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Wettability-Regulated Extracellular Electron Transfer from the Living Organism of *Shewanella loihica* PV-4**

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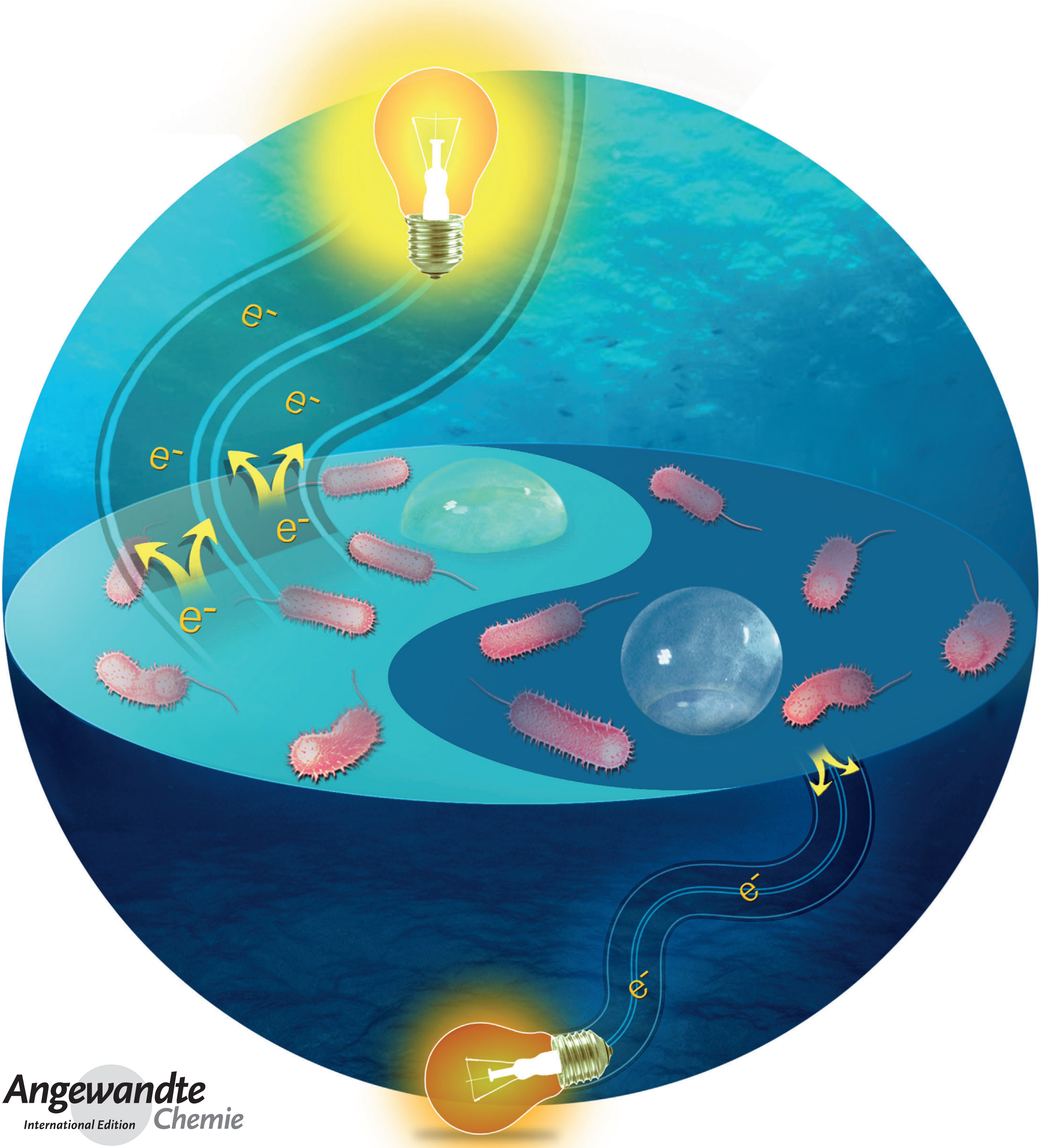

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Abstract: *C-type cytochromes located on the outer membrane (OMCs) of genus Shewanella act as the main redox-active species to mediate extracellular electron transfer (EET) from the inside of the outer membrane to the external environment: the central challenge that must be met for successful EET. The redox states of OMCs play a crucial role in dictating the rate and extent of EET. Here, we report that the surface wettability of the electrodes strongly influences the EET activity of living organisms of Shewanella loihica PV-4 at a fixed external potential: the EET activity on a hydrophilic electrode is more than five times higher than that on a hydrophobic one. We propose that the redox state of OMCs varies significantly at electrodes with different wettability, resulting in different EET activities.*

Extracellular electron transfer (EET) occurs at the interface between microbes and external solid state electron acceptors (such as metal oxides or electrodes). It is a significant natural phenomenon that is involved in many processes, including bioremediation and the biogeochemical cycling of minerals, and is applicable to microbial fuel cells (MFCs) and other electrochemical devices.[1] Not surprisingly, it has attracted considerable research attention in the last decade.[2,3] For the model organisms of the genus *Shewanella*, multiheme c-type cytochromes located on the outer membrane (OMCs) are the main redox-active species that mediate electron flow from the cell interior to the external insoluble electron acceptors across the insulated phospholipid bilayer cellular envelope.[4] Some of these OMCs can act either independently[5] or in concert with flavin cofactors[6] to shuttle electrons depending on its redox state.[7] Thus, controlling the redox state of OMCs appears to be a very important and effective strategy to regulate the EET activity, which was normally realized by altering the applied potential.[7,8] However, tuning the redox state of OMCs at a given potential through a non-electrochemical approach remains a challenge.

To this end, it is well known that the electrochemical properties of purified redox-active proteins can be varied by the surrounding local polar environment.[9,10] For example, the formal potential (E_f) of flavodoxins shifts depending on the general nonpolar environment and π–π stacking interactions provided by the aromatic amino acids.[9] The electron-transfer rate of purified c-type cytochromes showed more than 10^3-fold improvement on a COOH/OH-modified Au electrode compared to that on a COOH-modified one, presumably due to hydrophobic interactions.[10] Therefore, the local polarity, which is dependent on the surface wettability, is a crucial factor that can significantly alter the electrochemical properties of the redox-active proteins in vitro. Whether the same is true for similar proteins in vivo, in which the situation is far more complex (with membrane-bound proteins, and continuous electron flow), is not known.[11] Recently, we demonstrated that the variation in E_f of OMCs at different potentials was attributable to the different local polar environment surrounding bacterial OMCs.[12] Similarly, the redox potential of *Pseudomonas aeruginosa* Cytochrome c_{551} can be affected by the ionization state of the propionic acid side chain of heme 17.[13] Given these facts, it is reasonable to propose that the polarity of the local environment surrounding the OMCs in vivo could play a key role in regulating the rate and extent of EET, which remains largely unexplored.

Here we report that the surface wettability of the electrodes strongly impacts the EET activity of *Shewanella loihica* PV-4 (*S. loihica* PV-4) at a fixed external potential. The EET activity, namely, the ability of microbes to spit out electrons to the external electrode that can be directly visualized by measuring EET current, on a hydrophilic electrode is more than five times higher than that on a hydrophobic one. It was proposed that the redox state of OMCs varied significantly at electrodes with different wettability, which is assignable to different local polarities at the microbe/electrode interface. We believe that this finding will shed new light on the understanding of bacterial EET and also aid in the design and fabrication of electrodes with enhanced performance.

In our experiment, electrodes of tin-doped In_2O_3 (ITO) glass substrate with different wettability were fabricated by surface chemical modification of silanes, in which silanes of three-carbon length containing different terminal groups were used. In this way, the disadvantages caused by large molecules, namely the electron-transfer resistance and steric hindrance,[14] was largely avoided (Figure S1). Specifically, the ITO electrode coated with (3-mercaptopropyl)trimethoxysilane (SH-ITO) exhibited hydrophilicity with a water contact angle (CA) of 62.4° (Figure 1 a), whereas ITO coated with trichloro(propyl)silane (CH_3-ITO) was hydrophobic showing a water CA of 102.9° (Figure 1 b). X-ray photoelectron spectroscopy (XPS) confirmed the success of the surface modification (see the Supporting Information 2; SI 2).

The electrochemical behavior of the bacterium *S. loihica* PV-4 was characterized on these surface-modified ITO electrodes (see details in the Experimental Section and Figure S3). First, a linear sweep voltammetry (LSV) with a very slow scan rate of $10\ \mathrm{mV\,h^{-1}}$ was conducted to the cell suspension after 7 h preculture at −0.4 V, which can reflect the relationship between microbial respiration activity and the poised potential.[7] As shown in Figure 1 c, microbial

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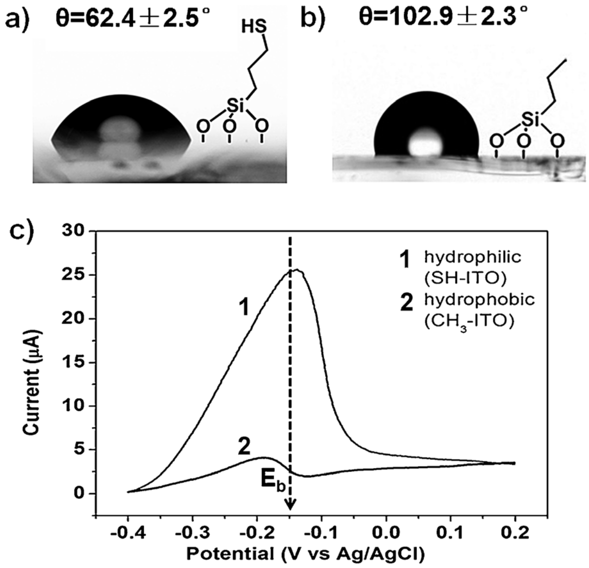

Figure 1. a,b) Contact angle measurements of a water droplet on SH-ITO and CH_3-ITO show the hydrophilicity and hydrophobicity, respectively. c) Linear sweep voltammetry curves obtained for SH-ITO (curve 1) and CH_3-ITO (curve 2) with a scan rate of 10 mV h^{-1}.

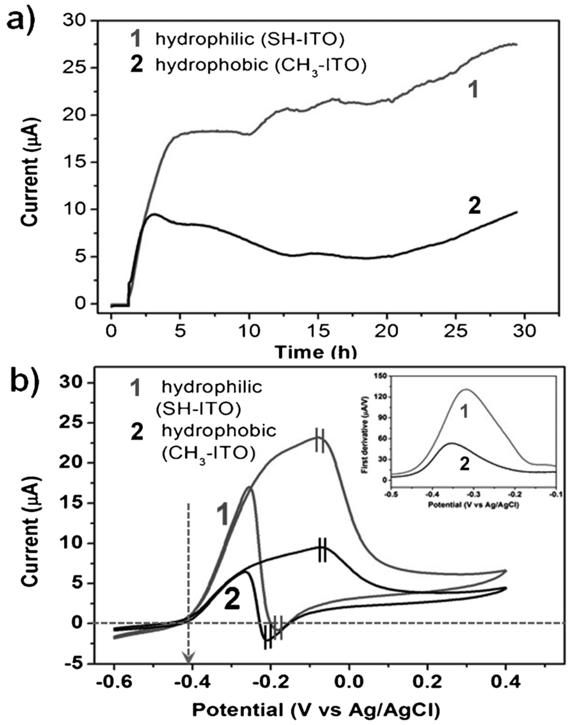

Figure 2. a) Current versus time curves at a constant potential of −0.2 V for *S. loihica* PV-4 on hydrophilic SH-ITO (curve 1) and hydrophobic CH_3-ITO (curve 2) electrodes, respectively. b) Cyclic voltammograms with a scan rate of 1 mV s^{-1} for microbes after 30 h electrochemical cultivation at −0.2 V on hydrophilic SH-ITO (curve 1) and hydrophobic CH_3-ITO (curve 2), the inset shows the first derivative of the according voltammetric curves. A pair of redox peaks with similar E_f of −0.13 V was observed on both hydrophilic SH-ITO and hydrophobic CH_3-ITO that is assignable to OMCs.[15]

respiration activity on two electrodes exhibited the similar potential-dependent behavior that we previously reported.[7] The current decreased rapidly in a narrow potential range (between −0.1 V and −0.2 V), in which the redox state of OMCs changed from the reduced to the oxidized one.[7,8a,b] Notably, this behavior was significantly enhanced on the hydrophilic SH-ITO: in the negative potential region (e.g., −0.2 V), the EET current on the hydrophilic SH-ITO was more than five times higher than that on hydrophobic CH_3-ITO. It clearly suggested that the surface wettability of the electrode significantly altered the bacterial EET activity.

To further examine the effect of surface wettability on EET activity, the EET currents at a constant potential of −0.2 V were measured (Figure 2 a). For hydrophilic SH-ITO, the current continued to increase with time and reached a maximum value of 27 μA after 30 h of operation (Figure 2 a, curve 1), more than three times higher than that generated on hydrophobic CH_3-ITO (Figure 2 a, curve 2). The results show good reproducibility: current values after 30 h cultivation on SH-ITO and CH_3-ITO electrodes were summarized in Figure S4.

Scanning electron microscopy (SEM) images of biofilms on hydrophilic SH-ITO and hydrophobic CH_3-ITO after 30 h of electrochemical culture show a similar coverage with *S. loihica* PV-4 cells on both electrodes (Figure S5), suggesting that the biofilm thickness is not the reason for the different EET current, in accordance with the result previously reported.[2d,15a] We proposed that it is rather attributed to the microdomain molecular interactions, that is, local polarity, since the surface wettability is directly related to the local polarity at the microbe/electrode interface.

For electrically active *Shewanella* strains, EET can proceed either directly to the external solid state electrode through oxidized OMCs or indirectly through self-secreted flavins that are stabilized by the reduced state of OMCs, and the efficiency for the latter case is much higher than that for the former one.[6a,7] Here, we propose that the different EET activities on electrodes with different wettability are attributable to the flavin-involved EET process. The whole-cell cyclic voltammetry (CV) with a fast scan rate of 50 mV s^{-1} taken after 30 h operation at −0.2 V, which can reflect the fast direct EET process, suggested a similar amount of the electrically active OMCs on both electrodes (Figure S6). However, CV with a scan rate of 1 mV s^{-1}, which reflects the flavin-associated slow diffusion process at the microbe/electrode interface, shows a visible difference. As shown in Figure 2 b, the anodic EET currents on both electrodes showed the same onset potential of −0.4 V, which is attributed to the nature of the terminal molecules of flavins.[16] It is notable that the slopes of CV curves near the onset potential were distinctly different, indicating the different EET activities due to flavins. The difference was more clearly visualized by the first derivative of the anodic CV curves (inset of Figure 2 b),[17] implying that the EET current through flavin is much more efficient on the hydrophilic electrode than that on the hydrophobic one.

To explore how the flavin-involved EET activity differs on hydrophilic and hydrophobic electrodes, differential pulse voltammetry (DPV) was conducted. As shown in Figure 3 a, the DPV curves contained two anodic peaks: the more

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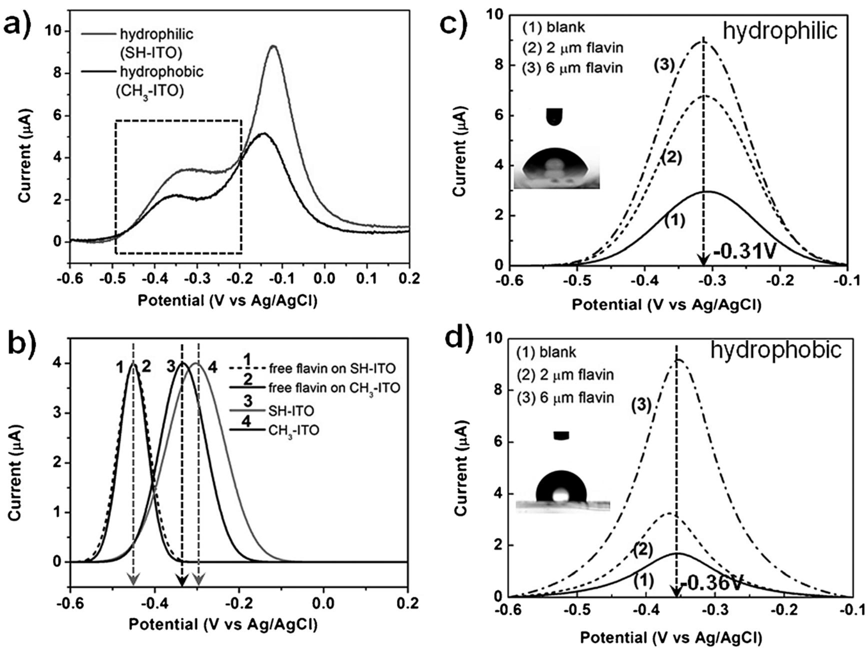

Figure 3. a) Differential pulse voltammetry for *S. loihica* PV-4 on hydrophilic SH-ITO and hydrophobic CH_3-ITO cultivated at −0.2 V versus Ag|AgCl (sat. KCl). b) Baseline-subtracted DPV for free riboflavin in bare DML media on hydrophilic SH-ITO (line 1) and hydrophobic CH_3-ITO (line 2), and for bacterial suspension cultivated at −0.2 V on hydrophilic SH-ITO (line 3) and hydrophobic CH_3-ITO (line 4) respectively. Baseline-subtracted DPV for flavin-OMC complex of *S. loihica* PV-4 on the c) hydrophilic SH-ITO and d) hydrophobic CH_3-ITO electrode in the presence of exogenously added riboflavins (a concentration of 0, 2.0, and 6.0 μM was used).

positive one was assigned to the OmcA-MtrCAB complex,[18] and the more negative one was assigned to self-secreted flavins, because the peak value increased upon addition of exogenous riboflavin (Figure 3c,d). To clarify the electron-transfer kinetics between microbes and electrodes, baseline-subtracted DPVs for the flavin-related peak were shown in Figure 3b. For a DML (defined medium with lactate as the carbon source, see SI) with only riboflavin (5 μM) in the absence of microbes, the curve was identical on both SH-ITO and CH_3-ITO electrodes with the redox potential (E_p) located at −450 mV and a half-width potential ($\Delta E_{p/2}$) of 60 mV (lines 1 and 2 in Figure 3b), suggesting a typical two-electron redox reaction of free flavins (oxidized form $+2H^+ + 2e^- \rightarrow$ hydroquinone).[6a] However, E_p performed a visible positive shift in the presence of microbes, and it is worth noting that E_p on the hydrophilic SH-ITO gave a value of ca. −303 mV, which is positively shifted by 50 mV compared with that on hydrophobic CH_3-ITO (ca. −350 mV). Moreover, the $\Delta E_{p/2}$ was enlarged in the presence of microbes, exhibiting a value of 136 mV on hydrophilic SH-ITO (Figure 3b, line 3) and 103 mV on hydrophobic CH_3-ITO (Figure 3b, line 4), respectively. The $\Delta E_{p/2}$ of 136 mV on the hydrophilic electrode indicates that the number of electrons involved in the flavin path was one, i.e., via an intermediate product of semiquinone (oxidized form $+H^+ + e^- \rightarrow$semiquinone).[6] Here, the $\Delta E_{p/2}$ of 103 mV on the hydrophobic CH_3-ITO is likely involved in a mixture of two- and one-electron transfer. As reported, the positive shift of E_p, along with the increase in $\Delta E_{p/2}$, is attributable to the formation of flavin-OMCs cofactor, by which the semiquinone can be stabilized and actually mediate EET.[6] The semiquinone can facilitate electron transfer through a one-electron process, resulting in a 10^3–10^5-fold faster process than that of the two-electron transfer via free flavin, thereby serving as a more efficient process.[6a] Therefore, the different EET processes on the hydrophilic and hydrophobic electrode were clearly suggested, which can be further confirmed by the fact that the 50 mV positive shift of E_p on hydrophilic SH-ITO was always observed, even with the exogenously added riboflavin (Figure 3c and 3d, Figure S8). Taken together, the hydrophilic electrode enables drastically facilitated EET through a one-electron-semiquinone path in comparison to that on the hydrophobic electrode.

Here we examined the electron-transfer kinetics and compared the rate constant (k) and generated current density (I) between microbes and electrodes with different wettability (SI 7). Assuming that a one-electron transfer process occurs in both cases, the ratio of k on hydrophilic SH-ITO to that on hydrophobic CH_3-ITO is about 2.5 (when $\alpha = 0.5$, and α is electron-transfer coefficient).[6,20] This result suggests that the electron-transfer rate through flavin-OMCs cofactor from microbes to hydrophilic SH-ITO was more than two-fold higher than that to hydrophobic CH_3-ITO, which is in good agreement with the results in Figure 2b. Furthermore, the ratio of I on hydrophilic SH-ITO to that on hydrophobic CH_3-ITO was calculated to be larger than 3, in accordance with the result in Figure 2a.

It has been reported that reduced OMCs is favorable for stabilizing semiquinones to form flavin-OMC cofactors, which therefore facilitate the bacterial EET by an one-electron path.[6,21] Here, the DPV results suggested that the flavin-OMC cofactor on hydrophilic SH-ITO and hydrophobic CH_3-ITO electrodes exhibited different electrochemical properties. Therefore, it can be concluded that the redox state(s) of OMCs was significantly varied on electrodes with different wettability.

We propose that the affinity between bacteria and hydrophilic SH-ITO is higher than that with hydrophobic CH_3-ITO, and as a result the strengthened electric interaction between microbe and hydrophilic SH-ITO was observed. It can be directly confirmed by measuring the charge-transfer resistance at the microbe/electrode interface,[22] which gives a value of 49.5 kΩ between microbe and hydrophilic SH-ITO, which is only half of that obtained for hydrophobic CH_3-ITO, based on the electrochemical impedance spectroscopy (EIS) Nyquist plot (Figure S9). Moreover, the surface of *S. loihica* PV-4 is hydrophilic,[18,23] thus one expects the cells to adhere tightly onto hydrophilic electrodes. Theoretical calculations

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also predicted that the bacterial binding ability is stronger on a hydrophilic electrode than on a hydrophobic one.[24] Here, with a poised potential of −0.2 V, the high bacterial affinity to the hydrophilic electrode facilitated electrical interactions, and consequently OMCs were mostly in the reduced state, as evidenced by the positive shift of E_p of the OmcA-MtrCAB complex on the hydrophilic SH-ITO compared with that on hydrophobic CH_3-ITO (Figure S10).

Taken together, we propose the mechanism that is schematically summarized in Figure 4. The physical affinity between microbes and hydrophilic SH-ITO enables a facilitated electrochemical interaction at the microbial/electrode interface. As a result, OMCs are mostly in the reduced state at −0.2 V. Self-secreted flavins are liable to bind to reduced OMCs arising from hydrophobic interactions between hydrophobic ferrous ion (reduced OMCs) and the hydrophobic flavins. Therefore, the as-formed flavin-OMC cofactor preferentially utilizes the semiquinone for the one-electron transfer process.[6] Thus, the electric interaction between microbes and the hydrophilic electrode is dramatically enhanced, and the high EET activity is observed at −0.2 V. In contrast, the affinity of the microbes to the hydrophobic electrode is much weaker, lowering the electrochemical interaction and resulting in only a part of the OMCs being in the reduced state even at −0.2 V. It should be noted here that the oxidized state of the OMCs is unfavorable for stabilizing semiquinone, and in this case only the two-electron transfer via free flavin is allowed.[6] As a result, the EET proceeds through a mixture of one- and two-electron transfer, leading to a lower EET activity.

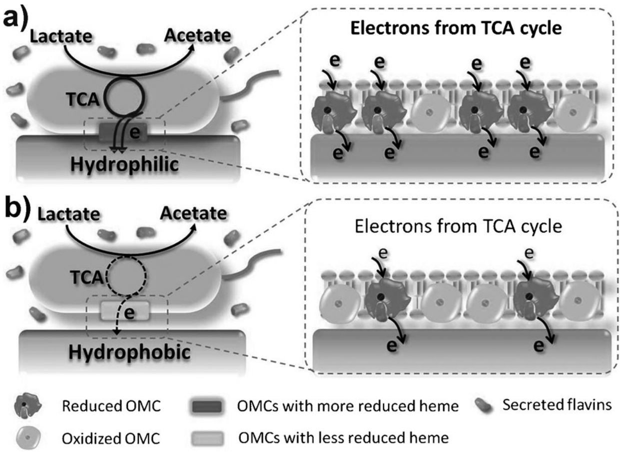

Figure 4. Schematic illustration for the hypothetic mechanism of electron transfer on a) hydrophilic and b) hydrophobic substrates at −0.2 V.

To further confirm that the redox state(s) of OMCs is different on electrodes with different wettability at a certain potential, the bacterial respiratory activity on both electrodes were characterized, since these two factors are closely related to each other.[6b,25] It is known that the respiratory activity of the genus *Shewanella* is under control of the tricarboxylic acid cycle (TCA cycle).[25] As shown in Figure S11 a, addition of the TCA-cycle inhibitor malonic acid[6b,25,26] into the system, resulted in a dramatic drop of the catalytic EET current on the hydrophilic SH-ITO, whereas only a slight decrease was observed for the hydrophobic electrode. Also, E_p of flavin-OMCs cofactor on hydrophilic SH-ITO shifted negatively after addition of the inhibitor but maintained the same value on hydrophobic CH_3-ITO (Figure S11 b), again suggesting that the redox state(s) of OMCs is varied by altering the surface wettability.

In summary, we have for the first time demonstrated that surface wettability can impact the rate and extent of EET of a living organism: the EET activity is five times greater on the hydrophilic electrode than that on a hydrophobic one. We have proposed that the surface wettability can directly alter the local polarity at the microbe/electrode interface, and thus affect the electrical interaction(s) with microbes. As a result, the redox state of OMCs varied, therefore the EET behavior changed drastically. Specifically, the hydrophilic electrode allows OMCs to be mostly in the reduced state at the fixed potential of −0.2 V, which facilitates one-electron transfer via flavin-OMCs. In this way, the EET activity was significantly increased on the hydrophilic electrode. We envision that this finding is not only meaningful for the understanding of the microbe/electrode interface, but can also provide new insight for the design and fabrication of other bioelectrical devices.

Experimental Section

Substrate modification: Smooth tin-doped In_2O_3 (ITO) electrodes were precleaned with detergent, acetone, ethanol, and deionized water. The plates were then immersed in a solution of $H_2O_2/NH_4OH/H_2O$ (1:1:5, *v*/*v*) for 30 min at 80 °C to obtain uniformly distributed OH groups on ITO surface, after which they were rinsed thoroughly with deionized water and dried. The silanization was followed by dipping ITO into a solution of (3-mercaptopropyl)trimethoxysilane or trichloro(propyl)silane (5 mM) dissolved in hexane for 50 min at room temperature. After reaction, the electrodes were rinsed with chloroform and water to remove physically absorbed silanes from the surface.

Microbe preparation: *Shewanella loihica* PV-4 were cultured aerobically in marine broth (MB, 20 $\mathrm{g\,L^{-1}}$) at 30 °C for 24 h. Then the cells were collected and washed three times with defined media (DML: contains $NaHCO_3$ (2.5 g), $CaCl_2{\cdot}2H_2O$ (0.08 g), NH_4Cl (1.0 g), $MgCl_2{\cdot}6H_2O$ (0.2 g), NaCl (10 g), and HEPES (7.2 g) per liter with sodium lactate (10 $\mathrm{mmol\,L^{-1}}$) as carbon source, pH 7.8) and finally placed in fresh DML medium for further aerobic cultivation at 30 °C for 24 h using lactate as a carbon source. The concentration of the cell suspension in the electrochemical cell was set to be an optical density of 2.0 at 600 nm.

Electrochemical characterization: A single chamber, three-electrode system was used to monitor the electrochemical behavior of the microbes. An Ag | AgCl (sat. KCl) and a platinum wire were used as the reference and counter electrode, respectively. The modified ITO plates were used as the working electrodes with the electrically active area of 3.14 cm^2. DML (4 mL) was used as electrolyte and was deaerated by N_2 bubbling for 30 min before the measurements. The temperature was maintained at 30 °C. The electrochemical measurement was performed using the electrochemical workstation CHI 1030B (CH Instruments, USA).

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