Catalytic biofilm formation by *Shewanella oneidensis* MR-1 and anode characterization by expanded uncertainty

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**A B S T R A C T**

In this study, high-density planktonic cultures of *Shewanella oneidensis* MR-1 are grown aerobically to early stationary phase. After washing the cells and removing from original medium, the culture is exposed to an anaerobic environment in an electrochemical cell. An applied potential of −0.30 V vs Ag/AgCl is applied to the working electrode and the corresponding current is measured via chronocoulometry. Current begins to increase within 2–3 h stabilizing at 5 h. Cyclic voltammetry was measured at 5 h indicating the initial stages a kinetically limited biofilm and again at 24 h with an apparently more stable catalytic biofilm. At this point, the biofilm appears to suffer mass transport limitation as the catalytic wave dominates the shape of the voltammogram, similar to voltammograms reported for *Geobacter* spp. Polarization curves are also reported herein, further demonstrating a large increase of current near the oxidation potential of what is believed to be the terminal protein complex (MtrC/OmcA) of the transmembrane cytochrome cascade, the Mtr pathway. Additional characterization and comparison between replicates of the biofilm is made using the idea of expanded uncertainty. This novel approach in reporting measured results for microbial fuel cells elucidates specific electrochemical parameters for appropriate comparison between systems and laboratories.

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1. Introduction

For microbial fuel cells based on the facultative anaerobic model organism, *Shewanella oneidensis* MR-1 (MR-1), forming catalytic biofilms anaerobically is essential for improved current density. In previous work, we have observed that an applied potential on the electrode during anaerobic cultivation enables MR-1 to grow and populate the surface and employ, what we believe to be, outer membrane cytochromes in direct reduction of the electrode coupled to the metabolic oxidation of a lactate (manuscript in preparation). We speculate that MR-1 exhibits an electrochemical taxis-like behaviour leading the organism to populate the surface; this taxis-like ability has also been observed with other groups based on the redox state of riboflavin [1,2]. The applied potential identified most effective in initial surface population was −0.30 V vs Ag/AgCl, approximately, the oxidized potential of electrode bound riboflavin. To that end, riboflavin, under carbon limitation has been observed in the extracellular medium by MR-1 cultures in our studies [3]. With these experimental data and observations from others, riboflavin redox potentials may lead the organism to biofilm formation on substrates that are known electron acceptors when extra-cellular electron transfer (EET) becomes essential for cell sustaining, stationary growth phase for example.

Within this study, *S. oneidensis* MR-1 was cultured aerobically in Luria Broth (LB) to early stationary phase growth, in order to procure a high-density culture. This culture, after being washed to remove from LB and resuspended in phosphate buffer containing 100 mM lactate, was exposed to anaerobic conditions in an electrochemical cell containing a working electrode having an applied potential of −0.30 V vs Ag/AgCl for varying time increments. The subsequent biofilms formed yielded catalytic activity as characterized by cyclic voltammetry and polarization curves after approximately 5 h with maximum current density being achieved within 24 h.

With the increasing number of electrochemical investigations into the metabolic processes of dissimilatory metal reducing bacteria (DMRB), the need to appropriately compare results between different laboratories becomes essential. Due to the fact that the performance of these bioelectrochemical systems (BES) or biofuel cells depends on several factors (metabolic state of the organisms, structure and composition of the biofilm and intrinsic electrode...
there is not a uniform approach to present the results. Two methods have been unofficially adopted within the literature: normalizing the current and power to the electrode geometric surface area or normalizing the measured electrochemical response to the volume of anode/cathode compartment and uncertainty of the measured ECSA. The reason for this is the hypothesis that these two parameters are the main factors influencing the biofuel cell overall current gain [7]. Normalizing the current and power to the electrode cross sectional area holds true only when the electrode is smooth with planar or rod shaped geometries (low surface morphology), in other words, when the geometric surface area is equal to the electrochemical surface area (ECSA). It is also proposed that the generated current is reversed, proportional to the distance between the electrode and the membrane (two-chambered MFC) or between the anode and cathode (membrane-less MFC), while being proportional to the diagonal of the anode/cathode chambers [8–11]. The volume of the fuel cell compartments plays a significant role when the ratio volume/ electrode surface is high, otherwise the MFC volume cannot be used as a normalization parameter.

The most common parameters used to represent the electrochemical response of MFCs are: the open circuit voltage, maximum power and short circuit currents [8,12–14]. These parameters are used to characterize the whole fuel cell performance; however, when the electrode (anode or cathode) is individually under interrogation, these parameters are less appropriate. In these instances, other parameters could be selected as the best representation of the system. In this study a statistical examination will help in identifying three defined potentials as being appropriately significant for our biofilms.

Another problem facing FMC studies is the irreproducibility of the measured results. There are two ways to overcome this problem: first, to minimize the influence of the factors leading to irreproducible results and secondly, to normalize the results to the main parameter contributing to the system’s uncertainty [8]. In both methods, the system should be examined in detail and the uncertainty of the main factors increasing the expanded uncertainty of the results should be evaluated.

Uncertainty is a term readily understood in other industries, pharmaceutical and food, for example; but this concept is new in the microbial fuel cell discipline and perhaps all biofuel cells. In this study we will evaluate the anodic electrochemical performance of novel biofilms of MR-1 using the theory of expanded uncertainty and will discuss how this methodology can be used to address a variety of standardization problems. This approach consists of: normalizing the current to the electrode electrochemical surface area (ECSA) and representing the measured current as μA cm⁻² (ECSA), accompanied by its expanded uncertainty. Using an expanded uncertainty analysis will provide insight towards the contribution of the intrinsic electrode material properties towards variation in measured results and provide validation in the need for better normalization techniques.

2. Experimental

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO) and are of highest available purity.

2.1. Strain and culturing conditions and biofilm formation

Frozen stocks of S. oneidensis MR-1 were obtained from a −80 °C freezer and plated on Luria Broth (LB) plates containing 1% agar by weight to ensure continuity of the stock cultures. Individual colonies were isolated and inoculated in 50 ml of liquid LB in 250 ml shaker flasks. The liquid cultures were incubated aerobically at 150 rpm and 30 °C for approximately 18 h. This time correlates to the start of early stationary phase corresponding to an optical density measured at 600 nm of 4.5 (~10¹³ CFU ml⁻¹). Cultures were then washed 3 times in 50 mM sodium phosphate buffered saline (PBS) (pH 7) and re-suspended to the original density. 100 mM of sodium lactate and 30 mM of sodium fumarate were added to the culture and the mixture is incorporated into an electrochemical cell. Sodium fumarate is intended to be a limiting electron acceptor in order to drive respiration with the electrode. Subsequent spiking of the culture with lactate occurs 10 h past inoculation. The 3-electrode set up consists of a carbon felt (Morgan AM&T Greenville, SC) working electrode (1.5 cm in diameter) wired with nickel wire as a current collector, platinum mesh (1 cm × 2 cm), and an Ag/AgCl (saturated KCl) reference electrode. Electrodes were connected to a Gamry 600 Potentiostat/Galvanostat/ZRA. The electrochemical cell was sealed and gassed with nitrogen to ensure an anaerobic environment during experiment.

2.2. Calculation of electrochemical accessible surface area (ECSA)

Electrodes were treated with Isopropyl alcohol (IPA) for 10 min and rinsed 3 times in sterile 1× PBS to clear away excess IPA, this ensured wettability of the normally hydrophobic material. Electrochemical accessible surface areas were then determined prior to inoculation with MR-1 culture by measuring a cyclic voltammogram at 0.01 V s⁻¹. Corresponding oxidation and reduction currents were used in the following equation to evaluate the ECSA for individual electrodes [15]:

\[
\text{ECSA} = \frac{I_{ox} - I_{red}}{2} \times c_{sp}
\]

where \(I_{ox}\) and \(I_{red}\) are the oxidation and reduction currents at a chosen potential, \(c_{sp}\) is the specific capacitance and \(v\) is the scan rate in V s⁻¹. The specific capacitance for the carbon felt is taken to be 35 μF cm⁻² and is used only for results comparison in the current study.

2.3. Calculation of expanded uncertainty

According to the International Vocabulary of Metrology “Uncertainty is a parameter associated with the results of measurements, characterizing the dispersion of values, which could be reasonably attributed to the measurand” [16]. The uncertainty combines random and systematic errors, but is not equal to them, and provides a realistic range of values within which the true value of a measured quantity probably lies [17]. In other words, the uncertainty is the range for which the true value of the quantity being measured lies.

Three symbols are used to express uncertainty [18]. Measurement or Standard uncertainty (\(u\)) which expresses the concept as a standard deviation. The combined uncertainty (\(U\)) combines the individual measurement uncertainties for complicated analytical procedures. Expanded uncertainty (\(U\)) defines a range that includes a large fraction of the values within which the quantity being measured will lie and is obtained by multiplying \(u\) by a coverage factor, \(k\), chosen according to the degree of confidence required for the range (Eq. (2)) typically being in the range of 2–3, with a \(k\) value of 2 corresponding to the 95% confidence interval and a \(k\) value of 3 corresponding to a confidence interval of 99%.

\[
U = k \times u
\]  

For this study, a \(k\) value of 2 was used, yielding our expanded uncertainty to be within a confidence interval of 95%.
2.4. Spectrophotometric determination of riboflavin content

Spectrometry was used as a fast and simple method for the determination of riboflavin concentration in the electrolyte at the end of the electrochemical measurements. The standard addition method is the optimal approach for the determination of an analyte that is in a complex matrix such as biological fluids and soil samples. Therefore due to the complexity of the electrolyte, at the end of the electrochemical experiments it contains intermediate and final products of the substrate oxidation; a standard addition method was used. The electrolyte was filtered through Nylon syringe filter (Fisherbrand, 0.2 μm, 25 mm, sterile) and the filtrate was spiked with riboflavin solution with known concentration (0.2, 0.8, 1 and 2 μM). The absorbance of the filtrate with and without the riboflavin additions at 420 nm was measured using spectrometer SpectraMax M5. The wavelength was selected based on the fact that riboflavin dissolved in 50 mM PBS has a maximum absorbance at 420 nm (data not shown).

2.5. Scanning electron microscopic examination of anodes

After electrochemical examination, 5 and 24 h polarized anodes were prepared for electron microscopy as described previously [3]. Briefly, the samples were fixed for 12 h in 2.5% (v/v) aqueous glutaraldehyde, followed by serial dehydration in ethanol. Samples were dried by immersion for 15 min in 50% (v/v) hexamethyldisilazane (HMDS): ethanol, followed by 15 min in 100% HMDS and finally air exposure overnight. The anodes were then coated with 15 nm Au/Pd by sputtering. Samples were examined under high vacuum using a JEOL JSM-5800L scanning electron microscope (accelerating voltage = 15 keV).

3. Results and discussion

3.1. Biofilm formation

Planktonic cultures were inoculated into the electrochemical cell with an applied potential of −0.30 V vs Ag/AgCl on the working electrode (via chronoamperometry) in an anaerobic environment, the current measurement obtained was zero for approximately 2.5 h (Fig. 1), after which the current started to increase and stabilized at 5 h. It is likely that this time is required for the culture to transition from an aerobic respiration based metabolism to one that is capable of electrode reduction as a function of metabolic oxidation of lactate. Initial biofilm formation is also occurring at this time.

The high-density culture (∼10^14 CFU ml^-1) contains 100 mM sodium lactate and 30 mM sodium fumarate at the onset with addition of only lactate (100 mM) after 10 h. The idea to add fumarate initially is to prevent the death of the culture during the beginning of the aerobic to anaerobic change. This prevents the loss of biomass from an arrested metabolism (inability to respire) until the biological structures are in place to begin biofilm formation and electrode respiration. With the addition of lactate after approximately 10 h, the measured current increases again and stabilizes within an hour.

Fig. 2 shows SEM micrographs of S. oneidensis MR-1 attached to the anodes after 5 and 24 h of electrode polarization. While few cells are observed attached at 5 h polarized electrodes indicating that this anode is at the initial stage of biofilm development. The 24-h samples have fully formed biofilm coverage with abundant exopolymeric substance encasing attached cells.

3.2. Cyclic voltammetry of biofilms

After 5 and 24 h, cyclic voltammetry was measured for the system to evaluate redox characteristics of the developing biofilm (Fig. 3). The 5-h voltammogram (obtained in turnover conditions) measured the beginning stages of a catalytic biofilm that appears to be engaged in kinetic limitation (Fig. 3a). In terms of a beginning biofilm, for this system, one must look at the voltammogram of the biofilm after a longer period has passed under anaerobic applied potential biofilm formation. After 24 h, the same measurement is made on the biofilm (again, under turnover condition) (Fig. 3b). The biofilm now appears to be under a mass transport based limitation, reaching saturation current above +0.40 V vs Ag/AgCl. When comparing the two time frames, we speculate that the 5-h biofilm is in the initial stages of biofilm formation (Fig. 2), leading to a system that suffers kinetic limitations. This is likely caused, at least in part,
in the lack of catalyst on the surface (low population density), with the orientation of the terminal reductases playing a role.

The CV for the 5-h biofilm exhibits pronounced catalytic activity beginning at 0.00 V vs Ag/AgCl. A zoom in graph (Fig. 3a - inserted graph) in the potential region between −0.8 and 0 V vs Ag/AgCl shows the presence of reduct peaks most likely associated with the electrochemical activity of riboflavin. Based on the small current densities due to the riboflavin redox reaction, it can be concluded that the catalytic activity of Shewanella at this stage appears to be dominated by DET mechanisms consistent with voltammograms for Geobacter spp. biofilms [19,20]. However, as the biofilm becomes more developed, complex and indeed larger (after 24-h of formation – Fig. 2), the presence of well-defined second DET mechanism appears at the onset of approximately −0.35 V vs Ag/AgCl (Fig. 3b). Likely, this is riboflavin, as the potential of the new catalytic feature corresponds to an onset potential near the redox potentials of riboflavin. The notable appearance of riboflavin is consistent with the theory that riboflavin becomes electrochemically detectable within carbon source limited cultures of S. oneidensis [3]. Thus, as this particular biofilm becomes more complex as a function time and applied potential on the electrode, the biofilm achieves greater thickness (Fig. 2); likely causing organisms near the electrode surface to switch to a carbon source limited metabolism and subsequent increased secretion of riboflavin significantly influencing the electrochemical character of the system.

Based on the CVs at the negative potential region (Fig. 3 – inserted graphs), it can be concluded that the amount of riboflavin increases with time and after 24 h its electrochemical oxidation on the electrode surface is expressed by well pronounced increase of the generated current starting at −0.4 V vs Ag/AgCl, followed by fast DET with onset potential of 0.00 V vs Ag/AgCl. Therefore the mechanism of the EET can be controlled by the potential at which the system will be operated: at negative potentials the MET will be dominating and at positive potentials the DET will be the major EET mechanism.

The assertion that the riboflavin content increases with the increased polarization time was confirmed by spectrophotometric determination of the riboflavin concentration using the standard addition method (Fig. 4). It was observed that the concentration of riboflavin after 5 h of polarization was 0.55 μM and after 24 h of polarization was 1.01 μM, which explains the differences in the electrochemical performance of the electrodes at the negative potential region.

3.3. Electrode polarization

To evaluate the achievable current densities of these biofilm systems, potentiostatic polarizations were measured beginning with the stabilized open circuit potential after the applied potential had been removed from the working electrode (biofilm formation). Under turnover conditions, the open circuit potentials for both systems stabilized within an hour to near −0.50 V vs Ag/AgCl. This low open circuit seems to correspond to the observations of other groups which propose an electron gradient perpendicular to the surface of the electrode as the biofilm is no longer able to respire with the electrode during open circuit conditions [21]. Multiple repeats of the same conditions on identically prepared electrodes (for both 5-h and 24-h biofilms) were evaluated and are plotted in Fig. 5.

The 5-h biofilms show little contribution towards current at potentials lower than −0.20 V vs Ag/AgCl with increased current measured near −0.10 to 0.00 V vs Ag/AgCl consistent with the previously measured CVs. Confirming results from the 24-h CVs are the 24-h polarization curves, with observed measurements increasing the current (compared to the 5-h biofilm) beginning at −0.30 V vs Ag/AgCl. However, the overall contribution to the total measured current within the region (riboflavin redox potential region −0.45 V vs Ag/AgCl) is little compared to the measured current contribution from the MtrC/OmcA terminal reductases (onset at 0.00 V vs Ag/AgCl). Indeed, the 24-h biofilm contributes to increased current within this region (above 0.00 V vs Ag/AgCl) compared to the 5-h biofilm, consistent with our hypothesis of a more developed, catalytic biofilm at this time point. The oxidation event near
–0.20 V vs Ag/AgCl in the 24-h biofilms is likely an intrinsic material property as this is evident in abiotic controls (data not shown).

3.4. Expanded uncertainty evaluation of abiotic electrodes

As previously shown, variation of the measured current is congruent between biofilms that are cultivated and grown in the same way on identically prepared electrodes. In order to determine an appropriate way to normalize the measured current to achieve less variability in the system, we first looked at the intrinsic material properties of the electrodes that were identically prepared. The uncertainty of the electrodes inhomogeneity was determined by measuring the mass of fifty carbon felt electrodes. The resistance and the ECSA of the same electrodes were also established. The uncertainties of these parameters were evaluated using Gaussian statistics and the results are found within Table 1.

It is obvious that the uncertainty due to differences in electrochemical surface area has the highest value; twelve times higher than the uncertainty due to variation in electrodes mass and more than four times higher than the electrode resistance uncertainty. Thus this parameter is the major factor limiting the results reproducibility. Therefore, normalizing the gained electrochemical results from the biocatalysts, one must consider the electrochemical accessible surface area as the most appropriate value.

3.5. Expanded uncertainty evaluation

In order to represent the results measured from the polarization curves in a statistically meaningful way, according to the requirements of the chemical metrology, and to define their reproducibility, the expanded uncertainty of the current at each point of the polarization curves were evaluated for both of the electrode systems – 5-h and 24-h biofilms (Fig. 6). Because of the complicated character of the system, Robust or nonparametric statistics were used as a statistical tool. As an estimation of the measured value, the median of all replicates is applied. The uncertainty is related to the median of absolute deviations (MAD) and normalized median of absolute deviation (MADN), which becomes compatible with the standard deviation [18,22].

When polarization curves are measured, the generated current is established. This current is used as a parameter in describing the electrochemical behaviour of the system. There are three major questions connected to it: (i) “How to best represent this current?”; (ii) “Can we compare it with other inter/intra laboratory results?”; and (iii) “Is this parameter reproducible?”. Resolving the last question will give us the answers to the other two.

For current reproducibility evaluation, the uncertainties of the main factors leading to irreproducible results were estimated, building an uncertainty budget [18]. The main factors having an impact on the current uncertainty, for these evaluated S. oneidensis anodes are: (i) electrode inhomogeneity; (ii) electrode resistance; (iii) electrode electrochemical accessible surface area; and (iv) biofilm growth and development. We can speculate that the last two factors are connected. The number of the cells attached to the electrode surface depends on the electrode surface area. Because of the fact that we observe direct electron transfer within S. oneidensis biofilms, the amount of cells, which can utilize the electrode as terminal electron acceptor, is proportional to the ECSA. Thus, for the purposes of the current uncertainty estimation the uncertainty due to the biofilm growth and development will not be discussed. We assumed that this uncertainty would be strongly correlated with

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**Table 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average</th>
<th>Standard deviation</th>
<th>Expanded uncertainty</th>
<th>Expanded uncertainty (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal resistance</td>
<td>3.28 Ω</td>
<td>± 0.25 Ω</td>
<td>± 0.49 Ω</td>
<td>14.9</td>
</tr>
<tr>
<td>ECSA</td>
<td>22.25 cm²</td>
<td>± 7.03 cm²</td>
<td>± 14.06 cm²</td>
<td>63.2</td>
</tr>
<tr>
<td>Mass</td>
<td>0.1039 g</td>
<td>± 0.0025 g</td>
<td>± 0.0050 g</td>
<td>4.9</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Multiple repeats of potentiostatic polarization curves for 5-h (black squares) and 24-h (red triangles) biofilms, including the median of all measurements for 5-h (black solid line) and 24-h (red solid line) biofilms. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

**Fig. 6.** Median polarization curves for (a) 5-h biofilms and (b) 24-h biofilms with corresponding percent expanded uncertainty for non-normalized current (blue bars) and electrochemical assessable surface area normalized current density (red bars). (For interpretation of the references to color in text, the reader is referred to the web version of this article.)
the uncertainty due to differences in ECSA. Furthermore, the factors previously described are not the only factors determining the anode electrochemical behaviour; however, they are the parameters that may have a substantial impact on the results’ uncertainty.

In the previous section we saw that the uncertainty due to differences in electrochemical surface area has the highest value (Table 1), which indicates this parameter as the major factor limiting the results reproducibility. Therefore, we expect that normalizing the measured results to the electrochemical accessible surface area will be the most appropriate approach. Before the uncertainty evaluation, the data from the polarization curves was checked for outliers using the linear dependence between current and ECSA. For each point of the polarization curves, the measured current is linearly proportional to the electrochemical surface area (data not shown), which is further evidence that this parameter has a large impact on the results’ reproducibility.

As it can be seen from Fig. 6a, the calculated expended uncertainty of the measured current is extremely high (blue bars), especially, at more positive potentials where a higher current is produced. This could be expected, due to the “living” characteristic of the anode, or from the high uncertainty due to variation in electrodes’ ECSA as discussed in the previous section. The same irreproducible results will be observed even if we represent the current density, when the current is normalized to the electrode geometric surface area (volume or cross-sectional area). The current densities uncertainties were therefore, reevaluated once normalized to the ECSA (Fig. 6b) and it is obvious that their magnitude is significantly decreased, especially for the 24-h biofilm polarization. This is further proof that this parameter (ECSA) has a significant impact on the results uncertainties. The smaller decrease of the uncertainty when the current is normalized to the ECSA for the 5-h biofilm polarization curve indicates that at the beginning of the biofilm formation, the number of electrode respiring bacterial cells (catalysts) on the electrode surface has an impact on the current generation and at that phase of the biofilm formation is still not fully limited by the ECSA. Consistent with the interpretation of the CV results. After 24 h when the biofilm is covering the electrode surface (Fig. 2), the number of cells attached to the surface can now be mostly defined by the ECSA and the uncertainty decreased significantly normalizing current to that parameter (Fig. 6b). Therefore the MFC’s electrochemical operational characteristics should be represented and compared when normalizing to the electrode’s electrochemical accessible surface area as a primary factor influencing the electrode’s electrochemical behaviour. This is especially recommended when the electrode material is inhomogenous and the geometrical surface area is inconsistent with the ECSA.

It is interesting to notice that three points with much lower uncertainty can be seen (Fig. 6). These points are approximately at the same potentials for both types of electrodes. For the 5-h biofilm they are at −0.275 V, 0.05 V and 0.275 V vs Ag/AgCl; and for the 24-h biofilm they are at −0.275 V, −0.10 V and 0.275 V vs Ag/AgCl. Obviously these points are not random (Fig. 7), but are closely related to the nature and diversity of the electron transfer mechanisms occurring within the system. Each point indicates the beginning of a particular change at the electrode surface and bacterial functionality.

The first point is associated with the “onset” potential of the electrochemical oxidation of riboflavin, thought to be respon-
Table 2

<table>
<thead>
<tr>
<th>Electrode</th>
<th>$E_{\text{MTR}}$ (µA cm$^{-2}$)</th>
<th>$E_{\text{ET}}$ (µA cm$^{-2}$)</th>
<th>$E_{\text{ML}}$ (µA cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-h biofilm</td>
<td>$1.20 \pm 0.08$</td>
<td>$3.42 \pm 0.37$</td>
<td>$15.37 \pm 0.21$</td>
</tr>
<tr>
<td>24-h biofilm</td>
<td>$1.60 \pm 0.04$</td>
<td>$6.02 \pm 0.31$</td>
<td>$32.15 \pm 0.04$</td>
</tr>
</tbody>
</table>

Table 2: Current densities (reported vs electrochemical accessible surface area) associated with specific potentials with lower current uncertainty for 5 and 24-h biofilms.

sible for mediated electron transfer. The second point occurs at the onset potential of the direct electron transfer accomplished by the cytochromes from the Mtr pathway. And the third point is at a potential corresponding to the beginning of mass-transport limitations. However, due to the nature of the electron transfer (DET, in this case), these limitations are no longer mass-transport. We speculate that instead of the system suffering a diffusion limitation (based on lactate and/or migration of ions from the biofilm/electrode interface) this potential is the region of “metabolic limitations”. Meaning, that the microorganisms are transferring electrons to the electrode at higher rates than they are produced by metabolic substrate oxidation. In essence, the electrons are consumed faster at the electrode (at higher potentials during polarization) than they are metabolically accumulated in the living cells, and as a result the current densities are being truncated.

In regards to the range of uncertainties corresponding to individual potentials, the fact that there are three electrochemical potential values with dramatically lower current density uncertainties and that these potentials are connected with defined electrochemical changes; means that the current densities generated at these potentials should be used for results comparison. In doing so, current densities can be associated with particular changes in the system and at the same time these current densities are reliable with high reproducibility. The results from this expanded analysis using the theory of uncertainty are summarized in Table 2.

The main mechanism for EET at negative potentials is mediated electron transfer utilized by riboflavin (Fig. 3 – inserted graphs). Due to the differences in the concentration of riboflavin the current densities at the beginning of the polarization measurements for both 5-h and 24-h biofilms increased by approximately 30% for the first comparison point ($E_{\text{MET}}$). With the increase of the potential during electrode polarization the current densities produced from the 24-h biofilm are significantly higher than those of the 5-h biofilm (75% $E_{\text{ET}}$ and 109% $E_{\text{ML}}$) due to the increased amount of electrochemically active microorganisms attached at or near the electrode surface. This is evidence that corroborate with our previous discussion that the 24-h biofilm (anaerobically formed on an electrode with applied $–0.30$ V vs Ag/AgCl potential for 24 h) is a more well-developed, complex and catalytic biofilm than the 5-h biofilm.

4. Conclusions

In this study, high-density planktonic cultures of S. oneidensis MR-1 are grown in a rich medium aerobically. The culture is then removed from this media and placed in an electrochemical cell, containing lactate and electrolyte and the culture is transitioned to an anaerobic metabolism. The biofilm was then formed by applying an external potential of $–0.30$ V vs Ag/AgCl on the working electrode for up to 24 h. At the onset of inoculation into the electrochemical cell/batch bioreactor, the measured current was zero for approximately 2.5 h when an increase was apparent. This increase of current reached stable maxima at approximately 5 h for multiple repetitions. The subsequent electrochemical characterization of the biofilms included CVs and polarization curves at time points of 5 and 24 h under turnover conditions. The 5-h biofilm exhibited a large current with the shape of the voltammogram lending to the analysis that at this particular time point the biofilm is in a region of kinetic limitation being defined by a small population in contact with the electrode surface or the initial stages of biofilm formation. The 24-h biofilm, however, exhibited more catalytic behaviour being a unique such CV reported for S. oneidensis MR-1. We speculate that the CV obtained from the 24-h biofilm formation exhibits a mass transport based limitation characteristic. However, despite being an initial observation leading to that conclusion, we speculate further that this limitation is in fact one based on the biofilm’s (or individual cell’s) metabolism. This metabolic limitation is occurring at high potentials in the electrode polarization as the metabolic oxidation of the carbon substrate can no longer “supply” electrons fast enough to keep pace with electrode respiration, and a truncated current is observed. Polarization curves in this study also confirm the results from the obtained CVs for each biofilm system.

In terms of uncertainty estimation of the data (polarization curves) between multiple identical experiments, three key concerns were addressed: (i) The generated current depends strongly on the electrochemical accessible surface area and the observed results from the polarization measurements should be represented as normalized to the ECSA. Normalizing the current to the ECSA leads to a significant decrease of the current expanded uncertainty; and (ii) there are three defined potentials in the polarization curves at which the generated current has remarkably lower uncertainty. These potentials are associated with particular changes in the electrochemical operation of the system. Considering the fact that the current values at these potentials have significantly higher reproducibility, it is highly recommended to use exactly these currents for results comparison and representation. In this study we did not quantify the biomass on the electrode surface, as we assumed that because each experiment was prepared in the same way (i.e. inoculum and culturing time) that the concentration of cells attached to the surface per unit area is the same (the planktonic concentration was identical between repetitions). Therefore, that surface area, defines the measured current and normalizing to the ECSA will be sufficient in results comparison; as it clearly is as described by the uncertainty analysis.

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References


