Improved Monitoring of *P. aeruginosa* on Agar Plates

SUPPLEMENTAL INFORMATION

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Calibration curve for pyocyanin in TSB

Stock pyocyanin at 5 mg/mL in dimethyl sulfoxide was diluted in TSB, TSB agar, and Kings A agar to concentrations from 0 to 100 μM. TSB samples were scanned using square wave voltammetry (SWV) on three separate disposable carbon electrodes three times each from -0.5 to 0.0 V at a frequency and amplitude voltage of 15 Hz and 50 mV, respectively. Agar samples were scanned three times on a single electrode at the same parameters The peak current values after baseline subtraction were averaged and are reported in Fig. S1. A linear fit over the range of pyocyanin concentrations was obtained, yielding a slope of 1.8×10^{-1}, 1.7×10^{-1}, and 1.3×10^{-1} A/M for pyocyanin in TSB, TSB agar, and King's A agar, respectively. The King's A agar fit was used to calculate the concentration of pyocyanin diffusing through the King's A agar during experiments.

To confirm that the above curve would work for pyocyanin detection in agar matrices, known concentrations of pyocyanin were prepared in agar. Agar and pyocyanin were prepared at twice the desired final concentrations. Equal volumes of each were mixed together and then placed onto the electrodes and allowed to solidify. One sample was scanned three times at each concentration (with the mean maximum current shown) and the error bars are the standard deviation of these measurements. All reported concentrations of pyocyanin in the manuscript are the approximate concentrations based on the calibration curve for solid King's A agar.

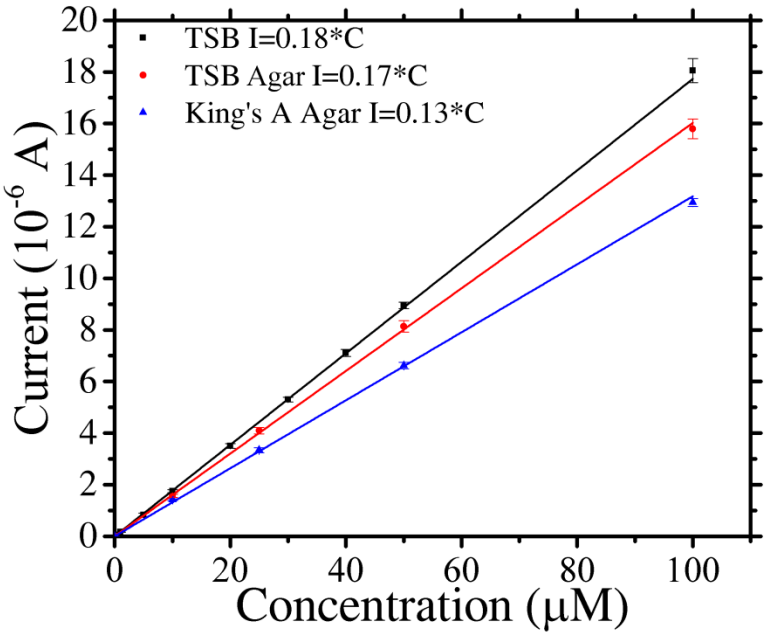

Fig. S1. Pyocyanin concentration curve fit in TSB liquid medium, solid TSB agar, and solid King's A agar. For TSB liquid medium, each data point represents the average of three scans with three different disposable electrodes. Additionally, two replicates per concentration were performed at each electrode. For solid TSB and King's A agar, error bars represent three scans with one disposable electrode at each concentration.

Effect of agar thickness on PA14 detection

10^8 *Pseudomonas aeruginosa* (PA14) cells were grown on King's A agar plates with embedded electrodes at three different agar thicknesses to determine what effect, if any, agar thickness would have on *P. aeruginosa* detection. Samples were analyzed with SWV approximately every hour. Samples showed a thickness dependent electrochemical signal change (Fig. S2). Thicker agar plates yielded lower electrochemical signals than thin agar plates at every time points, indicating that it took longer for pyocyanin to diffuse down to the electrode surface. All of the plates yielded a measureable signal, and the agar thickness can be optimized to achieve a faster signal response. The standard deviation of results is quite large for a given plate thickness as even slight variations in thickness result in a substantial change in diffusion time for these distances.

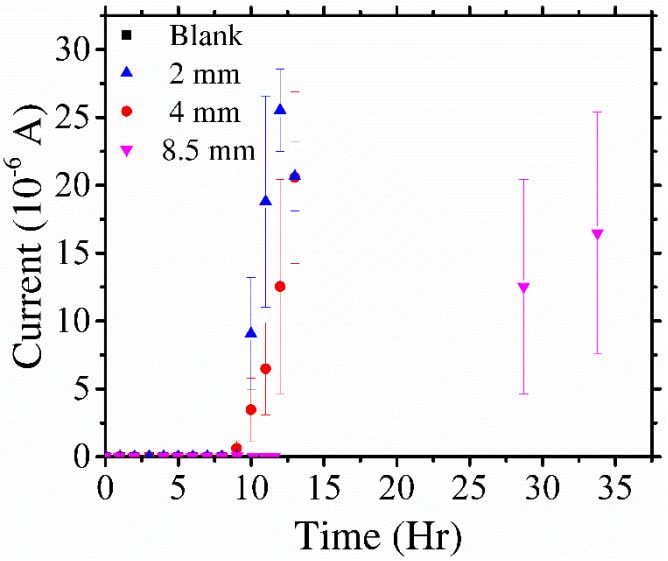

Fig. S2. Plots of average maximum current for 10^8 PA14 cells loaded onto agar that was approximately 2 (blue upward pointing triangles), 4 (red circles), or 8.5 (pink downward pointing triangle) mm thick. As a control experiment 5 µL of TSB solution without cells was placed onto a 2 mm thick agar slab and monitored with time. Over the course of experimentation the control showed no signal change (black squares). Current was measured via SWV from -0.5 to 0 V at a frequency of 15 Hz and an amplitude voltage of 50 mV.

COMSOL data of pyocyanin diffusing through Kings A Agar

Experiments with 100 µM solutions of pyocyanin diffusing through King's A Agar showed pyocyanin concentrations at the electrode surface that were significantly lower than the expected value of 100 µM. Simulations of pyocyanin diffusing through different thickness agars showed differing trends from the experimentally measured values (Fig. S3).

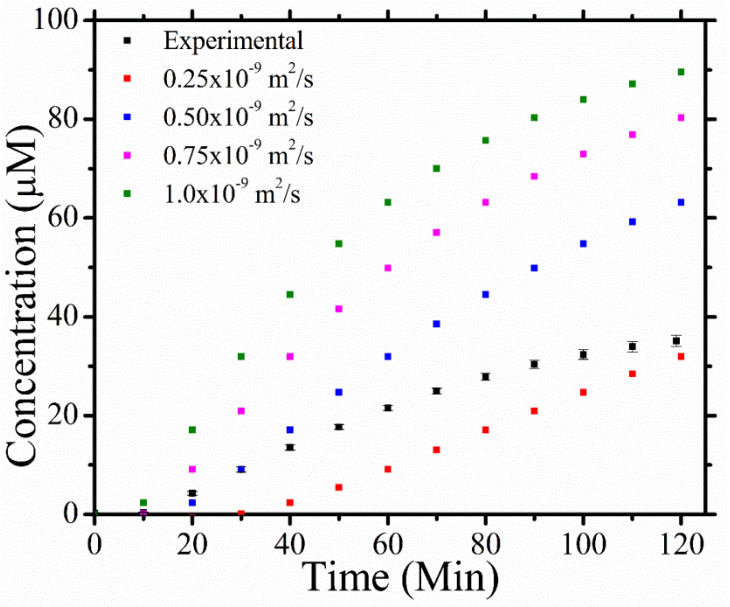

Fig. S3. Experimental measurement of pyocyanin (initially loading 200 µL at 100 µM) diffusing to the surface of the electrode (Black squares) through a 2.72 mm thick King's A agar slab. Error bars represent the standard error of the mean for triplicate samples. The remaining data points (red, blue, pink, and green squares) represent COMSOL simulations of a constant pyocyanin source at the top of a 2.72 mm thick agar block as it diffuses to the surface of the electrode (bottom of the block) evaluated with different diffusion coefficients.

From Fig. S3 it is clear that simulating the diffusion of pyocyanin using a 100 µM source does not correctly capture what is occurring within the system. The experimental data reaches an equilibrium value at a much faster rate and at a lower pyocyanin concentration. This indicates two things, one that the diffusion of pyocyanin is relatively fast in 2.72 mm thick agar, and that the pyocyanin is being diluted as it diffuses through the agar. This is not surprising given the large amount of water that is present in agar gels. When this dilution effect is taken into account for each agar thickness, the COMSOL simulations for match experimental results very well (Fig. 1A).

Effect of PA14 bacterial load on pyocyanin production and time to detection

To determine the limit of detection for PA14 cells, 5 µL of solution containing reconstituted PA14 cells was pipetted onto the agar directly above the working electrode. For example, if the desired bacterial load was 10^6 PA14 cells then 5 µL of a $2x10^8$ cells/mL solution was used. The samples were incubated at 37 ºC and analyzed using SWV (Fig. S4). The time to detection for cellular load was determined by the following equation:

$$I_{ave}(t_n)\text{-}S_{err}(t_n) > I_{ave}(t_{n-1})\text{-}S_{err}(t_{n-1})$$

Where I_{ave}, S_{err}, t_n are the average peak current, the standard error of the mean, and the time at which the measurement was taken, respectively. The results from Fig. 4A also make it possible to determine the maximum production rate of pyocyanin produced using the slope of the pyocyanin concentration over time (see **Measuring the maximum production rate of pyocyanin**).

Fig. S4 highlights the production of an electrochemical response for PA14 cultured at 23, 37, and 42 ºC at starting loads of 10^6 or 10^8 cells. A drastically reduced signal was obtained at 23 °C compared to PA14 cultures incubated at higher temperatures. The error bars show the standard error of the mean, based on three separate experiments at each temperature and starting amount of cells.

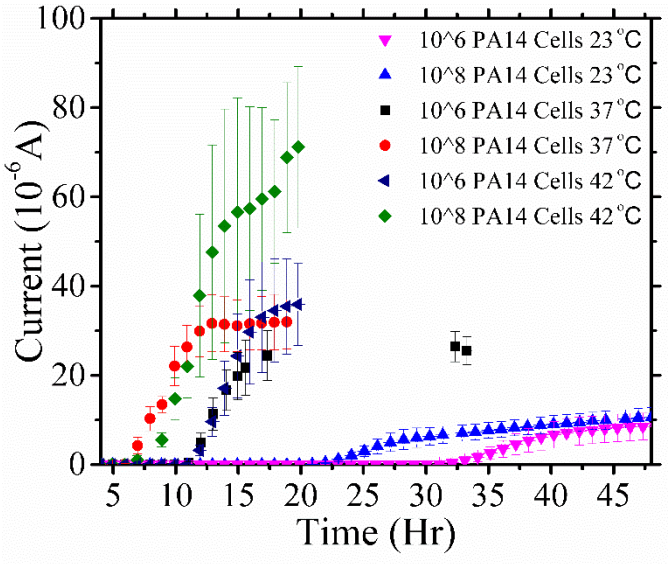

Fig. S4. Plot of baseline subtracted peak currents vs. time for cells cultured on King's A agar placed directly above the embedded working electrode at 23, 37, and 42 °C. Starting from 10^6 cells or 10^8 cells in 5 µL. Error bars are one standard error of the mean from the average of three separate experiments at each temperature and starting concentration.

Measuring the maximum production rate of pyocyanin

Fig. S5 plots the region containing the maximum slope for pyocyanin production for data shown in Fig. 4A and S4. The rate was determined from the slope of the line from Fig. S4 for 10^6 and 10^8 cells at all three temperatures. Additionally the production rate for 10^4 and 10^5 cells at 37 °C were calculated from the data presented in Fig. 4A. Production rates from 10^2 and 10^3 cells could not be obtained due to the inability to obtain a reproducible signal for these two bacterial loads. Significantly lower production rates for pyocyanin were measured electrochemically for samples cultured at 23 ºC compared to incubation temperatures of 37 and 42 ºC. The maximum production rate of pyocyanin (µM/Hr) at each temperature in Fig. 2B is reported as the average of the slopes at the applied temperature.

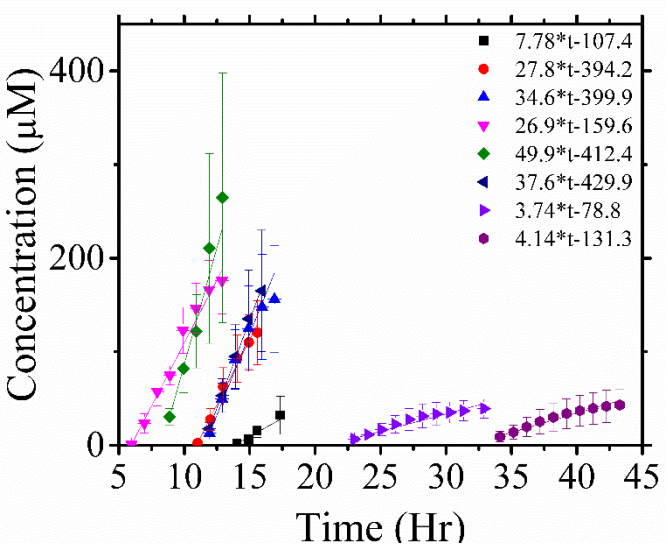

Fig. S5. Plots of the linear portions of the slope obtained from pyocyanin production rates by PA14 shown in Fig. S4 and Fig. S4. The slopes for each of the conditions are shown in units of µM/Hr. Black squares = 10^4, 37 °C; red circles = 10^5, 37 °C; blue upward pointing triangles = 10^6, 37 °C; pink downward pointing triangles = 10^8, 37 ºC; green diamonds = 10^8, 42 °C; navy blue left pointing triangles = 10^6, 42°C; purple right pointing triangles = 10^8, 23 °C; purple circles = 10^6, 23 ºC.

The x-offset seen in Fig. S5 is due to the time required for PA14 cells to begin producing measureable amounts of pyocyanin. Both temperature and cell load were shown to increase the amount of time required to produce measureable pyocyanin concentrations. Pyocyanin detection was observed to have a strong temperature dependence, as lower temperatures lead to the largest increase in the time required for a measureable electrochemical signal.

Improvement of electrochemical detection using embedded agar electrodes over optical detection

Liquid cultures of *P. aeruginosa* PA01, *Escherichia coli*, and *Staphylococcus aureus* were grown overnight at 37°C in 3 mL of LB. The cultures were streaked onto LB agar plates with embedded electrodes and grown at room temperature for 50 hours. Squarewave voltammograms were taken every 30 minutes from -0.5 to 0.5 volts at a frequency of 15 Hz and an amplitude voltage of 50 mV. Samples were run in triplicate and the average results after baseline subtraction are reported Fig. S6. Images of the culture plates at different time points are shown in Fig. S7. LB was used for these experiments since it is a commonly used growth medium for all three bacterial species.

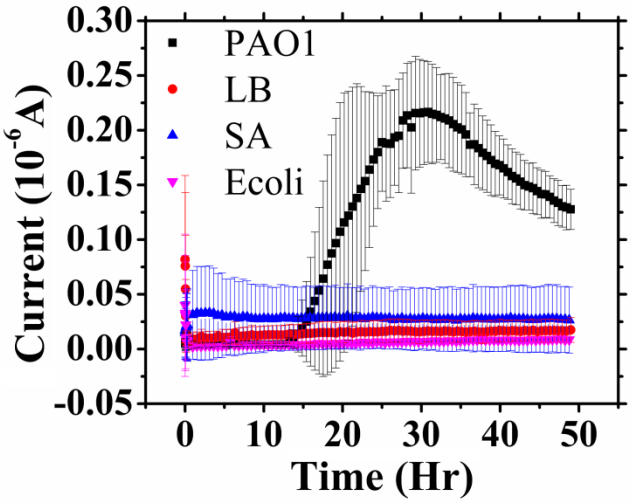

Fig. S6. Average peak currents after baseline subtraction for voltages between -0.4 to -0.2 V for embedded electrodes with no cells (red circles), PA01 (black squares), *S. aureus* (blue upward pointing triangles), and *E. coli* (pink downward pointing triangles).

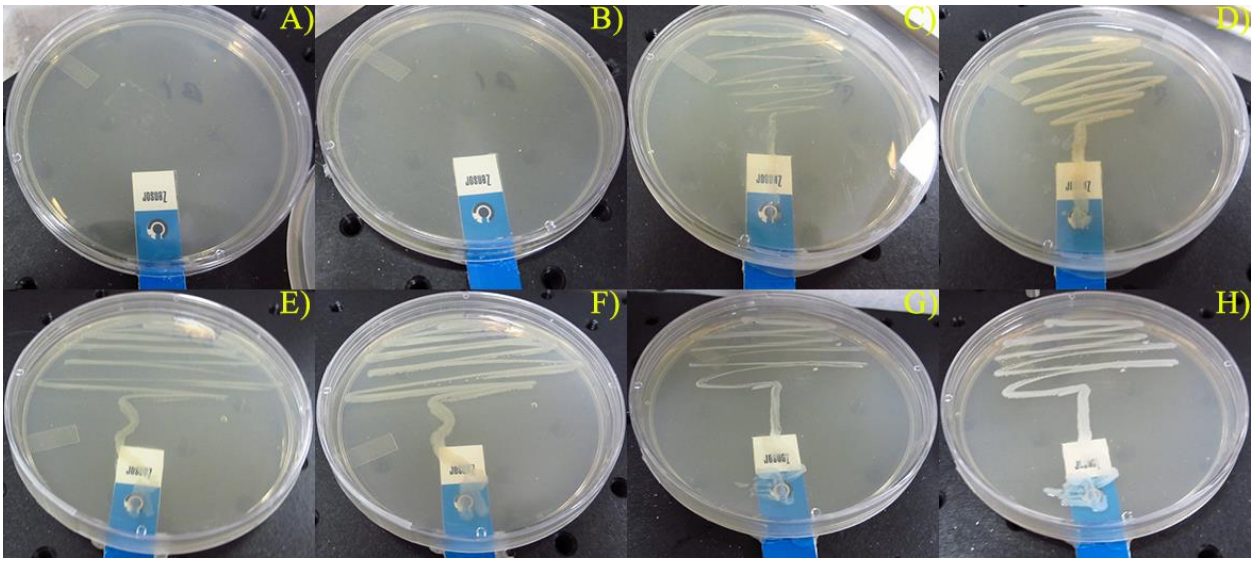

Fig. S7. Culture plates with embedded electrodes containing A) LB after 25 h, B) LB after 48 h, C) PAO1 after 25 h, D) PAO1 after 48 h, E) *E. coli* after 25 h, F) *E. coli* after 48 h, G) *S. aureus* after 25 h, and H) *S. aureus* after 48 h growth at room temperature.

Of note is the inability to distinguish optically between the different cell cultures shown in Fig. S7 after room temperature incubation. By comparison there is a clear signal increase in the electrodes exposed to *P. aeruginosa* growth, while there is no signal produced by the other bacterial species (Fig. S6). The lowered electrochemical response of *P. aeruginosa* on the LB agar plates was improved by using the King's A agar, which is optimized to increase pyocyanin production.

Selectivity of this approach to *P. aeruginosa* detection at 37 ºC

To confirm that higher temperatures would not allow other common bacteria to produce electrochemical molecules in the same potential window as *P. aeruginosa*, samples of *S. aureus* were cultured above the embedded King's A agar electrode assembly at 37 ºC. Comparing measured peak currents between PA14 and *S. aureus* cultured on King's A agar, it was clear that *S. aureus* was producing little to no interfering molecules that could potentially lead to false positive detection. The results in Fig. S6 and S8 suggest that this platform can selectively detect the presence of PA14 in a mixture of cells.

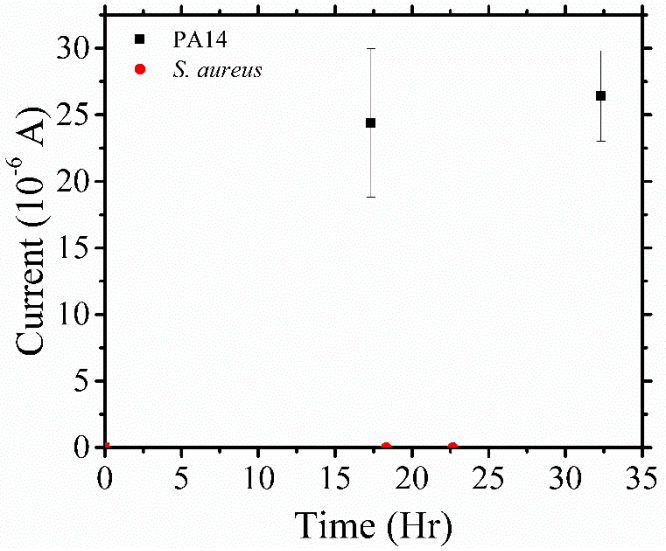

Fig. S8. Baseline subtracted maximum peak currents for PA14 and *S. aureus* cultured at 37 ºC on King's A agar.

Scanning electron micrographs of PA14 grown on Kings A Agar

After culturing PA14 on King's A agar, it was necessary to determine whether the measured current change was really due to pyocyanin diffusing through the agar or due to PA14 cells making their way to the surface of the working electrode and then excreting pyocyanin. To this end, the agar surrounding the disposable electrode was cut free and fixed in a 2.5% glutaraldehyde solution containing 0.1 M cacodylate buffer. After dehydrating in ethanol, the samples were dried further with hexamethyldisilizane, sputtered with platinum or palladium metal (to provide a conductive surface) and then inspected using scanning electron microscopy. Fig. S9 A) and B) shows the electron microscopy images of a PA14 colony that grew on top of the King's A agar, while Fig. S9 C) and D) shows the bottom of the agar that was in physical contact with the disposable electrode assembly. The magnified images show that the majority of the PA14 cells were found at the top surface of the agar (Fig. S9 A) and B)) compared to the bottom surface of the electrode (Fig. S9 C) and D)).

SEM scans of the electrodes (Fig. S10) highlight the lack of PA14 cells on the surface of the embedded electrode after the experiments. Together, Fig. S9 and S10 show that the majority of the cells are growing on the top surface of the agar, meaning that the large current detected is due to diffusing pyocyanin and not from PA14 cells growing on the electrodes and releasing pyocyanin at the sensor surface.

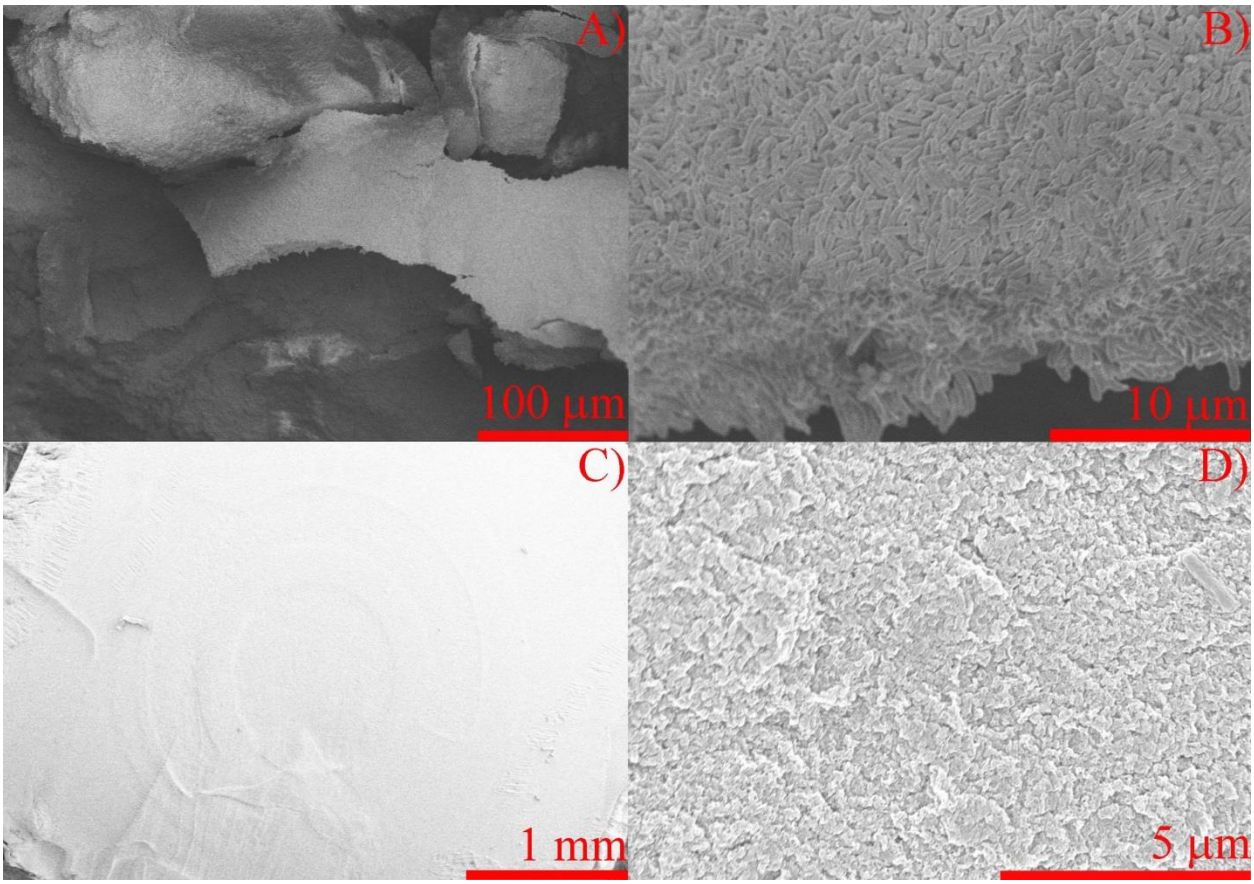

Fig. S9. Scanning electron microscopy images of A) the top surface of agar where 10^8 PA14 cells were incubated at 42 °C. B) 10X magnification of previous image. C) Surface of the agar that was in contact with the embedded electrode at the bottom of the petri dish. D) Magnified view of previous image. Images were obtained using an acceleration voltage and emission current of 3 kV and 10 µA, respectively.

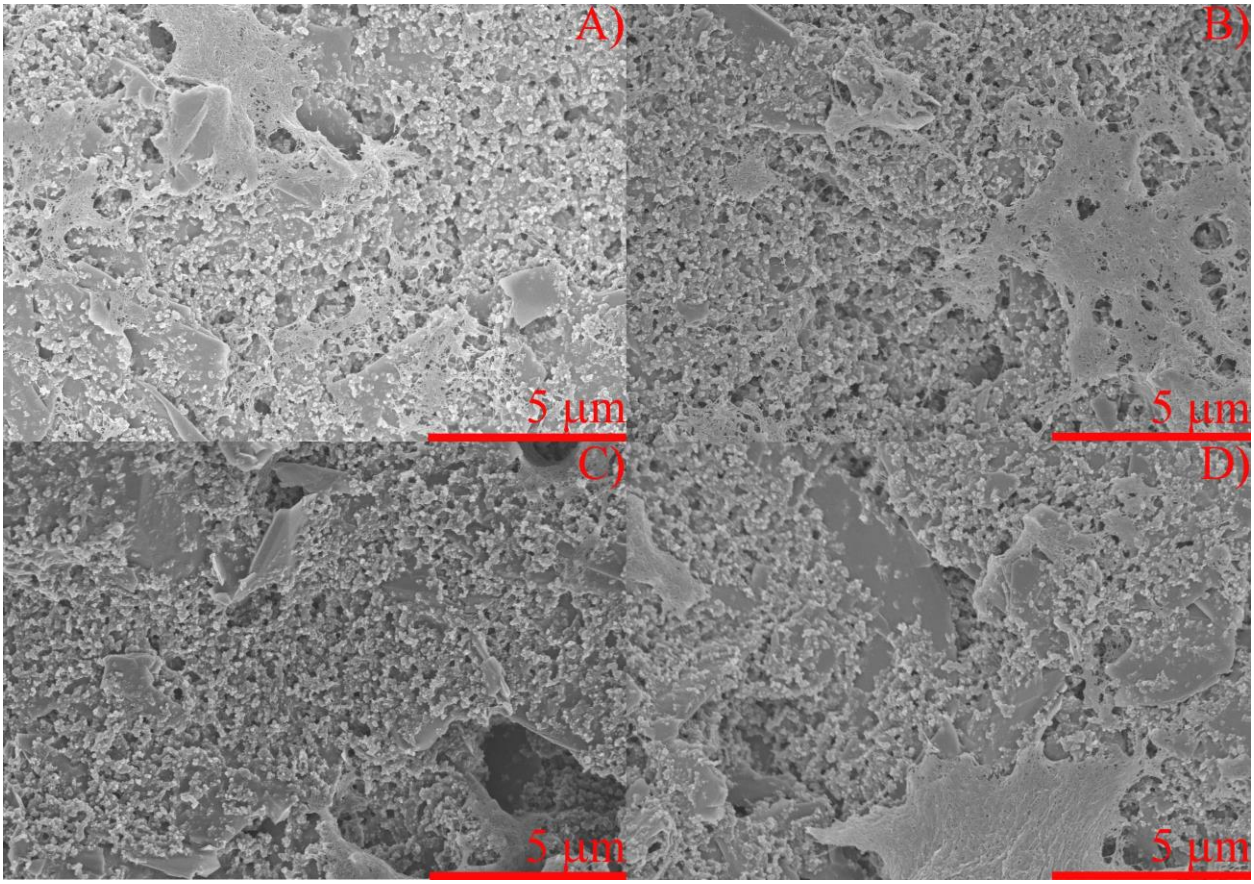

Fig. S10. Scanning electron microscopy images of embedded electrodes after growth of 10^6 and 10^8 PA14 cells on top of 2 mm thick Kings A Agar. The agar was separated from the electrode at the end of the experiment. A) 10^6 cells grown at RT, B) 10^6 cells grown at 42 °C, C) 10^8 cells grown at RT, and D) 10^8 cells grown at 42 °C. No cells are visible on the electrodes. Scans performed at an acceleration voltage and emission current of 3 kV and 10 µA, respectively.