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Mass Transfer Studies of *Geobacter sulfurreducens* Biofilms on Rotating Disk Electrodes

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Abstract

Electrochemical impedance spectroscopy has received significant attention recently as a method to measure electrochemical parameters of *Geobacter sulfurreducens* bio-films. Here, we use electrochemical impedance spectroscopy to demonstrate the effect of mass transfer processes on electron transfer by *G.sulfurreducens* biofilms grown in situ on an electrode that was subsequently rotated. By rotating the biofilms up to 530 rpm, we could control the microscale gradients formed inside *G.sulfurreducens* biofilms. A 24% increase above a baseline of 82 μA could be achieved with a rotation rate of 530 rpm. By comparison, we observed a 340% increase using a soluble redox mediator (ferrocyanide) limited by mass transfer. Control of mass transfer processes was also used to quantify the change in biofilm impedance during the transition from turnover to non-turnover. We found that only one element of the biofilm impedance, the interfacial resistance, changed significantly from 900 to 4,200 Ω under turnover and non-turnover conditions, respectively. We ascribed this change to the electron transfer resistance overcome by the biofilm metabolism and estimate this value as 3,300 Ω . Additionally, under non-turnover, the biofilm impedance developed pseudocapacitive behavior indicative of bound redox mediators. Pseudocapacitance of the biofilm was estimated at 740 μF and was unresponsive to rotation of the electrode. The increase in electron transfer resistance and pseudocapacitive behavior under non-turnover could be used as indicators of acetate limitations inside *G.sulfurreducens* biofilms.

Keywords

Geobacter sulfurreducens; rotating disk electrode; biofilm; electrochemical impedance spectroscopy; pseudocapacitance

Introduction

Geobacter sulfurreducens biofilms are a well-studied electro-chemically active biofilm system that utilizes conductive elements to respire on electrodes (Bond and Lovley, 2003; Reguera et al., 2005). The conductivity of *G.sulfurreducens* biofilms and the mechanism of conduction through the biofilm are novel concepts which deserve attention in order to understand the electrophysiology of microbially driven electrochemical systems where

electrons travel long distances to reach the electrode (Malvankar et al., 2012b; Snider et al., 2012). One of the more recent tools used to analyze the conductivity or electron transfer capability of *G.sulfurreducens* biofilms is electrochemical impedance spectroscopy (EIS). EIS measures the impedance response of the biofilm to small AC perturbations in polarization potential. We refer to the impedance response as the biofilm impedance. EIS has been used to monitor the biofilm impedance of *G.sulfurreducens* biofilms over time both in anodic half-cells as well as in microbial fuel cells. EIS was also used to compare the conductivities of different strains of *G.sulfurreducens* biofilms (Malvankar et al., 2012b). However, EIS will not discriminate between electron transfer impedances and mass transfer impedances in the overall biofilm impedance. To make accurate measurements of electron transfer resistance, mass transfer resistance must be accounted for and decoupled. Unfortunately, due to the conductive nature of *G.sulfurreducens* biofilms, limited information can be found on the importance of mass transfer resistances when employing EIS.

Several cases of mass transfer processes could limit the electron transfer capabilities of *G.sulfurreducens* biofilms since it was recently found that mass transfer is severely restricted by the dense layers of cells packed inside (Renslow et al., 2013). The mass transfer limitation could take the form of: electron donor not penetrating the whole of the biofilm, protons generated by electrode-respiration accumulating inside the biofilm and inhibiting respiration, or more generally counter-ion fluxes limiting the electron flux through the biofilm. A mini-review recently put forth covers the topic of mass transfer in biofilms as an important, non-negligible aspect of the biofilm mode of life (Stewart, 2012). To determine if such mass transfer limitations existed in *G.sulfurreducens* biofilms and how it could manifest in the biofilm impedance measured with EIS, we needed an electrochemical system that could enhance mass transfer (i.e., convection) in and around the biofilm.

Electrochemical systems used to assess the role of mass transfer processes are generally a variant of a flow cell where flow velocity is varied, a rotating electrode where rotation rate is varied, or an impinging jet electrode. A flow cell setup has been used to characterize the oxygen reduction capabilities of cathodic biofilms on biocathodes (Ter Heijne et al., 2011). A rotating disk electrode setup has been used to measure the thickness of river water biofilms (Bouletreau et al., 2011). However, impinging jet type systems may not be viable due to their inherent ability to remove biomass (Cense et al., 2006). To date, rotating disk electrodes have not been used to study both electron transfer and mass transfer processes in *G.sulfurreducens* biofilms respiring on electrodes. The advantage in using a rotating disk electrode is the flow pattern of fluid that is established above the electrode as it is rotated around its central axis. Nutrients are directed towards the biofilm with the fluid flow and flows laterally right near the electrode, enhancing mass transfer at the biofilm surface and reducing the magnitude of mass transfer resistance. Therefore, the mass transfer resistance can be controlled systematically by increasing the rotation rate of the electrode.

To test whether mass transfer limitations could be identified in *G.sulfurreducens* biofilms using EIS and a rotating disk electrode, we focused on reducing acetate delivery to the biofilm by controlling bulk acetate concentration. However, in the absence of sophisticated acetate measurements inside the biofilm (i.e., NMR methods), we assessed acetate

limitations by investigating biofilm impedance under completely turnover and completely non-turnover conditions. Turnover conditions and non-turnover conditions refer to the biofilm's ability to generate anodic current in the presence of acetate and inability to generate anodic current in the absence of acetate, respectively. However, the biofilm retains its ability to be oxidized/reduced by the electrode under either condition. We expect that the biofilm impedance will reflect the minimum (turnover) and maximum (non-turnover) increase in electron transfer resistance due to acetate depletion within the biofilm. The impedance information could be used to determine if a biofilm was experiencing acetate limitations in situ. To do so, however, requires an adequate biofilm impedance model.

Impedance spectra of *G.sulfurreducens* biofilms obtained using EIS have generally yielded a two-time constant response (Marsili et al., 2008), which refers to an impedance response similar to two resistor and capacitor elements in parallel (RC) (Agarwal et al., 1992). These similarities led to the use of equivalent electrical circuit (EEC) modeling to extract physical interpretations of electron transfer mechanisms in *G.sulfurreducens* biofilms. The distribution of how these RC elements can be arranged to model microbially driven electrochemical systems has been reviewed in detail (Dominguez-Benetton et al., 2012). Both parallel and series arrangements have been used previously (He and Mansfeld, 2009; Jung et al., 2011; Malvankar et al., 2012a). In this case, we have chosen the parallel arrangement as shown in Figure 1A because it approximates the porous film system as well as electron transfer mechanisms involving bound (adsorbed) redox mediators of *G.sulfurreducens* biofilms. Furthermore, real electrochemical interfaces experience non-ideality that cause “time-dispersion” effects. “Time-dispersion” effects can be approximated using a constant-phase element, Q , with a power of α (Macdonald, 1987). In Figure 1A, we expect that Q_1 and Q_2 will reflect the biofilm capacitance and double layer capacitance considering time-dispersion effects, respectively. R_1 , R_2 and R_3 will reflect the solution resistance, resistance through the biofilm, and electron transfer resistance at the biofilm/electrode interface, respectively.

Biofilm Impedance Equivalent Electrical Circuit

We use the EEC in Figure 1A to model the impedance data under turnover conditions. At a constant polarization potential, the lower branch of resistors, R_1 , R_2 and R_3 are the overall resistance to electron transfer in the biofilm. Under non-turnover conditions and a constant polarization potential, no electrons can be transferred to the electrode since the electron donor, acetate, is not available. In Figure 1B, the addition of a capacitor, C_1 , reflects the blocking of current at a constant polarization potential. Since bound redox mediators are assumed to be the carriers of electrons inside the biofilm, the capacitance of C_1 is expected to reflect the amount of bound redox mediators inside the biofilm (in the film and at the interface). Figure 1C maps the EEC in Figure 1A onto the physical biofilm system. We should note that the EEC model shown in Figure 1C represents an interpretation of the impedance elements that are likely to be dominant. Since each circuit element is likely comprised of many complex biochemical reactions, a combination of resistors and capacitors may not reflect all the impedance behavior in this system. Therefore, more complex and detailed models could be constructed; however, this is out of the scope of this work. The EEC and physical model shown in Figure 1C sufficiently fits the impedance data

presented and is used to draw conclusions. To emphasize the lack of uniqueness of EEC models, the EECs in Figure 1A and B can be transformed to different, but equivalent, circuits. For example, Wu et al. (1999) showed that the EEC in Figure 1A is equivalent to that shown in Figure SI-1 (Wu et al., 1999). Similar EECs to those shown in Figure SI-1 have been used previously to estimate the capacitance of *G.sulfurreducens* biofilms spanning across a gap (Malvankar et al., 2012b).

In this work, a *Geobacter sulfurreducens* biofilm was grown on the surface of an electrode that was subsequently rotated to quantify the role of mass transfer in the overall electron transfer rates of the biofilm during electrode respiration. EIS is a powerful electrochemical technique that enables the measurement of electron transfer resistances in redox-mediated systems and was therefore used to quantify biofilm impedance of *G.sulfurreducens* biofilms at select rotation rates. An EEC model was then used to fit the biofilm impedance obtained through EIS and quantify the change in electron transfer resistance over the growth of the biofilm and at select rotation rates. Rotation was also used to differentiate between finite Warburg responses and pseudocapacitive responses under non-turnover conditions where a pseudocapacitance could be measured inside the biofilm. Collectively, the parameters obtained through EEC fitting at both turnover and non-turnover conditions were used to estimate the overall electron transfer resistance that the biofilm metabolism overcomes and estimate the number of heme groups available that could facilitate electron transfer through the extracellular matrix. We compared the effect of rotation on the biofilm to a mass transfer-controlled soluble redox mediator, ferrocyanide, to make the distinction between Warburg and pseudocapacitive responses. Overall, we tested the hypothesis that the rotating disk electrode can be used as an electrochemical tool that controls mass transfer processes when studying electrochemically active biofilms and facilitates our understanding of EIS in microbially driven electrochemical systems.

Materials and Methods

Bioelectrochemical Cell

Biofilms were grown in a continuously fed, temperature controlled electrochemical cell as shown in Figure 2. The counter electrode was placed behind porous glass. The working electrode, on which *G.sulfurreducens* respired, was a 5 mm diameter glassy carbon rotating disk electrode (#970-00060, Gamry Instruments, Warminster, PA). The glassy carbon surface was polished with 0.1 μm alumina suspension on a felt pad followed by 5 min sonication in deionized (DI) water. A final polish using 0.05 μm alumina suspension was done followed by another 5 min sonication in DI water. The working electrode was mounted to the cell using a high-precision adapter with ball-bearing (Gamry Instruments #970-00089). The counter electrode was a graphite rod (Sigma-Aldrich #496545), and the reference electrode was a saturated KCl Ag/AgCl reference. The reactor body was a temperature-controlled electrochemical cell (Gamry Instruments #990-00249) modified to allow continuous feeding. Norprene tubing (Cole-Parmer #EW-06404-14 and #EW-06404-13) was used for the feed and waste streams, respectively. Flow breakers were used in the feed and waste streams to prevent back contamination. A 0.2-mm filter was used at the gas inlet to sparge a mixture of N_2/CO_2 (80%/20%). Gas inlet pressure was adjusted

slightly above the water column pressure in the cell to provide positive pressure without vigorous mixing by rising gas bubbles. Another 0.2- μm filter was used at the gas outlet to relieve pressure buildup. The entire setup except for the reference and working electrodes were autoclaved for 20 min at 121°C. The growth medium was autoclaved separately in a 1-L autoclavable glass bottle for 20 min at 121°C. Once the biofilm reactor and growth medium cooled to room temperature, the growth medium bottle was aseptically connected to the biofilm reactor feed stream. Working and reference electrodes were placed in 70% v/v ethanol in DI water for 45 min under UV exposure before being placed inside the cell. A temperature controller was used to maintain a cell temperature of 30°C using the glass jacket. A mixture of N_2/CO_2 (80%/20%) gas was then sparged for 24 h.

Growth Medium

Growth medium used to grow *G.sulfurreducens* strain PCA (ATCC 51573) biofilms consisted of: potassium chloride, 0.38 g/L; ammonium chloride, 0.2 g/L; sodium phosphate monobasic, 0.069 g/L; calcium chloride, 0.04 g/L; magnesium sulfate heptahydrate, 0.2 g/L; sodium carbonate, 2 g/L; Wolfe's vitamin solution, 10 mL/L; modified Wolfe's mineral solution, 10 mL/L. Acetate (20 mM) was provided as the electron donor. No fumarate or other soluble electron acceptor was added to the growth medium.

Growing the Biofilms

The cell was then inoculated with *G.sulfurreducens* inoculum prepared following a previously published method (Babauta et al., 2012). Cell volume was 115 mL. Within 24 h, the current began to increase and the feed pump was turned on. The dilution rate of the cell was 0.01 h^{-1} (or a flow rate of ~ 1 mL/h). Then, the system was operated in continuous mode and the biofilm was allowed to grow continuously. The biofilms were grown on the electrode without rotation. Rotation experiments were only conducted after a pseudo-steady current was observed. Throughout the growth of the biofilm, EIS was collected at selected current values.

Electrode Polarization

The rotating disk electrode was polarized continuously using a Gamry Reference 600TM potentiostat (Gamry Instruments, Warminster, PA). A selected potential, which provides maximum current, of 0.3 $\text{V}_{\text{Ag}/\text{AgCl}}$ was used. Cyclic voltammetry (CV) and EIS was run using the same potentiostat without any physical modification to the system. EIS and CV parameters are listed in the supplementary information for each individual experiment.

EIS Data Analysis

Impedance data was analyzed using Gamry Echem Analyst Software. The software uses a non-linear fitting routine using the simplex method to fit EEC models. An auto-fitting function within the software auto-adjusted the initial parameters to provide the best fit while minimizing user bias. For all EIS data analysis, the following generic initial parameters were used: 100 Ω for R_1 ; 500 Ω for R_2 and R_3 ; 1×10^{-5} F for Q_1 , Q_2 , and C_1 ; 0.8 for α_1 and α_2 . Software outputted fitted values with the regression error as well as the goodness of fit.

Kramers–Kronig transformations were performed on the impedance data using the software and example fits are provided in Figures SI-3 and SI-5 in the supplementary information.

Biofilm Under Turnover Conditions

Once a pseudo-steady current was observed, the electrode was rotated at 0, 10, 20, 40, 80, 160, and 530 rpm. At each rotation rate, the current was allowed to stabilize before further increasing the rotation rate. When running EIS, the electrode was rotated at each rotation rate for 5 min prior to running the experiment.

Biofilm Under Non-Turnover Conditions

After rotation experiments were finished under turnover conditions, acetate-free media was introduced into the cell to dilute out acetate. Initially, acetate was washed out by passing approximately nine cell volumes, or 1 L of acetate-free media. During the acetate washout process, current was observed to decrease. When current reached 28 μA , the acetate washout process was stopped temporarily to run EIS at 0 and 530 rpm. After 1 L of acetate-free media was continuously fed to the reactor ($[\text{Acetate}] < 0.1 \text{ mM}$), the current was 2 μA and a second EIS run at 0 and 530 rpm was made. However, to ensure completely non-turnover conditions, the cell was continually washed further with acetate-free media for 3 days. After the third day, the current was $\sim 1 \mu\text{A}$ and did not change with rotation rate. A final EIS run at 0 and 530 rpm was made. Normalized against a pseudo-steady current of 82 μA , the four EIS runs were made at 100%, 30%, 2%, and $< 1\%$ normalized current. At the end of the experiment, the electrode was removed from the cell and the biofilm was imaged using a stereomicroscope.

Biofilm Studies

G.sulfurreducens biofilms were grown in replicate on rotating disk electrodes and the observations were nearly identical. EIS over the growth of the biofilm as well as capturing turnover to non-turnover conditions with EIS were run at least three times in multiple reactors. In all cases, the results supported the presented conclusions.

Results and Discussion

EIS of *G.sulfurreducens* Biofilms

Prior to inoculation, the initial (background) current measured a steady sub-microamp current value. Figure 3A shows that within a few hours of inoculation, current began to increase. After 6 days, the current reached $\sim 80 \mu\text{A}$, which we considered as the pseudo-steady current. The inset shows an image of the *G.sulfurreducens* biofilm grown on the electrode at the end of the experiments. Half of the biofilm was removed to provide better contrast between bare glassy carbon and biofilm-covered glassy carbon. The biofilm appeared intact and covered the entire electrode surface as seen by the biofilm half on the Teflon shroud and on the electrode surface. Current production, a good indicator of biofilm viability, was stable throughout these experiments. From the initial attachment to the mature biofilm, we wanted to document the changes in impedance spectra as the current capacity of the biofilm increased. Therefore, from the time of inoculation to pseudo-steady current, we obtained several impedance spectra at select current values. Figure 3B shows impedance

data as a complex plane plot where increasing current production resulted in the formation of a typical “depressed semi-circle” shape commonly seen at electro-chemical interfaces (Macdonald, 1987). Higher current resulted in tighter semi-circles indicating that the biofilm impedance decreased as the biofilm matured on the electrode. Once the pseudo-steady current of 80 mA was reached, the impedance spectra did not change further (results not shown).

We fit the impedance data in Figure 3B to the EEC shown in Figure 1A. Collectively, the individual circuit elements are plotted against the current measured prior to EIS in Figure 4 with error bars representing the standard error derived from the fitting routine. Goodness of Fit for all parameters was always less than 20×10^{-5} . Example of the fit at a current of 82 μA is shown in Figure SI-3. The Kramers–Kronig transformations were used for each individual fit to assess whether data points satisfied the assumptions required for EIS (Orazem and Tribollet, 2008). Figure 4A and B track the changes in Q_1 and Q_2 along with their respective α_1 and α_2 values over increasing current. As described earlier, we interpret Q_1 and Q_2 as the film capacitance and interfacial capacitance, respectively. Not including the first point that represents the bare electrode prior to inoculum addition, Q_1 and Q_2 appear to be linear with respect to current. For Q_1 , the slope of the linear fit was $4.6 \times 10^{-7} \pm 2.5 \times 10^{-8} \text{ s}\alpha/\Omega \text{ } Q\text{A}$ ($R^2 = 0.991$). For Q_2 , the slope of the linear fit was $2.5 \pm 10^{-6} \text{ } 3.2 \times 10^{-7} \text{ s}\alpha/\Omega \text{ } \mu\text{A}$ ($R^2 = 0.952$). α_1 and α_2 remained relatively constant around 0.84 and 0.75, respectively. Constant values of both α_1 and α_2 with increasing current likely reflect that the nature of Q_1 and Q_2 remained capacitive (for ideal capacitor $\alpha = 1$). In Figure 4C and D, R_2 and R_3 decrease non-linearly with increasing current. The minimum values for R_2 and R_3 are $1,300 \pm 400 \text{ } \Omega$ and $800 \pm 430 \text{ } \Omega$, respectively. R_1 , the solution resistance, remained constant as current increased at a value of $130 \pm 16 \text{ } \Omega$.

The increase in both Q_1 and Q_2 indicate that the adsorption of charged species on the surface of the electrode and inside the film was increasing with current. However, Q_2 increased nearly fivefold higher than Q_1 with current. Therefore, the accumulation of charged species at the surface of the electrode was faster than that accumulated in the film. This could mean that either these charged species were being produced by the biofilm near the electrode surface, the biofilm was more dense near the electrode surface rather than in the film, or a combination of both. Recently, we have demonstrated that *G.sulfurreducens* are denser near the bottom and biofilm density decreases with depth in *G.sulfurreducens* biofilms (Renslow et al., 2013). Furthermore, the localization of OmcZ, an outer-membrane cytochrome implicated in current production in *G.sulfurreducens* biofilms, was found to be near the electrode surface under electrode-respiring conditions (Inoue et al., 2011). The larger increase in Q_2 over Q_1 likely reflects these two observations. In contrast to Q_1 and Q_2 , R_2 , and R_3 decrease to minimum values of $1,300 \pm 400 \text{ } \Omega$ and $800 \pm 430 \text{ } \Omega$, respectively. Since both film resistance and interfacial resistance are of the same order of magnitude despite OmcZ being localized near the electrode surface (Inoue et al., 2011), this could mean that the passage of current through the film is no more difficult than current passing from the biofilm to the electrode. However, direct measurements would be required to make such a distinction.

EIS of *G.sulfurreducens* During Rotation of the Rotating Disk Electrode

After pseudo-steady current was chosen, we began rotating the biofilm. The electrode was spun from 0 to 530 rpm in discrete steps. Higher rotation rates did not add any more useful information and was therefore not needed. We should note though that above 1,000 rpm, the current increase was negligible (results not shown). Figure 5A shows that rotating the biofilm up to 530 rpm did not affect current generation as two consecutive rotation sweeps yielded nearly identical current values, which corresponds to the biofilm remaining intact during rotation. The largest increase in current occurred when rotation was set to 10 rpm. Diminishing increases in current were observed upon increasing the rotation rate by a factor of two, up to 160 rpm. A current of 102 μA at 530 rpm over a baseline current of 82 μA at 0 rpm was measured. Figure 5B shows how the increased current is reflected in the biofilm impedance at each rotation rate.

In a separate experiment, we replicated the conditions in the biofilm case but replaced the biofilm with a well-known, mass transfer-limited redox mediator system consisting of ferrocyanide (7.8 mM) oxidation to ferricyanide on the electrode. The purpose was to show how mass transfer resistance in the form of a Warburg response manifests and behaves using EIS. The oxidation of ferrocyanide to ferricyanide at the same rotation rates used for the biofilm is shown in Figure 5C. Increasing the rotation rate from 0 to 530 rpm yielded a current increase from 45 to 196 μA or 340% increase over the baseline, respectively. Figure 5D shows how the increased current is reflected in the impedance at select rotation rates. We used selected representative rotation rates to improve figure clarity (Fig. 5D).

There are two key differences between the *G.sulfurreducens* biofilm case and ferrocyanide case when comparing Figure 5A/B and C/D. First, the magnitude of the current increase in response to an increase in rotation rate for the biofilm decreased as the rotation rate was increased to 530 rpm. For the ferrocyanide control, the magnitude of the current increase in response to an increase in rotation rate increased. Second, the biofilm impedance changed only slightly as the mid to low frequency regions shifted down with increasing rotation rate. The ferrocyanide impedance changed dramatically as the diffusion tail at low rotation rates (<40 rpm) shifted down towards the x -axis, forming the typical Warburg response seen at higher rotation rates (>160 rpm; Macdonald, 1987). The ferrocyanide impedance response reflects the shift from semi-infinite diffusion towards a planar electrode to finite diffusion through a stagnant film developed near a rotating electrode, which is sometimes referred to as the Nernst Diffusion Layer. This change would directly affect soluble electron transfer mechanisms such as ferrocyanide oxidation. However, bound electron transfer mechanisms would not be affected directly but could be indirectly affected through the enhanced transport of solvent or counter ions through the stagnant film near the electrode surface. For the *G.sulfurreducens* biofilm, we interpret the increase in current and change in the impedance spectra on the basis that rotation reduces the accumulation of protons and increases the acetate delivery towards the bottom of the biofilm (Babuta et al., 2012; Renslow et al., 2013; Torres et al., 2008). Rotation of the electrode did not directly affect the electron transfer mechanism inside the biofilm and reaffirms that *G.sulfurreducens* biofilms utilize conductive electron transfer. Though microscale gradients inside the biofilm can generate suboptimal conditions for electrode respiration, the fact that current did not

increase 2-fold, or even 10-fold, reiterates what has been found in the literature that it is not always diffusion of protons that limit *G.sulfurreducens* biofilms (Babauta et al., 2012). Considering the mass transfer-controlled current to be 82 mA, the maximum kinetic current in the absence of mass transfer limitations for the *G.sulfurreducens* biofilm under study was 102 μ A.

EIS of *G.sulfurreducens* Biofilms at Non-Turnover Conditions

To determine the minimum and maximum electron transfer resistance under acetate limitations, acetate was removed by washout and parts of the biofilm moved from turnover to non-turnover conditions. Figure SI-4 shows the gradual decrease in current as acetate is removed and its effect on the biofilm CV. The non-turnover CV in Figure SI-4 shows that the multiple redox reactions are still functioning under non-turnover conditions. As the normalized current decreases from 100% to 2% in Figure 6A–C, the difference in the low frequency biofilm impedance between 0 and 530 rpm increased. This reflects the increasing difficulty for current to pass with lower acetate concentration. However, when normalized current reaches less than 1%, rotation did not affect the biofilm impedance. Because the biofilm impedance is unresponsive to rotation in Figure 6D, it excludes the possibility that the low-frequency impedance region is a diffusion-tail (Warburg response) similar to the one shown in Figure 5D for a rotation rate of 10 rpm. The impedance response is more likely the result of the oxidation/reduction of bound mediators in the biofilm that can be modeled as a pseudocapacitance, C_1 , as shown in Figure 1B. Additionally, when acetate becomes current-limiting, we expect that the impedance spectra take the form of some combination of Figure 6A and D depending on the extent of the acetate-limitation. From this perspective, the impedance spectra in Figure 6B and C are logical transitions from turnover to non-turnover conditions. They represent instances where the bottom of the biofilm is limited by acetate while the top still had access to acetate (Renslow et al., 2013). Impedance measurements under these conditions are indicative of electron transfer limitations associated with acetate inside the biofilm *even though* the bulk acetate concentration could be as high as 20 mM.

For simplicity, we analyze only the purely turnover and non-turnover cases shown in Figure 6A and D by fitting the impedance data to the EECs shown in Figure 1A and B, respectively. When the impedance spectra shown in Figure 6D were fit to the EEC in Figure 1B, a relatively good fit was found and an example fit for the data taken at 530 rpm is shown in Figure SI-5. Table SI-1 lists the fitted parameters for the impedance spectra shown in Figure 6A and D. Interestingly, the only fitted parameter that showed a significant change from turnover to non-turnover conditions was R_3 , the interfacial resistance. R_2 and Q_2 showed marginal differences that could be attributed to fitting error whereas R_3 increased nearly five-fold from $900 \pm 360 \text{ } \Omega$ to $4,200 \pm 260 \text{ } \Omega$. For the non-turnover conditions, C_1 was found to be 740 ± 41 and $760 \pm 43 \text{ mF}$ at 0 and 530 rpm, respectively.

Any change in the fitted parameters is expected to reflect the single change in the state of the biofilm during the shift from turnover to non-turnover conditions, which is the biofilm metabolism. This reasoning can be justified by the observations that *G.sulfurreducens* biofilms can be resuscitated from non-turnover conditions readily without loss in current

(Bond and Lovley, 2003). More recently, it was shown that the conducted current—different from the catalytic current from acetate oxidation—in *G.sulfurreducens* biofilms grown on gold interdigitated microelectrode arrays was nearly identical under both turnover and non-turnover conditions (Snider et al., 2012). Furthermore, biofilm conductivity measurements spanning a gap showed marginal changes between turnover and non-turnover conditions (Malvankar et al., 2012a). Therefore, we interpreted the increase in R_3 as the resistance to transfer electrons to the electrode and that this resistance is overcome by the biofilm metabolism. Approximately 3,300 Ω of resistance is driven by the biofilm metabolism. Since the metabolic activity is affected by the acetate concentration, the increase in R_3 can also be thought of as the increase in electron transfer resistance due to acetate limitations. In the absence of the electron flux from acetate oxidation, the bound mediators can be oxidized/reduced (Figure SI-4) unlike turnover conditions where reduction is negligible (at low scan rates) and is the origin of the pseudocapacitance.

Pseudocapacitance of the Biofilm Redox Mediators

We have used the fitted parameter, C_1 , to represent the pseudocapacitance of the biofilm redox mediators, which we assume are essentially heme groups within cytochromes utilized in the overall electron transfer mechanism. We differentiate C_1 from both the film capacitance, Q_1 , and interfacial capacitance, Q_2 , on the basis that both Q_1 and Q_2 could contain elements of real, non-faradaic capacitances such as the double layer capacitance. Because C_1 appears only under non-turnover conditions, the capacitive behavior is likely due to the charge stored within the biofilm redox mediators during oxidation or reduction. Capacitive behavior that manifests in impedance spectra that is the result of Faradaic processes is a form of electrochemical pseudocapacitance. Conway (1999) related pseudocapacitance of this type via the Nernst equation and mathematical manipulation to arrive at:

$$C = Q \frac{F/RT \cdot \exp(\Delta E \cdot F/RT)}{[1 + \exp(\Delta E \cdot F/RT)]^2}, \quad (1)$$

where C is the pseudocapacitance (F), Q the total charge stored (C), F the Faraday's constant (96,485 C/mol e^-), R the universal gas constant (8.3145 J/mol K), T the temperature (K), and E is the difference between the equilibrium redox potential and the standard potential of the redox mediator (formal potential in this case; Conway, 1999). Experimentally, we treat the open circuit potential as the equilibrium redox potential and the half-current potential on the biofilm CV as the formal potential representing the overall electron transfer process, which has been used previously to estimate heme content in *G.sulfurreducens* biofilms (Malvankar et al., 2012b). The open circuit potential under turnover conditions at max current was measured to be $-460 \text{ mV}_{\text{Ag/AgCl}}$ whereas the formal potential was taken to be $-340 \text{ mV}_{\text{Ag/AgCl}}$. This yields a value for ΔE of 0.12 V, which is shown in Figure SI-6 as a solid vertical line. For a pseudocapacitance of 740 μF and a ΔE of 0.12 V, the estimated amount of heme, assuming 1 mol electron transferred per heme, is $\sim 20 \text{ nmol}$. This value is comparable with previous studies where a range of approximately 10–50 nmol heme content was measured (Esteve-Nunez et al., 2008; Malvankar et al., 2012b). The fact that the pseudocapacitance is only observable with decreasing normalized current in Figure 6A–D—

which followed the removal of acetate—suggests that it could be an indicator of acetate limitations inside *G.sulfurreducens* biofilms.

Conclusions

We used a rotating disk electrode to determine the biofilm resistance, biofilm capacitance, interfacial resistance, interfacial capacitance, and pseudocapacitance for *G.sulfurreducens* biofilms. We have shown that an EEC with two time constants in parallel adequately fit biofilm impedance over the growth of the *G.sulfurreducens* biofilm. Rotation of the biofilm electrode up to 530 rpm increased the current by only 24% above baseline of 82 μ A whereas a ferrocyanide control increased by over 340% above baseline of 45 μ A. The response of the biofilm electrode to rotation identifies that microscale gradients formed inside the biofilm do not dominate the electron transfer rates. Under non-turnover condition, we estimated the maximum electron transfer resistance that the biofilm metabolism overcomes as 3,300 Ω . Additionally, a pseudocapacitance of 740 μ F was measured representing the redox mediators bound inside the biofilm. An estimated heme content of 20 nmol was derived from the pseudocapacitance. When *G.sulfurreducens* biofilms become acetate-limited, electron transfer resistance will increase and bound redox mediators will be under-utilized and observable via a pseudocapacitance. This is true when the bottom of the biofilm experiences acetate limitations even in the presence of bulk acetate and highlights the importance of accounting for acetate limitations inside *G.sulfurreducens* biofilms while studying other bioelectrochemical phenomena.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Equivalent electrical circuit models used to fit impedance spectra under turnover (**A**) and non-turnover (**B**) conditions. **C:** Physical interpretation of the circuit elements in the model under turnover conditions.

Figure 2. Schematic of glass cell used to grow *G. sulfurreducens* biofilms on a glassy carbon rotating disk electrode (working electrode).

Figure 3.

A: Current production of the *G.sulfurreducens* biofilm on the rotating disk electrode. The inset shows a section of the biofilm removed by cutting. **B:** Impedance spectra of *G.sulfurreducens* biofilm at increasing current values. The legend shows the current generated by the biofilms before measurements were taken. The corresponding Bode plot showing frequency information is shown in Figure SI-2.

Figure 4.

Change in Q_1 (A), Q_2 (B), Q_2 (C), and Q_3 (D) with increasing current under turnover conditions. The equivalent electrical circuit shown in Figure 1C was used to fit the impedance data shown in Figure 3B. R_1 , the solution resistance, remained constant as current increased at a value of $130 \pm 16 \Omega$.

Figure 5.

Increasing current response to rotation of the *G. sulfurreducens* biofilm from 0 to 530 rpm (A). Corresponding impedance spectra at each discrete rotation rate (B). Ferrocyanide oxidation under identical setup and conditions as the biofilm (C). Corresponding impedance spectra for ferrocyanide oxidation (D). 0, 20, and 80 rpm were omitted to improve figure clarity.

Figure 6.

Impedance spectra of *G.sulfurreducens* biofilm at 100% (A), 30% (B), 2% (C), and less than 1% (D) normalized current. Impedance spectra were obtained during the removal of acetate from the reactor volume. Current was normalized by dividing by the pseudo-steady current of 82 μA .