A Fumarate Microbiosensor for Use in Biofilms

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Fumarate is one of the terminal electron acceptors which can be used by microorganisms growing as biofilms. Mass transport in biofilms is dominated by diffusion, which creates a fumarate concentration gradient and could be the limiting factor for the growth of biofilms respiring on fumarate. Bulk solution measurements cannot be used to determine fumarate limitations in fumarate-respiring biofilms. Therefore, new approaches are needed for measuring fumarate concentration in biofilms. The goal of this study was to develop a microbiosensor for in situ depth profiling in biofilms. The working principle of the microbiosensor is the correlation between fumarate concentration and current consumption during fumarate reduction by G. sulfurreducens grown on a carbon microelectrode tip. The microbiosensor consists of a carbon microelectrode with G. sulfurreducens biofilm on a tip with a diameter of ~30 μm as the working electrode and a built-in Ag/AgCl pseudo reference electrode, enclosed in a glass outer case. The microbiosensor responded in the 0–3.2 mM fumarate concentration range and had a detection limit of 258 ± 25 μM (S/N = 2). We quantified the stirring effect and the interfering effect of alternative substrates on the microbiosensor response. The utility of the fumarate microbiosensor was demonstrated by depth profile measurements of fumarate concentration in cathodic G. sulfurreducens biofilms. We found that fumarate was not a limiting factor for cathodic current generated by G. sulfurreducens biofilms.

Biofilms can utilize various electron acceptors, such as nitrate, sulfate, sulfur, insoluble Mn(IV) and Fe(III) oxides and fumarate, in the absence of oxygen. Fumarate respiration is one of the common types of anaerobic respiration for biofilms. Mass transport in biofilms is dominated by diffusion, which creates a fumarate concentration gradient and could be the limiting factor for the growth of biofilms respiring on fumarate. This limitation cannot be quantified from the bulk measurements, and new approaches are needed to measure fumarate depth profiles in biofilms.

To date, in situ depth profiling in biofilms has been limited to several studies using nuclear magnetic resonance (NMR) microscopy, spectroscopy, and combined NMR/confocal scanning fluorescence microscopy to study biofilm metabolism. For example, Majors et al. used NMR to measure in situ fumarate and lactate concentrations in biofilms. In this technique, the fumarate concentration profiles were measured with a spatial resolution of 22 μm × 2.5 mm × 2.5 mm (140 pL/voxel) and a detection limit of ~1 mM. The NMR technique is very expensive and its use is limited to those who have access to this instrument. We should note that this type of measurements is limited to the equipment located at the Environmental Molecular Sciences Laboratory (EMSL) located at Pacific Northwest National Laboratory (PNNL) located at Pacific Northwest National Laboratory (PNNL), Richland, WA. Therefore, an alternative measurement technique with a better detection limit and spatial resolution is needed.

Needle-type microsensors with small tip diameters—generally less than 30 microns—are primary tools for measuring microscale gradients noninvasively in biofilms with higher resolutions, faster response times, lower detection limits, and enhanced sensitivities compared to many other techniques. Therefore, a large number of electrochemical and optical microsensors have been developed for quantifying various chemical species and light intensity in biofilms. In addition, microbiosensors, which are a subclass of electrochemical microsensors, have been developed that employ immobilized microorganisms as biological transducers. These are used to measure glucose, methanate, nitrate, galactose and acetate concentrations in biofilms. Although many microsensors have been developed to detect selected chemical species, there is currently no microsensor capable of measuring fumarate concentrations in biofilms.

Bacterial species that are known to form biofilms and have well-studied electron transfer properties include Shewanella sp., Geobacter sp., and Pseudomonas sp. These biofilms are called electrochemically active biofilms (ECABs). ECABs can donate electrons to a solid electrode (anodic biofilm) and can accept electrons from a solid electrode (cathodic biofilm). Of these, only Geobacter sulfurreducens biofilms exhibit the stability, selectivity, and high current output required to operate as the biological transducer in a fumarate microbiosensor. G. sulfurreducens biofilms grown on electrodes can reduce fumarate using electrons provided through a cathodic current. It has been shown that fumarate reduction by G. sulfurreducens biofilms is dependent on cathodic current when the biofilm electrode is polarized to a sufficiently negative potential. Under these conditions, fumarate concentration decreases in the bulk solution, it cannot be determined whether fumarate is limiting the cathodic current. Even when fumarate concentrations are monitored in the bulk solution, it cannot be determined whether fumarate is limiting in G. sulfurreducens biofilms without measuring its variation in the biofilm. Since G. sulfurreducens biofilms can reduce fumarate using electrodes as their electron donor under anaerobic conditions, a needle-type microbiosensor based on the fumarate-current relationship can be developed to detect fumarate concentrations amperometrically.

The goal of this work was to develop a microbiosensor based on the direct conversion of fumarate to succinate using electrons from a solid electrode by G. sulfurreducens biofilm grown on the tip of a carbon microelectrode (this is referred to as a microelectrode-biofilm). We demonstrated the usefulness of the fumarate microbiosensor by measuring fumarate concentration depth profiles inside a G. sulfurreducens biofilm grown on a large glassy carbon electrode (referred to as a macroscale-biofilm). We expect that in addition to biofilm applications, the developed fumarate microbiosensor can be used in various anaerobic applications such as in a wastewater treatment system during anaerobic conversion processes in which fumarate is used as an electron acceptor. For example, fumarate can be used as an alternative electron acceptor by sulfate-reducing bacteria in biological wastewater treatment.
is composed of a carbon microelectrode and a Ag/AgCl pseudo reference electrode enclosed in a glass outer case. The carbon wire used to construct carbon microelectrodes was a 30-μm-diameter electrochemically active carbon fiber (World Precision Instruments, Sarasota, FL, USA, catalog #C3005). Briefly, the carbon microelectrode was constructed by sealing a carbon wire into a glass capillary made from Corning 8161 premium patch clamp glass (Warner Instruments, Hamden, CT, USA, catalog #G8616ST-4). A custom-made microelectrode puller with an O-shaped heating element was used to melt the glass so that it covered the carbon wire. Once the carbon wire was covered with glass, the tip of the carbon microelectrode was opened so that it had an exposed surface area. To achieve this, a diamond grinding wheel (Narishige, Tokyo, Japan, model #EG-44) was used to remove excess glass from the tip of the carbon microelectrode. The Ag/AgCl reference electrode was made of a disposable borosilicate glass Pasteur pipette (Loctite, Westlake, OH, USA, catalog #1365868). After complete drying of the epoxy, the prepared Ag/AgCl reference electrode was inserted into the outer case and glued with five-minute epoxy.

**Sensing mechanism of the proposed fumarate microbiosensor.**—The microelectrode-biofilm placed into the outer case (Figure 1a) is used as the biological transducer. Initially, fumarate diffuses through the ~30-μm open tip. Fumarate is then reduced to succinate by a pregrown G. sulfurreducens biofilm using the electrons from the carbon microelectrode (Figure 1b) according to Equation 1.

\[
\text{C}_4\text{H}_4\text{O}_4 \rightarrow \text{C}_4\text{H}_6\text{O}_4 + 2\text{H}^+ + 2e^- \quad \text{biofilm}
\]

\[
E^\circ = -169 \text{ mV}_{\text{Ag/AgCl}}
\]  

The electrons used in this reaction are harvested from the reference electrode (RE), which also acts as a counter electrode (CE). The standard reaction occurring on RE/CE is given in Equation 2.

\[
\text{AgCl} + e^- \rightarrow \text{Ag} + \text{Cl}^-, \quad E^\circ = 0 \text{ mV}_{\text{Ag/AgCl}}
\]  

In this case, electrons are liberated from Ag(s) and are consumed in Equation 1. It should be noted that operating the microbiosensor in this way is possible because the current passed between the working electrode and RE is on the order of nanoamps. The increase in [Cl\(^-\)] during one hour of operation at ~3 nA is only ~0.1 mM, which is insignificant when compared to the initial [Cl\(^-\)] of 9.45 mM. At the cost of an increase in complexity of construction and operation, a true CE (carbon wire) can be inserted. However, we observed that the response of the three-electrode system was identical to that of the two-electrode system (result not shown). Therefore, we operated our fumarate microbiosensor as a two-electrode system in which the true CE (carbon wire) can be inserted. However, we observed that the response of the three-electrode system was identical to that of the two-electrode system (result not shown). Therefore, we operated our fumarate microbiosensor as a two-electrode system in which the true CE (carbon wire) can be inserted.

![Figure 1.](image)

**Figure 1.** a) The fumarate microbiosensor consists of a carbon microelectrode with a tip diameter of ~30 μm, a Ag/AgCl reference electrode, and an outer case. b) Sensing mechanism of the fumarate microbiosensor. c) Photo of the carbon microelectrode with G. sulfurreducens biofilm grown on the tip. Figure not drawn to scale. The medium contains fumarate, which diffuses through the open tip and is then reduced by G. sulfurreducens biofilm using electrons from the microelectrode. The cathodic current is correlated with the fumarate concentration in the vicinity of the tip.
sulfurreducens biofilm is proportional to the fumarate concentration in the vicinity of the microbiosensor tip.

**Growth medium.**—The growth medium (acetate- and fumarate-free medium) used to grow *G. sulfurreducens* strain PCA (ATCC 51573) consisted of: KCl, 0.38 g/L; NH₄Cl, 0.2 g/L; NaH₂PO₄, 0.069 g/L; CaCl₂, 0.04 g/L; MgSO₄·7H₂O, 0.2 g/L; NaHCO₃, 2 g/L; Wolfe’s vitamin solution, 10 mL/L; modified Wolfe’s mineral solution, 10 mL/L. The details of Wolfe’s vitamin and mineral solution can be found somewhere else. The biofilms were initially grown using the growth medium in the presence of 20 mM (1.64 g/L) sodium acetate. This medium was used to grow *G. sulfurreducens* biofilms on both the macroelectrode and the microelectrode. After a mature biofilm was observed, the medium containing acetate was purged and replaced with a growth medium including 3 mM sodium fumarate (0.48 g/L) for macroscale-biofilms. Medium, free of acetate and fumarate was used as the electrolyte solution in the microbiosensor after the microelectrode-biofilms had grown on the tips.

**Growing microelectrode-biofilms.**—The microelectrode-biofilm was formed by growing *G. sulfurreducens* on a carbon microelectrode (after it was assembled to glass outer case). If the tip is used as the electron donor for growing *G. sulfurreducens*, the cells initially attach to the electrode but are unable to develop into thick biofilms. This does not provide the biomass needed for the microbiosensor to function properly. Therefore, we initially grew microelectrode-biofilms using a carbon microelectrode (polarized at +300 mV Ag/AgCl) as the electron acceptor while providing acetate as the electron donor. When *G. sulfurreducens* biofilm had grown on the carbon microelectrode tip, we then changed the polarization potential to −600 mV Ag/AgCl. Under this condition, the microelectrode-biofilm respired on fumarate with the electrode as the electron source and therefore could be used as a microbiosensor for fumarate detection.

A temperature-controlled bioelectrochemical cell (Gamry Instruments #990-00249) similar to the setup in Ref. 35 was modified to grow a *G. sulfurreducens* microelectrode-biofilm on a carbon microelectrode tip. The cell was operated with 150 mL of growth medium in batch mode. The heat jacket was maintained at 30 °C using a Fisher Scientific Model 910 water bath. The counter electrode was a 3-mm graphite rod (Sigma-Aldrich #496545), and the reference electrode was a saturated KCl Ag/AgCl reference electrode. A 0.2-μm filter was used at the gas inlet to feed in a mixture of N₂/CO₂ (80%/20%). A Gamry Interface 1000 (Gamry Instruments, Warminster, PA) was used to polarize the carbon microelectrode and to take cyclic voltammograms (CVs). The cell was then inoculated with 15 mL of *G. sulfurreducens* inoculum prepared according to a previously published procedure. The current started to increase within a few hours of inoculation, as shown in Figure 2a. After approximately 120 hours of growth, the current reached ~40 nA, which we considered the pseudo-steady current based upon prior work. CVs were taken for the carbon microelectrode at t = 24 hours, right before the inoculation, and at t = 145 h. The CVs were used to verify the biofilm activity on the microelectrode tip. Figure 2b shows a CV of a *G. sulfurreducens* microelectrode-biofilm grown on the carbon microelectrode tip (Figure 1c). The current response was flat before the inoculation at t = 24 h, while a CV taken after the growth (at t = 145 h) is characteristic of *G. sulfurreducens* biofilm. The limiting current was observed after −200 mV Ag/AgCl (Figure 2b). The response observed in Figure 2b verified that the microelectrode-biofilm was ready for the calibration. The polarization potentials of +300 mV Ag/AgCl and -600 mV Ag/AgCl, in the anodic and cathodic limiting current region were selected respectively.

**Calibration of the fumarate microbiosensor.**—As grown, the microbiosensor would not detect fumarate. To induce the sensing mechanism (Equation 1), the acetate was replaced with fumarate and the electrode potential was switched from +300 mV Ag/AgCl to -600 mV Ag/AgCl. This was done in a calibration cell identical to the bioelectrochemical cell used. A 150-mL volume of deoxygenated fumarate-free growth medium was added to the calibration cell. First the microbiosensor was removed from the bioelectrochemical cell, and then ~1 mL of deoxygenated acetate- and fumarate-free medium was used to purge the outer case of medium containing acetate. After two rinses, the microbiosensor was placed into the calibration cell and was polarized at -600 mV Ag/AgCl. The microbiosensor was allowed to reach a steady current at -600 mV Ag/AgCl polarization (which usually takes 2–3 hours) before calibration. Aliquots of sodium fumarate solution from 1 M (160 g/L) sterilized deoxygenated stock were added to increase the total fumarate concentration in the calibration chamber to 100, 200, 400, 800, 1600 and 3200 μM. After each addition, approximately 5 minutes were allowed for the microbiosensor to reach its final response. The calibration curves were produced by plotting the concentration against the average of the 90% final steady current response against corresponding fumarate concentration. We estimated
response time by measuring the time required to reach 90% of the final response.\textsuperscript{38,39}

**Interfering electron acceptors and stirring sensitivity.**—The fumarate microbiosensor response was tested for interference of ferric citrate, elemental sulfur, and acetate since they can be used as alternative electron acceptors or donors during the operation of the microbiosensor.\textsuperscript{40-42} The interference responses were observed when the microbiosensor was calibrated in solutions of ferric citrate, elemental sulfur, and acetate. Since we constructed the microbiosensor with an open tip filled with liquid medium, it was essential to test whether this membraneless tip is sensitive to stirring. A magnetic stirrer (ika, catalog #2669923) and a stir bar with a 2-cm diameter were used to mix the calibration solution at 50, 100, 150, and 200 rpm. The rpm value refers to the number of rotations completed in one minute at the base of the calibration cell. Calibration curves were obtained at each stirring rate, including the stagnant condition, and compared.

**Preparing macroscale G. sulfurreducens biofilms to measure in situ depth profiles.**—The microbiosensor was tested by measuring fumarate concentration profiles within a macroscale \textit{G. sulfurreducens} biofilm. The macroscale-biofilm was first grown anodically and then converted to cathodic operation for fumarate respiration, similar to the conversion of a microelectrode-biofilm from anodic to cathodic mode. The reduction of fumarate within the macroscale \textit{G. sulfurreducens} biofilm during cathodic operation was monitored with the fumarate microbiosensor. The biofilms were grown on a glassy carbon electrode (working electrode, SPI-Glas grade 11: 25 mm × 25 mm × 2 mm) placed in a three-electrode bioreactor made of polycarbonate. The bioreactor had a port (20-mm diameter) located at the base of the working electrode, which allowed the insertion of the microbiosensor for fumarate profile measurements. The counter electrode was a 6-mm diameter graphite rod electrode (Sigma-Aldrich, St. Louis, MO, USA, catalog #496553), and the reference electrode was a custom-made Ag/AgCl reference electrode constructed according to.\textsuperscript{7} The total working volume of the bioreactor was approximately 60 mL. During operation, the reactor was continuously fed with sterilized growth medium and sparged with the filtered gas mixture of N\textsubscript{2}/CO\textsubscript{2} (80%/20%). The potential of the working electrode was controlled using a custom-built potentiostat.\textsuperscript{43} The current was recorded directly from the potentiostat at 60-second intervals using a computer with custom LabVIEW VI software (National Instruments Corporation, Austin, TX, USA). Initially, the macroscale \textit{G. sulfurreducens} biofilm was grown on the glassy carbon electrode polarized at +300 mV\textsubscript{Ag/AgCl} as described previously.\textsuperscript{36} Acetate (20 mM) was used as the electron donor while the glassy carbon electrode served as an electron acceptor. After \∼1 month of operation, prior to microbiosensor measurements, the acetate was washed out from the biofilm by feeding the bioreactor with acetate- and fumarate-free growth medium until the current reached a steady level of <1 \textmu A at +300 mV\textsubscript{Ag/AgCl}. The bioreactor was then switched to cathodic operation by changing the polarization potential of the glassy carbon electrode to -600 mV\textsubscript{Ag/AgCl} and feeding the bioreactor with a growth medium containing 3 mM fumarate. At this potential, the electrode served as the electron donor to the \textit{G. sulfurreducens} macroscale-biofilm and fumarate (3 mM) was used as the electron acceptor.

Organic acids in the bulk liquid were determined using high-performance liquid chromatography (HPLC) with an Aminex HPX-87H column (Bio-Rad) operated with 5 mM H\textsubscript{2}SO\textsubscript{4} as the eluent (0.6 ml min\textsuperscript{-1}) and UV detection (210 nm). The percentage of fumarate reduced by the macroscale \textit{G. sulfurreducens} biofilm while it used the electrode as the electron donor was calculated as described in the literature (Gregory et al., 2004). HPLC measurements of acetate and fumarate concentrations were used to calculate electron balance and to verify that acetate was completely consumed in the bioelectrochemical cell.

**Measuring depth profiles of fumarate in \textit{G. sulfurreducens} biofilms.**—A custom-built microprofiling system equipped with a three-axis micromanipulator with a motorized precision linear actuator (PI M-230.10S, Physik Instrumente, Auburn, MA, USA) controlled using custom-made LabVIEW software (National Instruments, Austin, TX, USA) was used to position the microbiosensor tip. The vertical range of the motorized actuator was 8 mm with a submicron step resolution. The microbiosensor and the surface of the biofilm were located using a stereomicroscope (Zeiss Stemi 2000 microscope). Following the setup of the micropilling system, the microbiosensor was hooked up to the micromanipulator arm. The three-electrode bioreactor was then opened by removing the rubber stopper used to plug the sampling port. Subsequently, the microbiosensor tip was positioned ∼250 \textmu m from the macroscale-biofilm surface using the coarse knobs on the micromanipulator and software controls. Custom-developed micropoller software was then used to automate the measurement of fumarate by depth from ∼250 \textmu m above the macroscale-biofilm surface to the electrode surface in 5-\mu m increments. The depth profile experiments quantified the variation of fumarate concentration within the macroscale \textit{G. sulfurreducens} biofilm while the electrode potential was held constant.

**Results and Discussion**

**Calibration of the fumarate microbiosensor.**—Figure 3a shows the response of the microbiosensor to an increase of fumarate concentration from the baseline (0 mM fumarate) to a final concentration of...
3.2 mM. The average response time of the fumarate microbiosensor was 2.75 ± 0.6 min within a 0.1–3.2 mM calibration range, and the response time was concentration-dependent for fumarate concentrations above 0.8 mM. While the response time was 2.4 ± 0.32 min for 0.1, 0.2, 0.4, and 0.8 mM fumarate concentrations, it was 3.2 min and 3.7 min for 1.6 mM and 3.2 mM fumarate concentrations, respectively. Figure 3b shows the calibration curve of a fumarate microbiosensor correlating the bulk fumarate concentration and current consumption. The working range of the fumarate microbiosensor was between 0 mM and 3.2 mM fumarate concentrations with a detection limit of 258 ± 25 μM (S/N = 2) calculated from three independent calibrations. The calibration curve of the fumarate microbiosensor (Figure 3b) is described using the second-order polynomial curve fitting.44,45 This type of nonlinear concentration-dependent saturation response was expected because of delayed metabolite transport by carrier systems in the cell membrane.46 This behavior is also related to Michaelis-Menten kinetics, in which the reaction rate changes linearly with substrate concentration at low substrate concentrations, while the reaction rate is independent of substrate concentration at higher substrate concentrations.37

Based on the multiple constructions and calibrations, each microbiosensor had a unique calibration curve depending on the biomass of pre-grown G. sulfurreducens microelectrode-biofilm. We observed that the calibration range was consistent but that the variation in the maximum response at 3.2 mM concentration was -2308 ± 245 pA (n = 3) and the average background current was -146 ± 100 pA (n = 3). This was expected since it is difficult to grow the same mass of biofilm on the carbon microelectrode tip for each new microelectrode. In addition, while tips are ground during their construction, small variations in the diameters of the ∼30-μm tips of the carbon microelectrodes result in slightly different exposed surface areas for microelectrode-biofilms to grow on.

It should be noted that the maximum cathodic current density of the microelectrode-biofilm was 4.10 A/m², while the average current density in the replicated experiments was 3.44 ± 0.64 A/m² (n = 3). The maximum current density from the microelectrode-biofilm was ∼13 times higher than that of G. sulfurreducens biofilms grown on larger electrodes. For example, Dumas et al. reported a maximum current density of only 0.32 ± 0.08 A/m² on graphite cathodes polarized to -600 mV (Ag/AgCl).28 In addition, the maximum current density was 0.45 A/m² for the macroscale-biofilms in which we measured fumarate depth profiles. Although the variation of current density with electrode size has been previously studied, the reasons for the high current densities from smaller electrodes are mostly not well known and are still debated.48-50 The high current density produced by the microelectrode-biofilm enabled us to develop a microbiosensor with enhanced sensitivity and a faster response time. It is already well known that sensors at the micron scale provide better response times and lower detection limits than their macroscale counterparts.6,51

**Verification of sensing mechanism.**—To verify the sensing mechanism shown in Equation 1 and Figure 1b, we quantified the fumarate reduction and succinate production from a G. sulfurreducens macroscale biofilm under cathodic conditions. This biofilm was developed during previous anodic operation, which resulted in a 150-μm-thick biofilm on glassy carbon electrode. This thickness also provided enough spatial resolution for measuring fumarate concentrations in the macroscale-biofilm using the microbiosensor with a 30-μm tip diameter. When the medium containing acetate was purged from the bioreactor and replaced with growth medium containing 4.35 ± 1.42 mM fumarate (no acetate), we observed an immediate development of biofilm on the carbon microelectrode using the microbiosensor with a 30-μm-long, 30-μm-wide cylindrical open platform was located underneath the pores.55 Figure 4 shows the effect of stirring on the fumarate microbiosensor calibration from 0 mM to 3.2 mM bulk fumarate concentrations. The Reynolds numbers (Re) for stirring rates of 50, 100, 150, and 200 rpm were 333, 667, 1000, and 1333, respectively. It should be noted that 100 < Re < 1000 shown to be due to partial oxidation of fumarate when an alternative electron donor, such as an electrode, is used. Partial oxidation of fumarate was supported by the detection of organic acids which were expected to be metabolized when a portion of the fumarate was oxidized. These results, the development of cathodic current and the reduction of fumarate to succinate, verified the sensing mechanism of the microbiosensor that we proposed (Equation 1).

**Interfering effects on the microbiosensor response.**—Aside from fumarate, G. sulfurreducens biofilms can use several other compounds as alternative electron acceptors, including Fe(III) and elemental sulfur.40-42 The existence of these compounds in target samples could interfere with fumarate measurements with our microbiosensor. Therefore, we tested the current responses of the fumarate microbiosensor to these alternative electron acceptors at their equivalent concentrations of fumarate. We did not see any noticeable interfering effect from ferric citrate or elemental sulfur because of their low solubility in aqueous solutions (data not shown).52,53 The interfering effect of acetate in the medium was also tested. Since microelectrode-biofilms were initially grown in the presence of acetate, residual acetate in the outer case could have negatively affected the microbiosensor response. We found that acetate addition induced a -2.20% ± 1.91% decrease in cathodic current. This is because the addition of acetate provided additional electrons to the biofilm and therefore decreased the electron consumption from the carbon microelectrode. It was also previously shown that G. sulfurreducens can tolerate exposure to atmospheric oxygen for a day and use it as the sole electron acceptor at concentrations of 10%.54 However, experimentally we found that exposing the microbiosensors to oxygen for more than 15 minutes deactivated them. This could be because of the mass of biofilm and the surface area of our carbon microelectrode, which was significantly smaller than that of the large electrodes used in the published literature (7 × 10⁻¹⁰ cm² vs. 6.25 cm²).

**Interfering effect of stirring on the microbiosensor response.**—Since we employed a 200-μm-long, 30-μm-wide cylindrical open tip which acted as a diffusion barrier separating the microelectrode-biofilm from the external environment, it was necessary to verify that the microbiosensor response is not sensitive to stirring. A similar approach was previously used to ablate several-micron pores in insulating material where carbon conductive substrate as a sensing platform was located underneath the pores.55 Figure 4 shows the effect of stirring on the fumarate microbiosensor calibration from 0 mM to 3.2 mM bulk fumarate concentrations. The Reynolds numbers (Re) for stirring rates of 50, 100, 150, and 200 rpm were 333, 667, 1000, and 1333, respectively. It should be noted that 100 < Re < 1000.
indicates a transition from laminar to turbulent flow, so Re > 1000 indicates mixing is turbulent.50 We also tested the fumarate microbiosensor under stagnant conditions (0 rpm). Calibrations in a wide range of Re verified that we tested our microbiosensor in laminar and turbulent zones. The microbiosensor response was tested at various concentrations and stirring rates (from 0 rpm to 200 rpm). There was no detectable effect of stirring on the microbiosensor response. For example, the difference in the microbiosensor response between 50 rpm and 200 rpm was 4.07% at 3.2 mM fumarate concentration. We also tested microbiosensor response under stagnant conditions (0 rpm) for various fumarate concentrations. We did not see significant differences between no mixing and mixing at 50 rpm. The maximum difference we observed was 3.75% at 3.2 mM fumarate concentration, and this difference was not significant. However, for convenience, we used 50 rpm for the calibrations since it provides quicker calibration and a well-mixed (homogenized) solution. This verifies that the open tip functioned as a diffusion barrier and adequately isolated the internal medium of the fumarate microbiosensor from the external medium. It also confirms that the diffusion barrier functioned as a membrane. The open tip successfully eliminated convection between the bulk medium and the microbiosensor electrolyte solution. We verified experimentally that the biofilm that we probed did not pass through the open tip.

In another words, the large biofilm was firmly enough attached that it did not move into the open tip of the microbiosensor. After each measurement, the tip was clean.

Fumarate concentration depth profiles in a G. sulfurreducens biofilm.—Figure 5 shows the fumarate concentration depth profiles measured within a macroscale G. sulfurreducens biofilm 1) when no current was passed at the open circuit potential (OCP, −286 ± 3 mVAg/AgCl), and 2) when current (−293.1 ± 5 μA) was passed at a polarization potential of −600 mVAg/AgCl. At OCP, we observed that the fumarate concentration decreased from 2.60 mM near the biofilm surface to 2.06 mM at 150 μm below the biofilm surface. We observed identical profiles at OCP in replicated experiments (data not shown). This was not an expected result, since there was no detectable residual acetate in the medium (verified with HPLC) and the electrode did not provide any electrons to the biofilm at OCP. The reduction of fumarate in the absence of an electron donor could be attributed to the ability of biofilm to metabolize biofilm matrix components such as polysaccharides. Previously, McLean et al. also observed the utilization of fumarate in Shewanella oneidensis MR-1 biofilms when there was no exogenous electron donor.3 Their experiments using NMR showed that ~26 mM fumarate was reduced over a 40-min period by S. oneidensis MR-1 in the absence of any exogenous electron donor such as lactate. Our depth profile measurements at OCP and previous NMR results suggest that the cells can use endogenous cellular energy sources. When current was allowed to pass at −600 mVAg/AgCl, a decrease in fumarate concentration was observed both near the biofilm surface (1.16 mM) and at the bottom of the biofilm (0.53 mM). Overall, these results show that fumarate was reduced when the electrode was used as the electron donor. The availability of fumarate at the bottom shows that cathodic current generation by G. sulfurreducens biofilm is not fumarate-limited at 3 mM bulk concentration. More interestingly, the shape of the depth profile at −600 mVAg/AgCl shows that the area with the steepest concentration gradient, which translates to a higher consumption rate, was within the top 50 μm of the biofilm. The average flux calculated (following procedures given by 3) from three replicates was 0.71 × 10−7 ± 0.13 × 10−7 moles/cm² s). In addition, calculated fumarate fluxes from the bulk solution were compared with the electron flux from the electrode (4.86 × 10−10 moles of e−/cm²). During −600 mVAg/AgCl depth profiles; this showed that 34.89 ± 6.22% of fumarate was reduced by G. sulfurreducens biofilm using the electrons from the electrode. As stated above, our HPLC experiments in the bulk showed that 45.31% ± 17.52% of fumarate was reduced by electrons harvested from the electrode. These results are comparable to those of previous literature, which found 42% fumarate reduction by electrodes.27 At OCP, the concentration gradient was linear across the majority (150 μm) of the biofilm. The difference between the shapes of the curves with and without current suggests that only the top portion of the biofilm was highly active for fumarate reduction via the electrode. In a previous study, the metabolic activity of cells in G. sulfurreducens biofilms respiring on an electrode were tested using uranium as a redox-active component.27 Similar to our findings, it was found that cells near the top of the biofilm reduced uranium more actively than cells near the base. Also, high-resolution transmission electron microscopy images showed that healthy cells are located near the top while plasmolysed cells are located near the base of the biofilm during electron transfer between biofilm cells and the electrode.

Conclusions

A fumarate microbiosensor based on G. sulfurreducens biofilms was developed and tested for measuring in situ fumarate concentrations inside biofilms. The microbiosensor is composed of a carbon microelectrode working electrode (~30-μm tip diameter) with a pregrown G. sulfurreducens biofilm on the tip and a built-in pseudo Ag/AgCl reference electrode, enclosed in a glass outer case. The microbiosensor showed a working range between fumarate concentrations of 0 and 3.2 mM with a detection limit of 258 ± 17.52 μM (S/N ≥ 2). With the developed microbiosensor, we successfully measured depth profiles of fumarate concentrations inside a macroscale G. sulfurreducens biofilm operated cathodically on a glassy carbon electrode. The depth profile measurements showed that the fumarate concentration decreased to 2.06 mM at the bottom of the biofilm from 2.60 mM near the biofilm surface when no current was passed. When current was allowed to pass at −600 mVAg/AgCl polarization, a decrease of fumarate concentration was observed both at the biofilm surface (1.16 mM) and at the bottom of the biofilm (0.53 mM), suggesting that electrons from the electrode were responsible for the reduction of fumarate. The results from depth profile measurements revealed that fumarate was not limiting in G. sulfurreducens biofilm at 3 mM bulk concentration. Overall, depth profile measurements demonstrated that fumarate reduction was limited kinetically by the biofilm, not by diffusion to the electrode surface.

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References

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