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Redox and pH Microenvironments within *Shewanella oneidensis* MR-1 Biofilms Reveal an Electron Transfer Mechanism

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Abstract

The goal of this research was to quantify the variations in redox potential and pH in *Shewanella oneidensis* MR-1 biofilms respiring on electrodes. We grew *S. oneidensis* MR-1 on a graphite electrode, which was used to accept electrons for microbial respiration. We modified well-known redox and pH microelectrodes with a built-in reference electrode so that they could operate near polarized surfaces and quantified the redox potential and pH profiles in these biofilms. In addition, we used a ferri-/ferrocyanide redox system in which electrons were only transferred by mediated electron transfer to explain the observed redox potential profiles in biofilms. We found that regardless of the polarization potential of the biofilm electrode, the redox potential decreased toward the bottom of the biofilm. In a fully redox-mediated control system (ferri-/ferrocyanide redox system), the redox potential increased toward the bottom when the electrode was the electron acceptor. The opposite behavior of redox profiles in biofilms and the redox-controlled system is explained by *S. oneidensis* MR-1 biofilms not being redox-controlled when they respire on electrodes. The lack of a significant variation in pH implies that there is no proton transfer limitation in *S. oneidensis* MR-1 biofilms and that redox potential profiles are not caused by pH.

INTRODUCTION

Bacteria colonizing surfaces as biofilms consume electron donors and require electron acceptors to respire. The electron acceptor can be a solid surface or a soluble compound. In natural environments, a solid surface which bacteria use as an electron acceptor is generally a mineral phase, such as hematite.^{1,2} However, researchers have recently discovered that bacteria can use solid electrodes as electron acceptors and that they can respire on electrodes.^{3–6} When cells are grown as a biofilm on an electrode surface, electrons from the

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Supporting Information. A cyclic voltammogram of one of our biofilms (Figure SI-1), a representative confocal scanning laser microscopy image of *S. oneidensis* MR-1 biofilm grown on a biofilm electrode (Figure SI-2A), and a photograph of a microelectrode tip entering a cell colony to measure pH profile (Figure SI-2B). This material is available free of charge via the Internet at <http://pubs.acs.org>.

metabolic reactions can be transferred to the electrode (1) by direct electron transfer from the micro-organisms or (2) by electron transfer mediators.^{7–19} In this paper, we use the term *electron transfer* to describe extracellular electron transfer from cells in a biofilm to a solid electrode acting as an electron acceptor. Most of the research on electron transfer processes has focused on using them to produce electricity through microbial fuel cells (MFCs). However, electron transfer to solid electrodes by biofilms could be useful in many other applications, such as feeding electrons to bacteria and accepting electrons from them to control their metabolic reactions in bioelectrochemical systems (BES).^{20–22} Fundamental research is needed to understand electron transfer processes occurring in biofilms respiring on electrodes and potentially to help improve electron transfer efficiency for desired processes.

Electron transfer must be coupled with proton transfer to maintain electroneutrality in solution, which is expected to cause variation in pH. Furthermore, when electrons are transferred using a redox mediator, the solution redox potential should change because of the oxidation/reduction of the redox mediator. If the redox mediator is protonated during reduction then the redox potential should affect the pH as well. Therefore, if a biofilm is respiring on an electrode while oxidizing an electron donor, both the pH and the redox potential are expected to change.^{23,24} This concept can be illustrated by applying the Nernst equation, eq 2, to reaction 1, a general two-electron, two-proton redox reaction²⁵

$$E_{\text{h}} = E^{\circ} - (0.05916/2) \times \log([\text{H}_2\text{Red}]/[\text{Ox}]) - 0.05916 \times \text{pH} \quad (2)$$

where E_{h} is the redox potential (V), E° is the standard redox potential (V), $[\text{Ox}]$ is the concentration of the oxidized species (M), and $[\text{H}_2\text{Red}]$ is the concentration of the reduced species (M). According to eq 2, E_{h} depends linearly on pH. From eq 2, polarizing an electrode with an attached biofilm to a value above or below the open-circuit potential (OCP) of the electrode is expected to change the pH near the electrode surface—in the biofilm. However, the change will only be significant if proton transfer limits electron transfer. This effect has been demonstrated in mathematical models of biofilms respiring on electrodes.²⁶ At the same time, the redox potential can only change significantly if a large amount of the soluble chemical species is oxidized/reduced near the surface of the biofilm electrode, implying a significant current is passed by either the soluble chemical species or the redox mediators. As eq 2 implies, redox potential and pH are related and should be measured together in biofilms respiring on electrodes to determine whether mediated electron transfer critically controls overall electron transfer.

Research on redox potential and proton transfer in biological and biofilm systems is not new. In earlier research, Myers and Nealson (1990) linked proton translocation to electron transfer to manganese and iron oxides.²⁷ In a recent paper, Pinchuk et al. used constraint-based metabolic modeling to calculate the proton generation in *Shewanella oneidensis*.²⁸ Mahadevan et al. mathematically showed that *Geobacter sulfurreducens* biofilms accumulating protons during extracellular electron transfer acidified their cytoplasm.²⁹ Torres et al. also showed that increasing buffer strength increased maximum power density for an acclimated anode-respiring bacterial community.²⁶ In a different system, in which biofilms respired on soluble electron acceptors, Yu et al. (1998) measured pH, redox potential and nitrate, sulfate, and oxygen concentrations within mixed culture biofilms using microsensors and concluded that the profiles corresponded to the metabolic activity of the

biofilm.³⁰ These studies confirm that bacterial metabolism affects redox potential and pH within biofilms during electron transfer to electrodes, and we expect to observe these effects as variations in redox potential and pH in biofilms.

Franks et al. (2009) developed a method for measuring variation in pH within a *G. sulfurreducens* biofilm respiring on the anode of an MFC using a fluorophore, C-SNARF-4, optically. Inside the biofilm cluster, a change in pH of one unit was observed 60 μm from the electrode surface while the MFC was in operation. They also showed the dependence of current on bulk pH in a continuously fed reactor.²³ In addition to experimental work, there are mathematical models that predict variation in redox potential and pH in biofilms respiring on an electrode. Picioreanu et al. and Marcus et al. both modeled the anode of a single-species MFC during operation.^{24,31} Picioreanu et al. predicted variation in both redox potential (200 mV in a 30 μm thick biofilm) and pH (0.03 in a 100 μm thick biofilm) for a biofilm respiring on an electrode under batch conditions. The authors showed variation in pH at the highest modeled current density, 0.2 A/m². Marcus et al. predicted a pH profile of 1.5 pH units over 450 μm for an exchange current of 15 A/m² but did not provide a prediction of variation in redox potential. To the best of our knowledge, variations in redox potential and pH were not quantified in the same biofilms.

The goal of this research was to quantify variation in the redox potential and pH of biofilms respiring on electrodes. We grew *S. oneidensis* MR-1 on an electrode which was used to accept electrons for microbial respiration. The electrodes were polarized to selected potentials, and respiration rates were measured as current. We modified well-known redox and pH microelectrodes with a built-in reference electrode so that they could operate near polarized surfaces. In this paper, an electrode on which biofilms grew and respired is referred to as a biofilm electrode. We selected +400 and 0 mV_{AgAgCl} for polarizing biofilm electrodes because it has been shown that *S. oneidensis* MR-1 can respire on electrodes at these potentials.³² In addition, we used a ferri-/ferrocyanide redox system in which electrons are only transferred by mediated electron transfer to explain observed redox potential profiles in biofilms. Finally, pH profiles were measured to determine whether proton transfer was limiting respiration and whether variation in pH in the biofilms caused the observed redox potential profiles.

MATERIALS AND METHODS

Biofilm Reactor

Biofilms were grown in a continuously fed, dual-chamber flat plate biofilm reactor with recycle (Figure 1). The reactor was designed for growing biofilms, polarizing electrodes, and measuring current and using microelectrodes to measure redox potential and pH profiles in biofilms. One chamber contained the graphite (Graphitestore.com grade, GM-10) electrode (2.54 cm $\times$ 2.54 cm $\times$ 0.3 cm) on which *S. oneidensis* MR-1 biofilms grew and respired. The second chamber was separated from the other by a proton-exchange membrane (ESC-7000 Electrolytica Corp.) and contained a graphite counter electrode and a custom-made Ag/AgCl reference electrode. The body of the biofilm reactor was constructed of polycarbonate and autoclavable plastic connectors. It was held together by nuts and bolts to prevent leaking between chambers. Norprene tubing (Cole-Parmer, catalog EW-06404-14, EW-06404-16) was used for the feed, recycle, and waste streams, and 0.2 μm filters were attached to the small ports at the tops of the chambers to prevent pressure buildup and to purge nitrogen gas (99.999%). Flow breakers were used in the feed and waste streams to prevent back contamination. Once the reactor was assembled, it was filled with deionized (DI) water and autoclaved for 20 min at 121 $^{\circ}\text{C}$ to sterilize the entire system. The growth medium was autoclaved separately in a 10 L carboy (Thermo Scientific, 2250-0020) for 100 min at 121 $^{\circ}\text{C}$. After the reactor cooled down, the feed was connected to the reactor

aseptically, the DI water was replaced by growth medium, and the reactor was inoculated. The dilution rate of the reactor was 0.1 h^{-1} .

Inoculum and Growth Medium

We used different growth media for inoculation and biofilm growth. A *S. oneidensis* MR-1 stock culture was thawed at room temperature and placed into a flask containing 100 mL of 5 g/L LB broth in a 100 mM phosphate buffer at pH 7.0. The culture was grown for 24 h. The composition of the medium used to grow biofilms has been described previously.³³ The solution conductivity was 9.07 mS/cm. Briefly, we used lactate as the electron donor and a solid electrode as the electron acceptor during biofilm growth.

Growing Biofilms

The biofilm reactor was inoculated with 15 mL of the inoculum. Cells were allowed to attach for 2 h while the recycle pump was on. Then the system was continuously fed with fresh medium. *S. oneidensis* MR-1 biofilms were allowed to grow aerobically until biofilms approximately 100–200 μm thick formed on the graphite electrode. Then the system was operated under anaerobic conditions, with the biofilm electrode as the only electron acceptor.

Biofilms Respiring on the Electrode

The biofilm electrode was polarized to a desired potential against the Ag/AgCl reference electrode using a Gamry Series G 300 potentiostat. During the first 2 days of polarization, the bulk solution became visibly clear, indicating the suspended cells were washed out as only the cells on the electrode surface could respire and continue to grow. Even though the current remained constant after the second day, we measured redox potential and pH profiles after 7 more days of continuous operation while biofilms respired on the electrode.

Microelectrodes

Because of the electrical field generated during biofilm electrode polarization and the long distance between an external reference electrode and the working electrode, the previously described redox and pH microelectrodes were not useful in our system.²⁰ For our study, we constructed each microelectrode with a built-in reference electrode. The distance between the reference electrode and the working electrode was constant and less than several hundred micrometers, minimizing the effects of polarization and solution resistance. The redox microelectrode tip was constructed by depositing platinum on the platinum tip electrochemically. A porous Pt ball with a large surface area was formed at the tip; this increased sensitivity and response time (Figure 2A). The redox or pH microelectrode was placed in an outer casing which included an electrolyte solution (3.5 M KCl) and a Ag/AgCl reference. The space between the redox or pH microelectrode and the outer casing was filled with 1% agar 0.1 M Na_2SO_4 to ensure electrochemical conductivity. The redox microelectrodes were calibrated using YSI 3682 Zobell Solution (YSI Inc., Yellow Springs, OH), which contained potassium chloride (75%), potassium ferrocyanide trihydrate (14%), and potassium ferricyanide (11%). The sensitivity of the redox microelectrode was $\pm 1 \text{ mV}$. The pH microelectrode was a potentiometric sensor with a liquid ion-exchange tip ($\sim 10 \mu\text{m}$), as shown in Figure 2B. The pH microelectrodes were calibrated using buffer solutions (pH = 4, 7, and 10) from ACROS Organic. Linear calibration curves were obtained with slopes in the range of 54–57 mV/pH, and the detection limit was 0.02 units.

Microelectrode Measurements

Redox and pH measurements are based on potentiometric methods. Potentials were measured between the redox/pH working electrode and the built-in Ag/AgCl reference

electrode using a Keithley 6517A Electrometer/High Resistance Meter. The movement of the microelectrodes was controlled by a stepper motor controller (PI M-230.10S Part No. M23010SX, Physik Instrumente, Auburn, MA) controlled by custom Microprofiler software. Data were recorded on a Dell laptop using an Analog/Digital Converter (ADC, Measurement Computing USB-1608FS, Norton, MA).

After the biofilm thickness reached approximately 100–200 μm , the port above the biofilms was opened for microelectrode measurements. Ultrapure nitrogen gas (99.999%) was purged into the headspace of the biofilm reactor to maintain an anaerobic condition in the biofilm when the port was opened. With the port opened, the microelectrode was manually lowered into the reactor using micromanipulators until it reached the bulk solution above the biofilm. Then the pH or redox profile was measured. The surface of the biofilm was located using a stereomicroscope (Zeiss Stemi 2000 stereomicroscope), and the distance between the bottom and the surface was quantified as the biofilm thickness. The bottom of the biofilm was located by breaking the microelectrode tip.

Redox Potential Profiles in the Ferri-/Ferrocyanide System

YSI 3682 Zobell Solution (YSI Inc., Yellow Springs, OH) was used to measure redox potential profiles of ferri-/ferrocyanide in a clean, redox-controlled system. Instead of a biofilm electrode, we used a clean graphite electrode and polarized it to the desired potential. We used another graphite electrode as the counter electrode, and all potentials were measured against a Ag/AgCl reference electrode. The working electrode was polarized with a Gamry Ref600 potentiostat to +400 mV_{Ag/AgCl}. Oxygen was removed from the system by bubbling ultrapure nitrogen gas (99.999%) into the solution. Once the current reached a steady state, a redox microelectrode was inserted into the reactor (Figure 1) and the redox potential was measured from the bulk to the electrode surface as described above.

Quantifying Variation in Redox Potential after Spent Medium Was Replaced with Fresh Medium

In a second control experiment, we used a *S. oneidensis* MR-1 biofilm which had a bulk redox potential of approximately $-500 \text{ mV}_{\text{Ag/AgCl}}$ and the bulk solution was replaced with newly prepared medium with a bulk redox potential of $+250 \text{ mV}_{\text{Ag/AgCl}}$. Again, the biofilm electrode was polarized with a Gamry Ref600 potentiostat to $+300 \text{ mV}_{\text{Ag/AgCl}}$. Current was allowed to stabilize prior to microelectrode measurements.

RESULTS AND DISCUSSION

A typical cyclic voltammogram of one of our biofilms is shown in Figure SI-1, Supporting Information. A representative confocal scanning laser microscopy image of *S. oneidensis* MR-1 biofilm grown on a biofilm electrode is shown in Figure SI-2A, Supporting Information, and a photograph of a microelectrode tip entering a cell colony to measure the pH profile is shown in Figure SI-2B, Supporting Information. Redox potential and pH measurements were performed at least three different times at different locations in the biofilms. The results were all similar and led to identical conclusions. Here we show selected representative measurements.

Redox Potential Profiles in Biofilms Respiring on the Electrode

Figure 3 shows the variation of redox potential with depth inside a *S. oneidensis* MR-1 biofilm under polarized conditions. The steady state current was 19 and 33 μA for 0 and $+400 \text{ mV}_{\text{Ag/AgCl}}$, respectively. Regardless of the polarization potential of the biofilm electrode, the redox potential decreased from the top of the biofilm to the bottom. Approximately 5 μm from the electrode surface, the redox microelectrode tip touched the

electrode surface, causing the redox potential to increase and match the polarization potential of the biofilm electrode. For our purposes, we calculated the difference in redox potential between the top of the biofilm (bulk) and near the bottom of the biofilm (just before the dramatic change) and report this as the difference in redox potential between the bulk and the bottom of the biofilm.

At a polarization of the biofilm electrode of 0 mV_{Ag/AgCl}, the redox potential decreased 66 mV from the bulk (−207 mV_{Ag/AgCl}) to the bottom of the biofilm. At a polarization of the biofilm electrode of +400 mV_{Ag/AgCl}, the redox potential decreased 53 mV from the bulk (−0.164 mV_{Ag/AgCl}) to the bottom of the biofilm. The shapes of the redox potential profiles for biofilms polarized to 0 and +400 mV_{Ag/AgCl} were almost identical, and the redox potentials were shifted by approximately 40 mV. Polarization of the biofilm electrode above OCP, which was between −500 and −200 mV_{Ag/AgCl}, forced electrons to flow toward the biofilm electrode, oxidizing any electrochemically active species present. As electrons were accepted by the biofilm electrode, the electrochemical equilibrium was shifted according to eq 2: the concentration of the reduced species *decreased* closer to the biofilm electrode, forming a concentration profile that could be measured as an *increasing* redox potential near the biofilm electrode.

A decreasing redox potential was observed by Yu et al. (1998) in anaerobic zones in mixed culture biofilms and predicted by Picioreanu et al. (2009) for a monospecies biofilm utilizing redox mediators during mediated electron transfer to an electrode.^{24,30} Yu et al. (1998) showed that the redox potential decreased from +440 mV_{Ag/AgCl} in the bulk to −225 mV_{Ag/AgCl} at the bottom of the biofilm, where a significant drop in redox potential was due to depletion of oxygen. The decrease in redox potential was due to biofilm growth under anaerobic conditions tending to transfer electrons to the solution (instead of oxygen), thus reducing and *decreasing* the redox potential of the solution.

The negative redox potential decreasing toward the biofilm electrode in Figure 3 confirms reducing activity. Picioreanu et al. (2009) predicted that the redox potential would first decrease inside the biofilm and then increase approximately 10–15 μm from the biofilm electrode. They also predicted that redox mediators would tend to be reduced inside the biofilm and oxidized near the biofilm electrode surface. Because the biofilm electrode is polarized to a higher potential, the biofilm electrode removes electrons from the solution, thus oxidizing and *increasing* the redox potential of the solution. The *decreasing* redox potential change shown in Figure 3 indicates that there was an *increased* concentration of reduced species near the biofilm electrode, opposite to what is expected for redox-controlled and subsequently mediated electron transfer.

Redox Potential Profiles in the Ferri-/Ferrocyanide Control System

Figure 4 shows the redox potential profiles in the ferri-/ferrocyanide solution and in the biofilm when the electrode was polarized to +400 mV_{Ag/AgCl}. The biofilm data are identical to those shown in Figure 3. This allows us to directly compare the results between a fully redox-controlled system and the biofilm system. The redox potential in the ferri-/ferrocyanide solution increased from the bulk value of ~+220 to +310 mV_{Ag/AgCl}, which is unlike the biofilm redox profile shown in Figure 3. On the redox microelectrode tip, the ferri-/ferrocyanide redox couple equilibrated according to eq 3 and determined the redox potential.

When the electrode was not polarized, there was no profile: the redox potentials were constant (results not shown), and the redox potential was approximately +230 mV_{Ag/AgCl}. When the electrode was polarized to +400 mV_{Ag/AgCl}, redox potential variation was generated as ferrocyanide was oxidized to ferricyanide. Figure 4 confirms Piciooreanu et al.'s (2009) predicted redox potential profiles for mediated electron transfer. These control experiments demonstrate that if soluble redox mediators play a significant role in electron transfer, redox potential should *increase* rather than *decrease* because of electrode polarization. In another set of experiments, we found similar redox potential profiles using flavin mononucleotide (data not shown): the redox potential increased toward the bottom. In conclusion, if the current is generated through bound electrochemically active species in the biofilm, the redox potential profile is expected to decrease as seen in Figure 3, as only a redox-controlled system is expected to produce the redox potential profile shown in Figure 4.

Variation in Redox Potential after Spent Medium Is Replaced with Fresh Medium

In a second control experiment, we used a *S. oneidensis* MR-1 biofilm which had a bulk redox potential of approximately -500 mV_{Ag/AgCl}. The bulk solution was replaced with newly prepared medium with a bulk redox potential of +250 mV_{Ag/AgCl}, right after which the redox potential without polarization was measured from the bulk solution to the bottom of the biofilm. We found that the redox potential decreased by 90 mV (from 210 to 120 mV_{Ag/AgCl}) (Figure 5). The biofilm was then polarized to +300 mV_{Ag/AgCl} until a constant current (2.5 μ A) was measured. The redox potential was measured again during polarization from the bulk solution to the bottom of the biofilm. The variation in redox potential during polarization was very similar to the results without polarization, except the measured values were shifted by 20 mV. The redox potentials in the bulk and near the bottom were +239 and +160 mV_{Ag/AgCl}, respectively. However, near the surface, the redox potential increased from 160 to +300 mV_{Ag/AgCl}. These variations in redox potential are similar to those shown in Figure 3 except that the bulk redox potential was nearer to that of fresh growth medium.

Figure 5 provides insight into redox-mediated electron transfer. The current was 2.5 μ A at +300 mV_{Ag/AgCl} and dropped 0.5 μ A after the growth medium was replaced. Similar observations have been made by many other researchers.^{19,21} These researchers concluded this current drop was due to removal of redox mediators from the medium. We agree with the literature: if any soluble redox mediators were present in the spent growth medium, they should have been removed when the biofilm was placed in fresh growth medium. However, redox potential measurements bring a new explanation for the observed current drop. Our results show that replacing spent growth medium with new growth medium increased the bulk redox potential, which likely reduced the current drawn from the biofilm electrode. Because the bulk redox potential was more positive (+250 mV_{Ag/AgCl}) than growth in highly reduced conditions (~-500 mV_{Ag/AgCl} in our system), the tendency for the solution itself to oxidize any electrochemically active species was increased. Thus, replacing spent growth medium with fresh growth medium likely reduced the current drawn from the biofilm electrode. Since the redox potential profiles in Figures 3 and 5 are nearly identical in shape, removal of the soluble redox mediators had no observable effect on the shape of the redox potential profiles.

Does the Direction of Electron Flow Conflict with Thermodynamics?

One critically important observation from Figure 5 is that while the bulk redox potential was approximately +250 mV_{Ag/AgCl}, the OCP of the biofilm electrode was negative, indicating that the potential of the biofilm electrode was controlled by the biofilm and not by soluble compounds. According to thermodynamics, electrons are always transferred from a lower potential to a higher potential. The observed current of 0.5 μ A with a bulk redox potential

higher than the electrode potential can be explained as *S. oneidensis* MR-1 biofilms not being redox controlled when they respire on electrodes. Otherwise, the observed data would conflict with thermodynamics.

pH Profiles

Figure 6 shows that there was no detectable variance in pH in the biofilm at 0 mV_{Ag/AgCl} polarization. However, a variance in pH of 0.08 units from the bulk to the bottom of the biofilm was observed at +400 mV_{Ag/AgCl}. When we repeated the experiment with 100 mM phosphate buffer (10× original concentration) at +400 mV_{Ag/AgCl}, the variance in pH was absent, indicating that proton transfer was limited by buffer strength, although a variance in pH of 0.08 units is very minimal and only four times higher than our minimum detection limit. Interestingly, this is consistent with Picioroanu et al. and Marcus et al.'s modeling results, which predicted minimal variance in pH in biofilms.^{24,31} Variance in pH in biofilms can be observed only under proton transport limiting conditions, which may be achieved under low buffer strength or at high current flow. The pH varies because protons generated during microbial respiration are not consumed by soluble electron acceptors. Electrons pass through to the biofilm electrode, whereas protons must diffuse out of the biofilm and migrate to the counter electrode to be consumed. If this process of diffusion and migration of protons is limiting, we expect pH profiles to be measured.

Geobacter sp. systems have higher observed current densities than *Shewanella sp.* systems and thus potentially have larger pH differences between the surface and the bottom of the biofilm, as Franks et al. (2009) showed.³⁴ These higher current densities have also been shown to increase with increasing ionic strength of the growth media (decreasing internal resistance).³⁵ Thus, with increasing solution conductivity, pH changes inside the biofilm become more likely because of increased current. This warrants further investigations.

These pH profiles demonstrate that the redox potential profiles shown in Figure 6 were not caused by pH. Using eq 2, for a two-electron transfer reaction, the variation in redox potential caused by a variation in pH of 0.08 units inside the biofilm is estimated to be 9.5 mV and for a one-electron transfer reaction 4.8 mV. Thus, the variation in redox potential due to a variation in pH of 0.08 units is small in comparison to the overall variation in redox potential measured in Figure 3. Our conclusion is further confirmed by the presence of a redox potential profile without a pH profile while the biofilm electrode was polarized to 0 mV_{Ag/AgCl}.

Microelectrodes are one of the critical tools used to quantify local chemistry in biofilms. They have been used to measure carbon dioxide, dissolved oxygen, hydrogen peroxide, hydrogen sulfide, other dissolved gases and ions, pH, redox potential, and diffusion coefficients in biofilms. They are minimally invasive and do not impact biofilm structure.³⁶ They can provide information which cannot be obtained using any other method. For the first time, we applied them to characterize a biofilm grown on a polarized surface. We believe that the microelectrodes we developed and describe here will be used in the future to characterize electron transfer processes in biofilms used in microbial fuel cells and bioelectrochemical systems.

In summary, for *S. oneidensis* MR-1 biofilms grown under electrode respiring conditions, we have shown that regardless of the polarization potential of the biofilm electrode, the redox potential decreases from the top of the biofilm to the bottom. The decreasing redox potential in the biofilm is not due to pH changes. In a fully redox-mediated system, the redox potential increases toward the bottom when the electrode is the electron acceptor. The opposite behavior of redox potential profiles in biofilms and redox-controlled systems is

explained by *S. oneidensis* MR-1 biofilms not being redox controlled when they respire on electrodes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Diagram of the dual-chamber flat plate biofilm reactor showing the positions of the microelectrode and the biofilm during redox potential and pH measurements. The reactor was operated anaerobically, and the cells respired on the electrodes. PEM: proton-exchange membrane.

Figure 2.

Diagram of (A) redox and (B) pH microelectrodes. RE: Ag/AgCl reference electrode. LIX: liquid ion exchange.

Figure 3.

Variation in redox potential in a *S. oneidensis* MR-1 biofilm respiring on an electrode. Redox potential was measured inside a reactor, while the biofilm electrode was polarized to 0 or $+400\text{ mV}_{Ag/AgCl}$. The inset shows the data over a smaller range on the y axis to make the variation more noticeable.

Figure 4.

Redox potential measurements taken at the redox microelectrode in a ferri-/ferrocyanide solution while the electrode was polarized to +400 mV_{Ag/AgCl}. The redox profile taken in a *S. oneidensis* MR-1 biofilm shown in Figure 3 is added for comparison.

Figure 5.

Changes in redox potential in *S. oneidensis* MR-1 biofilm grown on an electrode after the bulk medium (with $-500 \text{ mV}_{\text{Ag/AgCl}}$ redox potential) was replaced with fresh medium (with $+250 \text{ mV}_{\text{Ag/AgCl}}$ redox potential). The profiles were measured at the open-circuit condition and after the biofilm electrode was polarized to $+300 \text{ mV}_{\text{Ag/AgCl}}$. The potentials at the bottom were -500 and $+300 \text{ mV}_{\text{Ag/AgCl}}$. These data points are not shown in the figure for clarity.

Figure 6.

Variation in pH in a *S. oneidensis* MR-1 biofilm respiring on an electrode shows that proton transport was not a limiting factor for electron transfer in our steady-state biofilms, while the biofilm electrode was polarized to 0 and +400 mV_{Ag/AgCl}.