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The Roles of Biofilm Conductivity and Donor Substrate Kinetics in a Mixed-Culture Biofilm Anode

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Abstract

We experimentally assessed the kinetics and thermodynamics of electron transfer (ET) from the donor substrate (acetate) to the anode for a mixed-culture biofilm anode. We interpreted the results with a modified biofilm-conduction model consisting of three ET steps in series: (1) intracellular ET, (2) non-Ohmic extracellular ET (EET) from an outer membrane protein to an extracellular cofactor (EC), and (3) ET from the EC to the anode by Ohmic-conduction in the biofilm matrix. The steady-state current density was $0.82 \pm 0.03 \text{ A/m}^2$ in a miniature microbial electrochemical cell operated at fixed anode potential of -0.15 V versus the standard hydrogen electrode. Illumina 16S-rDNA and -rRNA sequences showed that the *Geobacter* genus was less than 30% of the community of the biofilm anode. Biofilm conductivity was high at $2.44 \pm 0.42 \text{ mS/cm}$, indicating that the maximum current density could be as high as 270 A/m^2 if only Ohmic-conduction EET was limiting. Due to the high biofilm conductivity, the maximum energy loss for Ohmic-conduction EET was negligible, 0.085 mV . The energy loss in the second ET step also was small, only 20 mV , and the potential for the EC involved in the second ET was -0.15 V , a value documenting that $>99\%$ of the EC was in the oxidized state. Monod kinetics for utilization of acetate were relatively slow, and at least 87% of the energy loss was in the intracellular step. Thus, intracellular ET was the main kinetic and thermodynamic bottleneck to ET from donor substrate to the anode for a highly conductive biofilm.

Graphical Abstract

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Introduction

Electron transfer from a dissolved electron donor to a conductive solid as the bacteria's terminal electron acceptor is of interest to environmental scientists and engineers due to its role in bioremediation of contaminants in sedimentary environments and resource recovery in wastewater treatment.(1–3) In the latter case, microbial electrochemical cells (MxCs) exploit this unique form of respiration as a means to oxidize organic contaminants and generate an energy-rich output at the same time.(3–5) Current density to the anode of an MxC is one of the significant parameters that should be optimized, since anode kinetics typically is rate-limiting in high-performance MxCs.(6, 7)

Electron transfer from the soluble donor substrate to the anode in an MxC can be divided into intracellular and extracellular electron transfer (IET and EET). Although the Monod equation describes well the kinetics of IET,(8–10) EET kinetics depends on mechanisms that include direct contact, diffusion of soluble mediators, long-range electron hopping (or superexchange), and Ohmic conduction through the biofilm matrix.(6–9, 11–14) Direct contact and mediator-dependent EET cannot support high current density MxCs,(6) but electron hopping and Ohmic conduction can lead to high current density, i.e., > several A/m².(6, 7, 15) Although electron hopping and biofilm conduction have been portrayed as competing ways of understanding EET,(16, 17) they share the common features that electrons flow to the anode is extracellular and is driven by a potential gradient within the biofilm anode.(9, 12, 18)

Ohmic conduction describes electron transfer in conductors where the electron-transfer rate (the current) is directly proportional to potential gradient across the conductors and the biofilm's conductivity (K_{bio}). Ohm's law describes well the current response to voltage at scales of a few to hundreds of micrometers,(19, 20) which means that EET kinetics obeys Ohm's law in electronically conductive biofilms.(9, 13, 18)

A conduction-based approach using Ohm's law has been applied successfully to represent the kinetics of biofilm anodes having high current density.(6–10, 13, 18, 21) Marcus et al.(9) originally developed an Ohmic-conduction model for electron transfer from donor substrate to the anode by using a novel form of dual-limitation kinetics: Terms for the concentration

of the donor substrate and the potential of the anode acceptor were combined in the Nernst–Monod model. Torres et al.(6, 10) validated the Nernst–Monod model, and their analysis of experimental data suggested that biofilm conductivity (K_{bio}) was higher than 0.5 mS/cm. Malvankar et al.(13, 18) experimentally quantified K_{bio} for *Geobacter* pure and dominant cultures in biofilm anodes: It ranged from 0.2 to 5 mS/cm, values high enough that the EET by conduction could have been much faster than other electron-transfer steps. However, the Nernst–Monod model does not directly account for biofilm conductivity, but assumes that the conductivity is high enough that the potential drop across the biofilm is negligible.

For biological systems, electron hopping describes electron conduction at angstrom to nanometer scales in photosynthetic reaction centers(22) or cytochromes(23) in membranes. Due to the small-scale feature of electron-hopping, quantum physics becomes important, and the electron-hopping mechanism is described with Marcus’s theory.(23) If EET occurs through long-range electron hopping in a biofilm anode, a network of closely spaced extracellular cofactors (ECs) must be well-distributed throughout the biofilm to accept and pass along the electrons.(12, 24) The EC concentration in the biofilm determines EET kinetics when electron hopping is viable. If long-range electron hopping is active in a biofilm at the micrometer scale, Ohm’s law may be able to describe the kinetics of electron hopping, although quantum effects (i.e., electronic wave functions) are significant in atomic and molecular levels.(25)

Although Ohmic conduction often describes steady-state EET kinetics well, a number of observations point to the importance of ECs involved in EET when the system is not at steady state. For example, cyclic voltammetry (CV) experiments have shown “bumps” in the sigmoidal pattern of CVs for *Geobacter sulfurreducens* pure- and enriched-culture biofilm anodes experiencing substrate nonlimiting conditions;(8, 10, 26) deviations from the sigmoidal pattern cannot be explained and simulated with an Ohmic Nernst–Monod model. The bumps support a capacitance effect of ECs: Reduced ECs can rapidly discharge electrons due to an increase in the anode’s potential in the upward part of a CV test. Likewise, the oxidized EC can rapidly be charged with electrons as the anode potential declines.(12, 26) The literature directly and indirectly suggests the presence of the ECs associated with EET in *Geobacter sulfurreducens* pure- and enriched-culture biofilms.,(8, 10, 12, 26) and these biofilms can have high electrical conductivity.(13, 17, 18) However, the Nernst–Monod model does not consider ECs for electron transfer in biofilm anode. Kinetic and thermodynamic understandings of complex electron-transfer mechanisms from soluble electron donor to the anode are still limited for mixed-culture biofilm anode, although individual EET mechanisms have been explored.(9–18)

In this study, we assess the rate-limiting step for electron transfer from a donor substrate to the anode for a conductive mixed-culture biofilm by interpreting experimental results with a modified biofilm-conduction model; EET by shuttle-based mediators was not included. The new model consists of three electron-transfer steps: (1) intracellular electron transfer from the donor substrate to outer membrane proteins (OMP), (2) non-Ohmic electron transfer from an OMP to an EC, and (3) conductive EET from the EC to the anode governed by Ohm’s law; we include electron hopping within “non-Ohmic electron transfer”. We quantify biofilm conductivity and thickness for the biofilm anode of a steady-state MxC, identify the

bacterial community structure in the biofilm anode, and estimate Monod kinetic parameters for the ARB. We then evaluate the thermodynamics and kinetics for the three electron-transfer steps, identify the rate-limiting step, and finally discuss the implication of a highly conductive biofilm for biofilm-anode kinetics.

Electron-Transfer Model for a Biofilm Anode

Marcus et al.(9) proposed a conduction-based model for electron transfer from a soluble electron donor to the anode based on dual-limitation kinetics(27, 28) in which the biofilm matrix is considered a porous conductor:

$$j = \underbrace{0.14(1 - f_s^o)q_{\max,app}X_f L_f \frac{S_d}{K_{sd,app} + S_d}}_{\text{Monod term}} \underbrace{\left[\frac{1}{1 + \exp\left(-\frac{nF}{RT}(E_{\text{anode}} - E_{K_A})\right)} \right]}_{\text{Nernst-Monod term}} \quad (1)$$

where j is the current density (A/m^2), 0.14 is the conversion factor for converting substrate flux to current density ($0.14 \text{ A} = 1 \text{ g COD/d}$), f_s^o is the fraction of electrons used for cell synthesis, $q_{\max,app}$ is the apparent maximum specific substrate utilization rate (g COD/g VS-d), X_f is the concentration of active ARB in the anode biofilm (g VS/m^3), L_f is the biofilm thickness (m), S_d is the donor substrate concentration (mg COD/L), $K_{sd,app}$ is the apparent half-maximum-rate concentration of donor substrate (g COD/m^3), E_{anode} is the anode potential (V), E_{K_A} is the anode potential when $j = j_{\max}/2$ (V), R is the ideal gas constant (8.3145 J/mol-K), F is the Faraday's constant ($96,485 \text{ C/mol e}^-$), T is temperature (298.15 K), and n is the number of electrons transferred (assumed to be 1).(8, 9, 29)

The right-hand term in eq 1, which is called the Nernst-Monod term, is based on two hypotheses. The first is that the “concentration” of the electron acceptor, which is the anode, can be related to the anode potential via the Nernst Equations. The second hypothesis is that EET by conduction is fast enough that the potential within the biofilm does not deviate significantly from E_{anode} . Although a substantial amount of literature indirectly supports these hypotheses,(8, 10, 29, 30) direct evidence considering overall anode kinetics is absent. To evaluate the underpinning of the Nernst-Monod term in eq 1, we need to know the biofilm conductivity (K_{bio} , S/m), from which we can assess EET's impact on the potential in the biofilm and the kinetics of electron transfer.

Here, we modify the Nernst-Monod equation to include the effect of biofilm conductivity on the potential at locations in the biofilm away from the anode surface. We envision that donor electrons are transferred to the anode in three steps that are illustrated in Figure 1a: (1) from the substrate to outer membrane proteins (OMP), (2) from OMP to an EC, and (3) from the EC to the anode via Ohmic conduction. We assume that electrons transfer from the OMP to the EC in a non-Ohmic manner, since the distance z is in a nanometer scale in the proposed electron-transport model ($z \ll L_f$).(12, 31)

Applying potential drops in series, we modify the Nernst-Monod term of eq 1 to

$$\left[\frac{1}{1 + \exp\left(-\frac{F}{RT}(E_{\text{anode}} - \Delta E_{\text{EET}} - E_{\text{KA,EC}})\right)} \right] \quad (2)$$

where E_{EET} is an average energy loss in conductive EET of the third electron-transfer step (V), and $E_{\text{KA,EC}}$ is a rate-limiting EC potential at which current density is half of the maximum current density (V). $E = G/F$, where E is electrical potential energy (V), G is the Gibbs free energy (kJ/mol of electrons), and F is the Faraday constant (96.485 kJ/V-mol). $E_{\text{KA,EC}}$ is equal to E_{KA} in the original Nernst–Monod when $E_{\text{EET}} = 0$, indicating no potential drop in the biofilm anode. With the formulation of eq 1, E_{KA} is a composite potential that includes E_{EET} , which depends on biofilm conductivity, biofilm thickness, and current density. We provide the derivation of the modified Nernst–Monod equation in Supporting Information (SI).

Based on Ohm's law, $\Delta E_{\text{EET}} = \left(\frac{f}{2}\right)\left(\frac{L_f}{2}\right)\frac{1}{K_{\text{bio}}}$, where K_{bio} is the biofilm conductivity (S/m), $L_f/2$ is the midpoint of biofilm thickness (which is $\gg z$), and $j/2$ is an estimate of the current density at the biofilm's midpoint. We assumed that the acetate concentration gradient through the biofilm anode was approximately linear due to the high acetate concentration in bulk liquid and modest biofilm thickness.(8) With a linear substrate gradient, the average flux ($j/2$) corresponds to the average thickness ($L_f/2$). We also assumed that K_{bio} was constant across the biofilm anode. Then, eq 2 can be expressed as

$$\left[\frac{1}{1 + \exp\left(-\frac{F}{RT}(E_{\text{anode}} - \frac{jL_f}{4K_{\text{bio}}} - E_{\text{KA,EC}})\right)} \right] \quad (3)$$

Substituting eq 3 into eq 1 gives the Nernst–Monod expression that includes resistance in a steady-state biofilm:

$$j = 0.14(1 - f_s^o)q_{\text{max,app}}X_fL_f\left(\frac{S_d}{S_d + K_{\text{sd,app}}}\right) \left(\frac{1}{1 + \exp\left[-\frac{F}{RT}(E_{\text{anode}} - \frac{jL_f}{4K_{\text{bio}}} - E_{\text{KA,EC}})\right]} \right) \quad (4)$$

Materials and Methods

Microbial Electrochemical Cell (MxC) Design, Inoculation, and Operation

The dual-chambered, miniature MxC, illustrated in Figure 1, was specially designed and fabricated for measuring biofilm conductivity. Two gold electrodes (5 mm × 8 mm × 100 nm each) (Figure 1b) with a gap of 100 μm were placed on a glass base (Figure 1c), and copper tape was attached to the surface of each gold electrode for establishing an external

connection. We used a rigid graphite plate (16 mm × 32 mm) with an available electrode surface area of 0.8 cm² as the cathode and a titanium wire for the external connection. Two nonconductive polyethylene mats (16 mm × 32 mm × 3 mm) with rectangular holes (5 mm × 16 mm) were used as well layers for the anode and the cathode chamber, which created a working volume of 256 µL for each chamber. We employed a cation exchange membrane (Nafion 112, Dupont) as the separator between the two chambers and assembled all components together tightly with a clamp to prevent liquid leakage. A reference electrode (Ag/AgCl reference electrode, MF-2052, Bioanalytical System Inc.) was inserted in an effluent line of the anode chamber within 1 cm distance from the anode to fix the anode potential at −0.15 V vs the standard hydrogen electrode (SHE) using a potentiostat (BioLogic, VSP, Gamble Technologies, Canada) during experiments.(32) For continuous operation two needles were inserted into each chamber through the polyethylene mat for feeding medium and collecting effluent, as shown in Figure 1d.

The MxC was inoculated with the effluent from a dual-chamber mother MxC that had been run with 25 mM acetate medium(33) for more than 8 months. The genus *Geobacter* was dominant (98%) in the biofilm anode of the mother MxC. To facilitate biofilm formation on the gold anodes during acclimation, we continuously fed the MxC with a mixture of acetate medium (25 mM acetate and 50 mM phosphate buffer) and the mother MxC's effluent (5:1, v/v) until we observed positive current from the MxC. After that, we fed only the acetate medium to the MxC anodes. To avoid loss of water volume due to evaporation in the cathode chamber, where hydrogen gas was produced, we continuously fed tap water to the cathode chamber. Throughout all experiments, including acclimation, we maintained a hydraulic residence time (HRT) of 1 h for each chamber using the syringe pumps (Cole-Parmer programmable ten-syringe infusion/withdrawal pump, model 230, Cole-Parmer, Canada) at a flow rate of 6.15 mL/d. Current and electrode potentials were recorded at every 2 min using EC-Lab for Windows v. 10.12 software with the potentiostat connected to a personal computer. The MxC was operated in a temperature-controlled room at 26 ± 1 °C.

Biofilm Conductivity

Biofilm that spanned the two gold anodes established an electrical connection having a distance of 100 µm. We quantified biofilm conductivity across this 100-µm span using the two-probe conductance measurement method proposed by the Malvankar et al.(13, 18) The anodes and the cathode were disconnected temporarily (open-circuit mode), and then a small voltage was applied across the two gold anodes (0, 25, or 50 mV) to observe the biofilm conductance (G_{observed} , mS) using current–voltage curves. We measured biofilm conductance in five cycles with substrate nonlimiting conditions until a steady state current–voltage response (I–V characteristic) was obtained. The duration between individual cycles was 1 min, which allowed any effect of biofilm capacitance on current generated in K_{bio} measurement to be discharged.(34) To account for ionic current between the two gold anodes, we measured ionic conductance (G_{control} , mS) with an identical, abiotic MxC (control) using acetate medium ($G_{\text{biofilm}} = G_{\text{observed}} - G_{\text{control}}$). We recorded the current using a highly stable semiconductor characterization system (Agilent 4155C semiconductor parameter analyzer, Agilent Technologies, CA). Then, the conductivity of the biofilm

(k_{biofilm} , mS/cm) was computed from the biofilm conductance (G_{biofilm}) using conformal mapping (Schwarz-Christoffel transformation):(18, 35)

$$K_{\text{biofilm}} = G_{\text{biofilm}} \frac{\pi}{L} \ln \left(\frac{8L_f}{\pi a} \right) \quad (5)$$

where G_{biofilm} is the biofilm conductance (mS), L is the length of the electrodes (0.5 cm), and a is half of the nonconductive gap between two electrodes (100 μm). The biofilm conductivity was measured in quintuplicate.

Based on the nearly identical CV patterns for each anode after the anodes were connected via the conductive biofilm (shown at Figure S2 in SI), we treated the two anodes as one electrode serially connected through the conductive biofilm for cyclic voltammetry and kinetic experiments.

Cyclic Voltammetry (CV)

To determine the EC half-saturation potential ($E_{\text{KA,EC}}$) in the modified Nernst–Monod equation, we carried out CV tests with conditions in which the acetate concentration did not affect the current density (i.e., 25 mM acetate in the input medium, HRT of 1 h, and effluent acetate >20 mM).(6, 8, 10) Anode potential was ramped from -0.33 to $+0.01$ V vs SHE at a scan rate of 1 mV/s using the potentiostat. The current and anode potential were recorded every 5 s using EC-Lab for Windows v. 10.23 software. To ensure the reproducibility of the CV data, we conducted CV experiments in quadruplicate. To minimize capacitance effects, we averaged the latter three CVs to report j versus $E_{\text{KA,EC}}$ for fitting eqs 1 and 6.

$$j = j_{\text{max,app}} \left[\frac{1}{1 + \exp \left(-\frac{F}{RT} \left(E_{\text{anode}} - \frac{jL_f}{4K_{\text{bio}}} - E_{\text{KA,EC}} \right) \right)} \right] \quad (6)$$

where $j_{\text{max,app}}$ is the apparent maximum current density (A/m^2) ($j_{\text{max,app}} = 0.14(1 \text{ fs}^\circ) q_{\text{max,app}} X_f L_f$) under substrate nonlimiting conditions.

Estimating Apparent Monod Kinetic Parameters

We monitored current density and effluent acetate concentration in the MxC by changing the acetate concentration from 133 to 2000 mg COD/L. Using eq 4 and the measured parameters (L_f , $E_{\text{KA,EC}}$, j , and acetate concentration), we estimated the best-fit $K_{\text{s,app}}$ (mg COD/L) and $q_{\text{max,app}} X_f$ (g COD/ $\text{m}^3\text{-d}$) with a relative least-squares method(36) and MS 2007 Excel solver. We assumed $f_s^0 = 0.1$ for the estimation.(8, 10)

Measurement of Biofilm Thickness (L_f)

At the end of the experiments (i.e., for biofilm conductivity, CV, and Monod kinetic parameters), we measured L_f for individual anodes with a stainless steel conductive microelectrode connected with a motorized micromanipulator (MM33, Unisense A/S, Denmark). To minimize biofilm damage by the microelectrode, we only measured L_f one

time for each anode, and we averaged the two values. SI provides detailed procedures on the measurement of biofilm thickness.

Acetate Measurement

The acetate concentration was analyzed using a gas chromatography (GC) (Model: Hewlett-Packard HP 5890 Series II) equipped with flame ionization detector (FID).(33)

Microbial Community Analysis on Biofilm Anode

At the end of the experiments, we collected all biofilm on the two anodes by scraping with a sterilized spatula. Total RNA and DNA were extracted from the biofilm as previously described.(37) Briefly, the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany) was used to extract total nucleic acid. RNA was further purified using Ambion TURBO DNA-free DNase kit (Life Technologies, Grand Island, NY), and cDNA was generated using random hexamer primed Superscript III system (Life Technologies). We used barcoded 16S rRNA gene targeting primers (i.e., 515F and 806R)(38) and then sequenced the targeted product (i.e., 291bp) in both directions using an Illumina MiSeq PE250 approach. Sequence reads (16S rDNA- and 16S rRNA-based) were processed and analyzed using Mothur software.(39) Sequence reads were grouped at a 97% similarity, and the consensus sequences were then identified using Mothur and the Silva database(40) as a reference. Excel was used to determine the overall relative abundance of representative sequences at different taxonomic levels (e.g., class, order, family, and genus). Sequences were analyzed using Blast (<http://www.ncbi.nlm.nih.gov/BLAST/>) and RDP classifier(41) to further confirm their phylogenetic affiliation and to classify sequences at a low taxonomic level (genus and species).

Results and Discussion

Current Density, Biofilm Thickness, and Biofilm Community

The steady-state current density reached 0.82 ± 0.03 A/m², a modest value characteristic of mini microbial electrochemical cells equipped with gold electrodes.(42–44) The measured biofilm thickness (L_f) averaged 100 μ m based on duplicate measurements (85 and 115 μ m for each anode), which is relatively thick compared to other mini microbial electrochemical cells.(18, 45, 46) The biofilm thickness was relatively uniform in the MxC, probably due to the smooth surfaces of the gold anodes (see Figure S5). Hence, duplicate measurements with a range of only ± 15 μ m represented the average biofilm thickness well. We confirmed the relatively uniform thickness of biofilms on gold electrodes using six measurements with the equivalent devices and operating conditions; these results are in SI.

A total of 74 938 and 111 125 sequences were used for rDNA and rRNA libraries, respectively. Proteobacteria was the most abundant phyla in terms of DNA (about 68% of the reads) and RNA (94%) libraries (Table 1). *Geobacter* and *Rhodopseudomonas* genera and the *Rhodocyclaceae* family (close to *Azoarcus* and *Thauera* genera) were the most numerically abundant bacteria in RNA library, along with $\sim 10\%$ population of *Azospirillum* and *Aeromonas* genera. The literature has reported isolation of or genomic dominance by *Geobacter*, *Rhodopseudomonas*, *Azoarcus*, *Thauera*, *Azospirillum*, and *Aeromonas* genera

in biofilm anodes.(47–50) Except for the *Geobacter* genus, these ARB produce low to moderate ranges of current density, from 0.3 to 5 A/m² in acetate-fed MxCs(49, 51, 52, 54–57) or MxCs run with simple organics (C1 to C3).(53, 58, 59) Thus, our biofilm anode was dominated by known ARB, but *Geobacter* was only 27% (DNA) or 21% (RNA). We cannot completely exclude that a small amount of O₂ may have intruded into the anode chamber of the MxC, perhaps through the feeding tubing. Due to the small anode chamber (256 μL), a small O₂ intrusion might have had an impact on the microbial community, even though similar O₂ intrusion is insignificant for larger anodes in liter-scale MxCs.(60) This O₂-based scale effect might have led to a relatively diverse biofilm community on the anode. This lower dominance of *Geobacter* may have been at least partially the reason for the modest current density. The results for the 16S rRNA gene and 16S rRNA are similar and complementary, which supports that the dominant bacteria in the biofilm were metabolically active.

Biofilm Conductivity

The observed biofilm conductance was 1.00 ± 0.11 mS from quintuplicate measurements. Control tests without biofilm had ionic conductance of 0.37 ± 0.00 mS, indicating that the average G_{biofilm} was 0.63 mS (see Table 2). Using eq 5, the average biofilm conductivity (K_{bio}) was computed to be 2.44 ± 0.42 mS/cm, which is about 1 order of magnitude larger than the value for a mixed-culture biofilm anode dominated with *Geobacteraceae* family,(61) but smaller than the value for pure-culture of *G. sulfurreducens* biofilm anode.(13) We did not monitor anode potential during K_{bio} measurement, but supplementary tests using a potentiostat showed negligible variation of anode potential (<20 mV) when a potential of 50 mV was applied between the two gold electrodes. In addition, the literature(62) clearly proves that K_{bio} was not altered when the anode potential was changed from –0.2 V to +0.2 V vs SHE in steady-state mixed culture biofilm anodes, further supporting the validity of the K_{bio} value (2.44 mS/cm) obtained in this study. The K_{bio} value is large enough to confirm that the biofilm anode was electrically conductive.(9, 10, 13)

Using the measured L_f and K_{bio} values, we computed the average potential loss ($E_{\text{EET}} = jL_f/4K_{\text{bio}}$) for the final step of electron transfer: 0.085 mV. In comparison, the overall energy loss from donor substrate and the anode was much larger: 150 mV ($E_{\text{anode}} - E_{\text{acetate}}' = (-0.15 \text{ V}) - (-0.30 \text{ V}) = 0.15 \text{ V}$) for the steady-state MxC; see SI for computation of E_{acetate}' . Thus, E_{EET} was negligible for electron transfer from the donor substrate to the anode (only 0.06% of the total energy loss), which indicates that the conductive EET was not an energy bottleneck for electron transfer from donor substrate to the anode. We also calculated the maximum current density achievable in the final electron-transfer step using Ohm's law ($j = K_{\text{bio}} (E_{\text{anode}} - E_{\text{EC}}^{\circ'})/L_f$); see SI for details of the computation at standard conditions for a rate-limiting EC at pH 7 where EC concentration does not affect E_{EC} : ($E_{\text{EC}}^{\circ'} = -0.26 \text{ V vs SHE}$).(12) The maximum current density in the final step was calculated as 270 A/m², a value over 300-fold higher than our observed steady-state current density (0.82 A/m²) and about 20-fold higher than maximum current densities of 10–14 A/m² observed in high-performing MxCs equipped with carbon-based anodes.(10, 29, 33) A recent study reported that E_{EC} was –0.19 V in a *Geobacter sulfurreducens* biofilm anodes.(63) The maximum current density in the final step was computed to be 97 A/m² with the observed E_{EC} , which

still is 7- to 10-fold higher than seen with high-performing MxC anodes. This result further supports that conductive EET is not the current-limiting factor in microbial electrochemical cells.

Estimation of Half-Saturation EC Potential ($E_{KA,EC}$)

Figure 2 shows the average of three forward CVs for the steady-state biofilm under substrate-nonlimiting conditions. The sigmoidal CV matches the Nernst–Monod pattern well. $E_{KA,EC}$ for the steady-state biofilm was -0.28 ± 0.00 V vs SHE. Since E_{EET} (0.085 mV) was trivial, E_{KA} also equals -0.28 V. This small E_{EET} explains why CVs simulated with the original Nernst–Monod equation ignoring E_{EET} term matched the experimental CVs in Figure 2, as well as in the literature.(10) $E_{KA} = -0.28$ V is more negative than E_{KA} values reported in the literature, which range from -0.15 to -0.24 V vs SHE for *G. sulfurreducens*-enriched biofilms.(8, 10, 42) The more negative $E_{KA,EC}$ indicates that the current density saturated at a relatively low anode potential, a factor that favors energy recovery.(8–10) However, low current density would be limited by the first electron-transfer step. The literature shows more negative midpoint potential or E_{KA} as substrate concentration decreased in MxCs.(6, 7, 10)

Evaluation of Kinetics and Thermodynamics in the Second Electron-Transfer Step

The negligible E_{EET} indicates that E_{EC} at pH 7 (E_{EC}') was close to -0.15 V ($E_{EC}' = E_{anode} - E_{EET}$) for the steady-state current density. This computation accords well with tentative E_{EC}' values of -0.15 to -0.16 V reported by the literature.(64) According to the Nernst equation $\left(E_{EC}' = E_{EC}^{\circ'} - \frac{RT}{F} \ln \frac{EC_{red}}{EC_{ox}}\right)$, where EC_{red} is the reduced form of EC (mol/L) and EC_{ox} is the oxidized form of EC (mol/L), the high value of E_{EC}' means that EC_{ox} was 99% of the total EC for steady state (see SI for the computation). A recent work reported that E_{EC} was close to -0.19 V,(63) but EC_{ox} was still 94% of the total EC at E_{EC} of -0.19 V. Robuschi et al.(65) also showed that cytochromes were fully oxidized at the biofilm-electrode interface, supporting the dominance of EC_{ox} in our electron transfer model where the distance between OMP and EC is in nanometer scales. The dominance of EC_{ox} over EC_{red} means that the electrons transferred from an OMP to the terminal EC immediately moved to the final electron-transfer step. This implies that the second electron-transfer step (transfer from OMP to the EC) was much faster than the first electron-transfer step (donor–substrate oxidation with electron transfer to the OMP). This estimate accords well to literature suggesting that intracellular electron transfer would be slower than the electron transfer between ECs.(12, 14, 66)

Table 3 shows that the energy loss in the second electron-transfer step was 20 mV for the steady-state current density. This energy loss was only 13% of the overall energy loss for the anodic reaction (150 mV), meaning that the second electron-transfer step was not a significant energy barrier to electron transfer from donor substrate to the anode for the steady state.

Apparent Kinetic Parameters for Biofilm Anode

Based on the measured parameter values for L_f , $E_{KA,EC}$, j , and acetate, we estimated (using eq 6) the best-fit values of $K_{s,app}$ and $q_{max,app}X_f$ to be 274 g COD/m³ and 92×10^3 g COD/m³-d, respectively. The estimated $K_{s,app}$ is relatively high compared to a *Geobacter*-enriched biofilm anode, for which previous estimates ranged from 119 to 176 g COD/m³. (8, 10) The genus *Geobacter* accounted for only 21% (RNA) and 27% (DNA) in our biofilm anode (Table 1), whereas bacteria affiliated with the *Rhodopseudomonas* genus and the *Rhodocyclaceae* family (close to *Azoarcus* and *Thauera* genera) also were important. *Rhodopseudomonas*, *Azoarcus*, and *Thauera* genera can use electrodes as the terminal electron sink for anaerobic respiration, (51–59) but the high $K_{s,app}$ suggests that their kinetic features are inferior to those of *Geobacter*. Similarly, Kim et al. (58) reported that $K_{s,app}$ was as high as 466 g COD/m³ in a microbial fuel cell where the *Azoarcus* genus was the second largest in biofilm community. The best-fit $q_{max,app}X_f$ also was substantially lower than literature values, which range from 10^6 to 2×10^6 g COD/m³-d. (8, 29) This is another sign of inferior microbial kinetics of our biofilm anode as compared to *Geobacter*-enriched biofilm anode. The slow kinetics for utilization of acetate agrees well to the estimated predominance of EC_{ox} over EC_{red} in the second electron-transfer step.

Implication of a Highly Conductive Biofilm

Relatively poor biological kinetics (small $q_{max,app}X_f$ and high $K_{s,app}$) correlated with low abundance of *Geobacter* spp. (<30%) in the biofilm on the gold anodes. Nevertheless, biofilm conductivity was high at 2.44 mS/cm, and E_{EET} was trivial at 0.085 mV for the steady-state current density. Thus, the final electron-transfer step did not limit current density kinetically or thermodynamically.

Although the mechanism underlying the kinetics for the second electron-transfer is not resolved for mixed-culture biofilm anodes, (14–18) we were able to determine that EC_{ox} dominated (>99%) EC_{red}. This means that the kinetic “bottleneck” for electron transport from the donor substrate to the anode occurred at the beginning of catabolism, or steps involved in the transfer of electrons from donor substrate to the OMP. In parallel with the kinetic bottleneck, the potential loss in the first step (acetate oxidation with electron transfer to the OMP) accounted for 87% of the overall potential loss. While further studies are required to better gauge kinetics and thermodynamics of the second electron-transfer in biofilm anodes, our results clearly prove that the first electron-transfer step was the dominant kinetic and thermodynamic barrier to electron transfer from donor substrate to the anode in MxCs enriched with highly conductive biofilms, such as *Geobacter*-dominant biofilm anodes. Our study suggests that MxCs enriched with highly conductive biofilms could generate several hundred A/m² from oxidizing a donor substrate if substrate-utilization rate did not limit current density. Therefore, maximizing biofilm density (X_f) of ARB that are kinetically efficient and generate an electronically conductive matrix are apparent keys for maximizing the current density in MxCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic diagrams of a proposed electron-transfer model and the miniature, dual-chambered microbial electrochemical cell (MxC). (a) A three-step electron-transfer model from the donor substrate to the anode in a conductive biofilm anode. S: donor substrate, ARB: anode-respiring bacteria, OMP: outer membrane proteins, EC: the extracellular cofactor, z : an electron-transfer distance between the OMP and the EC (m), and L_f : biofilm thickness (m). The numbers 1, 2, and 3 indicate the three steps of electron transfer from substrate to the anode. Step 1: from the donor substrate to the OMP (intracellular catabolic

reactions described with the Monod equation); Step 2: from the OMP to the EC by non-Ohmic conduction (z is in nanometer scales); and Step 3: from the EC to the anode via Ohmic conduction. It is assumed that $L_f \gg z$. (b) Architecture of the dual-chambers, (c) schematic of the two gold anodes ($5 \text{ mm} \times 8 \text{ mm} \times 100 \text{ nm}$) on the glass base with a gap of $100 \text{ }\mu\text{m}$, and (d) schematic of test setup for operating the miniature microbial electrolysis cell.

Figure 2.

Experimental and simulated current-density profiles versus anode potential (j - E_{anode}). The blue line is the experimental forward CVs (1 mV/s) for the steady-state MxC, the red line is j vs E_{anode} simulated with the original Nernst–Monod equation, and the dotted black line is j vs E_{anode} simulated by the modified Nernst–Monod model. The red and dotted black lines are coincident.

Table 1.Distribution of Reads for the Bacterial 16S rDNA and 16S rRNA^a

class	genus	DNA (<i>n</i> = 74 938)	RNA (<i>n</i> = 11 1125)
alpha-proteobacteria	<i>Azospirillum</i>	–	9362 (8.4%)
	<i>Magnetospirillum</i>	775 (1.0%)	3396 (3.0%)
	<i>Ochrobactrum</i>	–	4573 (4.1%)
	<i>Pannonibacter</i>	2170 (2.9%)	2938 (2.6%)
	<i>Rhizobium</i>	5873 (7.8%)	–
	<i>Rhodopseudomonas</i>	–	18 149 (16%)
beta-proteobacteria	<i>Comamonadaceae</i> ^b	423	549
	<i>Rhodocyclaceae</i> ^c	19 476 (26%)	30 674 (28%)
delta-proteobacteria	<i>Desulfovibrio</i>	313	–
	<i>Geobacter</i>	20 210 (27%)	23 513 (21%)
gamma-proteobacteria	<i>Aeromonas</i>	2228 (3.0%)	10 818 (9.7%)
	<i>Stenotrophomonas</i>	403	2856 (2.6%)
bacteroidia	<i>Bacteroides</i>	596	–
	<i>Dysgonomonas</i>	4501 (6.0%)	1478 (1.3%)
cloacamonae	<i>Cloacamonaceae</i> ^b	439	473
clostridia	<i>Lachnospiraceae</i> ^b	1672 (2.2%)	–
	<i>Fusibacter</i>	696	229
flavobacteria	<i>Flavobacterium</i>	13 066 (17%)	214
spirochaetes	<i>Treponema</i>	649	328

^a–, not found or less than 200 sequences.^b Family.^c *Azoarcus* and *Thauera* belong to this family.

Table 2.Biofilm Conductance and Conductivity for the Steady-State Biofilm Anode^a

observed biofilm conductance (G_{observed} , mS)	1.00 ± 0.11
control conductance (G_{control} , mS)	0.37 ± 0.00
biofilm conductance (G_{biofilm} , mS)	0.63 ± 0.11
biofilm conductivity (K_{bio} , mS/cm)	2.44 ± 0.42

^a Conductance tests were carried out in quintuplicate.

Table 3.

Characterization of Energy Loss for the Three Electron-Transfer Steps in the Biofilm anode

electron-transfer step (Figure 1)	energy loss (V)
step 1	0.13 ^a (87%)
step 2	0.02 ^b (13%)
step 3	8.5×10^{-5} (<<1%)

^aEnergy loss = ($E_{OMP} - E_{acetate}'$).^bEnergy loss = ($E_{EC}' - E_{OMP}$).

^cEnergy loss = ($E_{anode} - E_{EC}'$). We assumed that the potential for the outer membrane protein (E_{OMP}) is close to the midpoint potential -0.17 V for periplasm c-type cytochromes of *Geobacter sulfureducens* at pH 7.^(67, 68) E_{EC}' was quantified at -0.15 V ($E_{anode} - E_{EET}$). Parentheses indicate the fraction of the overall energy loss that comes from an individual energy loss. The overall energy loss is computed at 0.15 V ($E_{anode} - E_{acetate}' = (-0.15 \text{ V}) - (-0.3 \text{ V}) = 0.15 \text{ V}$). Supporting Information provides $E_{acetate}'$ computation.