Supplementary Materials for

Extracellular polymeric substances are transient media for microbial extracellular electron transfer

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Supplementary Discussion

Derivation of eq. (1) and Estimate of Charge from Monolayer DPV

DPV has mainly been used for analytical purposes, and DPV theory in the diffusion mode is well established (39, 40). We provide here a model of thin-layer DPV. The one-electron current in reversible monolayer/thin layer voltammetry (41), \(i(E)\) can be expressed as

\[
i(E) = e \frac{F}{RT} \ell [P]_0 A_{elec} \frac{v \exp \left[ F \left( E(t) - E_0 \right) / RT \right]}{RT \left[ 1 + \exp \left[ F \left( E(t) - E_0 \right) / RT \right] \right]^2}
\]  \hspace{1cm} (A1)

where \(e\) is the electronic charge, \([P]_0\) the concentration of electroactive species is the monolayer or thin layer, \(v\) the scan rate, \(\ell\) the thickness of the layer, \(A_{elec}\) the electrode area, \(F\) Faraday’s constant, \(R\) the gas constant, and \(T\) the temperature. \(E(t)\) is the electrode potential at time \(t\), and \(E_0\) the standard reduction potential.

We wish to estimate the formal charge, \(Q_{\text{formal}}\) in the adlayer

\[
Q_{\text{formal}} = \int_{-\infty}^{\infty} i(t) dt = \frac{1}{v} \int_{-\infty}^{\infty} i(E) dE
\]  \hspace{1cm} (A2)

This is not directly observable in DPV such as in cyclic voltammetry. Introducing

\[
x = \exp\left[ F(E - E_0)/RT \right]; \quad E = E(t)
\]  \hspace{1cm} (A3)

gives

\[
dx = \exp\left[ F(E - E_0)/RT \right] (F/RT) dE \quad \text{and} \quad dE = \frac{1}{x} dx (RT/F)
\]  \hspace{1cm} (A4)

\(Q_{\text{formal}}\) is then

\[
Q_{\text{formal}} = e \ell [P]_0 A_{elec} \int_{0}^{\infty} \frac{x}{(1 + x)^2} \frac{1}{x} dx = e \ell [P]_0 A_{elec} \frac{RT}{\nu F} \int_{0}^{\infty} \frac{dx}{(1 + x)^2}
\]  =
\(-e\ell[P]_0 A_{elec} \left[ \frac{1}{1 + x} \right]_0^\infty = e \ell A_{elec} [P]_0 \) \hspace{1cm} (A5)

Imposing a pulse increment, \( \Delta E \) at time \( t \) invokes a current rise, \( i_{\text{pulse}} \)

\[
i_{\text{pulse}}(E) = eP_{\text{red}}(t)k_0 \exp\left[ \alpha F \left( E(t) - E_0 + \Delta E \right) / RT \right] - eP_{\text{ox}}(t)k_0 \exp\left[ \beta F \left( E(t) - E_0 + \Delta E \right) / RT \right]
\]

where \( P_{\text{ox}} \) and \( P_{\text{red}} \) are the concentration of the oxidized and reduced species, respectively, \( k_0 \) the standard electrochemical electron transfer (ET) rate constant, while \( \alpha \) and \( \beta \) are the transfer coefficients of the cathodic and anodic ET process, respectively.

The following equations apply

\[
\alpha + \beta = 1 ; \quad P_{\text{ox}} + P_{\text{red}} = [P]_0
\]

(A7)

The ratio of the concentrations of oxidized and reduced species is

\[
\frac{P_{\text{ox}}(t)}{P_{\text{red}}(t)} = \exp\left[ F \left( E(t) - E_0 \right) / RT \right] \quad \text{or}
\]

\[
P_{\text{red}} = [P]_0 \frac{1}{1 + \exp\left[ F \left( E(t) - E_0 \right) / RT \right]}
\]

\[
P_{\text{ox}} = [P]_0 \frac{\exp\left[ F \left( E(t) - E_0 \right) \right]}{1 + \exp\left[ F \left( E(t) - E_0 \right) / RT \right]}
\]

(A9)

The pulse current is then

\[
i_{\text{pulse}}(E) = e([P]_0 \frac{\exp\left[ \alpha (E(t) - E_0) \right]}{1 + \exp\left[ F \left( (E(t) - E_0) \right) / RT \right]} \left[ \exp\left( \frac{\alpha F \Delta E}{RT} \right) - \exp\left( -\beta F \Delta E / RT \right) \right]
\]
If $\Delta E$ is small, then
\[
    i_{pulsa}(E) = e\ell[P]_0 A_{elec} k_0 \frac{F \Delta E}{RT} \frac{\exp\left[\alpha F\left(E(t) - E_0\right)\right]}{1 + \exp\left[F\left(E(t) - E_0\right) / RT\right]}
\]  

The integral under the DPV curve is
\[
    Q_{DPV} = e\ell[P]_0 A_{elec} k_0 \frac{F \Delta E}{RT} \frac{1}{v} \int_{-\infty}^{\infty} \frac{\exp\left[\alpha F\left(E(t) - E_0\right)\right]}{1 + \exp\left[F\left(E - E_0\right) / RT\right]} dE
\]  

From the substitution in eqs. (A3) and (A4) we can rewrite eq. (A12) as
\[
    Q_{DPV} = e\ell[P]_0 k_0 \frac{\Delta E}{v} \int_{-\infty}^{\infty} \frac{x^{\alpha-1}}{1 + x} dx, \quad \text{or for } \alpha = \frac{1}{2},
\]
\[
    Q_{DPV} = e\ell[P]_0 k_0 \frac{\Delta E}{v} \int_{-\infty}^{\infty} \frac{1}{\sqrt{x} (1 + x)} dx
\]  

Introducing
\[
    y = \sqrt{x} \quad ; \quad dy = \frac{1}{2y} dx \quad ; \quad dx = 2y dy
\]  

gives for $Q_{DPV}$
\[
    Q_{DPV} = e\ell[P]_0 A_{elec} k_0 \frac{\Delta E}{v} \int_{-\infty}^{\infty} 2 \frac{1}{1 + y^2} dy =
\]
\[
    e\ell[P]_0 A_{elec} k_0 \frac{\Delta E}{v} 2\arctan\frac{y}{y_0} = Q_{formal} \frac{k_0 \Delta E}{v}
\]  

as in eq. (1) in the text.

The total charge in the electrode/membrane or electrode/EPS adlayer, $Q_{formal}$ and
The total charge in the electrode/membrane or electrode/EPS adlayer, $Q_{\text{formal}}$ and therefore the number of electroactive molecular ET units can be determined from eq.(A15) if the rate constant $k_0$ (s$^{-1}$) is known. The CV peak separation for the anodic and cathodic cyt peaks was 227 mV (fig. S3). Using Laviron’s method (42), we obtain $k_0 = 0.026$ s$^{-1}$ for EPS retained MR-1 and $k_0 = 0.056$ s$^{-1}$ for EPS depleted MR-1. In comparison, Marsili et al.’s data (8) give $k_0 = 0.013$ s$^{-1}$. These values are for the cytochromes directly in the whole cell and much smaller than the reported value of 100 s$^{-1}$ for purified MtrC protein on a basal plane graphite electrode (9).

The potential pulse increment $\Delta E$ is 0.006 V and the scan rate 15 mV s$^{-1}$, giving $Q_{\text{DPV}} = 1.26 \times k_0 \times Q_{\text{formal}}$, or $Q_{\text{DPV}} \approx k_0 \times Q_{\text{formal}}$. The DPV peak areas ($Q_{\text{DPV}}$) are summarized in table S3 and are in the range $(1-10) \times 10^{-9}$ C for MR-1 in the presence of EPS, $(1-2.5) \times 10^{-8}$ C for EPS depleted MR-1, and $(1.5-7) \times 10^{-9}$ C for isolated EPS. Using $k_0 = 0.026$ s$^{-1}$ gives the $Q_{\text{formal}}$ values in table S3. These are in the range $(3-40) \times 10^{-13}$ moles or $(2-25) \times 10^{11}$ molecules of one-ET units for EPS retained MR-1.

The average number of single-ET redox carriers over a 3 mm electrode surface is thus $(3-40) \times 10^{-13}$ moles or $(2-25) \times 10^{11}$ molecules. If these were evenly distributed over a 35 nm membrane/EPS layer on the electrode surface, i.e. 0.245 nl EPS, a formal molar concentration of $10^{-3}$-$10^{-2}$ M or $(8-100) \times 10^{20}$ molecules/L would emerge, or an average distance between the molecular redox units of 5-10 nm. This value is subject to the following reservation. The 10 µL of MR-1 cells was used to form the biofilm. If 50 % is water, the actual cell volume is 5 µl. We can calculate the total volume of the EPS layer from the radius and radius decrease of the equivalent cell cylinders in table S1, suggesting about 0.25 % volume decrease after EPS extraction. The EPS volume in the biofilm can therefore be up to 12.5 nl, giving up to five times lower cyt c concentration, i.e. $\approx 10^{-4}$-$10^{-3}$ M or about twice longer average inter-site distance.
**fig. S1. 3D AFM images of *S. oneidensis* MR-1 cells.** (A) MR-1 cells treated at 30 °C are enveloped by EPS, and the substrate i.e. Pt sheet covered by EPS. (B) Details of the cells in A (indicated by the dotted box). Flagella on the cell surface can be observed (marked by an arrow). (C) MR-1 cells treated at 38 °C have little EPS on the surface, and the substrate is also very clean. (D) Details of the cells in C (indicated by the dotted box) show a very smooth surface of these cells. These images are constructed by SPIP 6.6.2 (Image Metrology A/S, Denmark) based on the images shown in **Fig. 2 B** and C.
**fig. S2. Yields of different EPS components from MR-1.** No nucleic acid was detected from EPS extraction. The group 30 °C indicates EPS from MR-1 cells treated at 30 °C for 30 min (control group), while the group 38 °C indicates EPS from MR-1 cells treated at 38 °C for 30 min. The error bar refers to triplicate determination.
**fig. S3.** CV of *S. oneidensis* MR-1 cells in the presence (blue line) and absence (red line) of EPS. MR-1 cells in the absence of EPS were those treated at 38 °C for 30 min, and cells in the presence of EPS those treated at 30 °C for 30 min (i.e. the control group). In the presence of EPS the CV shows only the reduction peak of c-type cytochromes. Two oxidative peaks (-270 and -66 mV) are seen for cells in the absence of EPS, and the enhanced peak of c-type cytochromes (-205 mV) can also be observed. Scan rate 10 mV/s.
**fig. S4.** UV-vis spectra of EPS from MR-1. (A) UV-Vis spectra of EPS, 0.9 % NaCl (w/v), and pure riboflavin in the wavelength range 200 to 600 nm. No peak is observed for 0.9 % NaCl (w/v) ‘blank’. Pure 5 mg/L riboflavin in 0.9 % NaCl shows 4 peaks at 223, 267, 374 and 446 nm. EPS from both 30 and 38 °C show a very strong absorbance peak around 208 nm. Two other peaks at 267 and 410 nm were also observed (indicated by red dashed lines). The peak at 267 nm is the characteristic riboflavin peak. Other characteristic riboflavin peaks could not be observed in the EPS spectra due to strong background absorption. This result indicates that riboflavin is present in EPS. (B) UV-Vis spectra of EPS, 0.9 % NaCl (w/v), and pure riboflavin in the wavelength range 380 to 440 nm. An absorption peak from EPS can be observed at 410 nm which is the characteristic Soret peak of c-type cytochromes (23). The result indicates that c-type cytochromes are present in EPS.
**fig. S5. Chronoamperometry $i$-$t$ test on MR-1 cells.** Arrows indicate the time point when 1 ml 5.7 mol/L of lactate in 25 mL phosphate buffer solution (50 mM, pH=7.0) was added in the electrochemical cell. All tests were performed by an Autolab electrochemical workstation using the same glassy carbon electrode.
fig. S6. DPV curves for MR-1 cell pellets treated at 30° and 38°C. After being cultured in LB broth at 30 °C for 48 h and washed with 0.9 % NaCl solution twice, MR-1 cells were collected by centrifugation. These cell pellets were not re-suspended but were directly treated at 30 and 38 °C for 30 min, respectively. The pellets were then used immediately for DPV measurements and gave very similar DPV curves. In this experiment, the EPS were not removed. This result indicated that the heat treatment did not change the redox protein expression and the activity of MR-1.
fig. S7. DPV curves for MR-1 cell culture treated at 30° and 38°C. After being cultured in LB broth at 30 °C for 48 h, some MR-1 culture was washed with 0.9 % NaCl solution twice and subjected immediately to DPV. Other MR-1 culture was further cultured for 30 min but at 38 °C. These MR-1 cells were then washed with 0.9 % NaCl solution twice and immediately subjected to DPV. Very similar DPV profiles were observed for the two groups of MR-1. This result indicates that short-term heat treatment does not change the protein expression.
fig. S8. Effect of different heating temperatures on microbial viability evaluated by flow cytometry. (A) WS-XY1; (B) Ps. After EPS extraction, the cells were fluorescence stained with PI and characterized by flow cytometry (Quanta SC, Beckman Coulter, USA). PI stained cells emit red fluorescence and indicate dead cells. 45 and 55 °C were chosen as the optimal temperatures for EPS extraction from WS-XY1 and Ps, respectively.
fig. S9. Yields of different EPS components from strains WS-XY1 and Ps strains. (A) WS-XY1; (B) Ps. The optimal temperatures are 45 and 55 °C for WS-XY1 and Ps, respectively. The group 30 °C indicates EPS from cells treated at 30 °C for 30 min (control groups). No nucleic acid was detected from EPS extraction.
fig. S10. Live/Dead staining of WS-XY1 and Ps cells after heating treatments.

Live/Dead staining shows similar ratios of live cells at 30 (A) and 45 °C (B) treated WS-XY1 cells and in 30 (C) and 55 °C (D) treated Ps cells. Yellow cells are dead cells, while green cells are live cells. Live/Dead staining showed similar ratios of live cells to total cells in the presence (A and C) and absence (B and D) of EPS. Cells in the absence of EPS were those treated at 38 °C for 30 min, cells in the presence of EPS those treated at 30 °C for 30 min (control group).
fig. S11. CV of pure and EPS-depleted cells of WS-XY1 and Ps. CV of WS-XY1 cells (A) and Ps cells (B) with (blue line) and without (red line) EPS. Cells in the absence of EPS were WS-XY1 cells and Ps cells treated at 45 and 55 °C for 30 min, respectively. Cells in the presence of EPS were those treated at 30 °C for 30 min (control group). Scan rate 10 mV/s.
table S1. Size determination of MR-1 cells using a laser particle size analyzer.

<table>
<thead>
<tr>
<th>Diameter of equivalent sphere/µm</th>
<th>Average intensity*/%</th>
<th>Equivalent cylinder</th>
<th>Radius decrease, i.e. thickness of removed EPS layer**/µm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In the presence of EPS</td>
<td>In the absence of EPS</td>
<td>Radius/µm</td>
</tr>
<tr>
<td>0.214</td>
<td>0.000</td>
<td>0.000</td>
<td>0.065</td>
</tr>
<tr>
<td>0.243</td>
<td>0.000</td>
<td>0.000</td>
<td>0.074</td>
</tr>
<tr>
<td>0.276</td>
<td>0.226</td>
<td>0.180</td>
<td>0.095</td>
</tr>
<tr>
<td>0.314</td>
<td>0.770</td>
<td>1.898</td>
<td>0.139</td>
</tr>
<tr>
<td>0.357</td>
<td>1.924</td>
<td>3.418</td>
<td>0.139</td>
</tr>
<tr>
<td>0.405</td>
<td>3.720</td>
<td>5.266</td>
<td>0.139</td>
</tr>
<tr>
<td>0.523</td>
<td>6.004</td>
<td>7.232</td>
<td>0.139</td>
</tr>
<tr>
<td>0.594</td>
<td>8.422</td>
<td>9.050</td>
<td>0.139</td>
</tr>
<tr>
<td>0.675</td>
<td>10.532</td>
<td>10.436</td>
<td>0.139</td>
</tr>
<tr>
<td>0.767</td>
<td>11.898</td>
<td>11.150</td>
<td>0.139</td>
</tr>
<tr>
<td>0.872</td>
<td>12.210</td>
<td>11.054</td>
<td>0.139</td>
</tr>
<tr>
<td>0.991</td>
<td>11.418</td>
<td>10.154</td>
<td>0.139</td>
</tr>
<tr>
<td>1.130</td>
<td>9.754</td>
<td>8.626</td>
<td>0.139</td>
</tr>
<tr>
<td>1.280</td>
<td>7.638</td>
<td>6.768</td>
<td>0.139</td>
</tr>
<tr>
<td>1.450</td>
<td>5.520</td>
<td>4.934</td>
<td>0.139</td>
</tr>
<tr>
<td>1.650</td>
<td>3.730</td>
<td>3.390</td>
<td>0.139</td>
</tr>
<tr>
<td>1.880</td>
<td>2.442</td>
<td>2.260</td>
<td>0.139</td>
</tr>
<tr>
<td>2.130</td>
<td>1.634</td>
<td>1.514</td>
<td>0.139</td>
</tr>
<tr>
<td>2.420</td>
<td>1.186</td>
<td>1.052</td>
<td>0.139</td>
</tr>
<tr>
<td>2.750</td>
<td>0.970</td>
<td>0.792</td>
<td>0.139</td>
</tr>
</tbody>
</table>

* The percentages shown represent the total volume of all particles with diameters in this range to the total volume of all particles in the distribution. The values were calculated based on five repeats.

** These values are calculated from the contiguous radius of equivalent cylinder. For example, 0.019 µm was the result of 0.158 µm minus 0.139 µm. The radius decrease was calculated in this way because, with similar intensity, the diameter of equivalent spheres for cells in the absence of EPS was one size gradient smaller than those in the presence of EPS. We therefore calculated the radius decrease using the sizes with intensity higher than 5 %, i.e. those indicated by bold and red font in the table. These sizes had a total intensity of about 80 %.
Table S2. Amperometric $i-t$ test for MR-1 cells. The potential of $+0.3 \text{ V} \text{ vs. Ag/AgCl}$ was applied to the glassy carbon electrode.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of lactate added (s)</th>
<th>Current when lactate added (nA)</th>
<th>Peak current after lactate added (nA)</th>
<th>Time when peak current appears (s)</th>
<th>Maximum current increment (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR-1 in the presence of EPS (control)</td>
<td>10480</td>
<td>1.497</td>
<td>3.712</td>
<td>11024</td>
<td>2.215</td>
</tr>
<tr>
<td></td>
<td>7624</td>
<td>3.916</td>
<td>8.272</td>
<td>8304</td>
<td>4.356</td>
</tr>
<tr>
<td></td>
<td>10960</td>
<td>1.094</td>
<td>4.078</td>
<td>11480</td>
<td>2.984</td>
</tr>
<tr>
<td>MR-1 in the absence of EPS</td>
<td>10482</td>
<td>2.237</td>
<td>6.444</td>
<td>11228</td>
<td>4.207</td>
</tr>
<tr>
<td></td>
<td>7619</td>
<td>4.613</td>
<td>12.450</td>
<td>8233</td>
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<td></td>
<td>14033</td>
<td>1.492</td>
<td>4.675</td>
<td>14698</td>
<td>3.183</td>
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</table>
Table S3. Charge calculation based on DPV shown in Fig. 3.

<table>
<thead>
<tr>
<th></th>
<th>Peak potential/mV</th>
<th>Peak area $Q_{DPV}$/C</th>
<th>Charge $Q_{formal}$/C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MR-1 in the presence of EPS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidative peak</td>
<td>-450</td>
<td>1.14E-09</td>
<td>4.38E-08</td>
</tr>
<tr>
<td>Reductive peak</td>
<td>-416</td>
<td>8.24E-10</td>
<td>3.17E-08</td>
</tr>
<tr>
<td>Oxidative peak</td>
<td>-138</td>
<td>7.31E-09</td>
<td>2.81E-07</td>
</tr>
<tr>
<td>Reductive peak</td>
<td>-170</td>
<td>1.01E-08</td>
<td>3.88E-07</td>
</tr>
<tr>
<td><strong>MR-1 in the absence of EPS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidative peak</td>
<td>-306</td>
<td>8.51E-09</td>
<td>3.27E-07</td>
</tr>
<tr>
<td>Reductive peak</td>
<td>-302</td>
<td>7.32E-09</td>
<td>2.82E-07</td>
</tr>
<tr>
<td>Oxidative peak</td>
<td>-102</td>
<td>1.29E-08</td>
<td>4.96E-07</td>
</tr>
<tr>
<td>Reductive peak</td>
<td>-182</td>
<td>2.40E-08</td>
<td>9.23E-07</td>
</tr>
<tr>
<td><strong>EPS</strong></td>
<td></td>
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<tr>
<td>Oxidative peak</td>
<td>-456</td>
<td>4.62E-09</td>
<td>1.78E-07</td>
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<tr>
<td>Reductive peak</td>
<td>-422</td>
<td>6.66E-09</td>
<td>2.56E-07</td>
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<tr>
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