Charge Transport through Geobacter sulfurreducens Biofilms Grown on Graphite Rods

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ABSTRACT: Biofilms of the electroactive bacterium Geobacter sulfurreducens were induced to grow on graphite-rod electrodes under a potential of 0 V (vs Ag/AgCl) in the presence of acetate as an electron donor. Increased anodic currents for bioelectrocatalytic oxidation of acetate were obtained when the electrodes were incubated for longer periods with periodic electron-donor feeding. The maximum current density for acetate oxidation increased 2.8-fold, and the biofilm thickness increased by 4.25-fold, over a time period of 83–147 h. Cyclic voltammetry in the presence of acetate supports a model of heterogeneous electron transfer, one electron at time, from biofilm to electrode through a dominant redox species centered at −0.41 V vs Ag/AgCl. Voltammetry performed under nonturnover conditions provided an estimate of the surface coverage of the redox species of 25 nmol/cm². This value was used to estimate a redox species concentration of 7.3 mM within the 34-μm-thick biofilm and a charge-transport diffusion coefficient of 3.6 × 10⁻⁷ cm²/s. This value of diffusion coefficient is greater than that observed in traditional thin-film voltammetric studies with redox polymer films containing much higher surface concentrations of redox species and might be associated with proton transport to ensure electroneutrality within the biofilm upon electrolysis.

INTRODUCTION

Microbial electrogenesis harnesses the oxidation of fuel (waste) substrates by biofilms of electroactive bacteria (EAB) on anodes to generate electricity. During this process, the anodic microbial communities transfer their electrons not to their characteristic terminal electron acceptor, but rather to a solid electrode.1–6 Increased interest is now focused on understanding the behavior of electroactive biofilms to facilitate efficient energy generation in microbial fuel cells or offsetting energy in microbial electrolysis cells to produce high-value feedstocks, chemical, or fuels. To date, a variety of mechanisms of electron transfer from bacteria to anode have been proposed, such as indirect transfer using low-molecular-weight electron shuttles and direct electron transfer from cell-surface redox-active proteins (e.g., c-type cytochromes) or electron-conductive “nanowires” (pili) produced by the bacteria.5–28 However, the mechanisms and complexity involved in wiring EAB to electrodes and the physiology of electron transfer released from the central metabolism of each EAB to the outside cell environment (for example, to the anode or to the biofilm) are not yet completely understood.27–29

In recent years, the use of electroanalytical techniques to aid in the understanding of EAB–anode interactions, using different inocula as the source of EAB, has been explored.12,30–39 Traditional three-electrode electrochemical cell configurations can be used to induce biofilm growth on anodes, under an applied potential versus a reference electrode, to achieve better control of the anode potential than can be obtained in the widely used closed-circuit systems with anode and cathode connected across a simple fixed resistance load.30–32,34,39–41 Monocultures of Geobacter sulfurreducens are used extensively as a model organism for biofilm formation on anodes to probe electrical communication between the EAB and the anode, because of the demonstrated enrichment of this species in the most efficient electricity-producing anodic biofilms.7,12,27–29,32,34,42–45 Although the exact mechanism of electron transfer within biofilms of Geobacter sulfurreducens on anodes and between the biofilm and the anode is not yet understood, recent results suggest that Geobacter sulfurreducens biofilms are interconnected and wired to electrodes through outer-cell-surface-expressed c-type cytochromes during electricity generation.5,10,12,13,19–21,23,27–29 Distinct roles of membrane- and pili-bound cytochromes (omcB, omcE, omcS, omcZ) and of nanodimensional pili have been examined during different stages of biofilm growth and in response to short- and long-term changes in anode potential.5,7–10,12–14,17,19–23,25,42,46

Herein, we report on the cyclic voltammetric behavior of G. sulfurreducens biofilms grown on graphite-rod electrodes in batch-fed mode under an applied potential and correlate electron transfer within the biofilms and from biofilms to electrode with biofilm thickness.

MATERIALS AND METHODS

Preparation of Graphite-Rod Electrodes for the Formation of Biofilms. Custom-built graphite-rod (0.3-cm Ø, Goodfellow,
Huntingdon, U.K.) electrodes were used to form electrode-attached G. sulfurreducens biofilms. These were made by shrouding graphite rods of various dimensions in glass tubes using heat-shrink plastic tubing (Alphawire, Sunbury-on-Thames, U.K.) and establishing an electrical connection at the rear with a 0.3-cm-diameter copper rod (Farnell Electronic Components, Dublin, Ireland) and silver epoxy adhesive (Radionics Ltd., Dublin, Ireland). Prior to use, these electrodes were sterilized in boiling 0.1 M H2SO4 for 15 min and washed several times with distilled water, after which they were placed in absolute ethanol overnight.

**G. sulfurreducens Biofilm Preparation.** G. sulfurreducens (ATCC S1573) was used as a source of electroactive bacteria. The strain was subcultured in 100 mL airtight, rubber septa-sealed, anaerobic syringe bottles containing 70 mL of growth medium, prepared according to the protocol supplied by the culture center (http://www.dsmt.de, medium no. 826). The bacteria were cultured in fumarate-containing Geobacter growth medium for ~2 weeks (three subcultures) prior to inoculation in the electrochemical cell. Biofilms were formed on graphite-rod electrodes, with four electrodes in the same electrochemical cell, under a constant applied potential (0 V vs Ag/AgCl) using a multichannel potentiostat (CHI-1030a, CH Instruments, Austin, TX), a common platinum gauze (5 cm Ag/AgCl) using a multichannel potentiostat (CHI-1030a, CH Instruments, Austin, TX), and silver epoxy adhesive (Radionics Ltd., Dublin, Ireland). Prior to use, these electrodes were sterilized in boiling 0.1 M H2SO4 for 15 min and washed several times with distilled water, after which they were placed in absolute ethanol overnight.

**Nonturnover Cyclic Voltammetry (CV) Analysis.** Prior to nonturnover CV analysis, acetate was gradually removed from the electrode-attached biofilms by washing the electrodes (×4), under anaerobic conditions, in acetate-free culture medium. The washed electrodes were subsequently transferred into a separate vessel containing 100 mL of acetate-free culture medium and were incubated for 1 h under anaerobic conditions to dilute the acetate concentration in the biofilm matrix. The electrodes were subsequently transferred into a separate electrochemical cell containing 100 mL of acetate-free culture medium and polarized continuously at 0 V (vs Ag/AgCl) until the anodic current approached zero. Polarization was then stopped to undertake nonturnover CV analysis in the same cell.

**Biofilm Imaging.** Electrodes were removed from the electrochemical cell at two time intervals following initiation of biofilm growth (83 and 147 h) and sectioned into two pieces for subsequent scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) imaging. Prior to SEM imaging, fixation was undertaken by placing the electrode in the following solutions: (a) 1% glutaraldehyde, 2% paraformaldehyde, 0.2% picric acid, 10 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES, pH 7.4) for 1 h, (b) 50 mM NaNO3 for 1 h, (c) 2% tannic acid for 1 h, (d) 1% osmium tetroxide for 2 h, (e) 1% thiocarbohydrazide for 30 min, and (f) 1% osmium tetroxide overnight, with washing using 10 mM HEPES buffer (pH 7.4) between steps (all Sigma-Aldrich). The samples were then dehydrated in a graded series of aqueous ethanol solutions (10–100%) and oven-dried (2 h at 40 °C) to remove residual moisture. The dried samples were mounted over SEM stubs with double-sided conductivity tape and a thin layer of gold metal applied using an automated sputter coater (Emitech, K550) for 1 min and imaged using a model 4700 SEM instrument (Hitachi, Japan). For CLSM analysis, electrode sections were transferred into sterile vessels containing 50 mL of anaerobic acetate-free growth medium. The graphite rod was cross-sectioned into pieces (~3 mm in height) using a scalpel and stained, by incubation for 15 min in 10 mL of 10 mM potassium phosphate buffer, pH 7.0, containing 1 μL of propidium iodide and 1 μL of Syto 9 from a Molecular Probes BacLight LIVE/DEAD L7012 stain kit (Invitrogen Corp., Carlsbad, CA) in the dark.

The samples were then gently washed in phosphate buffer (10 mM, pH 7.0) to remove unbound residual dye from the biofilm matrix. The sectioned face of the rod was placed on a multiwell microscope slide to examine horizontal growth of biofilm through a Zeiss LSM 510 Axiovert inverted confocal microscope with a 40× Achromplan oil immersion lens. A minimum of 10 fields of biofilm views were imaged, and Z-series images were processed and analyzed with Zeiss LSM510 operating software for biofilm thickness measurements. The differences in biofilm thickness observed between biofilm images showed no statistical significance (P > 0.05; t test), suggesting uniform biofilm formation on the carbon rod. Images were obtained using an excitation wavelength of 488 nm and a BP500–550 emission filter for green fluorescence. The excitation wavelength was 543 nm, and emission filter LP605 was used to obtain images for red fluorescence.

**RESULTS AND DISCUSSION**

In biological fuel cells, with current flow between the anode and the cathode across a fixed resistance load, the potential imposed on the anode is difficult to control, because of variations in the anode potential as a function of the load resistance and mass transport to, and catalytic activity at, the anode and cathode. Growth of EAB biofilms can be induced using three-electrode electrochemical cells, to allow precise control of the anode potential versus a reference electrode, to examine biofilm growth and activity on the anode, in an attempt to understand microbial fuel cell bioelectrochemistry.30–33,47 In this study, we focus on biofilms of G. sulfurreducens induced to form on anodes during fed-batch-mode operation in a three-electrode electrochemical cell using acetate as the electron donor and graphite-rod anodes held at a potential of 0 V vs Ag/AgCl as the electron acceptor (Figure 1). The applied potential was...
A current of approximately 0.3 A/m² was reached for the first two fed-batch cycles, with replacement of acetate-containing cell culture medium when the current dropped to baseline levels. This trend is related to biofilm electrode attachment and acclimatization during an initial phase, followed by growth as a function of time and subsequent current decrease indicating electron-donor (acetate) depletion. Once attachment and acclimatization occurs, current density rises at a higher rate for the next fed-batch cycle (from 125 h). The fed-batch amperometric response profiles in Figure 1 are similar to those observed by others for *G. sulfurreducens* biofilm growth on electrodes, although the pattern of growth can vary depending on a range of factors, such as feed concentration, inoculum concentration and stage, electrode material, and potential. More recently, current densities for acetate oxidation of 4–7 A/m² were achieved after 72 h under an applied potential of 0.04 V vs Ag/AgCl at graphite or roughened glassy carbon electrodes, which compares well with the 3.8 A/m² after 68 h at 0 V vs Ag/AgCl applied potential in Figure 1 for films on graphite-rod electrodes. Low-resistance indium tin oxide (ITO) electrodes were used to grow biofilms of *G. sulfurreducens* to provide acetate oxidation current densities of between 2.8 and 4.4 A/m² after approximately 60 h, under applied potentials of 0.04 V vs Ag/AgCl. Self-limiting films of *G. sulfurreducens*, developed in a plug-flow microbial fuel cell operated under a fixed resistance load in acetate medium versus an oxygen or ferricyanide-reducing cathode, reach maximum current densities of approximately 3 A/m² after periods of up to 15 days, depending on the cathode. From Figure 1, the maximum current density after two feed cycles was close to 5 A/m² and

**Figure 2.** Slow-scan (1 mV/s) cyclic voltammetry of *G. sulfurreducens* biofilms grown on graphite-rod electrodes at 0 V vs Ag/AgCl over (A) 83 and (B) 147 h (black traces) showing CV data corrected for a 100-Ω iR drop (gray traces). The fits to eq 1 are for *n* = 1 (dashed lines) and *n* = 2 (dotted lines) with (A) $E^0 = -0.41$ V vs Ag/AgCl and $j_{\text{lim}} = 1.8$ A/m² and (B) $E^0 = -0.44$ V vs Ag/AgCl and $j_{\text{lim}} = 5.0$ A/m².
rapidly reached a level of 9.2 A/m² during the third feed cycle. Such a rapid increase in current production, following the initial period of bacterial attachment, has been reported by others.31,48 For example, the G. sulfurreducens biofilms induced to grow on ITO generated a maximum current density only 3 h after a medium change following depletion of acetate during an initial fed-batch cycle.38

To probe the correlation between current density and biofilm thickness, the voltammetric behavior of biofilms was studied at two time intervals following initial inoculation, namely, 83- and 147-h-aged biofilms, as indicated in Figure 1 with arrows, by placing the electrode-attached biofilms in fresh culture medium containing 10 mM acetate as an electron donor, leaving them for a 1-h incubation period with no applied potential, and recording the cyclic voltammograms, presented in Figure 2. The CV response at this low scan rate is sigmoidal in shape, indicative of an electrochemical redox transition coupled to a catalytic reaction, as observed previously for bioelectrocatalytic responses at enzyme and microbial biofilm-modified electrodes.12,20,32,38−52 The first derivatives of the cyclic voltammograms (not shown) indicate that one dominant redox transition is responsible for the catalytic wave, with a redox potential centered at −0.41 V vs Ag/AgCl. Previous research has established that, although several redox transitions are apparent in the first derivative of cyclic voltammograms recorded at the early growth stages of G. sulfurreducens biofilms on electrodes in the presence of acetate as an electron donor, the moiety responsible for the redox transition at −0.41 V vs Ag/AgCl becomes dominant after longer times, such as those investigated here. For example, Katuri et al.32 reported that the first-derivative cyclic voltammogram changed between 48 and 64 h after initial inoculation with G. sulfurreducens from multiple peaks to one broad peak centered at −0.41 V vs Ag/AgCl. The amperometric and CV results reveal that higher anodic currents, as depicted in Figures 1 and 2, were obtained when the electrodes were incubated for longer periods, with periodic feeding, in the presence of an electron donor at this applied potential. The midpoint potential of the observed dominant redox couple is in good agreement with that reported by others.12,13,30,31,33,34,47,49 G. sulfurreducens produces multiple cytochromes29,53 that can be differentially expressed in the organism during electrogensis, depending on the prevailing culture conditions17,18,19,21,22,29,46,54,55 and nature of the terminal electron acceptor present during the growth period.56−58 The midpoint redox potential of expressed extracellular and membrane-associated G. sulfurreducens cytochromes, under Fe(III)-reducing conditions, were characterized as −0.370 V vs AgCl,59 −0.300 and −0.390 V vs AgCl,60,61 −0.370 V vs AgCl59, and, more recently, in the range from −0.620 to −0.260 V vs AgCl for the octa-heme OmcZ cytochrome deemed to be essential for production of a high current density by G. sulfurreducens biofilms.21 The midpoint redox potential of the dominant redox couple observed here falls within this range.

Although direct comparison is difficult because of the sampling protocol used, the steady-state limiting catalytic oxidation currents observed in the slow-scan cyclic voltammograms correlate with the amperometric currents observed at fixed applied potential during growth. For example, the CV catalytic currents increased by a factor of 2.8, from 1.8 to 5 A/m², between 83 and 147 h, whereas the maximum amperometric current density immediately preceding the sampling time increased by a factor of 2.4, from 3.8 to 9.2 A/m², between 83 and 147 h. The increase in the steady-state CV and amperometric currents over this time period might be due to an increase in the number of electrically contacted bacteria in the biofilm or the number of electrically contacted redox species in each bacterium in the biofilm as a function of time—or, more realistically, a combination of both of these effects—and was previously observed for G. sulfurreducens films grown under potential control in fed-batch conditions.31,32

Bioelectrocatalytic oxidation of acetate by biofilms is assumed to proceed through an EC mechanism (i.e., an electrode process followed by a catalyzed chemical process), analogous to bioelectrocatalytic reactions involving mediated electron transfer from redox enzymes to electrodes.12,13,27−29,51,52 A simple model of the response at low scan rates can thus be used to compare steady-state voltammetric signals, assuming that the current at each potential in a scan reflects a Nernstian equilibrium distribution of the oxidized and reduced dominant redox species responsible for transferring electrons between the biofilm and the electrode.50 The current density can be modeled as

$$j = \frac{j_{lim}}{1 + \exp\left(\frac{nF(E^0 - E)}{RT}\right)}$$

where $j_{lim}$ is the limiting current density and $E^0$ categorizes the formal redox potential of the dominant redox species, approximated here as the midpoint redox potential and classified by others66,38,62 as an empirical parameter, termed variously as an operating or catalytic potential, and represents the potential at which the current density reaches its half-maximum value in a Nernst−Monod relationship.36,38 This approach, as previously demonstrated,12 permits qualitative fitting of the observed cyclic voltammogram (solid line), in Figure 2A, at 83 h to the model for $n = 1$ (dashed line), rather than $n = 2$ (dotted line), once a correction for the $ir$ drop between the working and reference electrodes is applied (gray line, vide infra). AHeyrovský−Ilkovitch plot50 provides confirmation of the fit, indicating that electrons are transferred one at a time by the dominant redox species from the biofilm to the electrode. Comparison of the observed cyclic voltammogram to this model resulted in an estimate of −0.41 V vs Ag/AgCl for the formal redox potential of the dominant species and extraction of a limiting current density of 1.8 A/m² for the oxidation of acetate by the biofilm, at this growth stage. In addition, the fit between the observed cyclic voltammogram and this simple model confirms that heterogeneous electron transfer is fast relative to the time scale of the experiment, as reported previously.12,13 When a biofilm that had been allowed to age for a longer period (147 h, Figure 2B) was used, a slight shift in formal redox potential, from −0.41 to −0.44 V vs Ag/AgCl, possibly due to a change in the pH of the medium or within the biofilm as a function of time17,32,60 and an increase in steady-state catalytic currents for acetate oxidation, at the same acetate concentration, to 5.0 A/m² was observed. The observed CV response at the biofilm allowed to age for a longer period indicates distortion in the CV wave, indicative of resistive losses between the working electrode and the reference electrode. The effect of resistance on the cyclic voltammogram shape can be evaluated by correction for an ohmic $ir$ drop in the voltage applied to the working electrode, versus the Ag/AgCl reference electrode (Figure 2B, gray trace). The estimate of the resistance was achieved by correcting at each applied voltage for cyclic

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voltammograms recorded for the films grown for 147 h (Figure 2B, black trace) so as to achieve the best fit between the corrected cyclic voltammogram (Figure 2B, gray trace) and the simple model represented by eq 1 with $n = 1$ (Figure 2B, dashed black trace). Such an approach yielded an estimate of 100 $\Omega$ for the resistance, and inserting this resistance as a correction in Figure 2A (gray trace) confirmed that this was a reasonable estimate of the uncompensated iR drop in the electrochemical cell. The value of the resistance conforms to an estimate of 104 $\Omega$ uncompensated resistance using a conductivity of 1.44 S/m for the electrolyte culture medium,36 an electrode area of $1 \times 10^{-4}$ m², and an approximate distance between working and reference electrodes of $\sim 0.015$ m. Thus, as has been noted by others,38 it appears that the biofilm conductivity, under these conditions of cell dimensions, electrode spacing, and biofilm thickness, is sufficiently high not to limit electronic conductivity within the electrochemical cell, and that the distortion of the cyclic voltammogram is simply from iR drop due to the electrolyte and cell configuration, a subject that warrants further investigation.

From the microscopy images in Figure 3, using (A,C) SEM and (B,D) CLSM, thicknesses of 8 $\pm$ 2 and 34 $\pm$ 6 $\mu$m were estimated for the biofilms sampled at 83 and 147 h, respectively. This increase in thickness is correlated with an increase in limiting current density over the time period, suggesting that not only those bacteria in the inner layers of the biofilm closest to electrode surface but also peripheral-layer bacteria can exchange electrons with the electrode by wiring cell-to-cell interactions through outer-membrane-expressed cytochromes and/or using bacterial nanowires.10,13,27–29,44,65,64 This behavior corresponds to that reported for G. sulfurreducens biofilms grown under similar growth conditions.12,13 It is interesting to note that, for a 4.25-fold increase in film thickness (from 8 to 34 $\mu$m), the maximum current density increases by only 2.8-fold (CV, from 1.8 to 5.0 A/m²) or 2.4-fold (amperometry immediately preceding removal of films for CV and microscopy, from 3.8 to 9.2 A/m²) over the same period. Marsili et al.31 attempted to correlate limiting currents for acetate oxidation by G. sulfurreducens biofilms with protein biomass on carbon electrodes and observed that, whereas protein mass scales with current, a rate of mass increment to current density increment of approximately 1.35 was observed for exponentially growing electrodes, similar to the ratio of the film thickness increment to the current density increment, approximately 1.5, reported here. For example, from the data presented in Figure 2 of their report,31 a 3-fold increment in protein biomass resulted in only a 2.3-fold increment in current density for acetate oxidation. The study of biofilms under nonturnover conditions (i.e., in the absence of an electron donor in the growth medium) can help provide details on the surface concentrations of redox species and lead to an estimate of charge-transport rates within these films. We attempted to address the electroactive redox centers within the biofilm under nonturnover conditions by removing the biofilm, after 147 h of growth, from the growth medium and placing it into a medium without acetate as an electron donor. To qualitatively confirm that the physiological and electron-transfer behavior of the biofilms remained unaltered during the nonturnover experiments (i.e., under starvation conditions for the bacteria in the biofilm for the time period of these experiments), voltammograms were recorded in the culture medium in the absence of acetate both before and after removal of the biofilm, and CVs were recorded both immediately before and after removal of the biofilm, to confirm that the biofilm remained intact over these timescales.

Figure 3. SEM and CLSM images after (A,B) 83 h (biofilm thickness = 8 $\pm$ 2 $\mu$m) and (C,D) 147 h (biofilm thickness = 34 $\pm$ 6 $\mu$m) of growth of G. sulfurreducens on graphite-rod electrodes at an applied potential of 0 V vs Ag/AgCl. CLSM images (B,D) show peripheral growth of Geobacter on the graphite-rod electrode, where the dashed white line represents the graphite rod/biofilm interface.
after the series of voltammetric scans completed to evaluate single-turnover voltammetry. The voltammetric waveshape and redox transitions remained qualitatively similar (Figure 4), although from the integration of the total charge passed under the voltammetric wave, a decrease of 35% was observed in the quantity of redox species that can be addressed by the electrode within the time frame of the 20 mV/s scan rate used. In addition, once the electrode, following examination of single-turnover voltammetry, was placed in fresh culture medium containing acetate as an electron donor and subjected to an applied potential of 0 V vs Ag/AgCl, a rapid return of current production for bioelectrocatalytic oxidation of acetate was evident after a short 2–3-h lag time (Figure 5) to yield maximum current densities that were approximately 50% of those achieved in the fed-batch cycle immediately preceding the experiments in the absence of acetate (see Figure 1). Such a behavior was also recently reported for biofilms of *G. sulfurreducens* induced to grow on ITO electrodes.48 Thus, it seems that, although starvation of the biofilm for periods can result in a diminished acetate oxidation current response, as a consequence of either biofilm detachment or cell death, no qualitative alteration was observed in the voltammograms, indicating that the mechanism of electron transfer was conserved.

Typical slow-scan cyclic voltammograms of a *G. sulfurreducens* biofilm under nonturnover conditions are presented in Figure 6 and clearly show at least three pairs of redox couples centered at −0.51, −0.38, and −0.32 V vs Ag/AgCl, supporting the likelihood that at least three different redox transitions occur between the biofilm and the electrode, as observed by others.3,30–33,65 The peak-to-peak separations for these redox couples were observed to be 30, 75, and 80 mV at a scan rate of 10 mV/s and increased slightly with increasing scan rate, possibly indicative of some limitation of heterogeneous electron transfer to current generation at the higher scan rates. The redox potential of the dominant redox couple shifted to more negative potentials with increasing pH in the range of 6.0–8.0, with a slope of ∼50 mV/pH (close to the theoretical value expected at 25 °C for reversible proton-coupled electron transfer), suggesting that the electron transfer from *G. sulfurreducens* biofilms to the electrode was proton-coupled.13,29,32 Moreover, the decrease in the peak current densities of the redox couples centered at −0.38 and −0.32 V vs Ag/AgCl upon exposure of the biofilm to carbon monoxide, due to the formation of an Fe–CO complex (Figure 7), suggests that the redox couples contained iron as a prosthetic group and implies that the species was a c-type cytochrome, supported by recent spectro-electrochemical and genetic analyses of *G. sulfurreducens* biofilms.17,23,35,48,58,66

![Figure 4. Linear sweep voltammograms recorded at *G. sulfurreducens* biofilm anodes in culture medium in the absence of acetate, immediately before (solid line) and after (dotted line) the sequence of scans for nonturnover analysis (Figure 6). Scan rate = 20 mV/s.](image)

![Figure 5. Amperometric response (0 V vs Ag/AgCl) of biofilms, previously grown for 147 h, placed in 10 mM acetate medium after nonturnover CV analysis in culture medium in the absence of acetate.](image)

![Figure 6. Cyclic voltammetry under nonturnover conditions (in the absence of acetate) for *G. sulfurreducens* biofilms after 147 h of growth at 0 V vs Ag/AgCl, at scan rates of 1 (black), 10 (dark gray), 20 (medium gray), and 30 (light gray) mV/s in culture medium.](image)
observed in the cyclic voltammogram, within the potential window from $-0.6$ to $-0.2$ V at the 1 mV/s scan rate, can nonetheless provide an estimate of the charge passed upon electrolysis of the redox species within the biofilm. This charge was used to estimate a surface coverage of $\sim 25$ nmol/cm$^2$ of redox species per geometric projected electrode area. However, not all of the charge integrated under the slow-scan voltammograms can result from electron exchange with redox species that can contribute to electron donation to the electrode upon oxidation of acetate. It should also be noted that integration of the CV peak areas provides an underestimate of the total amount of redox species on the electrodes, as finite-diffusion conditions were not observed (i.e., peak current did not scale directly with scan rate). Considering these two counterbalancing effects, the $\sim 25$ nmol/cm$^2$ coverage can provide a balanced estimate of redox species that can participate in electron exchange with the electrode to transfer electrons generated upon oxidation of acetate. Given a film thickness of $\sim 34$ $\mu$m for this biofilm, a surface concentration of redox species, assuming a homogeneous distribution of redox sites within the biofilm, of 7.3 mM was thus estimated. It should be noted that these redox sites might not be homogenously distributed within the biofilm.

As the scan rate was increased, the electrode sampled only a portion of the biofilm within the CV time frame, indicative of a semi-infinite diffusion regime, resulting in CV signals that were similar to those obtained for solution-phase redox species being observed. Under these conditions, the peak currents scaled linearly with the square root of the scan rate, as observed in the inset of Figure 8, for scan rates above 40 mV/s. Assuming that the rate of heterogeneous electron transfer to the biofilm is high and that diffusion is planar, the CV peak current density response can be modeled by the Randles–Sevcík equation,$^{50,71}$

$$j = 0.4463 n F C_{\text{redox}} \left( \frac{nF}{RT} \right)^{1/2} D^{1/2} \nu^{1/2}$$  \hspace{1cm} (2)

where $C_{\text{redox}}$ represents the concentration of redox species (mol/cm$^3$) in solution or within the film, in this case; $\nu$ is the scan rate (V/s); and $D$ is an apparent diffusion coefficient (cm$^2$/s). Treating the concentration of redox species within the biofilm determined from slow-scan CV as an average concentration of the dominant redox species centered at $-0.385$ V, an apparent diffusion coefficient of $3.6 \times 10^{-7}$ cm$^2$/s can be evaluated from the slope of the current density versus square root of scan rate plot (inset of Figure 8). Using this value for $D$ and Fick’s laws of diffusion provides an estimate of 10 s for the time taken to electrolyze a film of $34-\mu$m thickness. This value for $D$ might be an overestimate, as the scan-rate-dependent studies (Figure 6) showed that thin-layer, surface-confined, finite-diffusion behavior was not observed even at scan rates of 1 mV/s (i.e., time scale of several hundred of seconds for electrolysis), and the concentration of redox species might therefore be underestimated. In solution-phase voltammetry, this diffusion coefficient represents the physical diffusion of redox species through a solution, as a result of a concentration gradient created at the electrode solution interface, to reach the electrode surface to permit heterogeneous electron transfer to occur. Physical diffusion of redox species is sometimes not possible, for example, within redox polymer films where the redox complex is tethered to the polymer or within biofilms when the redox species is membrane-bound or associated, as has been shown to be the case for $G. \text{sulfurreducens}$ biofilms. In these cases, the apparent diffusion coefficient can be assigned to the diffusion-like process that limits charge transport through the film. Charge transport within redox polymer films is postulated to occur through electron-hopping, through self-exchange of electrons as a result of bimolecular interactions between reduced and oxidized forms of the redox species.$^{50}$ The apparent diffusion coefficient can, however, also be characteristic of the associated charge-transport diffusion of counterions into or out of a film to maintain electroneutrality within the film as a consequence of charge introduced during redox reactions. Within tethered redox polymer films, diffusion coefficients have also been suggested to be characteristic of the segmental motion of polymer chains necessary to bring redox sites within close enough proximity to permit self-exchange electron transfer to occur.$^{50,52}$ The apparent diffusion coefficient of $3.6 \times 10^{-7}$ cm$^2$/s is lower than the value of $1.5 \times 10^{-6}$ cm$^2$/s estimated recently by Richter et al.$^{12}$ that was assigned to proton diffusion.
within the biofilm but higher than values of apparent diffusion coefficients estimated for charge transport through redox polymer films containing much higher surface concentrations of redox species (molar, instead of millimolar concentration levels).57–69,72 Such a high diffusion coefficient might be indicative of proton transport limiting charge transport within these films, rather than electron self-exchange or electron transport through conductive pili, as proposed by others.5,10,27

■ CONCLUSIONS

Current generation for acetate oxidation by Geobacter sulfurreducens biofilms induced to grow on graphite-rod electrodes under a potential of 0 V (vs Ag/AgCl) increased as biofilm thickness increased. Use of cyclic voltammetry lends support to a model of heterogeneous electron transfer of one electron at a time from the biofilm to the electrode through a dominant redox species. CV analysis under nonturnover conditions can provide an estimate of the redox-species surface coverage, concentration, and charge-transport diffusion coefficients of ∼25 mmol/cm², 7.3 mM, and 3.6 × 10⁻⁷ cm²/s, respectively. This value of the diffusion coefficient is much higher than values observed in traditional thin-film voltammetric studies with redox polymer films containing higher surface concentrations of redox species and might be indicative of proton transport limiting charge transport within Geobacter sulfurreducens biofilms.

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Notes
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