Polymer-based protein engineering grown ferrocene-containing redox polymers improve current generation in an enzymatic biofuel cell

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**A B S T R A C T**

Enzymatic biofuel cells (EBFCs) are capable of generating electricity from physiologically present fuels making them promising power sources for the future of implantable devices. The potential application of such systems is limited, however, by inefficient current generation. Polymer-based protein engineering (PBPE) offers a unique method to tailor enzyme function through tunable modification of the enzyme surface with functional polymers. In this study, we report on the modification of glucose oxidase (GOX) with ferrocene-containing redox polymers to increase current generation efficiency in an enzyme-modified anode. Poly(N-(3-dimethyl(ferrocenyl)methylammonium bromide)propyl acrylamide) (pFcAc) was grown from covalently attached, water-soluble initiator molecules on the surface of GOX in a “grafting-from” approach using atom transfer radical polymerization (ATRP). The covalently-coupled ferrocene-containing polymers on the enzyme surface promoted the effective “wiring” of the GOX active site to an external electrode. The resulting GOX-pFcAc conjugates generated over an order of magnitude increase in current generation efficiency and a 4-fold increase in maximum EBFC power density (≈1.7 μW cm⁻²) with similar open circuit voltage (0.27 V) compared to native GOX when physically adsorbed onto paddle-shaped electrodes made up of electrospun polyacrylonitrile fibers coated with gold nanoparticles and multi-wall carbon nanotubes. The formation of electroactive enzyme-redox polymer conjugates using PBPE represents a powerful new tool for the improvement of mediated enzyme-based bioelectronics without the need for free redox mediators or anode/cathode compartmentalization.

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1. Introduction

Enzymatic biofuel cells (EBFCs) have been intensely studied as prospective power sources for the future of implantable devices (Cosnier et al., 2014; Rasmussen et al., 2016). These fuel cells capitalize on the biocompatibility, specificity and mild operating conditions of enzymes to generate electrical power from physiologically present fuels without added toxicity concerns (Barton et al., 2004; Katz, 2014). The general operation of an EBFC consists of two separate redox reactions occurring at enzyme-functionalized electrodes connected through an external circuit (Bullen et al., 2006; Minteer et al., 2007). The magnitude of power capable of being produced is governed by the density of current generated and the potential difference between the anodic and cathodic redox reactions (Bullen et al., 2006; Luckarift et al., 2014). A major limiting factor in these systems is the inefficient generation of current at the anode (Kim et al., 2006; Osman et al., 2011).

The density of current produced (J max) at enzyme-functionalized anodes is proportional to the density of anodic working enzyme activity. This activity density depends on the loading of working enzyme incorporated onto the electrode material per unit area and the rates of two reactions: the turnover of substrate (i.e., fuel) by the anodic working enzymes and the transfer of electrons
from the active sites of these enzymes to the supporting electrode material. Consequently, there are two main strategies to improving anodic $J_{\text{max}}$ in EBFCs. One approach is to maximize enzyme loading density through the design of electrode materials with greatly increased available surface area for enzyme attachment through the incorporation of nanomaterials such as metal nanoparticles, graphene and carbon nanotubes (Filip and Tkac, 2014; Holzinger et al., 2012; Walcarius et al., 2013). These materials have further been fabricated into three-dimensional conducting matrices with extremely high specific surface area (SSA), which have produced some of the highest performing EBFCs to date with power densities reaching 2 mW cm$^{-2}$ (Prasad et al., 2014; Zebda et al., 2011).

The other approach to improving anodic $J_{\text{max}}$ is to enhance the observed reaction rates of the immobilized enzyme. The turnover rate constants ($k_{\text{cat}}$) of enzymes are impacted by the process of immobilization dependent on the characteristics of the support material, as well as on the immobilization method employed (Asuri et al., 2006; Campbell et al., 2014, 2016; McMillan et al., 2013). Similarly, the heterogeneous electron transfer rate constants ($k_{\text{e}}$) of electroactive enzymes are a function of support material, immobilization method and target working enzyme (Campbell et al., 2016; Ivnitski et al., 2007; Palanisamy et al., 2012). Much of the resistance to electron transfer limiting $k_{\text{e}}$ in such systems stems from the location of the enzymatic active sites being deeply buried within the protein shell (Falk et al., 2012; Goran et al., 2013). The transfer of electrons to the electrode surface also must compete with electron transfer to the natural enzyme co-substrate. Thus, the observed $J_{\text{max}}$ is generally limited by inefficient electron transfer between the enzyme active sites and the electrode surface. Attempts to mitigate electron transfer resistances have focused on the use of small molecule redox mediators and directed orientation immobilization strategies (Kavanagh and Leech, 2013; Milton et al., 2015; Reuillard et al., 2013). Also, polymer nanocomposite materials containing carbon nanotubes or metal nanoparticles have been shown to facilitate direct electron transfer (DET) with electroactive enzymes due to the capability of these nanomaterials to interact with the enzyme active site (Baghayeri, 2015; Baghayeri et al., 2013, 2014a, 2014b, 2015).

In mediated electron transfer (MET) type systems, the anodic working enzymes are re-oxidized by small molecule redox mediators that then transfer electrons to the electrode. These mediators are capable of reaching the buried active site more readily than the electrode materials, thus, increasing the percentage of available electrons that are transferred to the circuit rather than the natural electron acceptor. For instance, Reuillard et al. reported that the addition of free naphthoquinone to a glucose oxidase (GOX)/multi-walled carbon nanotube (MWCNT)-based anode resulted in a 14-fold increase in current output (Reuillard et al., 2013). However, the use of free mediators within EBFCs raises additional toxicity and stability concerns, and also necessitates membrane separation of anode and cathode (Kavanagh and Leech, 2013; Prasad et al., 2014).

To minimize the rate at which mediator leaches into solution, groups have incorporated redox moieties into polymer networks to effectively “wire” redox enzymes to electrodes (Heller, 2004; Mao et al., 2003). Specifically, the incorporation of anodic working enzymes into ferrocene, osmium and quinone containing polymer networks have been shown to enhance electron transfer efficiency between the enzymes and the electrode surfaces without the presence of freely diffusing mediator molecules (Abdellaoui et al., 2016; Chen et al., 2015; Osadebe et al., 2015). Polymer-based protein engineering (PBPE) using atom transfer radical polymerization (ATRP) offers a method to tailor enzyme function through tunable modification of the enzyme surface with rationally designed functional polymers. ATRP is a type of controlled radical polymerization that provides the formation of polymers with low polydispersity indices (PDI), enzyme-friendly reaction conditions and a large library of available monomers including ferrocene-containing monomers (Cummings et al., 2014; Hardy et al., 2011; Herfurth et al., 2012; Matyjaszewski and Tsarevsky, 2014; Matyjaszewski and Xia, 2001). A large density of polymer can be grown from the surface of target enzymes using the “grafting-from” method in which polymerization is initiated directly from the enzyme surface eliminating steric limitations of pre-grown polymers (Lele et al., 2005). Application of these methodologies to model enzymes has been shown to not only enhance enzyme activity and stability, but also allow predictable engineering of enzyme function through modification with temperature and pH responsive polymers (Cummings et al., 2013, 2014; Murata et al., 2013, 2014). However, the impact of redox mediator-containing polymers grown from the surface of enzymes has not been previously investigated.

Herein, we report on the functionalization of the anodic working enzyme GOX with ferrocene-containing redox polymers through PBPE (Fig. 1). We chose GOX because it is notoriously difficult to achieve DET between the flavin adenine dinucleotide (FAD) cofactor active site within GOX and an electrode surface.

![Fig. 1. Schematic representation of GOX-pFcAc formation using PBPE. (1) Preparation of FcAc monomer, (2) ATRP initiator modification of native GOX and (3) “grafting from” ATRP reaction to produce GOX-pFcAc conjugates.](image-url)
with bilirubin oxidase (BOD) prepared as previously described. The capabilities of PBPE to promote the effective performance of GOX-pFcAc conjugates were thoroughly characterized in terms of biochemical and electrochemical properties. We further determined the EBFC performance of GOX-pFcAc-gold/MWCNT fiber paddle anodes when coupled with gold/MWCNT fiber paddle cathodes modified with bilirubin oxidase (BOD) prepared as previously described (Campbell et al., 2016). The objectives of this study were to examine the capabilities of PBPE to promote the effective “wiring” of the buried GOX active sites to an external electrode by thorough characterization and comparison of the performance of GOX-pFcAc conjugates to that of native GOX.

2. Experimental

2.1. Materials

Sodium phosphate buffer (0.1 M, pH 7.0) prepared from phosphate salts and ultrapure milliQ grade water (resistivity of 18.2 MΩ cm) was used in all experiments unless otherwise stated. GOX type X-S from Aspergillus niger and horseradish peroxidase type VI-A were purchased from Sigma Aldrich. BOD from Myrothecium sp. was purchased from Amano Enzyme Inc. Tris[2-(dimethylamino)ethyl]amine (Me6TREN) was synthesized as described previously (Ciampoli and Nardi, 1966), MWCNTs with average diameter of 11.5 nm and average length of 30 μm were purchased from Cheap Tubes, Inc. Dialysis tubing (25 kDa molecular weight cutoff, Spectra/Per, Spectra Laboratories Inc.) was purchased from Fisher Scientific. All chemicals were of analytical grade and used as received.

2.2. Preparation of GOX-pFcAc conjugates

N-(3-dimethyl(ferrocenyl)methylammonium bromide)propyl acrylamide (FcAc) was prepared using 3-bromopropylamine hydrobromide, trimethylamine and (dimethylaminomethyl)ferrocene (details in supporting information). Synthesis of ATRP initiating molecules was carried out as previously described (Cummins et al., 2014; Murata et al., 2013). Synthesized initiating molecules (50 mg, 0.18 mmol) and GOX (300 mg, 0.002 mmol protein, 0.06 mmol primary amine) were dissolved in 0.1 M sodium phosphate buffer (pH 8.0) and stirred for 3 h at 4 °C. The solution was then dialyzed against 0.1 M sodium phosphate buffer (pH 7.0) for 48 h at 4 °C. We determined the resulting initiator modified GOX (GOX-Cl) concentration and number of initiating sites per GOX molecule via standard bicinchoninic acid (BCA) assay kit (ThermoFisher Scientific) and fluorescamine protein assay, respectively (details in Supporting Information). We modeled the expected sites of modification using computational analysis via lysine available surface area and predicted pKa (details in Supporting Information). UV–vis spectra were recorded using a UV–vis spectrometer (Lambda 2, Perkin Elmer).

To synthesize GOX-pFcAc conjugates, the GOX-Cl initiator complex (110 mg, 0.017 mmol initiator) and FcAc (73 mg, 0.17 mmol) were first dissolved in a 20 mL mixture of 80% ultrapure water and 20% 1,4-dioxane and bubbled with Argon for 1 h. In a separate flask, Me6TREN (5.36 μL, 0.02 mmol) was dissolved in ultrapure water (2 mL) and bubbled with Argon for 10 min. Copper(I) chloride (1.98 mg, 0.02 mmol) was then added to the Me6TREN solution and bubbled with Argon for 50 min prior to the addition of the GOX-Cl/FcAc solution to the copper/Me6TREN solution. Upon combining the two solutions, the reaction mixture was incubated at 4 °C for 5 h with stirring. The resulting solution purified by dialysis against 0.1 M sodium phosphate buffer (pH 7.0) using 25 kDa molecular cutoff dialysis tubing for 48 h at 4 °C (final 2 h of dialysis against ultrapure water) and then lyophilized.

2.3. GOX-pFcAc conjugates characterization

We determined the enzyme content of lyophilized GOX-pFcAc powder using the standard BCA assay kit. Hydrodynamic diameters (Dh) of native GOX and GOX-pFcAc were determined via dynamic light scattering (DLS) using a Nanoplas zeta/nano particle analyzer (Particulate Systems). GOX and GOX-pFcAc kinetic analysis was performed using the standard GOX 2,2'-azino-bis(3-ethylthiazoline)–6-sulfonic acid (ABTS) activity assay.

2.4. Electrode Fabrication

We prepared gold/MWCNT fiber paddle electrodes as previously described (Campbell et al., 2016; Jose et al., 2012). Enzyme-modified electrodes were formed by incubating individual gold/MWCNT fiber paddle electrodes in 1 mg mL⁻¹ enzyme solution (10 mL, GOX, BOD or GOX-pFcAc) in 0.1 M sodium phosphate buffer (pH 7.0) at 4 °C for 4 h to allow for physical adsorption of enzyme/conjugate. Electrodes modified with both native GOX and FcAc monomer were formed by similar incubation with both 1 mg mL⁻¹ GOX and 0.26 mg mL⁻¹ FcAc monomer. We then gently washed each fiber paddle to remove loosely bound enzyme prior to individual electrode characterization of EBFC testing. Scanning electron microscope (SEM) images were taken using a Hitachi S-2460 N SEM.

2.5. Electrode Characterization

Total GOX loadings were determined as previously described (Campbell et al., 2016). We performed all electrochemical measurements using a conventional three-electrode electrochemical cell utilizing a KCl saturated Ag/AgCl reference electrode and a 0.5 mm diameter platinum wire counter electrode. EBFC performances were tested in 200 mL of air saturated 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M glucose with stirring. A Fluke 287 True RMS multimeter was used to measure circuit voltage with circuit resistance varied manually with an IET Labs RS-200 resistance decade box.

3. Results and discussion

3.1. Preparation of GOX-pFcAc conjugates

PBPE has proven capable of adding functionality to enzymes through modification of the enzyme surface with stimuli responsive polymers (Cummins et al., 2013; Murata et al., 2013, 2014). We investigated the extension of these capabilities to ferrocene-containing redox polymers grown from the anodic working enzyme GOX (Fig. 1). To ensure highly modified enzyme-polymer conjugates, we utilized a water-soluble, NHS-functionalized ATRP initiator to react with primary amines on the surface of GOX (Murata et al., 2013). Each GOX dimer possessed 32 available...
primary amines including the N-termini (Wohlfahrt et al., 1999). Upon ATRP initiator modification, we determined that there was an average of 25 initiating sites per GOX molecule using a fluoroscinemamine protein assay (details in Supporting Information). Thus, there were 25 separate sites on each enzyme from which pFcAc polymers could be grown. We modeled the expected sites of initiator modification using computational analysis of predicted primary amine accessible surface area (ASA) and pKa (details in Supporting Information; Fig. S1) (Ahmad et al., 2004; Li et al., 2005). This analysis showed 6 lysine residues per monomer were buried with a relative ASA below 0.4, which is the average value for lysines within proteins (Lins et al., 2003). Thus, it was predicted that the 10 exposed primary amines per monomer (ASA greater than 0.4; Fig. S1) were modified in each GOX molecule. Discrepancy between predicted (20 accessible) and observed (25 modified) number of initiator sites per GOX molecule was likely due to the computer analysis using a static model with actual ASA fluctuating in solution. Nevertheless, it was predicted that at least one lysine per monomer as well as the N-termini within 4 Å of the GOX active site were modified, which may lead to interference with biocatalytic activity but would also be conducive to charge collection (Fig. S1).

GOX-pFcAc conjugates were prepared using a “grafting-from” approach in which FcAc monomers were extended from the chlorine functionalized ATRP initiators (Cummings et al., 2014). Enzyme content of the prepared GOX-pFcAc was determined using a standard BCA protein assay kit. From this information, and assuming all initiating sites participated in polymerization, we estimated the molecular weight of the conjugates to be approximately 204 kDa, assuming equivalent polymerization from each ATRP initiator modified GOX molecule (GOX Mw = 160 kDa; FcAc Mw = 435.19 Da) (Bankar et al., 2009). Details for BCA determined molecular weight calculations are provided in our previous reports (Cummings et al., 2013; Murata et al., 2013). The structure of pFcAc grown from free ATRP initiator without NHS functionality was estimated the molecular weight of the conjugates to be approximately 42 kDa. We have previously reported on the fabrication and thorough electrochemical characterization of GOX physically adsorbed onto gold/MWCNT fiber paddle electrodes (Campbell et al., 2016; Jose et al., 2012). Briefly, the electrodes were prepared by the electrospinning of polyacrylonitrile fibers containing gold salt followed by reduction and deposition of AuNPs with subsequent electrodeposition of MWCNTs (Campbell et al., 2016; Jose et al., 2012). In depth characterizations of the resulting electrode morphology can be found in our previous reports (Campbell et al., 2016; Jose et al., 2012). Characteristic SEM images of gold/MWCNT fiber paddle electrodes before and after GOX-pFcAc functionalization showed retention of electrode morphology upon conjugate immobilization (Fig. S6).

3.3. Characterization of GOX-pFcAc-modified electrodes

We have previously reported on the fabrication and thorough electrochemical characterization of GOX physically adsorbed onto gold/MWCNT fiber paddle electrodes (Campbell et al., 2016; Jose et al., 2012). Briefly, the electrodes were prepared by the electrospinning of polycrylonitrile fibers containing gold salt followed by reduction and deposition of AuNPs with subsequent electrodeposition of MWCNTs (Campbell et al., 2016; Jose et al., 2012). In depth characterizations of the resulting electrode morphology can be found in our previous reports (Campbell et al., 2016; Jose et al., 2012). Characteristic SEM images of gold/MWCNT fiber paddle electrodes before and after GOX-pFcAc functionalization showed retention of electrode morphology upon conjugate immobilization (Fig. S6).

Cyclic voltammetry (CV) traces of gold/MWCNT fiber paddle anodes showed a single reduction peak at 0.5 V versus Ag/AgCl, which was attributed to further gold salt reduction to AuNPs within the fibers (Fig. 2A) (Gotti et al., 2014; Hezard et al., 2012). GOX-pFcAc-gold/MWCNT fiber paddle anodes exhibited obvious oxidation and reduction peaks having a formal potential of 0.44 V versus Ag/AgCl and peak separation of 0.02 V indicating reversible electron transfer between ferrocene and the anode surface (Fig. 2A). Upon the addition of 0.01 M glucose to solution, an obvious shift in anodic current was observed, which signified MET between bioactive GOX and the electrode through attached pFcAc polymer. The observed formal potential was stable over a wide range of pH, which was consistent with the pH independence of ferrocene redox activity (Fig. 2B) (Chen and McCreery, 1996; Kumar et al., 2014). The slightly increasing formal potential exhibited at pH lower than 4.0 could result from a greater resistance to oxidation caused by positively charged GOX under those conditions (GOX pI = 4.2) (Koide and Yokoyama, 1999).

We performed electrochemical impedance spectroscopy (EIS) to examine the surface characteristics of the gold/MWCNT fiber paddle anodes with various adsorbed materials (Fig. 2C). Nyquist plots of the impedance spectra showed nearly straight trends for gold/MWCNT fiber paddle anodes and FcAc monomer-gold/MWCNT fiber paddle anodes, which were characteristic of diffusion limited systems (Zhang et al., 2014). Upon adsorption of native GOX, an insulating protein layer was formed at the electrode surface, evident from the appearance of the semicircular portion of the resulting spectrum (Fig. 2C). The diameter of this semicircular region corresponded to the resistance to electron transfer of GOX-gold/MWCNT fiber paddle anodes with the Fe(CN)63−/4− redox probe. We then fit this data to an equivalent circuit (Fig. 2C inset) to determine the corresponding electron transfer resistance (Ret), which was calculated to be 96.8 ± 2.8 Ω (Katz et al. and Willner, 2003), the Randles equivalent circuit incorporated (Ret), electrolyte/solution resistance (R), double layer capacitance (Cdl) and Warburg impedance (W) (Katz and Willner, 2003). EIS examination of GOX-pFcAc-gold/MWCNT fiber paddle anodes revealed a Ret of 3.5 ± 0.2 Ω, which proved decreased resistance to electron transfer by modification of GOX with pFcAc (Fig. 2C).

Next, we examined the properties of the GOX-pFcAc-gold/MWCNT fiber paddle anodes by running CV traces at varying scan
rates (Fig. S7). The dependence of ferrocene faradaic peak current on scan rate allowed for the calculation of ferrocene loading at the anode surface for GOX-pFcAc-gold/MWCNT fiber paddle anodes and gold/MWCNT fiber paddles modified with both native GOX and free FcAc monomer (GOX/FcAc monomer-gold/MWCNT fiber paddle anodes; Fig. S7A and C) (Wang, 2000). The FcAc monomer concentration used for GOX/FcAc monomer-gold/MWCNT fiber paddle anodes was selected according to the estimated molar ratio consistent with the native GOX concentration in the incubation. The linear dependence of peak current on scan rate showed that both systems were limited by electron transfer at the ferrocene-electrode interface rather than by diffusion (Fig. S7A and C) (Wang, 2000). Additionally, we determined the total GOX loading at the anodes by removing adsorbed GOX or GOX-pFcAc using sodium dodecylbenzenesulfonate surfactant and calculating the enzyme content of the resulting supernatant via standard BCA assay kit (Table 1). The roughly 2-fold greater ferrocene loading and 1.6-fold greater total GOX loading observed for GOX-pFcAc conjugates compared to the GOX/FcAc monomer mixture suggested polymerization of pFcAc from the surface of GOX allowed ferrocene groups preferential interaction with the anode surface due to the lack of native GOX adsorption while also providing greater GOX retention at the anode surface. Further, the total GOX loading of GOX-pFcAc was found to be similar to that of native GOX absorbed with no FcAc present (Table 1) (Campbell et al., 2016).

The dependence of ferrocene faradaic peak potential on the logarithm of scan rate allowed for the determination of $k_\text{s}$ in each configuration (Fig. S7B, D; Table 1) (Laviron, 1979; Laviron and Roullier, 1980). The 1.9-fold lower $k_\text{s}$ of GOX-pFcAc-gold/MWCNT fiber paddle anodes compared to GOX/FcAc monomer-gold/MWCNT fiber paddle anodes suggested slightly increased electron transfer resistances stemming from polymerized pFcAc despite the increased ferrocene loading. The observed $k_\text{s}$ of GOX-pFcAc was 3-fold higher, however, compared to GOX-gold/MWCNT fiber paddle anodes determined by the dependence of GOX faradaic peak potential on the logarithm of scan rate (Campbell et al., 2015).

We characterized electrical current generation via biocatalytic turnover of glucose in GOX-pFcAc-gold/MCWCNT fiber paddle anodes through amperometry at varying glucose concentrations and cell potentials (Fig. 3). Monitoring the increases in current density in Ar saturated solution with the cell potential held at the determined formal potential of pFcAc (0.44 V versus Ag/AgCl) provided characterization of glucose oxidation by GOX and MET.

Table 1

<table>
<thead>
<tr>
<th>Gold/MWCNT fiber paddle anode</th>
<th>Ferrocene loading $\times 10^{-20}$ (mol cm$^{-2}$)</th>
<th>Total GOX loading $\times 10^{-10}$ (mol cm$^{-2}$)</th>
<th>$k_\text{s}$ (s$^{-1}$)</th>
<th>$J_{\text{max}}$ (mA cm$^{-2}$)$^a$</th>
<th>$K_\text{M}^{\text{app}}$ (mM glucose)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOX-</td>
<td>N/A</td>
<td>$^b$2.60</td>
<td>$^b$0.95 $\pm$ 0.01</td>
<td>0.24 $\pm$ 0.01</td>
<td>41 $\pm$ 7</td>
</tr>
<tr>
<td>GOX-pFcAc-</td>
<td>6.24</td>
<td>2.25</td>
<td>2.86 $\pm$ 0.46</td>
<td>0.50 $\pm$ 0.02</td>
<td>50 $\pm$ 8</td>
</tr>
<tr>
<td>GOX/FcAc monomer-</td>
<td>3.18</td>
<td>1.37</td>
<td>5.35 $\pm$ 0.41</td>
<td>0.18 $\pm$ 0.01</td>
<td>52 $\pm$ 8</td>
</tr>
</tbody>
</table>

$^a$ $J_{\text{max}}$ and $K_\text{M}^{\text{app}}$ values relative to amperometry in oxygen saturated solution with cell potential held at 0.8 V versus Ag/AgCl

$^b$ Campbell et al. (2016)
through ferrocene to the electrode surface (Fig. 3A). The highest current density was observed for non-functionalized gold/MWCNT fiber paddle anodes due to the direct oxidation of glucose by AuNPs at this potential (Fig. S8) (Lang et al., 2014; Pasta et al., 2010). However, this activity was eliminated upon adsorption of FcAc monomer and inhibited upon adsorption of native GOX (Fig. 3A). GOX-pFcAc- and GOX/FcAc monomer-gold/MWCNT fiber paddle anodes exhibited reproducible current responses upon successive glucose injections (Fig. 3A). The steady-state current densities reached after glucose injection allowed the calculation of apparent Michaelis-Menten kinetics characteristic of MET (Fig. S9).

The apparent Michaelis-Menten constants ($k_{cat}$ and $K_{M}$) for the enzyme within GOX-pFcAc-gold/MWCNT and GOX/FcAc monomer-gold/MWCNT fiber paddle anodes were 19 ± 10 mM glucose and 13 ± 5 mM glucose, respectively, which showed similar affinities for glucose in both configurations. The maximum current density ($J_{max}$) of MET for GOX-pFcAc-gold/MWCNT fiber paddle anodes was somewhat higher at 0.09 ± 0.01 mA cm$^{-2}$ than that for GOX/FcAc monomer-gold/MWCNT fiber paddle anodes at 0.05 ± 0.01 mA cm$^{-2}$ revealing a slightly decreased resistance to electron transfer between FAD in GOX and the electrode surface through the ferrocene moieties. Apparent activity from GOX-gold/MWCNT fiber paddle anodes under these conditions was attributed to retained AuNPs glucose oxidation at due to incomplete coverage by enzyme alone (Fig. S9A).

We previously reported on electrical current generation by GOX-gold/MWCNT fiber paddles in oxygen saturated solution with cell potential held at 0.8 V versus Ag/AgCl (Campbell et al., 2016). At this potential, hydrogen peroxide produced from GOX was oxidized at the electrode surface, effectively using oxygen as a natural electron mediator. In configurations containing ferrocene, MET was also observed at these conditions, which allowed for a measure of total current generation capabilities (Fig. 3B) (Jose et al., 2012; Wang, 2008). Again, examination of increasing current density upon successive glucose injections provided for calculation of apparent Michaelis-Menten kinetics (Fig. S10; Table 1). Glucose oxidation by AuNPs was not observed under these conditions (Fig. 3B). The increased $K_{M}$ for all configurations was indicative of similar GOX/substrate interactions at each functionalized anode. GOX-pFcAc-gold/MWCNT fiber paddle anodes exhibited the highest overall $J_{max}$ despite a ten-fold lower $k_{cat}$ than native GOX and a nearly 2-fold lower $k_t$ than ferrocene at GOX/FcAc monomer-gold/MWCNT fiber paddle anodes (Table 1). The decreased overall current generation of GOX/FcAc monomer-gold/MWCNT fiber paddle anodes compared to GOX-gold/MWCNT fiber paddle anodes was consistent with the inhibition of native GOX biocatalytic activity (Fig. S5.).

Assuming the determined $k_{cat}$ values were the maximum rates of electron production from the 2-electron oxidation of glucose, the maximum specific current generation rates for GOX and GOX-pFcAc were 9.10 × 10$^7$ A mol GOX$^{-1}$ and 9.25 × 10$^6$ A mol GOX$^{-1}$, respectively. Combining these rates with the calculated total GOX loadings, the maximum theoretical $J_{max}$ values for GOX-gold/MWCNT fiber paddle anodes and GOX-pFcAc-gold/MWCNT fiber paddle anodes became 23.7 mA cm$^{-2}$ and 2.1 mA cm$^{-2}$, respectively. Thus, the current generation efficiency of GOX-pFcAc-gold/MWCNT fiber paddle anodes calculated as the percent of $J_{max}$ observed relative to the maximum possible $J_{max}$ was 24%, whereas the same value for GOX-gold/MWCNT fiber paddle anodes was only 1%. These results highlighted the effective “wiring” of the GOX active site to the electrode by pFcAc grown from the surface of GOX while also maintaining a portion of native GOX activity. The
dramatic increase observed in anodic efficiency motivated us to examine the performance of GOX-pFCAc-gold/MWCNT fiber paddle anodes in an EBFC.

3.4. GOX-pFCAc EBFC performance

GOX-pFCAc-based EBFCs were constructed with a single GOX-pFCAc-gold/MWCNT fiber paddle anode and single BOD-gold/MWCNT fiber paddle cathode connected through an external circuit without membrane separation. BOD is a multicopper oxidase that catalyzes the reduction of oxygen to water and is commonly used as a cathodic working enzyme in EBFCs due its proven capability of DET (Brocato et al., 2012; Shleev et al., 2005). The performances of all EBFCs were tested through the manual variation of circuit resistance while monitoring circuit voltage (Fig. 4). For EBFCs utilizing GOX-pFCAc-gold/MWCNT fiber paddle anodes, the observed maximum power density was 1.66 ± 0.47 μW cm⁻², which was 4-fold greater compared to EBFCs using GOX-gold/MWCNT fiber paddle anodes (Campbell et al., 2016). Further, the open circuit voltage (OCV) of EBFCs with GOX-pFCAc-gold/MWCNT fiber paddle anodes was 0.27 ± 0.01 V, which was similar to those modified with native GOX (Campbell et al., 2016). These results showed the capability of pFCAc modification through PBPE to increase current density without limiting the cell voltage, a common disadvantage in MET-type systems (Kavanagh and Leech, 2013; Reuillard et al., 2013). Further, EBFCs constructed with GOX-pFCAc monomer-gold/MWCNT fiber paddle anodes exhibited maximum power densities of only 4 ± 2 nW cm⁻² with decreased performance likely a result of inhibited GOX activity by free FcAc and detachment of free FcAc in the membrane-less system (Fig. S11). Non-functionalized gold/MWCNT fiber paddle anodes were previously shown to demonstrate negligible power generation (Campbell et al., 2016). These results confirmed the benefits of pFCAc “wiring” GOX to the electrode surface and thus increasing electron transfer rates for greater power generation despite decreased GOX biocatalytic activity.

A major issue in the development of EBFCs has been the instability of power generation over time (de Poulpiquet et al., 2014; Kim et al., 2006; Shrier et al., 2014). The use of free redox-mediators within EBFCs introduces additional stability concerns due to the tendency of these small molecule mediators to diffuse away from the working system (Kavanagh and Leech, 2013). The output power density of our EBFCs utilizing GOX-pFCAc-gold/MWCNT fiber paddle anodes steadily decreased during continuous operation (Fig. 5A). This instability was likely caused due to enzyme activity loss at the functionalized gold/MWCNT fiber paddle anodes. Indeed, consecutive CV traces of GOX-pFCAc-gold/MWCNT fiber paddle anodes confirmed a continuous decrease in ferrocene faradaic current density, which suggested detachment of GOX-pFCAc from the electrode surface over time (Fig. 5B; S12). This configuration did not solve the issue of power generation stability, but conjugation of redox polymer directly to the enzyme surface provides the potential for simultaneous covalent attachment of working enzyme coupled with redox mediator for increased stability, which will be a goal of our future work.

The characterizations described in this study allowed us to evaluate the use of PBPE to grow ferrocene-containing redox polymers from the surface of GOX via “grafting from” SI-ATRP as a new method toward the development of MET-type EBFC anodes. Indeed, the prepared GOX-pFCAc-gold/MWCNT fiber paddle anodes exhibited a dramatically increased current generation efficiency compared to unmodified GOX despite a lower biocatalytic turnover rate. This improvement in performance provided a 4-fold increase in EBFC power density over native GOX with a large loss of power generation observed when free FcAc monomer was adsorbed along with GOX. Our immediate next steps are to fully...
characterize EBFC stability and to report on strategies we are developing to overcome the low stability observed within our systems.

4. Conclusions

We have developed and thoroughly characterized a GOX-based electrode system formed by the growth of poly(N-(3-dimethyl(ferrocenyl)methylammonium bromide)propyl acrylamide) from the enzyme surface via PBPE techniques followed by the physical adsorption of these GOX-pFcAc conjugates onto gold/MWCNT fiber paddle electrodes. The final GOX-pFcAc-gold/MWCNT fiber paddle anodes proved capable of MET through the covalently attached redox polymer chains while maintaining GOX biocatalytic activity. The effective “wiring” of GOX through pFcAc led to a 24-fold increase in current generation efficiency compared to native GOX adsorbed onto the same electrode material. This performance enhancement extended to the capability of GOX-pFcAc-gold/MWCNT fiber paddle cathodes to produce a 4-fold greater EBFC power density (1.7 μW cm⁻²) compared to GOX-gold/MWCNT fiber paddle anodes without the presence of free mediator and thus no need for compartmentalization. With a variety of potential polymer types, mediator groups and working enzymes to select from, PBPE represents a powerful new tool in the development enzyme- mediator conjugate synthesis toward improved MET-type EBFCs.

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Appendix A. Supplementary material

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References