A voltammetric flavin microelectrode for use in biofilms

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ABSTRACT

Biofilms used in bioelectrochemical systems are expected to transfer electrons using electron transfer mediators. One mediator type, flavins, which includes flavin mononucleotide, riboflavin, and flavin adenine dinucleotide, has been found to be endogenously produced by Shewanella oneidensis MR-1. However, the presence and concentration of flavins inside a S. oneidensis MR-1 biofilm have never been reported. The goal of this study was to develop a flavin microelectrode capable of measuring flavins inside a living biofilm and apply it to a biofilm which produces flavins. Because flavins are electrochemically active molecules, the flavin microelectrode was based on detection via square-wave voltammetry. The microelectrode consisted of a carbon working electrode with a 10–30 μm tip diameter, a built-in platinum counter electrode, and a Ag/AgCl reference electrode, all enclosed in a glass outer case. The microelectrode was calibrated between 0.1 μM and 10 μM flavins and showed a linear correlation between flavin concentration and peak currents located at −424 mVAg/AgCl on a square-wave voltammogram. We also developed a model to explain the electrochemical mechanism of flavin detection, and to determine the effective surface area of the microelectrode, the standard reduction potential, and the transfer coefficient. We found that the effective surface area of the microelectrode was close to 100 times the projected surface area. The model predicted a standard reduction potential for RF/RH2 of −419 mVAg/AgCl at 20 °C and a transfer coefficient of 0.45. Lastly, we measured flavin concentration inside a S. oneidensis MR-1 biofilm grown on a glass surface using oxygen as the electron acceptor. The flavin concentration reached 0.7 μM, increasing near the bottom of the biofilm, where no oxygen was present. This shows the possibility that flavins are produced in the anaerobic zone to act as intermediate electron acceptors in the deeper parts of the biofilm, where there is no oxygen.

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1. Introduction

Microelectrodes have played a significant role in the expanding field of biofilm research. To date, microelectrodes have been used to measure numerous chemical species and microenvironment properties in biofilms, such as dissolved oxygen and pH [1–6]. Microelectrodes have many advantages in biofilm research: microelectrodes are (1) able to measure microscale and high-temporal resolution profiles, (2) able to respond quickly, (3) capable of taking measurements in live biofilms, and (4) they do not impact biofilm structure. It is well accepted that understanding biofilm microenvironments and microscale phenomena is the key to controlling and manipulating biofilms at the macroscale, as these microenvironments are the primary source of the advantages of biofilm formation over the planktonic mode [4].

Recently, significant research has focused on microbial fuel cells (MFCs) and bioelectrochemical systems (BESs), which utilize the ability of some bacterial biofilms to transfer and/or accept extracellular electrons [7,8]. These devices have been studied because of their potential role in (1) niche energy applications, such as powering remote sensors and aquatic instruments [9–12], and (2) the production of high-value molecules such as hydrogen, 1,3-propanediol, and ethanol [7,13–15]. These technologies utilize biofilms comprised of a class of microorganisms termed electrochemically active bacteria (EAB), which have been found to possess the ability to use insoluble conducting surfaces, via extracellular electron transfer (EET), as terminal electron acceptors for respiration or as electron donors for energy [16–19]. EET is one of the microscale phenomena in these biofilms that is still not well understood, and it is an important key for making BES and MFC technologies commercially viable. This is because research has shown that EET plays an important role in the chemical microenvironments of electrochemically active biofilms, affecting microscale electrochemical gradients, pH, chemical concentrations,
and metabolic rates [16,20]. Furthermore, EET from electrochemically active biofilms has been proposed to cause certain types of metal corrosion and the solubilization of solid Fe(III) minerals [21,22]. Thus, understanding EET may have an impact on a wide array of research areas.

The mechanisms by which EET occurs are not currently fully known, but one identified mechanism is electron mediation. Electron mediation occurs by the following steps: (1) terminal electrons from the electron transport chain are first transported to the cell surface via a pathway of redox-active proteins and low molecular weight compounds, (2) the electrons are then passed to electron mediators, most likely utilizing outer-membrane cytochromes as intermediaries, (3) the reduced electron mediators are transported to a solid electron acceptor such as insoluble oxides or an anode, where (4) they finally are oxidized and transfer the electrons. Electron mediators have been found to be low molecular weight organic molecules which are shuttled between the electrochemically active cells and the electrode by diffusive, advective, and/or electromigrative transport [23]. Scientific studies have revealed that EABs can utilize both exogenous (added) and endogenous (self-produced) mediators for EET [24]. In some cases endogenous mediators are used to accept electrons in deeper locations in biofilms where there is no oxygen. One mediator type that has been found to play a role in EET is flavins [25–29].

Flavins are electrochemically active organic compounds featuring a tricyclic ring dimethyl isalloxazine skeleton with a substituent off the center ring nitrogen (10 position) [30]. Three flavins, shown in Fig. 1, have been studied for their role in biofilm EET: flavin mononucleotide (FMN), riboflavin (RF), and flavin adenine dinucleotide (FAD). The flavin moiety can undergo a two-step redox reaction. When fully oxidized, or in the flavoquinone form, it can undergo a single-electron reduction to the flavosemiquinone form and then a second single-electron reduction to the flavohydroquinone form. As an example, this is shown for RF, but the reaction form is identical for all of the flavins:

\[
RF + H^+ + e^- \rightleftharpoons RFH^+
\]

(1)

![Fig. 1. The structures of the three primary flavins detected in S. oneidensis MR-1 biofilms are shown. Flavins are electrochemically active organic compounds featuring a tricyclic ring dimethyl isalloxazine skeleton with a substituent off the center ring nitrogen (10 position). The fully oxidized flavoquinone form undergoes a two-step reduction to the flavohydroquinone form (shown in the inset box), gaining two hydrogen atoms in the process. The change in double bonds to protonated nitrogen atoms is shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)](image)

RFH + H^+ + e^- \rightleftharpoons RFH_2

(2)

It has been found that flavosemiquinones generally only exist in solution in a low concentration (around 2%) [31], although they were previously believed to be present in much higher concentrations [32], as they are rapidly converted through the fast reaction of two flavosemiquinones to one flavoquinone and one flavohydroquinone [33]:

\[
RFH + RFH \rightleftharpoons RF + RFH_2
\]

(3)

Furthermore, the full reduction of flavoquinones to flavohydroquinones is very rapid and can be considered a single two-electron reduction step as shown:

\[
RF + 2H^+ + 2e^- \rightleftharpoons RFH_2
\]

(4)

Shewanella oneidensis MR-1 has recently been found to produce actively secreted flavins, particularly RF and FMN, as endogenous electron shuttles [25,26,29,34–36]. Von Canstein et al. found that both FMN and RF were excreted by S. oneidensis MR-1 and were used as an electron mediator during the reduction of Fe(III) oxides [29]. Marsili et al. also detected the secretion of flavins by S. oneidensis cells and confirmed their electron mediator activity [26]. Velasquez-Orta et al. found that flavin concentrations increased continuously in the bulk liquid of S. oneidensis MFCs, with FMN as the predominant flavin [36]. In their study, MFCs that were operated with membranes that restricted cell contact with the anode still produced current, highlighting the ability of flavins to mediate EET to remote electrodes, without the cells having direct access to the surface. Furthermore, they showed that when exogenous flavins were added to the MFC both current and power output increased. Covington et al. also confirmed the presence of endogenous flavins in S. oneidensis cultures [25]. They hypothesized that FAD was produced inside the cell and then converted to FMN in the periplasmic space, after which the FMN diffused through outer-membrane porins. Once outside the cell, a fraction of the FMN underwent spontaneous conversion to RF, allowing both FMN and RF to be detected.

Flavins have primarily been detected in biofilm research via high-performance liquid chromatography (HPLC) [25,29,36–40], liquid chromatography–mass spectrometry (LC–MS) [26], cyclic voltammetry (CV) [26,41,42], or differential voltammetry [43]. Other methods used to detect flavins include polarography [44,45], flow injection analysis with fluorescence [46], square-wave voltammetry (SWV) using a mercury drop electrode [47], and spectrophotometry [48,49]. Most of these methods require extracted liquid samples, and the analyzed data therefore represent the macroscale or bulk concentrations. To date, analysis of flavins inside a biofilm has never been performed in situ. This is critical for determining the limitations of EET in MFCs and BESSs and understanding the role of flavins as EET mediators.

Our goal was to develop a microelectrode capable of measuring flavins inside a living biofilm. Flavins are electrochemically active molecules and are therefore detectable using electroanalytical methods, such as voltammetry. The flavin microelectrode is based on detection via square-wave voltammetry, which is one of the most sensitive voltammetric techniques; it is much more sensitive than CV due to its ability to minimize charging current [50,51]. SWV utilizes a potential square wave superimposed on a staircase sweep, and the SWV signal is comprised of the difference between the current measured at the end of the peak potential and that measured at the end of the trough potential. We developed and optimized a flavin microelectrode for use in biofilms and tested it in a S. oneidensis MR-1 biofilm.
2. Materials and methods

2.1. Operational principles of the flavin microelectrode

Our flavin microelectrode works by performing SWV on a glass-covered carbon wire using a Reference 600™ potentiostat (Gamry® Instruments, Warminster, PA, USA). Only the wire tip (10–30 μm diameter) is exposed, acting as the working electrode, as shown in Fig. 2. During the experiment, the microelectrode is scanned from +700 mV Ag/AgCl to −700 mV Ag/AgCl. Note that all potentials in this study are given against a saturated Ag/AgCl reference electrode. We begin at +700 mV Ag/AgCl to insulate that all flavins near the surface of the electrode are fully oxidized to the flavoquinone form, which is generally the case for the bulk solution under aerobic conditions. This is because the standard redox potentials of flavins are located around ~400 mV Ag/AgCl, and any potential more positive than this will begin to oxidize any flavoquinones near the electrode surface. When SWV begins, flavins will therefore not produce any current signal. Upon approaching ~400 mV Ag/AgCl, the characteristic peak of flavin species will appear, as flavoquinones undergo the reaction exemplified by RF in Eq. (4). The actual standard reduction potential depends on the flavin(s) present, and the characteristic peak height center of the SWV voltammogram will match that potential. However, using SWV to discriminate between different flavins is not practical as the standard reduction potentials of the various flavins only differ by a few millivolts. Furthermore, standard reduction potentials can shift slightly in biofilms. Determining flavin type using SWV is not possible, as the differential current peaks are not distinguishable. Thus, the differential current peak height is proportional to the total flavin concentration.

2.2. Flavin microelectrode construction

Fig. 2 shows a diagram of the constructed flavin microelectrode. The flavin microelectrode is composed of a carbon wire working electrode, a platinum wire counter electrode, and an Ag/AgCl reference electrode encompassed in a pulled Pasteur pipette.

2.2.1. Making the glass capillary

A glass capillary is used to cover the carbon wire in order to isolate it from the KCl electrolyte solution surrounding the reference and counter electrodes. The glass capillary is made from Corning 8161 premium patch clamp glass (Model #: C86165T-4, Warner Instruments, Hamden, CT, USA). Its relatively low softening temperature of 600 °C allows for easy pulling and complete embedding of the carbon wire. The glass tube is pulled over a propane torch flame by hand to form a capillary. The capillary is cut in the middle to separate the two sides of pulled glass, creating two capillaries.

2.2.2. Covering the carbon wire with glass

The carbon wire is a 30 μm diameter electrochemically activated carbon fiber (Catalog #: C3005, World Precision Instruments, Sarasota, FL, USA). A ~5 cm long piece of wire is inserted into one of the glass capillaries, leaving 1 cm of carbon wire exposed at the end to be soldered to a copper wire. A custom-made microelectrode puller with an O-shaped electrical resistance heating element, shown in figure 4.28 of Lewandowski and Beyenal [4], is used to melt and pull the glass around the carbon wire. The heat is applied ~1 cm below the carbon wire tip inside the capillary, which allows the glass to flow down slowly and uniformly to cover the carbon wire. Because of the high temperatures present during the glass pulling, the carbon wire tip is partially burned and the tip diameter is therefore generally smaller than the starting 30 μm.

A diamond grinding wheel (Narisige, model EG-44, Tokyo, Japan) is used to remove glass from the tip of the carbon wire in order to expose it. Micromanipulators are used to control the capillary, and a stereomicroscope (Stemi 2000, Carl Zeiss Microlaiming GmbH, Göttingen, Germany) is used to monitor progress. Part of the carbon wire is also ground away, to refresh the burned tip and increase electrochemical activity. The final tip diameter is between 10 μm and 30 μm. The larger the diameter, the lower the detection limit will be. The tip of the carbon wire is then gently rinsed with nanopure water to remove glass and carbon particles accumulated during the grinding.

2.2.3. Making the outer case

The outer case is constructed from a borosilicate glass Pasteur pipette (Catalog #: 22-183-632, Fisher Scientific, Pittsburgh, PA, USA). The base of the pipette is cut so that the pipette is ~9 cm long, and the cut end is fire-polished with a propane torch, keeping the rough edge in the flame until the edge of the glass is smooth. To make the outer diameter of the electrode small enough to be useful in probing biofilms and to allow for enough space for an agar salt bridge, the outer case is tapered down to a 100 μm tip diameter. This is achieved by using the O-shaped heating element of the micropipette puller. The pipette is lowered by a micromanipulator to the tapered region to start the necking down process. Heat is applied until the glass begins to melt and the pipette begins to drop; then the heat delivery is quickly stopped. The pipette is then pulled back up to the next necked position. This sequence is repeated until the pipette tip is a few hundred microns in diameter. For the final thinning of the tip we use a smaller heating element, made of a 100 μm Pt wire, powered by a DC power supply (Model HY3005, RSR Electronics, Rahway, NJ, USA). We repeat the procedure for thinning the tip, applying the heat and stopping the current flow through the heating element when the pipette begins to drop. Usually just two applications of this procedure are enough to produce tip diameters less than 100 μm. Finally, using a micromanipulator, the tip of the outer case is broken under a 40× microscope by jamming it against a glass ball which has been made on the tip of a Pasteur pipette. Jamming the casing into the glass ball is continued until the tip of the outer case is ~100 μm in diameter.
2.2.4. Making the silver/silver chloride reference electrode

An 8 cm length of silver wire (0.25 mm diameter, ≥99.99% pure, Product #: 327034, Sigma–Aldrich, St. Louis, MO, USA) is polished using very fine 220 grit silicon carbide sandpaper (Ace Hardware, Pullman, WA, USA) and then rinsed with nanopure water. The silver wire and a 10 cm graphite rod cathode (Product #: 496545, Sigma–Aldrich, St. Louis, MO, USA) are dipped into a 0.1 M HCl solution and connected to a DC power supply (Model HY3005, RSR Electronics, Rahway, NJ, USA) at 0.5 V DC for 24 h. The finished silver/silver chloride wire is rinsed with nanopure water.

2.2.5. Final assembly of the flavin microelectrode

The carbon wire microelectrode and the outer case are assembled under 40× microscope magnification using manipulators. The carbon wire working electrode is inserted into the casing, with the tip positioned ~400 μm outside the casing tip. It is then glued to the casing using five-minute epoxy near the fire-polished end. At least two hours are provided so that the drying process is completed and the bond between the two glass walls is strongly formed.

A salt bridge is used to separate the working electrode from the reference and counter electrodes. In order to form the salt bridge, an agar (R-2A agar, Product #: 17209, Sigma–Aldrich, St. Louis, MO, USA) and 0.1 M Na2SO4 (reagent grade, Sigma–Aldrich, St. Louis, MO, USA) solution is first boiled and then allowed to cool to ~50 °C. This insures that the air bubbles inside the agar have disappeared. It is then applied inside the tip of the casing to a thickness of 1–1.5 mm, using a custom-made plastic capillary [4]. The agar salt bridge becomes solid after 10 min. Note that if the agar is applied directly after boiling, air bubbles will form, which will make the salt bridge less conductive and ultimately cause overflow current during use. If the temperature of the agar is too low during application, the agar will solidify too quickly, making it difficult to apply. The outer case is then filled with the electrolyte filling solution: saturated KCl saturated with AgCl (Fisher Scientific, Pittsburgh, PA, USA). Finally, the silver/silver chloride reference electrode and counter electrode (two twisted 100 μm platinum wires, 99.9999% pure, catalog #21884, California Fine Wire Company, Grover Beach, CA, USA) are inserted and glued using five-minute epoxy.

2.3. Microelectrode preconditioning

In order to enhance the signal, the microelectrode is preconditioned. We use chronoamperometry and CV to pretreat the flavin microelectrode, following these steps: (1) the clean microelectrode is polarized at a potential of +1800 mVAg/AgCl for 300 s in a 10 mM phosphate buffer (0.5075 g/L NaH2PO4 and 0.8195 g/L Na2HPO4). Then CV is performed at 100 mV/s from −800 mVAg/AgCl to +1000 mVAg/AgCl in the same solution for 30 cycles. The purpose of the preconditioning step is to create hydroxyl functional groups on the carbon surface [52–54]:

\[ \text{–C=O} + \text{H}^+ + \text{e}^- \rightarrow \text{–C–OH} \] (5)

Hydroxyl functional groups increase the number of active sites for flavin oxidation/reduction at the electrode surface and enhance the electron transfer capacity [54]. Additionally, previous studies have shown that the electrode surface structure becomes more porous after such treatment and the effective electrode surface area increases [54,55]. The flavin microelectrode can be regenerated by repeating the preconditioning. We obtained reproducible calibration curves after each use. A similar procedure was used by Brendel and Luther (1995) [2].

2.4. Calibration and optimization

Square-wave parameters, including equilibrium time, pulse potential, and scan frequency, are optimized in a 5 μM riboflavin solution to hone the microelectrode sensitivity specifically for flavins, similar to the approach used by Çakır et al. [47]. The flavin microelectrode is calibrated in riboflavin solutions with concentrations ranging from 10⁻⁶ M to 10⁻⁵ M. Riboflavin powder (Catalog #: R4500, Sigma–Aldrich, St. Louis, MO, USA) is diluted in the biofilm growth medium and used for both the optimization and the calibration [56].

2.5. Dissolved oxygen microelectrode

We followed the procedures described by Lewandowski and Beyenal to construct and calibrate a dissolved oxygen microelectrode [4]. The microelectrode had a tip diameter of 15 μm and was calibrated in sodium sulfite (Catalog #: 31454, Sigma–Aldrich, St. Louis, MO, USA) for the zero oxygen concentration and in air-bubbled nanopure water for the saturated oxygen concentration.

2.6. Biofilm growth

S. oneidensis MR-1 biofilms were grown on glass coverslips using a constant depth film fermenter (CDFF) as described by Renslow et al. [56].

2.7. Microelectrode measurements and data analysis

The biofilms were harvested from the CDFF and placed in a sterile Petri plate filled with growth medium at 20 °C. A flavin microelectrode and a dissolved oxygen microelectrode were used to measure concentration profiles near the center of a large cell cluster in the biofilm. The movement of the microelectrode was managed using a precision linear actuator (PI M-230.10S, Physik Instrumente, Auburn, MA, USA) controlled using custom-made LabVIEW software (National Instruments, Austin, TX, USA). The microelectrode measurements started in the bulk liquid, penetrated into the biofilm and stopped at the bottom of the biofilm. The setup is shown in Fig. 3. For the oxygen profile measurement, the microelectrode was moved downwards from the bulk phase to S. oneidensis MR-1 biofilm by a step size of 10 μm. For each flavin profile measurement, the microelectrode was moved downwards from the bulk phase to S. oneidensis MR-1 biofilm by a step size of 20 μm. After each step, the flavin microelectrode was scanned in SWV mode and the data were recorded in the computer with Gamry software.

![Fig. 3. Diagram of the microelectrode setup. A linear actuator controlled by custom LabVIEW software was used to create the flavin depth profiles inside the biofilms.](image-url)
3. Model development and implementation

A mathematical model for the flavin microelectrode was developed in order to explain the electrochemical mechanism of flavin detection, and to determine system parameters relating to our microelectrode. For this model, the microelectrode is assumed to be in the form of an inlaid disk microelectrode. O’Dea et al. demonstrated that the net current voltammogram is not dependent on the diffusion field at the electrode tip, so nonplanar diffusion does not need to be considered in SWV modeling, even for microelectrodes [57]. This was further verified by Komorsky-Lovrić et al., who showed that when performing SWV for reversible redox reactions, the effects of radial and hemispherical diffusion are negligible for electrodes of our size [58]. Their study demonstrates that the current peak, peak potential, and half-peak width values are nearly identical regardless of whether linear or hemispherical diffusion is assumed. Therefore, we assume a linear Fickian diffusion of flavins, given by:

\[
\frac{dC_{f,r}}{dt} = D_{f,r} \frac{d^2C_{f,r}}{dx^2} \tag{6}
\]

where \(C_{f,r}\) is the concentration (M) of flavin type \(f\) (FMN, RF, or FAD) in the r redox form (ox or red, referring to the flavoquinone or flavohydroquinone form, respectively), \(t\) is the time (s), \(D_{f,r}\) is the diffusion coefficient (\(m^2 \cdot s^{-1}\)) of flavin type \(f\) in the r redox form, and \(x\) is the distance (m) from the microelectrode tip. At the surface of the microelectrode tip, flavins are oxidized and reduced according to Butler–Volmer kinetics. The reduction kinetic rate constant, for the forward reaction described in Eq. (4), is given by [50]:

\[
k_{for,f} = k_0^f \exp \left[ -\alpha_{f} \frac{nF}{RT} (\varepsilon - E_{f}^{0}) \right] \tag{7}
\]

where \(k_{for,f}\) is the reduction kinetic rate constant (m/s) of flavin type \(f\), \(k_0^f\) is the standard heterogeneous rate constant (m/s), \(\alpha_{f}\) is the transfer coefficient (unitless), \(n\) is the number of electrons transferred during the redox reaction, \(F\) is the Faraday constant \((Coul/mol)\), \(T\) is the temperature (K), \(\varepsilon\) is the time-dependent potential \((V)\) applied to the microelectrode tip, and \(E_f^{0}\) is the standard reduction potential of flavin type \(f\). The oxidation kinetic rate constant, for the reverse reaction described in Eq. (4), is given by [50]:

\[
k_{rev,f} = k_0^f \exp \left[ (1 - \alpha_{f}) \frac{nF}{RT} (\varepsilon - E_{f}^{0}) \right] \tag{8}
\]

where \(k_{rev,f}\) is the oxidation kinetic rate constant (m/s) of flavin type \(f\). The oxidation and reduction of the flavins only occur at the electrode surface; therefore, the following boundary and initial conditions apply for Eq. (6):

Flavoquinone boundary and initial conditions

\[
\frac{dC_{f,ox}(t, x = 0)}{dx} = -C_{f,red}(t, x = 0) = C_{f,ox}(t, x = \infty) = C_{f,ox,bulk}
\]

Flavohydroquinone boundary and initial conditions

\[
\frac{dC_{f,red}(t, x = 0)}{dx} = C_{f,red}(t, x = \infty) = C_{f,red,bulk}
\]

where \(C_{f,ox,bulk}\) is the initial bulk concentration of the flavin solution. It is assumed that initially all of the flavins are in the flavoquinone form. The total current can be calculated using:

\[
i = AjF \frac{dC_{f,red}(t, x = 0)}{dx} \tag{9}
\]

where \(i\) is the current (A), \(j\) is the current density (A/m²), and \(A\) is the effective surface area of the electrode (m²).

The finite element model (FEM) model was implemented using a MATLAB (MathWorks, Natick, MA, USA) client connection to run Comsol Multiphysics (COMSOL Inc., Burlington, MA, USA) programming language code. Comsol was used to generate a backbone m-file based on the chemical engineering module diffusion application mode for a surface reaction, which also included the model geometry and finite element meshing. The m-file was expanded to handle all model parameter values and post-processing visualization. The square-wave potential sweep was implemented in an independent m-file, which was used to control the time-dependent micro electrode polarization potential, \(\varepsilon\). To avoid a jump discontinuity, which would prevent model convergence, the square-wave signal changes between low and high potentials were approximated by a differentiable, and thus continuous, spline function consisting of two quarter-sine waves connected by a straight line, as shown in Fig. 4. This was chosen over a Fourier series expansion to avoid Gibbs ringing artifacts in the model. The final SWV signal, \(\Delta \varepsilon\), is comprised of the difference between the current measured at the end of the peak potential and that measured at the end of the trough potential.

The model was fit to the calibration data by minimizing the following objective function:

\[
SSD = \sum_{y=1}^{Y} \sum_{z=1}^{Z} \left( \frac{i_{exp,y,z} - i_{calc,y,z}}{i_{exp,y,z}} \right)^2
\]

where \(Y\) is the total number of concentrations used for calibration, \(y\) is the arbitrary index value (from 1 to \(Y\)) of the calibration concentration, \(Z\) is the total number of discrete potentials at which the differential current is recorded, \(z\) is the arbitrary index value (from 1 to \(Z\)) of the discrete potential, \(i_{exp,y,z}\) is the experimentally obtained differential current for calibration concentration \(y\) at discrete potential \(z\), and \(i_{calc,y,z}\) is the model-calculated differential current for calibration concentration \(y\) at discrete potential \(z\). The difference between \(i_{exp,y,z}\) and \(i_{calc,y,z}\) is normalized against \(i_{exp,y,z}\) so each data point is weighted equally: the data points obtained at higher concentrations are not favored over those obtained at lower concentrations in the fit. The standard reduction potential, \(E_f^{0}\), the electrode surface area, \(A\), and the transfer coefficient, \(\alpha_{f}\), were used as fitting parameters. The electrode surface area could not be assumed to be the cross-sectional area of the carbon wire.
because carbon wire has been shown to be naturally highly porous and the preconditioning steps increase both the porosity and the functional groups on the surface [59].

Table 1 shows the model parameter values. The model is simplified to assume that the flavoquinone and flavohydroquinone forms of RF have the same parameter values. The riboflavin diffusion coefficient was calculated based on the Hayduk and Laudie method at 20 °C [60]. This method is based on the viscosity of the solvent and the Le Bas molar volume of the solute [61]. Calculations were performed using an online calculator developed by the United States Environmental Protection Agency for modelers with methods taken from Tucker and Nelken [62,63]. The FEM mesh included 1200 elements, with nodes 0.25 μm apart. The maximum time step was 0.01 ms, and the relative and absolute tolerances were set to 10−15. These FEM settings were more than rigorous enough to handle the rapid changes in the SWV waveform and the precision required for the flavin concentrations at the electrode surface.

4. Result and discussion

4.1. Electrode preconditioning

The chronoamperogram for the first preconditioning step is shown in Fig. 5A. The current begins high and decreases over time, except for a reproducible increase in current that peaks at 80 s. The voltammogram for the second preconditioning step is shown in Fig. 5B. During the first few cycles, a strong oxidation peak visible at +1000 mVAg/AgCl and a strong reduction peak visible at −800 mVAg/AgCl are present. However, after 30 cycles, a stable voltammogram is obtained and these peaks are significantly smaller. The species responsible for the redox peaks centered approximately at 0 mVAg/AgCl and +140 mVAg/AgCl are thought to be involved in the formation of the hydroxyl functional groups during the electroactivation preprocessing.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Model parameter values.</th>
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<tbody>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>F</td>
<td>Faraday constant</td>
</tr>
<tr>
<td>R</td>
<td>Molar gas constant</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>DZF₂</td>
<td>Diffusion coefficient for</td>
</tr>
<tr>
<td>kₚₑₛ</td>
<td>Standard heterogeneous</td>
</tr>
<tr>
<td>constant for RF/RFH₂</td>
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4.2. Optimization of the microelectrode response

The SWV parameters were optimized to give the best response for the detection of flavins. The effect of the SWV scan frequency is shown in Fig. 6A. As the frequency is increased from 1 Hz to 40 Hz, the flavin redox peak centered around −425 mVAg/AgCl increases. However, when the frequency is greater than 20 Hz significant noise appears. This effect was reproducible in multiple flavin microelectrodes; thus 20 Hz was chosen as the optimum frequency. Fig. 6B shows the effect of the equilibrium time on the SWV response. The flavin current peak had a small width at half-height and a symmetric shape when the equilibrium time was set to 5 s. Also, at 5 s and above, there was no noticeable difference in the SWV response as the equilibrium time changed; therefore, 5 s was chosen as the optimum equilibrium time. Fig. 6C shows the effect of the pulse height on the SWV response. We chose a pulse height of 40 mV because at 40 mV and above, a clear current peak develops. This is identical to the value used by Cakir et al. for riboflavin detection [47]. For the potential step height, O’Dea et al. demonstrated that 5 mV is the optimum value for two-electron transfer redox couples to provide good resolution and peak shape [65]. This value was also recommended later by Osteryoung and O’Dea [50,66]. Therefore, we chose 5 mV for the potential step height. All of the final SWV parameters chosen for the best flavin response are listed in Table 2.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Parameter values used for flavin microelectrode SWV. Equilibrium time, potential step height, pulse potential, and scan frequency were chosen to provide an optimized response for flavin detection.</th>
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<td>Parameters</td>
<td>Optimized values</td>
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<td>Starting potential</td>
<td>+700 mVAg/AgCl</td>
</tr>
<tr>
<td>Final potential</td>
<td>−700 mVAg/AgCl</td>
</tr>
<tr>
<td>Equilibrium time</td>
<td>5 s</td>
</tr>
<tr>
<td>Potential step height</td>
<td>5 mV</td>
</tr>
<tr>
<td>Pulse potential</td>
<td>40 mV</td>
</tr>
<tr>
<td>Scan frequency</td>
<td>20 Hz</td>
</tr>
</tbody>
</table>

4.3. Calibration of the microelectrode

Fig. 7 shows example SWV responses for several RF concentrations and the calibration curve obtained using the SWV peak currents. The linear fit calibration line had a coefficient of determination (R²) of 0.97. Interpolation of this calibration curve was used to determine the concentration of flavins in biofilms. Every flavin microelectrode uses a new, unique calibration curve.
4.4. Modeling the square-wave voltammetry response of the flavin microelectrode

Fig. 8 shows the model fit to the background-subtracted square-wave voltammograms. Five voltammograms and their corresponding model fit are shown for the riboflavin concentrations used to calibrate a flavin microelectrode. The model fitting parameters and the coefficient of determination are shown in Table 3. The model successfully fit the data, having a coefficient of determination of 0.99. The effective surface area was calculated to be just over 100 times the projected surface area of the 30 μm diameter tip. This was expected because of the high porosity of carbon wire and our preconditioning steps, which were used to further increase the surface area and active redox sites. The standard redox potential for riboflavin in the biofilm growth medium was calculated by the model to be $-419 \text{ mV}_{\text{Ag/AgCl}}$, which is slightly higher than the literature value of $-391 \text{ mV}_{\text{Ag/AgCl}}$ at 20°C (not in biofilm growth medium). The literature value was derived from experimental results reported in K senzhek and Petrova [32]. For all of the values in their study, we corrected the potential for temperature if it was different from 20°C using the Nernst equilibrium equation ($\ln K = nFEo/RT$, the van’t Hoff equation, and the enthalpy change values for flavin reductions cited by Beaudette and Langerman [67] and Watt and Burns [68]. Note that the literature value that is commonly cited is $-396 \text{ mV}$, corrected to 20°C, which we believe to be outdated [69]. The calculated transfer coefficient was 0.45, which is slightly lower than the generally assumed value of 0.5 for fully reversible redox reactions [50]. Note that calculating transfer coefficients from SWV in this manner is not a common method and is not a very sensitive technique. However, it does demonstrate that the flavin redox couple is not fully reversible, which had been shown by Verhagen and Hagen [33]. Based on their experimental work on FMN/FMNH2, they calculated a transfer coefficient as low as 0.34.

Table 3

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R^2$</td>
<td>Coefficient of determination</td>
<td>0.99</td>
<td>unitless</td>
</tr>
<tr>
<td>$A$</td>
<td>Effective electrode surface area</td>
<td>$8.16 \times 10^{-8}$</td>
<td>m$^2$</td>
</tr>
<tr>
<td>$E^{\circ}_{\text{RF}}$</td>
<td>Standard reduction potential for RF/RFH$_2$</td>
<td>$-419$</td>
<td>mV$_{\text{Ag/AgCl}}$</td>
</tr>
<tr>
<td>$a_{\text{RF}}$</td>
<td>Transfer coefficient for RF/RFH$_2$</td>
<td>0.45</td>
<td>unitless</td>
</tr>
</tbody>
</table>

Fig. 9. Flavin and oxygen concentration profiles in a S. oneidensis MR-1 biofilm. The top of the biofilm was approximately 300 μm from the bottom.
4.5. Measuring flavin concentrations in biofilms

Fig. 9 shows the concentration profiles measured in a S. oneidensis MR-1 biofilm. The biofilm was approximately 300 μm thick. The dissolved oxygen concentration started slightly under the oxygen saturation concentration at ~8 mg/L and dropped to 0 mg/L near the bottom of the biofilm. This trend is common in the literature [5,7,11]. The flavin concentration was below detection at all places except for a spike in concentration near the bottom of the biofilm, where oxygen was depleted. We did not detect any interfering peaks inside the biofilms that prevented flavin peak quantification. We hypothesize that flavins are produced in the anaerobic zone to act as intermediate electron acceptors in the deeper parts of the biofilm. The reduced flavins can then be transported to the aerobic zones, where they deposit the electrons to oxygen, which acts as the terminal electron acceptor.

5. Conclusions

In this study we successfully developed and tested an optimized flavin microelectrode. The microelectrode consisted of a carbon working electrode with a 10–30 μm tip diameter, a built-in platinum counter electrode, and an Ag/AgCl reference electrode, all enclosed in a glass outer case. The microelectrode was calibrated between 0.1 μM and 10 μM flavins and showed a linear correlation between flavin concentration and peak currents located at ~424 mV/Ag/AgCl on a SWV voltammogram. Using a computational model, we found that the effective surface area of the microelectrode was close to 100 times the projected surface area. The model also predicted a standard reduction potential for RF/RH2 of ~419 mV/Ag/AgCl at 20 °C and a transfer coefficient of 0.45. With this microelectrode, we successfully measured flavin inside a S. oneidensis MR-1 biofilm. The flavin concentration reached 0.7 μM, increasing near the bottom of the biofilm, where no oxygen was present.

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