increases cell resistance as well as cost. However, high substrate specificity also limits the degree of oxidation of possible fuel sources by a single enzyme and the ability to use fuel mixtures without the incorporation of multiple enzymes. The choices for fuels for enzymatic biofuel cells are effectively limited to substrates for which an isolatable redox enzyme exists. The use of only one anodic enzyme also limits the extent of oxidation of the fuel.

Much of the current research on enzymatic biofuel cells utilizes a multicopper oxidase enzyme or platinum metal at the cathode to reduce molecular oxygen to water[5−8] and an oxidase or dehydrogenase enzyme at the anode to oxidize a small sugar[8,9] or short-chain alcohol.[8,10,11] Most redox enzymes used at the anode catalyze only a single two-electron oxidation per molecule of substrate and leave the remainder of the substrate unreacted, which limits the efficiency and energy density of the biofuel cell.[12] Recent research has attempted to catalyze deeper oxidation of substrates through the use of immobilized enzyme cascades.[13−16] It was found that multiple enzymes can be immobilized at a single electrode in which the product of one enzymatic reaction can be used as a substrate for a subsequent enzymatic reaction.[10,17,18] Enzyme cascade-based bioanodes allow for deeper substrate oxidation in which multiple pairs of electrons can be extracted from one molecule of substrate, thus a larger current can be generated per molecule.

Several studies of deep substrate oxidation for enzymatic biofuel cells have examined methanol or glycerol as substrates because both can be completely oxidized using only three enzymes.[19,20] Methanol is oxidized to CO_2 using three nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenases to extract six electrons, while glycerol is oxidized to

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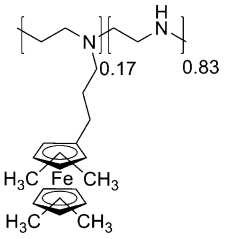
CO_2 using two pyrroloquinoline quinone (PQQ)-dependent dehydrogenases and oxalate oxidase to extract fourteen electrons per molecule of substrate.[10,18,21] Only a few studies have focused on larger sugar molecules as the primary substrate for an enzyme cascade-based oxidation.[16,22,23] Despite the prevalence of glucose in single-enzyme biofuel cell research, little work has been done using it as a parent substrate for deep fuel oxidation. This is because the primary oxidative pathway for glucose, glycolysis, contains only one oxidoreductase from which electrons can be transferred. Multiple alternative enzymatic pathways have been used to achieve four and six electron oxidation of glucose, however these methods are hindered by low maximum current densities (30−80 μA/cm^2)[16,22] and require the use of enzymes that are either genetically engineered or are otherwise not commercially available.

An alternative to more complete oxidation of glucose is the use of upstream metabolic targets such as sucrose. Sucrose is a common sugar (table sugar) and is a disaccharide. Amperometric sucrose biosensors have previously been reported that operate by the indirect detection of sucrose via the oxidation of fructose or glucose.[24,25] Invertase is used to hydrolyze sucrose into glucose and fructose, while either fructose dehydrogenase (FDH) or glucose oxidase (GOx) is used to oxidize fructose or glucose respectively. This method has been successfully used in the amperometric detection of sucrose; however, the low current densities generated have limited its potential use in biofuel cells.

In this work, we demonstrate a high current density bioanode which uses invertase, FDH, and GOx that have been coimmobilized in a polymer film to extract four electrons from one molecule of sucrose. Cross-linked films of a tetramethylferrocene-modified poly(ethylenimine) ($FcMe_4$-C_3-LPEI), shown in Scheme 1, were used to both immobilize the enzymes and enhance the electrochemical communication between the enzymes and the electrode surface.[26] The combination of the three enzymes resulted in improvement in the bioelectrode performance compared to bienzyme (hydrolyase/oxidoreductase) electrodes. To our knowledge, this is the first use of a bioanode that utilizes glucose and fructose simultaneously as substrates. The effects of pH and temperature on the current density, as well as the stability of the bioanode, are examined.

Scheme 1. Chemical Structure of 3-(Tetramethylferrocenyl)propyl-Modified LPEI (Me_4Fc-C_3-LPEI)



MATERIALS AND METHODS

Materials. Sucrose and D-glucose (anhydrous) were purchased from Macron Chemicals. D-Fructose was obtained from Sigma-Aldrich. Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4, type X-S, 157 U/mg of solid, 75% protein) and invertase glycoprotein from *Saccharomyces cerevisiae* (EC 3.2.1.26, 332.8 U/mg of solid) were purchased from Sigma-Aldrich. Fructose dehydrogenase from *Gluconobacter sp.* (EC 1.1.99.11, grade III, 169 U/mg of solid) was purchased from Toyobo Enzymes. Ethylene glycol diglycidyl ether (EGDGE) was purchased from Polysciences Inc., Washington, PA. All chemicals used in the synthesis of the redox polymer were purchased from Sigma-Aldrich. All chemicals were used as received unless otherwise noted. Tetramethylferrocenes and octyl-modified linear poly(ethyleneimine) (C_8-LPEI) were synthesized as previously reported.[26,27] Stock solutions of glucose and fructose were allowed to mutarotate for 24 h and stored at 4 °C. Toray paper electrodes were purchased from Fuel Cell Earth (190 μm thick, nonwet proof, Prod. No. TGP-H-060).

Preparation of 3-(Tetramethylferrocenyl)propyl-Modified LPEI ($FcMe_4$-C_3-LPEI). *3-(Tetramethylferrocene)-propanoyl Bromides (**1**).* 3-Bromopropanoyl chloride (0.85 g, 5.0 mmol) was added to a suspension of aluminum chloride (0.66 g, 5.0 mmol) in CH_2Cl_2 (20 mL) at 0 °C and stirred for 1 h. The mixture was added slowly to a stirring solution of tetramethylferrocenes (1.00 g, 4.0 mmol) in CH_2Cl_2 (50 mL) at 0 °C. The resulting purple solution was stirred for 18 h at room temperature. This solution was diluted with CH_2Cl_2 (20 mL) and poured over an equivalent volume of ice. The product was extracted with CH_2Cl_2 and the organic phase was washed with a saturated aqueous solution of $NaHCO_3$ and brine. The organic portion was filtered over $MgSO_4$ and concentrated under reduced pressure. 0.93 g of crude product mixture was obtained. No further analysis was performed prior to the next step in the reaction sequence.

*3-(Bromopropyl)tetramethylferrocene (**2**).* Borane-*tert*-butylamine complex (0.65 g, 7.4 mmol) in CH_2Cl_2 (10 mL) was added to a suspension of aluminum chloride (0.49 g, 3.7 mmol) in CH_2Cl_2 (20 mL) at 0 °C. After the mixture was stirred for 1 h, the 3-(tetramethylferrocenyl)propanoyl bromide product from the previous reaction (0.93 g) in CH_2Cl_2 (10 mL) was added slowly over 15 min. The resulting solution was stirred for 18 h at room temperature under a slow stream of nitrogen gas. The reaction mixture was hydrolyzed with water and extracted with CH_2Cl_2. The crude product was purified by flash chromatography (silica gel; CH_2Cl_2) to afford a yield of 48% (0.43 g, 1.2 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.75−1.95 (overlapping singlets, 12H, 4[$FcCH_3$]), 1.90 (m, 2H, $-CH_2-$), 2.4 (m, 2H, Fc-CH_2-), 3.4 (td, 2H, $-CH_2-$Br), 3.50−3.70 (m, 5H, Fc-H).

*3-(Tetramethylferrocenyl)propyl-Modified LPEI (**3**).* Tetramethylferrocene-modified linear poly(ethylenimine) (LPEI) was prepared according to a previously reported procedure.[26] LPEI (0.14 g) was dissolved in a mixture of acetonitrile and methanol (10:1, 10 mL) and heated to reflux solvent. 3-(Bromopropyl)tetramethylferrocenes (0.20 g, 0.6 mmol) in methanol (1 mL) were added to the refluxing LPEI solution. The reaction mixture was stirred for 24 h at reflux temperature. The solvent was removed under reduced pressure and the product was extracted using diethyl ether to remove any excess starting material. The final polymer was determined to be ∼17% substituted by ^{1}H NMR analysis, which was consistent with the polymer previously described.[26]

Electrode Fabrication. Electrode film solutions were prepared by combining aqueous solutions of $FcMe_4$-C_3-LPEI (60 μL, 12 mg/mL), enzyme mixture (25.74 μL total), and EGDGE (3.22 μL, 2 μL of EGDGE per 45 μL of H_2O).

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Enzyme mixture solutions consisted of invertase (8.58 μL, 20 mg/mL), fructose dehydrogenase (8.58 μL, 13 mg/mL), and glucose oxidase (8.58 μL, 13 mg/mL) in 18 MΩcm deionized H_2O. Toray paper electrodes were cut into L-shapes. The connecting ends of the electrodes were coated in paraffin wax (so that the exposed geometric electrode area was 1 cm^2) to prevent wicking of the electrolyte solution to the potentiostat lead. The electrode film solutions were mixed together and vortexed for 1 min until the solution was homogeneous, containing 1.25 mg/mL GOx, 1.25 mg/mL FDH, and 1.9 mg/mL invertase; then 25 μL of this mixture was drop-coated onto the Toray paper electrodes and evenly spread across the exposed electrode area using a plastic pipet tip. The electrodes were allowed to cure open to the atmosphere overnight at 25 °C. The cured electrode films contained 15.5 wt % GOx, 15.5 wt % FDH, and 24 wt % invertase with respect to the polymer weight.

For films that contained only one (or two) enzymes, the same procedure was used except the omitted component of the enzyme mixture was replaced by 8.58 μL (or 17.16 μL) of 18 MΩcm deionized H_2O. This was done to ensure a constant volume and relative concentration of the electrode film solution. Control experiments were performed by substituting for the $FcMe_4$-C_3-LPEI redox polymer with the nonredox active C_8-LPEI.

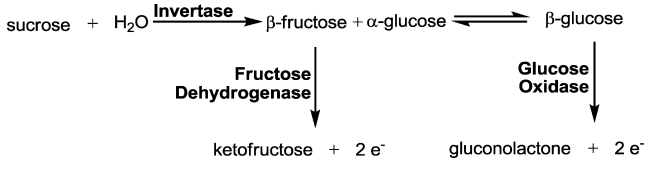
Voltammetric and Amperometric Characterization of FDH, GOx and Inv/FDH/GOx Electrodes. Electrodes were tested using a conventional three-electrode setup, and the potential was scanned from −0.2 to 0.5 V versus a saturated calomel electrode (SCE) using a platinum mesh counter electrode at 1 mV/s. CV experiments were performed using 3 mm glassy carbon electrodes as the working electrode, all other experiments were performed on 1 cm × 1 cm Toray electrodes. Experiments were performed in 50 mmol/L citrate buffer pH 5.5 (unless otherwise stated) using a VSP Multichannel Analyzer (Biologic), a CH650 (CH Instruments) potentiostat or a DY2100 (Digi Ivy) potentiostat. Each experiment was performed in triplicate using separately constructed electrodes (n = 3). Electrodes were analyzed by allowing them to soak in a 50 mmol/L citrate buffer solution, pH = 5.5, for 5 min before performing cyclic voltammetry experiments to determine the oxidation potential for each film as well as to allow them to equilibrate in solution prior to performing amperometry experiments. Amperometric studies were performed by allowing the films to reach a steady state at a potential that is +0.05 V (vs SCE) above the peak oxidation potential (E_{pa}) at 25 °C. The solutions were continuously stirred at 400 rpm. The charging current was allowed to dissipate for 400 s, and sequential injections from 1 M substrate solution in 50 mM citrate buffer pH 5.5 were made as current was recorded as a function of time. The substrate concentration in the bulk solution was increased by 0.5 mmol/L (two times), 1 mmol/L (four times), 5 mmol/L (one time), 10 mmol/L (one time), 30 mmol/L (one time), 50 mmol/L (one time), and 100 mmol/L (one time, FDH and GOx experiments only) for determination of Michaelis−Menten kinetics. Inv/GOx film kinetics were determined by testing the current response to each concentration of sucrose independently to allow steady state to be reached after mutarotation to minimize the effects of enzyme degradation; sample results of these experiments are shown in Supporting Information Figure S1. The substrate concentration was brought to 100 mmol/L for the stability, temperature, pH and efficiency studies. For the stability studies, electrodes were tested once per day for 2 h per test, and stored in buffer at 4 °C when not being used.

Fuel Cell Bioanodes Characterization. Bioanodes were constructed as described above using 25 μL castings of $FcMe_4$-C_3LPEI/enzymes on 1 cm × 1 cm Toray electrodes. The cathode consisted of a gas permeable ELAT electrode with 20% Pt on Vulcan XC-72 (E-Tek) pressed against the Nafion NRE-212 (Sigma) polymer electrolyte membrane as reported previously.[17] Sucrose was injected during the open circuit potential (OCP) measurements into the bulk electrolyte at t = 600 s to bring the sucrose concentration of the solution to 100 mmol/L. The OCV was allowed to reach steady state for two hours to build-up the concentration of both glucose and fructose. Slow scan polarization (1 mV/s from the measured open circuit potential to 1 mV) was used to obtain polarization and power curves by monitoring current as a function of potential. Controls were performed by omitting invertase in the bioanode or by substituting the $FcMe_4$-C_3-LPEI redox polymer with the C_8−LPEI nonredox polymer. It should be noted at the outset that amperometric results are reported as current densities; this value is based on the planar geometric electrode area rather than the true microscopic surface area of the Toray paper.

RESULTS

Our first objective was to determine the function of a sucrose enzyme cascade contained in a single polymer film as a bioanode. Previously, it was shown that ferrocene-modified poly(ethylenimine) films can be used to effectively immobilize and "wire" GOx onto the surface of an electrode.[26,28−30] Immobilization of GOx allows the oxidation of glucose to occur near the electrode surface, while the ferrocene moiety acts as a redox mediator to efficiently shuttle electrons from the flavin adenine dinucleotide (FAD) cofactor of GOx to the electrode surface. A similar approach is used in this work to immobilize three enzymes in a single polymer film; $FcMe_4$-C_3-LPEI was cross-linked with EGDGE in the presence of invertase, GOx, and FDH and coated onto a 1 cm × 1 cm Toray paper electrode. As illustrated in Scheme 2, invertase is used to hydrolyze sucrose to form glucose and fructose, which are subsequently oxidized by GOx and FDH, respectively.

Scheme 2. Simplified Outline of the Enzymatic Pathway Used to Extract Electrons from Sucrose by Hydrolyzing It to Fructose and Glucose and Then Electroenzymatically Oxidizing Fructose and Glucose to Ketofructose and Gluconolactone, Respectively



Sucrose Cascade Characterization. Cyclic voltammetry was used to characterize the electrocatalysis of fructose and glucose oxidation as well as the hydrolysis of sucrose by FDH, GOx, and Inv/FDH/GOx-based bioelectrodes, respectively, as shown in Figure 1. The representative CV for each bioelectrode has the same shape in the absence of substrate which is due solely to the ferrocene redox moiety, however the peak current of the FDH electrode is significantly lower than that of the

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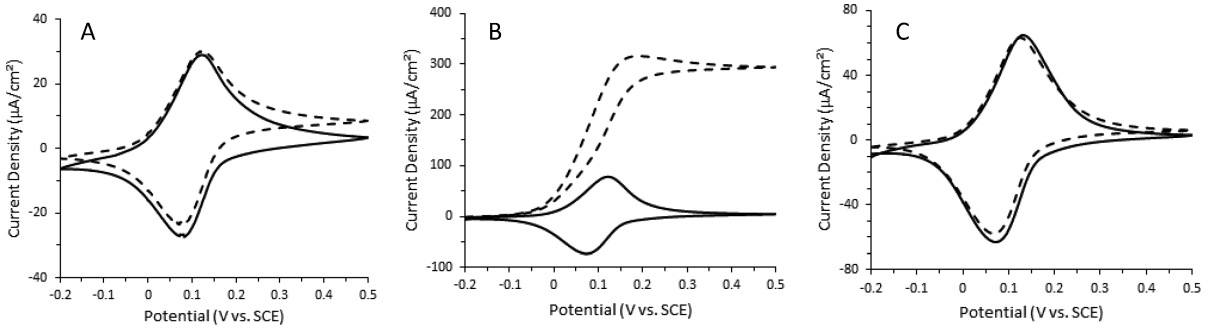

Figure 1. Representative cyclic voltammograms of (A) FDH-modified electrodes in the absence (solid line) and presence (dashed line) of 100 mmol/L fructose, (B) GOx-modified electrodes in the absence (solid line) and presence (dashed line) of 100 mmol/L glucose, and (C) Inv/FDH/GOx-modified electrodes in the absence (solid line) and presence (dashed line) of 100 mmol/L sucrose. All CVs were performed at 25 °C in 50 mmol/L citrate, pH 5.5, with a scan rate of 1 mV s^{-1}.

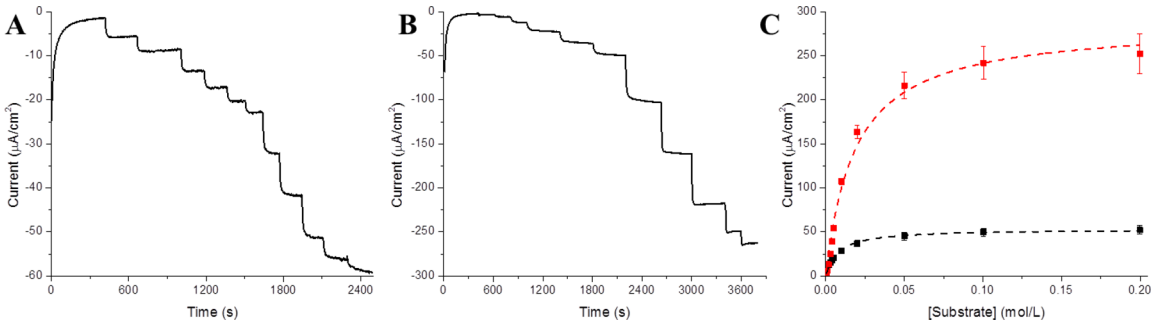

Figure 2. (A) Amperometric response for FDH-modified electrodes in increasing concentrations of fructose (0−200 mmol/L). (B) Amperometric response for GOx-modified electrodes in increasing concentrations of glucose (0−200 mmol/L). (C) Calibration curve for FDH-modified electrodes (black) and GOx-modified electrodes (red). Performed in 50 mmol/L citrate buffer, pH 5.5, at 25 °C. Error bars represent the standard deviation.

GOx or Inv/FDH/GOx electrodes. A visible complex forms between $FcMe_4$-C_3-LPEI and GOx when they are mixed together in solution. This complexation could cause a restriction of the polymer film swelling, thus resulting in a smaller hydrogel volume and a higher effective redox site concentration. In the absence of GOx, the FDH electrode would have a lower peak current due to a decrease in the effective redox site concentration.

Both the FDH and the GOx electrodes show an electrochemical response to the addition of substrate; however, there is no difference between the CVs of Inv/FDH/GOx films in the presence and absence of sucrose; this is likely because of the low activity of invertase which results in a large amount of time required to produce the two substrates essential for electrooxidation at the electrodes. Additionally, previous studies have shown that invertase can be reversibly inhibited by glucose and fructose via competitive product inhibition; which could be leading to further decrease its activity.[31]

Since observed evidence of enzymatic activity using cyclic voltammetry at the Inv/FDH/GOx electrodes was limited, amperometric measurements were taken to determine kinetics parameters of both oxidoreductases and invertase. Representative amperometric traces of fructose or glucose injections for FDH or GOx-modified electrodes held at 50 mV above the potential of the peak anodic current (E_{pa}) are shown in Figure 2A and 2B, and the corresponding calibration plots are depicted in Figure 2C. Fast increases in oxidation current were obtained, and both enzymes displayed a Michaelis−Menten profile. For FDH- and GOx-based electrodes, apparent K_m values were determined to be 7.9 ± 0.5 mmol/L and 18.7 ± 2.3 mmol/L and the currents at enzyme saturation (J_{max}) were 53.2 ± 0.9 $\mu A/cm^2$ and 286.5 ± 11.0 $\mu A/cm^2$, respectively. Since invertase does not involve an electrochemical process during the hydrolysis of sucrose, bienzyme electrodes (Inv/FDH or Inv/GOx) were used to indirectly characterize the kinetic behavior of invertase. Figure 3 presents the amperometric response of Inv/FDH and Inv/GOx-based electrodes to various concentrations of sucrose when held at 50 mV over E_{pa} of the redox polymer. Unlike for the single oxidoreductase-modified electrodes, the response for Inv/FDH- and Inv/GOx-based electrodes was much slower following the addition of sucrose into the bulk

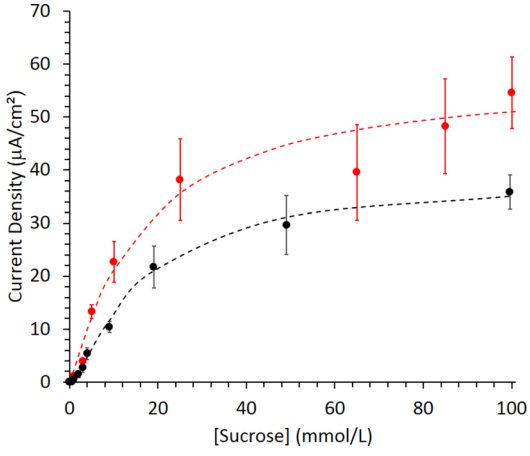

Figure 3. Calibration curves for Inv/FDH-modified electrodes (black) and Inv/GOx-modified electrodes (red); the dotted lines represent the fitted Hill function. Performed in 50 mmol/L citrate buffer, pH 5.5, at 25 °C. Error bars represent the standard deviation.

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solution. The calibration curves shown in Figure 3 did not display a typical Michaelis–Menten response, but rather a sigmoidal response. Deviation of Inv/FDH and Inv/GOx electrodes from Michaelis–Menten behavior indicates that the presence of the invertase in the polymer matrix alters the kinetics of the overall electrode reaction.

The sigmoidal response of both bienzyme electrodes can be mathematically interpreted using the Hill equation.[32] This equation takes into account a possible cooperativity or allosteric interactions in the enzymatic activity with the substrate concentration and is defined as

$$J = J_{\text{initial}} + \Delta J \frac{x^n}{K_m + x^n}$$

where J_{initial} is the initial current density, ΔJ is the current density variation, x is the sucrose concentration, K_m is the apparent constant, and n is the Hill coefficient. In the case of positive cooperativity, the enzyme activity is enhanced as the substrate concentration increases and its representation is a sigmoidal curve.[32] For negative cooperativity, its activity decreases as the substrate concentration increases. It can be claimed that a positive cooperativity occurs when invertase is an active component of electrode films based on calculated Hill coefficients of $n = 1.33 \pm 0.21$ for Inv/FDH films and $n = 1.12 \pm 0.61$ for Inv/GOx films; however, it remains unclear if this effect comes from the invertase only or from an allosteric behavior when the two enzymes are present. For the Hill coefficient to be interpreted in terms of cooperativity, an enzyme must contain multiple associated binding sites. Invertase has been shown previously to form functioning oligomers in solution,[33] therefore it is reasonable to consider that these oligomers can be present within the polymer matrix. Alternatively, the Hill coefficient can be interpreted in terms of a concerted transition model (or CT model) in which the conformation of the polymer matrix is being affected by reactions of either of the two redox enzymes.[34] This conformational change would then be favorable for invertase so that the catalytic hydrolysis of sucrose would be slow initially and then enhanced as the cascade reaches a minimum activity threshold. These results are congruent with previous findings which indicate that apparent K_m for invertase changes with the concentration of substrate; a concentration threshold must be reached before efficient hydrolysis can take place.[31] UV–vis assays performed on invertase immobilized in nonredox polymer films, C_8-LPEI, show that there is a slightly sigmoidal response even in the absence of other enzymes (Supporting Information Figure S2 and S3). This result indicates that the cause of the unique kinetics is due to an interfacial or conformational interaction between invertase and the PEI matrix and that the addition of either FDH or GOx enhances this interaction. However, the exact cause of this result is not fully understood, and experiments are ongoing to determine the exact nature of the cooperativity/allostery of the immobilized cascade system.

Kinetic parameters were analyzed for both bienzyme systems; apparent K_m values were determined to be 18.1 ± 4.2 and 16.3 ± 1.1 mmol/L, the currents at enzyme saturation (J_{max}) were 40.2 ± 4.3 μA/cm^2 and 57.7 ± 2.5 μA/cm^2 and the relative Hill coefficients (n) were 1.33 ± 0.21 and 1.12 ± 0.61 for Inv/FDH and Inv/GOx electrodes, respectively. Values of J_{max} for Inv/FDH-based electrodes were similar to the current density for the single FDH-based electrodes, and any variation in the J_{max} can be explained by the lower activity of invertase. However, J_{max} for Inv/GOx electrodes was much lower than J_{max} for the GOx electrodes (87% loss). This decrease may be the result of an apparent partial inhibition of GOx by fructose accumulation after sucrose hydrolysis; however, this is highly speculative and further studies are ongoing to investigate this result.

The chronoamperometric response of Inv/FDH/GOx films was compared to films containing invertase and only one redox enzyme (Inv/FDH or Inv/GOx), when 100 mmol/L sucrose was added in solution (injection at t = 400 s). The chronoamperometric results are shown in Figure 4. It is known that cross-linked $FcMe_4$-C_3-LPEI films form a hydrogel through which counterions, substrates, and products can easily diffuse.[26] Previous studies have shown that high substrate diffusion through enzymatic electrode films constructed using $FcMe_4$-C_3-LPEI allows for a single rapid amperometric response to substrate. However, the Inv/FDH/GOx films constructed for this study displayed a current response that occurred in two distinct events: an initial increase of ~50 μA/cm^2 from the sucrose injection over 1000 s, followed by a much larger subsequent increase that was observed over 5000 s. In the case of the Inv/FDH-based electrodes, the current increased about 50 μA/cm^2 after sucrose injection and slowly reached a steady state current over 1500 s. For the Inv/GOx-based electrodes, the current increased steadily over 2000 s and stabilized after 2 h incubation. These results indicate that the initial current response in Inv/FDH/GOx electrodes is a result of fructose oxidation, and the delayed current response is caused by the oxidation of glucose. Invertase catalyzes the hydrolysis of sucrose into β-D-fructose and α-D-glucose; and although FDH has a relatively high activity for both forms of fructose, GOx activity for α-glucose is only 0.64% of that for β-D-glucose.[35] Therefore, the delayed increase in current caused by glucose oxidation is likely due to the time required for thermal mutarotation from α-D-glucose to β-D-glucose. It should also be noted that slow current responses indicate the

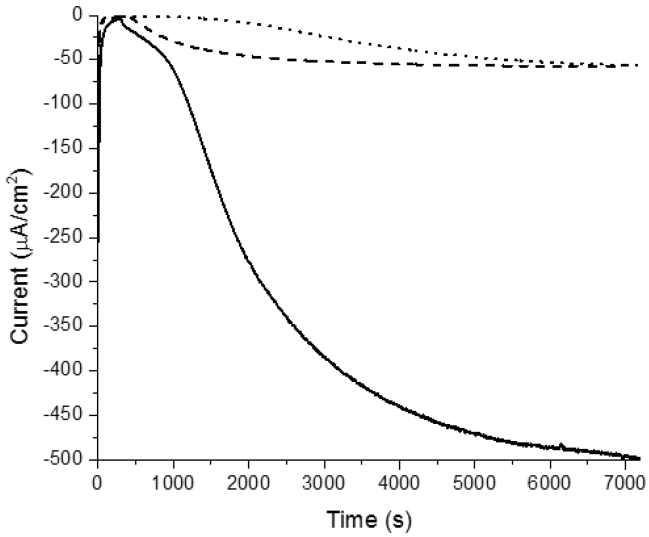

Figure 4. Amperometric response for Inv/FDH-modified electrodes (dashed line), Inv/GOx-modified electrodes (dotted line), and Inv/FDH/GOx-modified electrodes (solid line) in 100 mmol/L sucrose solution ($t_{\text{injection}}$ = 400 s). Performed in 50 mmol/L citrate buffer, pH 5.5, at 25 °C.

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Inv/FDH/GOx film requires a significantly longer amount of time to reach steady state than was expected. The amperometric responses of Inv/FDH/GOx films to injections of a mixture of 100 mmol/L glucose and 100 mmol/L fructose are shown in Figure 5. Rapid amperometric response time to

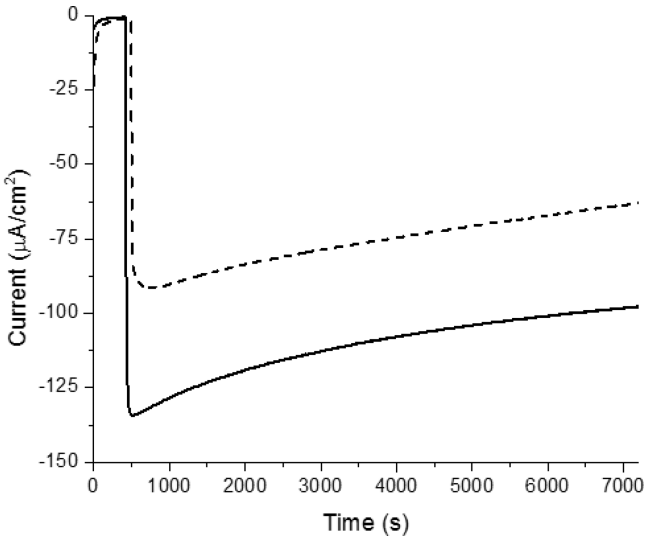

Figure 5. Amperometric response for Inv/FDH-modified electrodes (solid line), Inv/GOx-modified electrodes (dashed line) in a mixture of 100 mmol/L fructose, 100 mmol/L glucose solution ($t_{injection}$ = 400 s). Performed in 50 mmol/L citrate buffer, pH 5.5, at 25 °C.

equivalent amounts of fructose and glucose lead us to reason that the hydrolysis of sucrose by invertase is the rate limiting step in the cascade.

Control experiments were performed by using a nonredox polymer, octyl-modified linear polyethylenimine (C_8-LPEI), as the polymer matrix for enzyme immobilization. Chronoamperometric experiments performed in 100 mmol/L sucrose (Supporting Information Figure S4) shows the need for use of a redox mediator to help shuttle electrons from the enzymes to the electrode surface. A small amount of current density is obtained in the presence of the three enzymes cascade without a redox mediator which is attributed to the direct electron transfer of the FDH from its active site (pyroloquinoliquinone, PQQ) through the heme *c* cofactor.[36] Similar control experiments show that the polymer does not exhibit any electrochemical response to 100 mmol/L sucrose in the absence of FDH, GOx, and invertase.

The effect of substrate composition on the amperometric responses of various films is given in Table 1. Invertase catalyzes the hydrolysis of sucrose into one molecule of fructose and one molecule of glucose. The overall cascade-electrode current was presumed to be a result of the additive currents from each of the two redox enzymes. Therefore it would be expected that the current response of Inv/FDH/GOx films to 100 mmol/L sucrose should be equal to the sum of the current response of Inv/FDH and Inv/GOx to 100 mmol/L sucrose; however, this is not observed. The amperometric response of films containing both redox enzymes (Inv/FDH/GOx) to sucrose is much higher (351 ± 99 μA/cm^2) than the sum of the response of films containing only one of the redox enzymes (54 ± 5 μA/cm^2 for Inv/FDH; 51 ± 8 μA/cm^2 for Inv/GOx). A similar trend is observed when an equilibrated mixture of 100 mmol/L glucose and 100 mmol/L fructose are added as substrates instead of 100 mmol/L sucrose. This trend indicates that there is a synergistic effect of immobilizing FDH and GOx in the same film.

Single oxidoreductase-based electrodes (GOx and FDH) were made, and amperometric measurements were taken using their respective substrate to try to understand the unexpectedly high current response of Inv/FDH/GOx electrodes. The current response of GOx-based electrodes to glucose (242 ± 19 μA/cm^2) is significantly higher than that of analogous Inv/GOx-based electrodes to either sucrose or a glucose-fructose mixture. Additionally, there is a proportional difference in current response of FDH-based electrodes to fructose (50 ± 5 μA/cm^2) compared with the response of Inv/FDH-based electrodes to either sucrose or a glucose−fructose mixture. This indicates that the observed synergism between FDH and GOx is the result of an apparent product inhibition of Inv caused by the presence of fructose or glucose. When both GOx and FDH are present, fructose and glucose can be rapidly oxidized to prevent Inv inhibition which results in a higher than expected current response from Inv/FDH/GOx-based electrodes.

Optimization of Temperature and pH. The overall activities of Inv/FDH/GOx films were measured amperometrically as a function of both temperature and pH, as shown in Figure 6. The maximum current responses to sucrose by Inv/FDH/GOx films increase linearly with temperature between 21 and 37 °C. These experiments show that a current of 302 ± 57 μA/cm^2 was obtained at 25 °C, and a current of 602 ± 62 μA/cm^2 was obtained when the temperature was increased to 37 °C. This increase in current response agrees with the previously reported increase in activity per temperature for each enzyme.[37−39] All of the enzymes used in this study exhibit an increase in activity with an increase in temperature; however, this is not true for the dependence of pH on activity. Previous studies report that both invertase and FDH have a maximum activity for their respective substrates between pH 3.5−4.0 and activity of both are inhibited by 50% above pH 6.5; however, the maximum activity of GOx is observed at pH 7.4 while maintaining 80% of the maximal activity over the range of pH 5.5−9.0.[40−42] A pH profile of the Inv/FDH/GOx electrode film clearly showed that an optimum pH 5.5 allowed for all three enzymes to maintain a reasonable amount of activity.

Table 1. Comparison of the Amperometric Responses of Different Enzyme-Modified Electrodes in the Presence of Variable Substrate Mixtures

	J_{max} (μA/cm^2)				
	Inv/FDH/GOx	Inv/FDH	Inv/GOx	GOx	FDH
100 mmol/L sucrose	351 ± 99	54 ± 5	51 ± 8		
100 mmol/L glucose and 100 mmol/L fructose	330 ± 40	100 ± 20	61 ± 7		
100 mmol/L glucose				242 ± 19	
100 mmol/L fructose					50 ± 5

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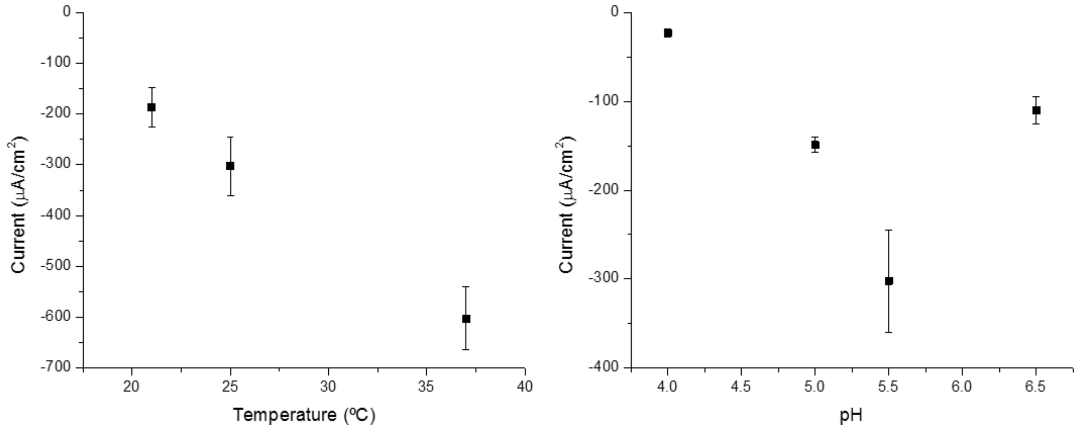

Figure 6. (A) Amperometric response recorded after 2 h for Inv/FDH/GOx-modified electrodes in 100 mmol/L sucrose solution at different temperatures. (B) Amperometric response recorded after 2 h for Inv/FDH/GOx-modified electrodes in 100 mmol/L sucrose solution at different pH solutions.

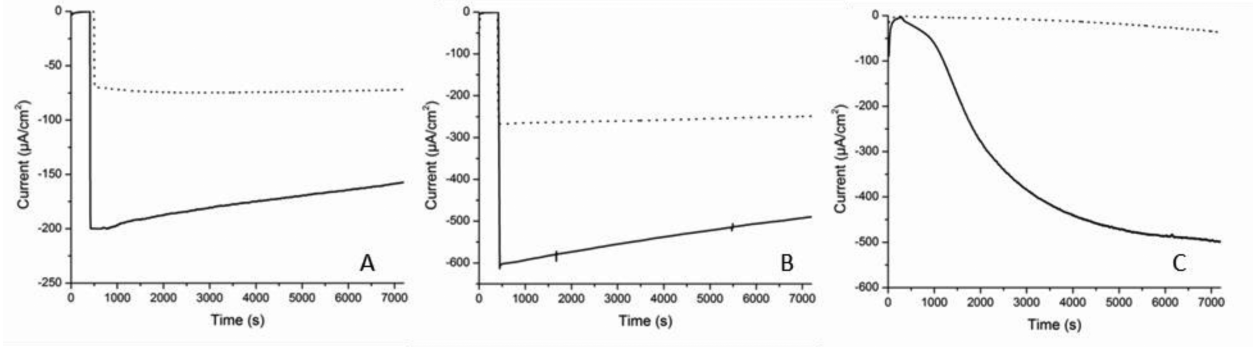

Figure 7. (A) Amperometric responses recorded at FDH-modified electrodes in 100 mmol/L fructose solution, day 1 (solid) and day 6 (dot). (B) Amperometric responses recorded at GOx-modified electrodes in 100 mmol/L glucose solution, day 1 (solid) and day 6 (dot). (C) Amperometric responses recorded at Inv/FDH/GOx-modified electrodes in 100 mmol/L fructose solution, day 1 (solid) and day 2 (dot).

Stability of Sucrose Cascade Anode. Comparative stability experiments on Inv/FDH/GOx films (Figure 7) show that both FDH and GOx maintain a reasonable fraction of their maximum current response for up to six days; the amperometric response to fructose decreased from 150 to 75 μA/cm^2 (50% decrease), while the response to glucose decreased from 500 to 250 μA/cm^2 (50% decrease) over a six day period. However, the maximum current response to sucrose decreased from 302 ± 57 to 41 ± 19 μA/cm^2 (87% decrease) in only 24 h. This loss in current response is likely caused by a conformational instability of invertase that occurs, when it is immobilized in $FcMe_4$-C_3-LPEI films, as stability experiments performed with fresh invertase in solution (i.e., not immobilized) result in a decrease from 387 ± 37 to 138 ± 20 μA/cm^2 (64.5% decrease) over a three day period. It has been shown previously that the polyamine backbone of cross-linked ferrocene-modified LPEI films is significantly protonated in an aqueous buffer at an acidic pH.[43] We hypothesize that the positively charged polymer backbone complexes favorably with the negatively charged surfaces of some enzymes. The occurrence of such a complexation was qualitatively confirmed in this study by a rapid formation of precipitate that occurs when $FcMe_4$-C_3-LPEI is mixed with GOx or FDH; however, no precipitate is observed upon the mixing of $FcMe_4$-C_3-LPEI and invertase. This lack of precipitate formation with invertase could be an indication that it is not fully incorporated into the polymer film, but rather randomly immobilized between several complexes of $FcMe_4$-C_3-LPEI/FDH and $FcMe_4$-C_3-LPEI/GOx near the surface of the film.

Sucrose Cascade Operation in a Biofuel Cell. To determine the effectiveness of the cascade electrode film in a sucrose/O_2 biofuel cell, $FcMe_4$-C_3-LPEI was used to immobilize invertase, FDH and GOx on a Toray paper electrode as the anode while an air-breathing Pt electrode was used as the cathode. A commercially available air-breathing Pt electrode was chosen as the cathode to ensure that it would not be the limiting electrode in the fuel cell. The resulting power curves are shown in Figure 8.

A summary of the fuel cell characteristics is shown in Table 2. The Inv/FDH/GOx anode was equilibrated in a mixture of 100 mmol/L sucrose for two hours prior to use; this was done to account for the time required for production of an amount of fructose and glucose sufficient enough for oxidation to occur at the anode. The constructed fuel cell was able to generate 42 ± 15 μW/cm^2 of power at ca. 172 mV with a maximum current density (short circuit current density) of 344 ± 25 μA/cm^2 at 25 °C. A decay in the current density is observed when the potential is approaching short circuit (0 V); this is most likely a result of a buildup of converted substrate within the polymer film decreasing the diffusion of new substrate to be oxidize at the electrode.

A separate bioanode was constructed by omitting the incorporation of invertase into the film. In this case, both maximum current density and power density dropped to 13 ±

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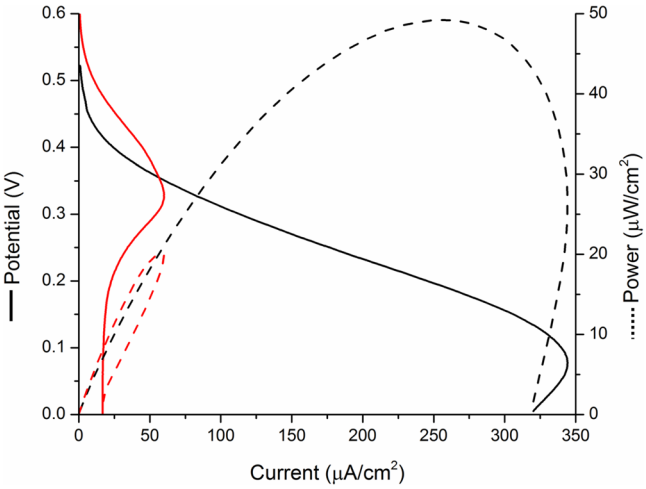

Figure 8. Representative polarization (solid line) and power (dashed line) curves obtained from a FDH/GOx-modified electrode in 100 mmol/L sucrose in absence (red) and in presence of invertase in solution (black). Scan rate = 1 mV s^{-1}.

Table 2. Comparison of Sucrose/Oxygen Biofuel Cells Composed of an Air-Breathing Pt Cathode with One of Three Different Inv/FDH/GOx Anodes: (1) C_8-LPEI Film with Inv in Solution, (2) $FcMe_4$-C_3-LPEI Film without Invertase, (3) $FcMe_4$-C_3-LPEI[a]

	FDH/GOx + Inv C_8-PEI	FDH/GOx $FcMe_4$-C_3-LPEI	Inv/FDH/GOx $FcMe_4$-C_3-LPEI
open circuit voltage (mV)	413 ± 1	610 ± 20	518 ± 14
maximum current density ($\mu A/cm^2$)	0.57 ± 0.04	13 ± 3	344 ± 25
maximum power density ($\mu W/cm^2$)	0.04 ± 0.01	19 ± 1	42 ± 15

[a]All fuel cells were run in a citrate buffer, pH 5.5, with 100 mmol/L sucrose at 25 °C.

3 $\mu A/cm^2$ and 19 ± 1 $\mu W/cm^2$, respectively. C_8-LPEI was used in separate experiments as a mediator-less polymer analogue to determine the effect of the ferrocene redox moiety. The resulting power curve shows significant decrease in both maximum power density (0.04 ± 0.01 $\mu W/cm^2$) and maximum current density (0.57 ± 0.04 $\mu A/cm^2$) because of insufficient electron transfer from the FDH and GOx active sites to the electrode surface, as has been shown in literature that GOx does not readily exhibit direct electron transfer, but FDH has been shown to exhibit direct electron transfer.[42,44]

CONCLUSION

$FcMe_4$-C_3-LPEI can be cross-linked to immobilize invertase, FDH, and GOx onto the surface of an electrode and operate as an enzymatic sucrose cascade. Inv/FDH/GOx-based electrode films exhibit a current response of 302 ± 57 $\mu A/cm^2$ in 100 mmol/L sucrose at 25 °C and 602 ± 62 $\mu A/cm^2$ when the temperature is increased to 37 °C. When poised against an air-breathing Pt cathode, Inv/FDH/GOx-based sucrose biofuel cells are able to reach a maximum power density of 42 ± 15 $\mu W/cm^2$ at ca. 172 mV with a maximum current density of 344 ± 25 $\mu A/cm^2$ on a 1 cm^2 Toray paper electrode at 25 °C. FDH/GOx films maintain almost 50% of their amperometric activity for three days when invertase is in solution.

The reduction potential of the cathode is fixed while using a Pt cathode to reduce molecular oxygen, however the oxidation potentials of both GOx and FDH are lower than the oxidation potential of the ferrocene redox mediator. Therefore future work to increase the overall cell voltage must be focused on lowering the oxidative overpotential of the ferrocene redox moiety in the bioanode. Strategies must also be devised to account for the relatively low activity of invertase and for the slow rate of mutarotation of α-glucose in order to achieve higher current densities and thus higher power densities, however this is one of the first reported sucrose bioanodes and, to our knowledge, the first report of a bioanode that utilizes both glucose and fructose as simultaneous fuel sources. Further examination of the overall kinetics of the cascade is currently underway.

ASSOCIATED CONTENT

Supporting Information

Experimental data for sucrose kinetics of Inv/FDH, Inv/GOx, and Inv/mediator-less electrode films, as well as single oxidoreductase stability. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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