

Supplementary Information

Deciphering single-bacterium adhesion behavior modulated by extracellular electron transfer

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Supplementary Note 1-10

Supplementary Table 1-3

Supplementary Figure 1-10

References

Supplementary note 1: Construction of the *in situ* ESCFS measurement system and the calibration of the reference electrode

We integrated a three-electrode electrochemical cell and an IOM into AFM to construct the *in situ* ESCFS measurement system, as shown in Figure 1a. The three-electrode electrochemical cell was fabricated as follows. A piece of transparent anodic bonded glass plate (45*45 mm², thickness 0.5 mm, Borofloat 33, Germany Schott) was utilized as the substrate of the electrochemical cell (Figure S1). Two interdigitated gold film electrodes with a thickness of 300 nm were deposited on the upper surface of the glass plate via vacuum vapor deposition, where a 50 nm Ti buffer layer was deposited on the anodic bonded glass plate by thermal evaporation first, followed by a 300 nm gold layer, with an evaporation rate of 1 Å/s by a high vacuum resistance evaporation coating instrument (VZZ-300S, VNANO, China). These two interdigitated gold film electrodes served as the working electrode (WE) and counter electrode (CE) (Figure 1b). A Ag wire (diameter: 0.5 mm) with a AgCl coating served as the reference electrode (RE). A polytetrafluoroethylene (PTFE) cell was fixed on the sample table by four screws, and an ~3.2 mL electrochemical cell was thus completed. The potential of the Ag/AgCl wire was calibrated relative to that of Ag/AgCl (saturated KCl) by cyclic voltammetry in 5 mM K₃[Fe(CN)₆] in the presence of 0.1 M KCl. Figure S2 illustrates that the potential of the RE was 0.077 V vs. Ag/AgCl (saturated KCl). All potentials mentioned are relative to Ag/AgCl (saturated KCl), unless otherwise specified.

Supplementary note 2: Bacterial strain and growth conditions

S. oneidensis MR-1 was used in this study and was preserved at -80 °C. Before use, it was transferred to a -20 °C refrigerator for 2 h and then transferred to a 0 °C refrigerator for 2 h. The bacterial suspension was prepared by transferring 400 µL of bacterial solution with a sterilized pipette to a 50 mL centrifuge tube containing 20 mL of LB broth (Miller, Sigma-Aldrich). Then, the bacterial suspension was incubated at 30 °C with 200 rpm shaking for 24 h until mid-exponential growth phase ($OD_{600\text{ nm}} \approx 1.0\text{--}1.2$). Next, 400 µL of the suspension was inoculated into a 50 mL centrifuge tube containing 20 mL of M9, followed by incubation at 30 °C and 200 rpm for 24 h ($OD_{600\text{ nm}} \approx 0.6\text{--}0.8$). Then, it was centrifuged at 5000 rpm for 5 min and suspended in PBS (pH 7.2-7.4, Biosharp), and this process was repeated three times. All of the reagents and materials used in this process were autoclaved at 121 °C for 20 min (ZEALWAY, DR85DF) before use. Details of the preparation of M9 nutrient solution are shown in supplementary note 3-4.

Supplementary note 3: Preparation of M9 nutrient solution.

The composition of the M9 nutrient solution used in this work is shown in Table S1.

Table S1. Composition of the M9 nutrient solution

Component	Content
Disodium hydrogen phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)	15.036 g/L
Potassium dihydrogen phosphate (KH_2PO_4)	3 g/L
Sodium chloride (NaCl)	0.5 g/L
Ammonium chloride (NH_4Cl)	1 g/L
Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.246 g/L
100x CaCl_2 Stock Solution	2.06 mL/L
Minerals Solution	12.5 mL/L
Vitamin Solution	12.5 mL/L
Sodium L-lactate ($\text{C}_3\text{H}_5\text{O}_3\text{Na}$)	2.017 g/L

Since vitamin solution and sodium L-lactate cannot be sterilized by autoclaving, the vitamin solution and sodium L-lactate were first filtered through 0.22 μm filters and then placed in a UV sterilizer. The solution without the addition of vitamin solution and sodium L-lactate was sterilized at 121 $^{\circ}\text{C}$ for 20 min. Finally, the vitamin solution and sodium L-lactate filtered by 0.22 μm filters were added to the solution in the sterilization cabinet. Then the solution was marked and placed in the sterilization cabinet.

Supplementary note 4: Preparation of the mineral and vitamin solutions.

The composition of the mineral solution in Table S1 is shown in Table S2.

Table S2. Composition of the mineral solution

Component	Content
Nitrilotriacetic acid ($\text{N}(\text{CH}_2\text{COOH})_3$)	1.5 g/L
Magnesium sulphate (MgSO_4)	3 g/L
Manganese sulfate monohydrate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$)	0.5 g/L
Sodium chloride (NaCl)	1 g/L
Ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.1 g/L
Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	0.1 g/L
Cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$)	0.1 g/L
Zinc chloride (ZnCl_2)	0.13 g/L
Copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	0.01 g/L
Aluminum potassium sulfate dodecahydrate ($\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$)	0.01 g/L
Boric acid (H_3BO_3)	0.01 g/L
Sodium molybdate Nickel chloride hexahydrate (NaMoO_4)	0.025 g/L
Nickel chloride hexahydrate ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$)	0.024 g/L
Sodium tungstate dihydrate ($\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$)	0.025 g/L

The composition of the vitamin solution in Table S1 is shown in Table S3.

Table S3. Composition of the vitamin solution

Component	Content
Biotin ($C_{10}H_{16}N_2O_3S$)	2 mg/L
Pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)	10 mg/L
Riboflavin ($C_{17}H_{20}N_4O_6$)	5 mg/L
Folic acid ($C_{19}H_{19}N_7O_6$)	2 mg/L
Nicotinic acid ($C_6H_5NO_2$)	5 mg/L
Pantothenic acid ($C_9H_{17}NO_5$)	5 mg/L
Thiamin ($C_{12}H_{17}ClN_4OS \cdot HCl$)	5 mg/L
p-Aminobenzoic acid ($C_7H_7NO_2$)	5 mg/L
Thioctic acid ($C_8H_{14}O_2S_2$)	5 mg/L
Vitamin B12 ($C_{63}H_{88}CoN_{14}O_{14}P$)	0.1 mg/L

The mineral and vitamin solutions do not need sterilization operation after preparation, and they should be marked and stored in the refrigerator at 4 °C.

Supplementary note 5: Functionalization of the cantilever with a single *S. oneidensis* MR-1

The anodic bonded glass plate deposited with gold film electrodes was ultrasonically cleaned in ultrapure water ($18.2 \text{ M}\Omega \cdot \text{cm}$, Heal Force) for 15 min and dried under a continuous and steady N_2 flow for 15 min. Then the glass plate was UV/ O_3 -cleaned for 15 min. Subsequently, a 10- μL droplet of the bacterial suspension was dropped directly on the glass plate between working electrode and counter electrode, and the droplet was allowed to stand for 30 min to allow *S. oneidensis* MR-1 to attach to the glass surface (Figure 1c)¹. The concentration of the bacterial suspension needs to be diluted in order to ensure the *S. oneidensis* MR-1 attach to the glass surface separately. Then the glass plate was tilted 30 degrees and rinsed with 3 mL of PBS to remove the unattached bacteria and prevent the *S. oneidensis* MR-1 from contacting the working electrode. Subsequently, the as-prepared glass plate was mounted on the electrochemical cell and 2.5 mL of test solution was immediately added to the electrochemical cell.

Single *S. oneidensis* MR-1 was immobilized on the end of cantilever by using polydopamine (PDA) as the wet adhesive². The PDA solution was prepared by dissolving dopamine hydrochloride powder (0.4 g, Sigma-Aldrich) to 100 mL of Trizma buffer (10 mM, BioReagent, Sigma-Aldrich), then the solution was buffered to pH=8.5 with 1M NaOH^{2-3} . Silicon nitride atomic force microscope (AFM) probes (nominal $k = 0.06 \text{ N/m}$, Novascan Technologies, Ames, IA, USA) with a SiO_2 micro-ball (diameter: 2.5 μm) on the head of triangle cantilever were used. To ensure the adhesiveness, the PDA solution and the probes with a PDA coating need to be prepared right before every experiment. The AFM probes were put into the UV/ O_3 -cleaner and cleaned for 10 min, followed by being immersed into 10 mL of PDA solution.

After shaking at 60 rpm for 15 min on a biological shaker, the probes were rinsed with ultrapure water (18.2 M Ω •cm, Heal Force) and dried under a continuous and steady N₂ flow for 30 min.

Functionalization of the cantilever with a single *S. oneidensis* MR-1 was conducted at 25°C utilizing AFM (Agilent 5500, USA), which was integrated into an IOM (LEICA DMi1). An AFM probe with a PDA coating was mounted on the AFM scanner and the electrochemical cell was mounted on the AFM subsequently. The electrochemical cell was then elevated slowly by manual to make the probe immerse into the electrochemical cell, allowing the triangle cantilever deflection to reach a steady deflection value (typically observed within 60 min). After that, the electrochemical cell was elevated at a slow speed of 0.5 μ m/s. Then force-curves measurements were conducted over the bare glass plate using contact mode AFM at a cantilever speed of 0.2 μ m/s. The deflection sensitivity of the cantilever was obtained from the slope of the contact region of deflection signal (V) vs piezo extension (nm) curves collected. Then the cantilever was withdrawn. With the help of a 60 \times objective of IOM, the electrochemical cell was moved horizontally and slowly in order to keep one single *S. oneidensis* MR-1 directly under the PDA-coated SiO₂ micro-ball that was attached to the head of cantilever. Then the electrochemical cell was elevated at a slow speed of 0.5 μ m/s, allowing the single *S. oneidensis* MR-1 to approach the PDA-coated SiO₂ micro-ball. The cantilever was engaged in contact mode over *S. oneidensis* MR-1 for 5 min with a 0.5 nN loading force, after which the cantilever was withdrawn, and a single *S. oneidensis* MR-1 was functionalized on the SiO₂ microball.

Supplementary note 6: Force measurements between a single *S. oneidensis* MR-1 and a gold film electrode.

The electrochemical cell was moved horizontally and slowly to keep the gold film electrode directly under the as prepared cantilever that was functionalized with single *S. oneidensis* MR-1. This electrochemical cell was connected to a potentiostat (Autolab PGSTAT204) that was controlled by a computer through GPIB-to-USB cables through a software suite (Metrohm Autolab Nova 2.1). In this study, chronoamperometry (CA) was performed in order to hold gold film electrode at different electrode potentials. The force-curves measurements were performed at 25°C in an electrochemical cell that was filled with different test solutions under aerobic and anaerobic conditions. And these experiments were conducted using contact mode AFM at a cantilever speed of 0.2 $\mu\text{m/s}$ and force distance of 1 μm . When the loading force of the cantilever reached a set value of 0.5 nN, the cantilever stopped and remained constant for 5 s, and then the *S. oneidensis* MR-1 was pulled from the gold film electrode by the cantilever. These force-distance curves data were obtained at some randomly chosen positions on the gold film electrode surface. Notably, just three reproducible force curves would be collected over each position in order to avoid the deposition of extracellular polymeric substances. For each measurement condition, at least three individual bacteria (from three independent bacteria cultures) were utilized to obtain a total of 100~250 force-distance curves for statistical analyses.

Raw data (i.e., cantilever deflection signal (V) versus piezo Z position) were converted into force-distance curves by multiplying the cantilever deflection signal (V) with the spring constant (N/m) of cantilever and the deflection sensitivity (nm/V). The maximum adhesion force (F_{Ad})

between *S. oneidensis* MR-1 and gold film electrode was obtained from each force-distance curve. After each experiment, L7012 live/dead BacLight bacterial viability kits were used to check the viability of the *S. oneidensis* MR-1 that was immobilized on the cantilever. 7.5 μ L of component A and 7.5 μ L of component B of kits L7012 were mixed thoroughly in a microfuge tube. 15 μ L of the dye mixture was added to 5 mL of the PBS and mixed thoroughly, then the probe functionalized with *S. oneidensis* MR-1 on cantilever was dismounted from the scanner and immersed into the dye solution immediately, which was incubated at room temperature in the dark for 15 min. After that, the probe was observed in a fluorescence microscope (Nikon ECLIPSE E200, Japan). Live bacteria stained fluorescent green, whereas dead bacteria stained fluorescent red.

Supplementary note 7: Biofilm formation on a gold electrode.

Biofilm formation on a gold electrode was performed in a three-electrode reactor. A 10×20 mm² gold electrode served as the WE, a 10×10 mm Pt mesh served as the CE and Ag/AgCl (saturated KCl) served as the RE. All components of this three-electrode system, including the reactor, WE, CE, RE and magnetons, were thoroughly cleaned and autoclave sterilized for 20 min at 121°C. After cooling, they were put into a UV sterilizer for use. *S. oneidensis* MR-1 in the mid-exponential growth phase ($OD_{600\text{ nm}} \approx 1.0\text{--}1.2$) in 20 mL of LB broth was centrifuged at 5000 rpm for 5 min and then suspended in M9, and this process was repeated three times to remove the LB residue. Finally, *S. oneidensis* MR-1 was suspended in 75 mL of M9 ($OD_{600\text{ nm}} \approx 0.8$) and added to the reactor. Biofilm formation experiments were performed under the conditions of different electrode potentials and solution compositions with 200 rpm magnetic stirring for 24 h at 25°C. After each experiment, L7012 live/dead BacLight bacterial viability kits were used to check the viability of the *S. oneidensis* MR-1 biofilm. Component A (4.5 μ L) and component B (4.5 μ L) from the L7012 kits were mixed thoroughly in a microfuge tube. 9 μ L of the dye mixture was added to 3 mL of PBS and mixed thoroughly. Then, the WE was dismantled from the three-electrode reactor and gently rinsed with PBS to remove the unattached bacteria and immersed into the dye solution immediately and incubated at room temperature in the dark for 15 min. Then, the WE was covered by an 18 mm square coverslip and observed under a fluorescence microscope (Nikon, DS-Qi2) to obtain fluorescence images of the biofilm.

Supplementary note 8: Calculation of the bacterial density of the biofilm formed on the gold electrode.

The bacterial density of the biofilm formed on the gold electrode was estimated from the fluorescence images by using Adobe Photoshop. First, we selected a representative fluorescence image of the biofilm formed on the gold electrode. Second, the magic wand tool in continuous mode with a tolerance value of 32 was used to determine the pixel number (N_s) of every *S. oneidensis* MR-1 cell. Furthermore, pixel number histograms of a single *S. oneidensis* MR-1 cell in the fluorescence image were obtained. The average pixel number of every *S. oneidensis* MR-1 cell was 60.6. Then, the magic wand tool in noncontinuous mode with a tolerance value of 32 was used to determine the total pixel number (N_T) of the green area throughout the fluorescence image of the biofilm formed on the gold film electrode under various conditions. Finally, the number (N_c) of *S. oneidensis* MR-1 cells in every fluorescence image was obtained by calculating the quotient of N_T and N_s (N_T/N_s), and then the bacterial density was estimated by calculating the quotient of N_c and the image area S (N_c/S , $S = 3.6 \times 10^{-3} \text{ cm}^2$). The average bacterial density of the biofilm formed on the gold film electrode under various conditions was calculated from five randomly selected $600 \times 600 \text{ }\mu\text{m}^2$ scanning areas under various conditions.

Supplementary note 9: Numerical WLC modelling.

To elucidate the underlying mechanism of the unfolding behaviour of extracellular proteins, the force-distance curves measured by ESCFS are modelled using a WLC model. Eq. (5) was fitted to the region of unfolding events in the force-distance curves, which show saw-toothed unfolding peaks.

Supplementary note 10: Data and statistical analysis

Statistical significance was calculated using a two-tailed unpaired t-test when more than two groups were compared. *P* values and statistical parameters are indicated in the figures and figure legends, respectively. The error bars represent the standard deviation throughout. For all experiments, the statistical significance level α was set to < 0.05 .

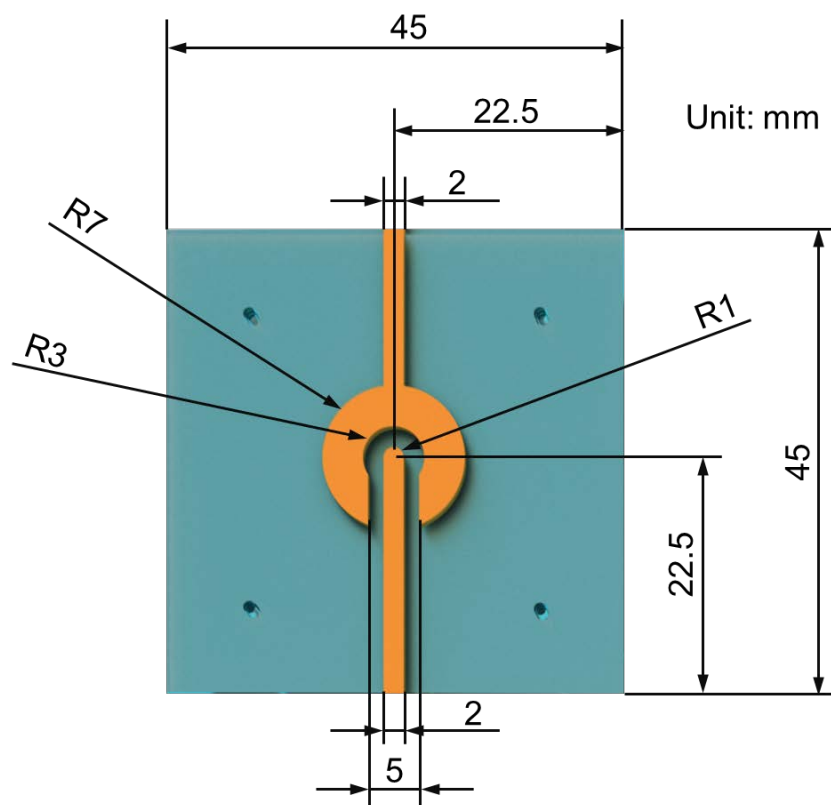


Figure S1. Geometric size of the transparent anodic bonded glass plate (45*45 mm², thickness 0.5mm, Borofloat 33, Germany Schott) that was utilized as the substrate of the electrochemical cell in this study (R_x: radius = x mm).

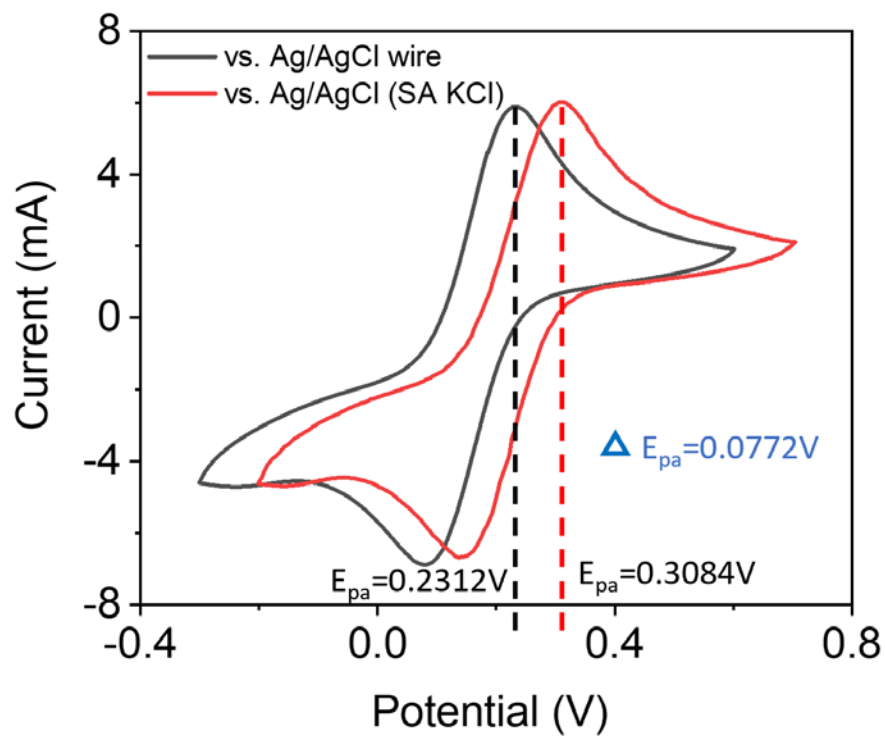


Figure S2. Cyclic voltammetry scans (50 mV/s) for Pt sheet electrode in $K_3[Fe(CN)_6]$ (5 mM) with a Ag/AgCl wire and a Ag/AgCl (saturated KCl) as the reference electrode respectively in the presence of 0.1M KCl. Temp: 25 °C.

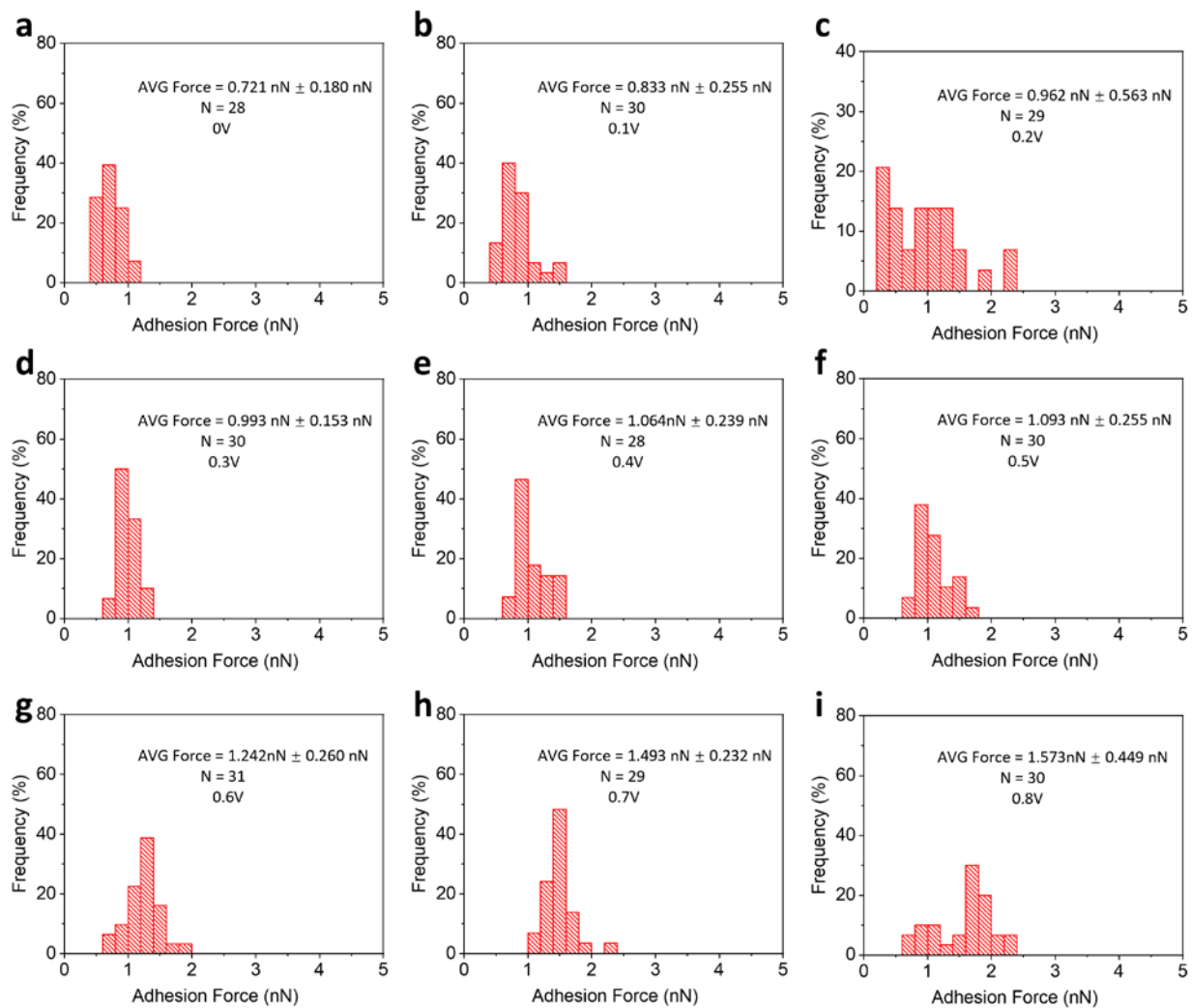


Figure S3. Histogram of the maximum adhesion force (F_{Ad}) between a single *S. oneidensis* MR-1 and a gold film electrode (WE) in PBS solution under aerobic conditions holding different electrode potentials. (a) 0V, (b) 0.1V, (c) 0.2V, (d) 0.3V, (e) 0.4V, (f) 0.5V, (g) 0.6V, (h) 0.7V, (i) 0.8V vs. Ag/AgCl (saturated KCl). The inset shows the histogram average (F_{Ad}), standard deviation, and number of measurements (N). Temp. 25°C.

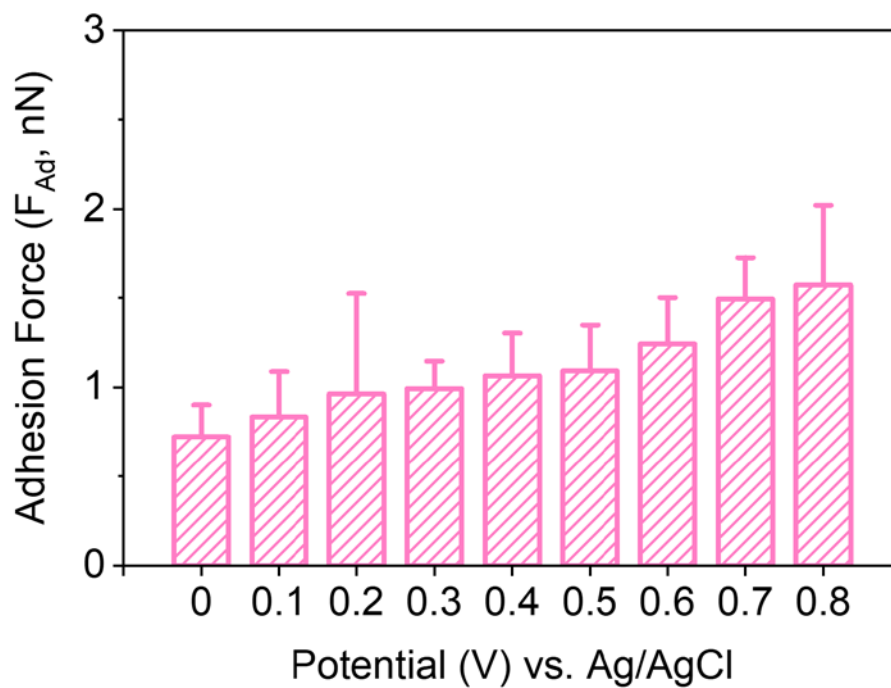


Figure S4. Maximum adhesion forces (F_{Ad}) between a single *S. oneidensis* MR-1 and a gold film electrode (WE) in PBS solution under aerobic conditions as a function of electrode potential vs. Ag/AgCl (saturated KCl). Temp. 25 °C.

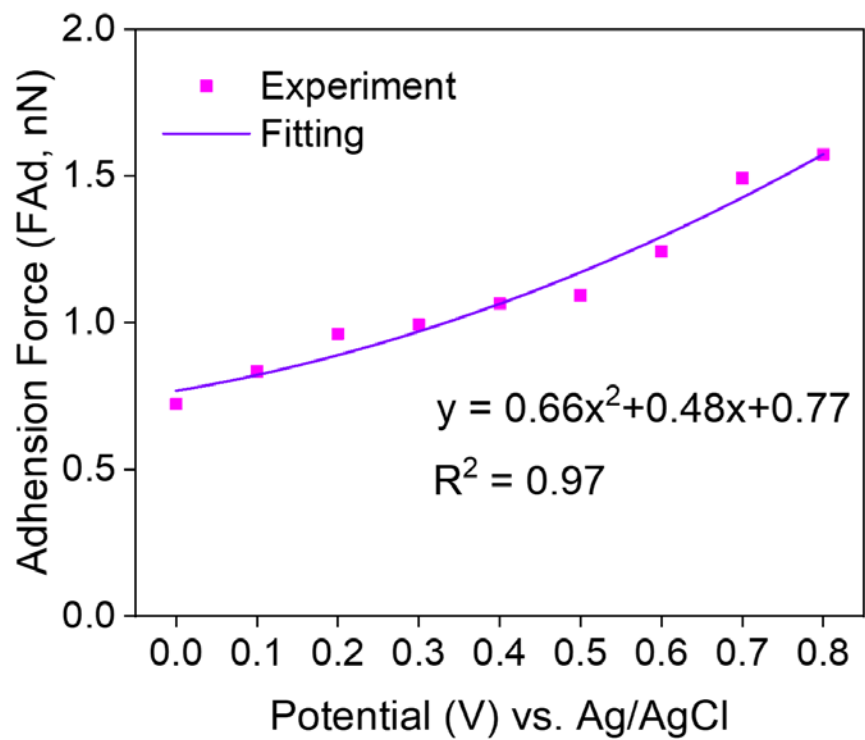


Figure S5. Fitting of the F_{Ad} data as a function of the potential with electrostatic force expression in PBS solution under aerobic conditions.

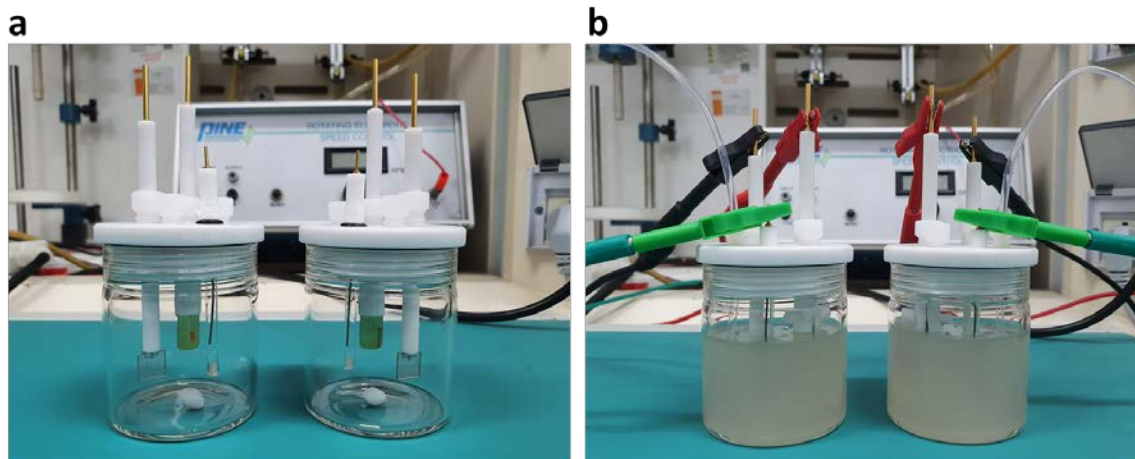


Figure S6. Images of the three-electrode reactors used to perform the biofilm formation experiments.

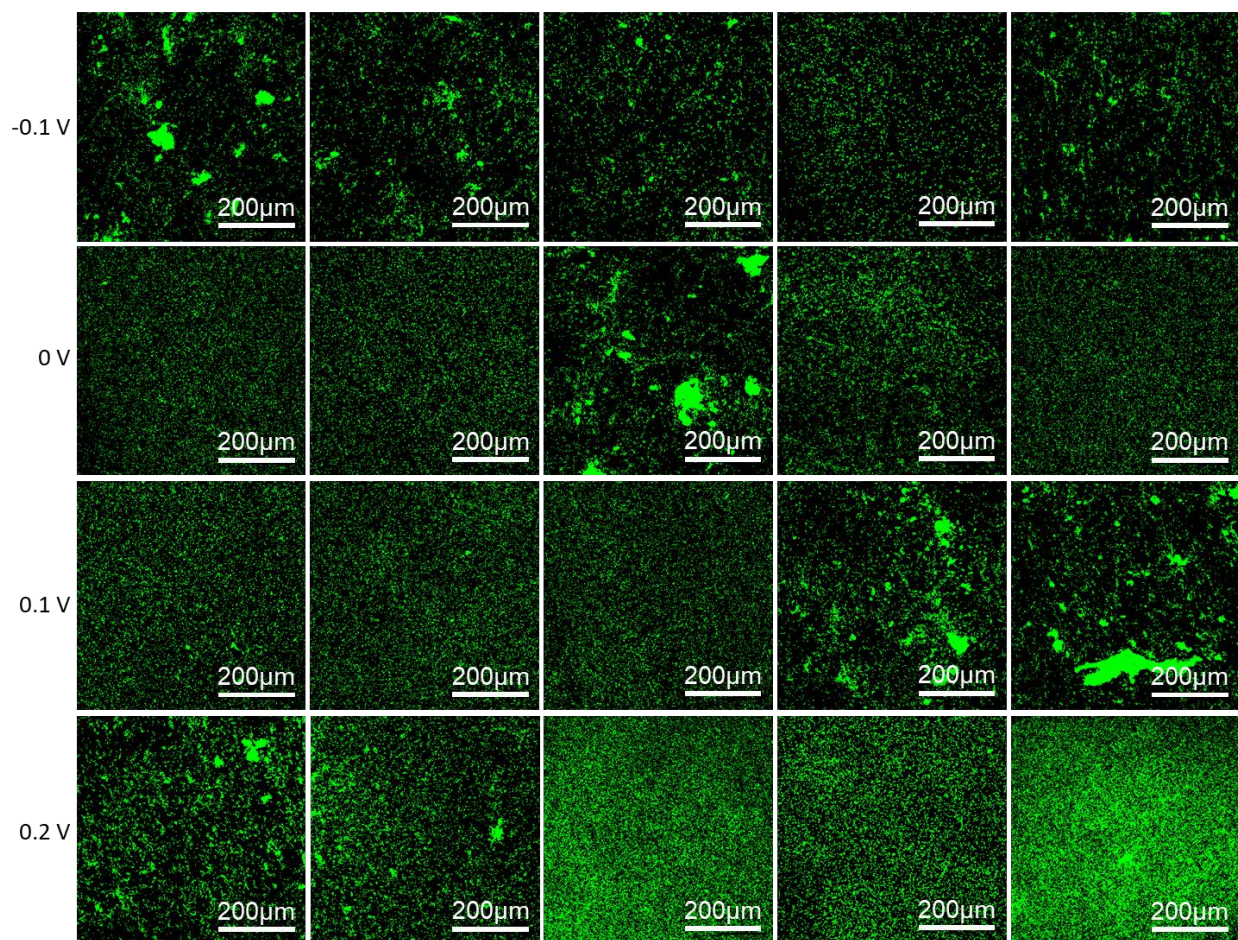


Figure S7. Microscope fluorescence images of *S. oneidensis* MR-1 biofilm formed on a gold film electrode (WE) under different electrode potential (vs. Ag/AgCl). Biofilm formation was conducted in a three-electrode reactor filled with M9 and 18 mM sodium lactate (M9+SL), under anaerobic conditions with 200 rpm magnetic stirring for 24h at 25°C. For every sample, five $600 \times 600 \mu\text{m}^2$ scanning areas were selected randomly.

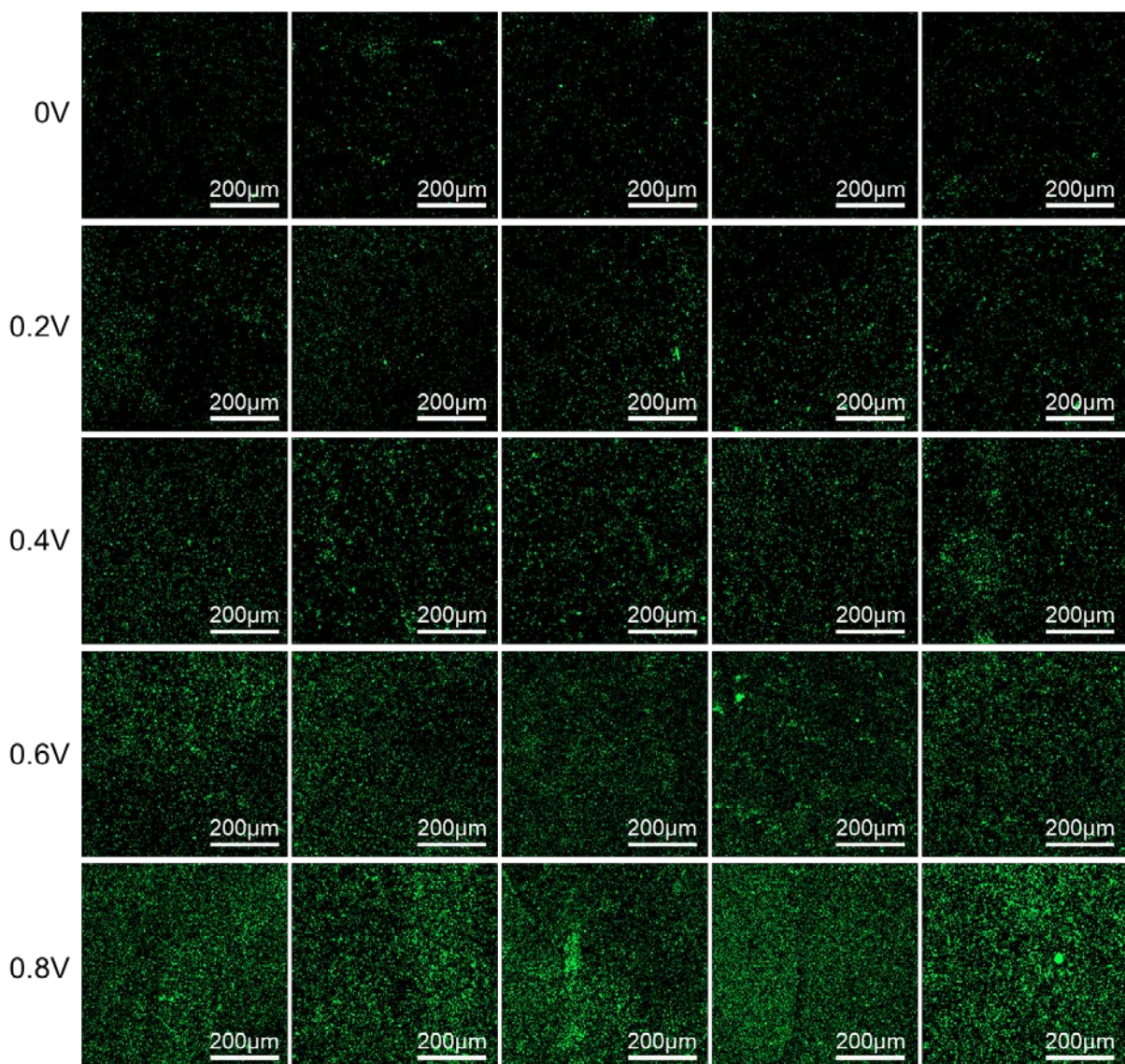


Figure S8. Microscope fluorescence images of *S. oneidensis* MR-1 biofilm formed on a gold film electrode (WE) under different electrode potential (vs. Ag/AgCl). Biofilm formation was conducted in a three-electrode reactor filled with M9 and 18 mM sodium lactate (M9+SL), under aerobic conditions with 200 rpm magnetic stirring for 24h at 25°C. For every sample, five 600 × 600 µm² scanning areas were selected randomly.

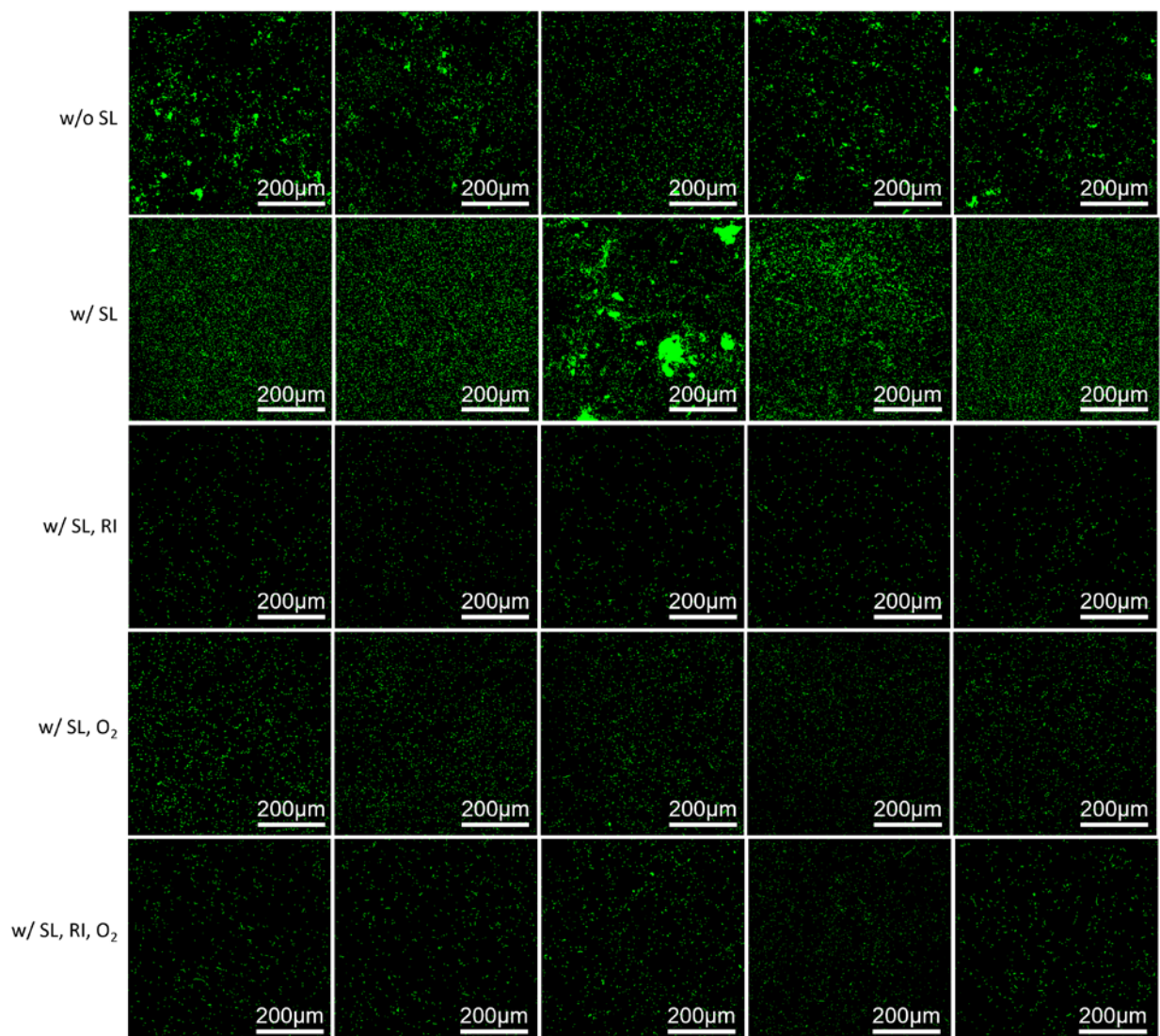


Figure S9. Microscope fluorescence images of *S. oneidensis* MR-1 biofilm formed on a gold electrode surface in M9 under different solution chemical conditions. Biofilm formation was conducted in a three-electrode reactor, holding WE at a potential of 0V vs. Ag/AgCl (saturated KCl) with 200 rpm magnetic stirring for 24h at 25°C. For every sample, five $600 \times 600 \mu\text{m}^2$ scanning areas were selected randomly.

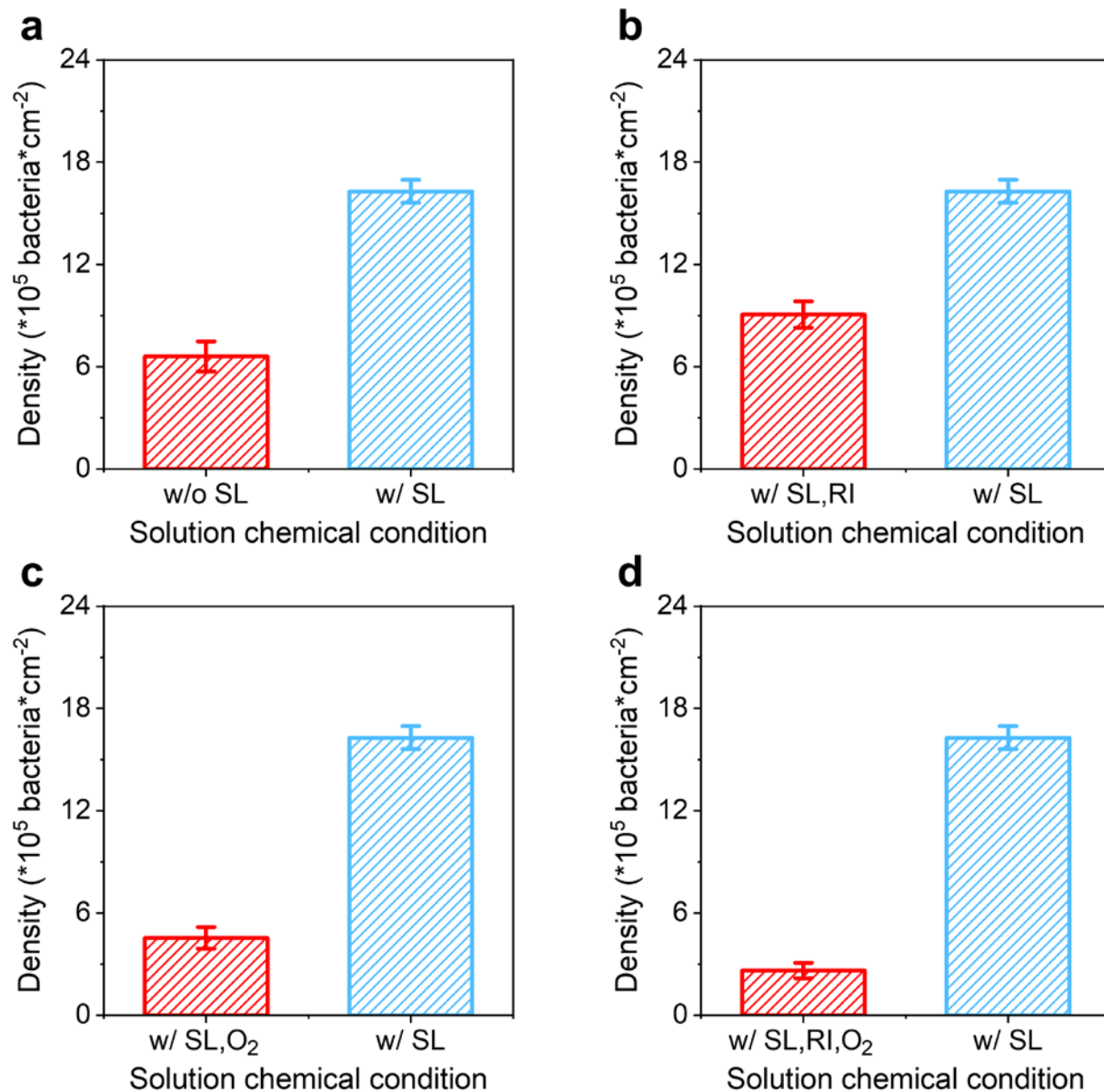


Figure S10. Bacteria density of the biofilm formed on a gold electrode under different solution chemical conditions. Biofilm formation was conducted in a three-electrode reactor filled with M9, holding WE at a potential of 0V vs. Ag/AgCl (saturated KCl) with 200 rpm magnetic stirring for 24h at 25°C.

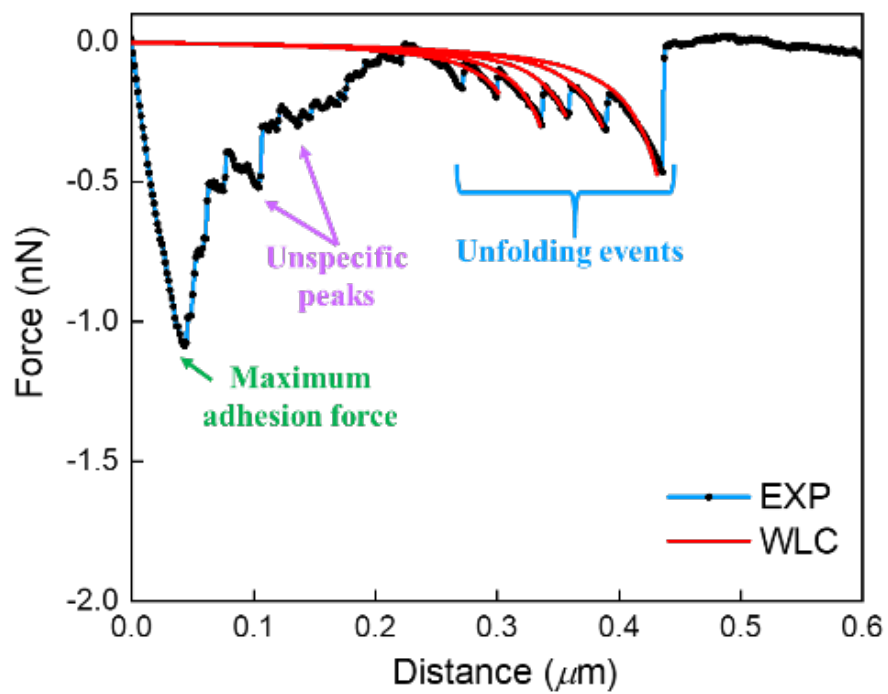


Figure S11. Fitting of the force-distance curve using the worm-like chain (WLC) model

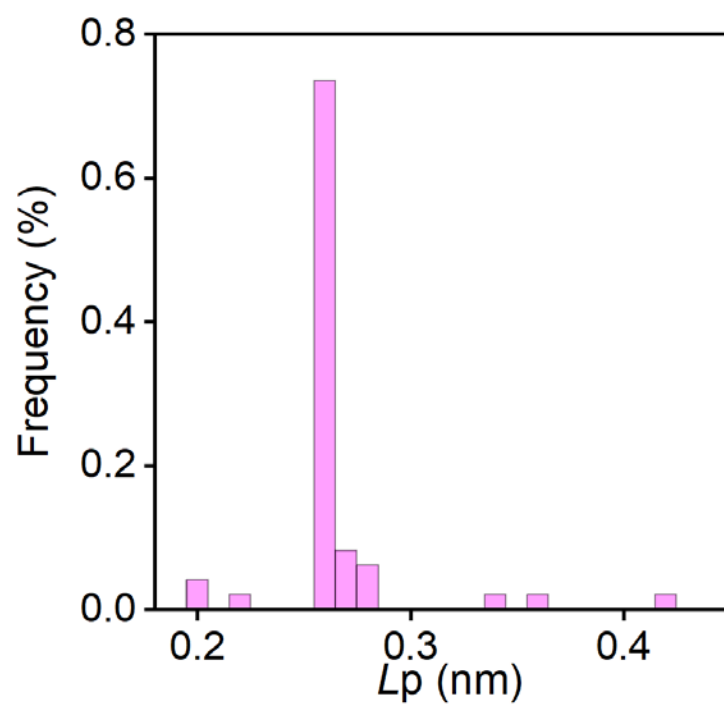


Figure S12. Distribution of the persistent length L_p calculated from fitting the force-distance curves with a worm-like chain (WLC) model.

REFERENCES

- (1) Wuolo-Journey, K.; BinAhmed, S.; Linna, E.; Romero-Vargas Castrillón, S. Do graphene oxide nanostructured coatings mitigate bacterial adhesion? *Environmental Science: Nano* **2019**, *6*, 2863-2875.
- (2) Xue, J.; Binahmed, S.; Wang, Z.; Karp, N.; Stottrup, B.; Castrillón, S. Bacterial Adhesion to Graphene Oxide (GO)-Functionalized Interfaces Is Determined by Hydrophobicity and GO Sheet Spatial Orientation. *Environmental Science & Technology Letters* **2018**, *5*, 14-19.
- (3) BinAhmed, S.; Hasane, A.; Wang, Z.; Mansurov, A.; Romero-Vargas Castrillón, S. Bacterial Adhesion to Ultrafiltration Membranes: Role of Hydrophilicity, Natural Organic Matter, and Cell-Surface Macromolecules. *Environmental Science & Technology* **2018**, *52*, 162-172.