Microbial Fuel Cells

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C-Type Cytochromes Wire Electricity-Producing Bacteria to Electrodes**

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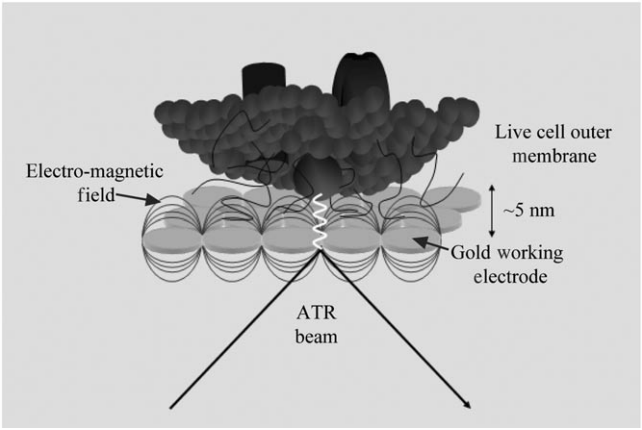
A new form of green energy based on the efficient conversion of organic matter into electricity is now feasible using microbial fuel cells (MFCs).[1] In these devices, microorganisms support their growth by oxidizing organic compounds and an electrode serves as the sole electron acceptor, so electricity can be harvested.[2–4] Most of these electricity-producing microorganisms are Fe^{III}-reducing bacteria.[5,6] Among them, the genus *Geobacter* is being extensively studied because of its outstanding electrogenic capacity.[7–11] Nevertheless, present understanding of the electron-transport mechanism and the specific interactions between bacteria and the electron-accepting electrode are far from complete. By using a combined approach that includes direct current (DC) electrochemistry, surface enhanced infrared absorption spectroscopy (SEIRAS), and subtractively normalized interfacial Fourier transform infrared spectroscopy (SNIFTIRS), we have analyzed in vivo the intimate contact interface between the electricity-producing bacteria *Geobacter sulfurreducens* and a gold electrode at the nanometer scale. We show herein that upon potential cycling, reversible changes in the spectra produce a band pattern that clearly resembles the electrochemical turnover of oxidized/reduced states in c-type cytochromes. The unique presence of these signals associated to the cell/electrode interface demonstrates that outermost membrane cytochromes in *Geobacter sulfurreducens* are responsible for the direct electron transfer to electrodes during electricity production.

Elucidation of the mechanism by which bacteria transport electrons to solid electrodes can accelerate the evolution of technological applications as microbial fuel cells, whole-cell biosensors, and bioreactors to a new generation of precisely nanostructured devices with an optimized cell–material communication.

It has been documented that the electrochemical activity of *Geobacter sulfurreducens* attached to a graphite electrode is manifested typically as a single redox pair related to the applied potential,[11] demonstrating the selective production of external redox molecules for optimizing the electrical connection and current production. To gain insight into the identity of molecule(s) involved in these reactions, we have implemented a combined spectro-electrochemical approach to study for the first time the interface between *G. sulfurreducens* cells and a polarized thin-film gold electrode by attenuated total reflection–SEIRAS (ATR-SEIRAS).[12] In this method, an IR beam totally reflects on the back side of a thin-film electrode producing an evanescent wave that protrudes into the solution side (white wavy line in Scheme 1). A near-field enhancement of the evanescent wave through the interaction with the electromagnetic field of the polarized film provides a unique opportunity to discriminate from the background the vibrations coming from molecules within the decaying field length (typically 5 nm; Scheme 1).[13] It is recognized that in spite of the complexity of the bacterial surface this technique allows the selective resolution of bacterial molecules (or molecular domains) in direct contact with the electrode with high specificity (Scheme 1).[12] As a matter of comparison, the evanescent field is notably thinner than the protruding redox domains (18–25 nm) previously detected on the surface of



Scheme 1. Representation of the cell/electrode interface. The position of relevant elements is shown during the in vivo detection of electrode-reducing molecules by ATR-SEIRAS, see text for details.

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another electrogenic bacteria such as *Shewanella oneidensis*,[14] and suggested to be heme-containing proteins.

The interaction of *G. sulfurreducens* cells with a gold electrode polarized at 0.2 V (vs. Ag/AgCl–NaCl 3 M) produces an electric current that increases with time (Figure 1 a),

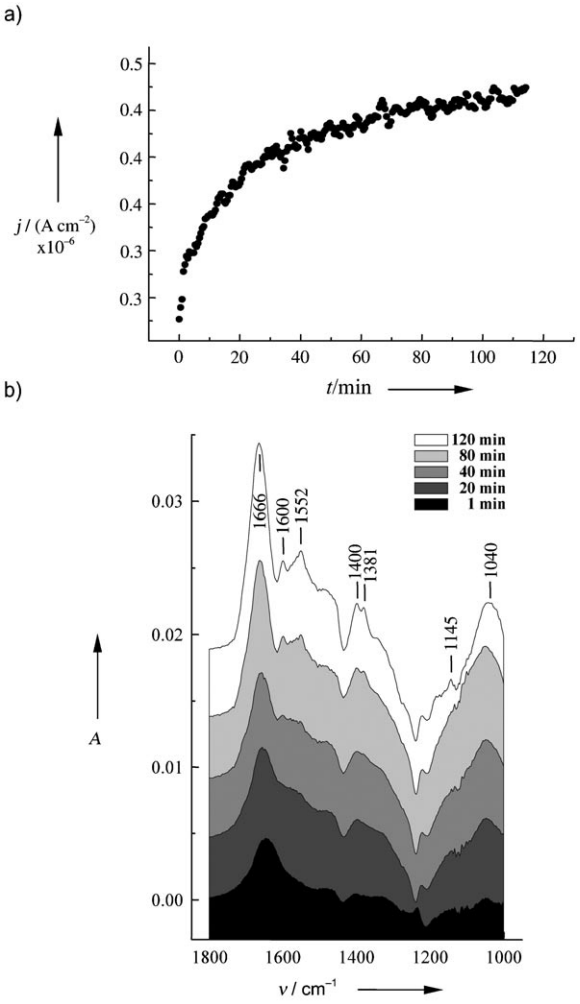

Figure 1. *Geobacter sulfurreducens* adaptation to produce current. a) Electricity production and b) ATR-SEIRAS spectra during the adsorption process of cells on a thin-film gold electrode. The electrode was initially polarized to 0.2 V in the acetate-containing electrolyte in a N_2:CO_2 (80:20) atmosphere. After acquiring the spectrum corresponding to the electrode/electrolyte interface a bacterial suspension (10^9 cell mL^{-1}) was added to the cell and current produced by bacteria were continuously measured. Single-beam spectra were recorded 5 min after the addition of cells.

demonstrating that a gold electrode can act as electron acceptor for these bacteria. In addition, the intensity of bacterial IR signals also increases (Figure 1 b), thus showing the progressive interaction between attached cells and the polarized surface. In agreement with reports on the IR fingerprint of other bacteria,[12,15] the spectra of *G. sulfurreducens* becomes rapidly dominated by a broad amide I band at 1666 cm^{-1}. Together with the lower amide II and amide III bands at 1552 and 1400–1380 cm^{-1}, respectively (Figure 1 b), all these bands indicate the increasing presence of proteins at the interface.[16] The prominent band at 1040 cm^{-1} (Figure 1 b) corresponds to lipopolysaccharides contacting the gold surface as described elsewhere.[12,15]

G. sulfurreducens is the organism known to contain the highest number of genes coding for c-type cytochromes (111 in the whole genome). Genome-sequence analysis has also revealed that most of these c-type cytochromes possess more than one heme group, in sharp contrast to the c-type cytochromes found in other microorganisms and eukaryotes, which typically contain only one heme.[17] Thus, a heme network is thought to exist in this bacterium to either shuttle the electrons to the exocellular electron acceptors or serve as an electron-storage structure in the absence of them.[18] Those multi-heme c-type cytochromes allocated in the outer membrane are expected to contribute to the protein signals in the ATR-SEIRAS spectra (Figure 1 b). In that sense, a band detected at 1600 cm^{-1}, not previously described for other bacteria, could be assigned to the ν_{37} stretching vibration of the heme moiety, according to previous in vitro studies on pure preparations of c-type cytochromes.[19] Importantly, the amide III band splits into two minor bands at 1400 and 1381 cm^{-1}, that jointly with the one at 1145 cm^{-1}, could also be related to reported vibrations of the heme ring.[20]

Heterogeneous electron transport requires active molecules close enough to the electrode to facilitate the tunneling of electrons, thus ensuring the occurrence of the reaction within the distance range of the SEIRA evanescent field. Taking advantage of this fact, we are able to detect conformational changes in the "electrode-reducing" molecules.

When analyzed through a differential absorption approach, IR or Raman spectroscopic data can provide valuable information about the functionally relevant vibrational changes of molecules during a conformational change.[16] This data is particularly useful in the case of cytochromes, whose conformational changes upon redox transition have been studied in detail.[19,21] With the goal to visualize the IR profile changes of the cell molecules contacting (or very near to) the electrode upon the transference of electrons, we collected the SEIRA spectra of attached *G. sulfurreducens* cells while performing in vivo cyclic voltammetry.

The voltammetric response of the interface shows a clearly defined oxidation peak located at about 0.24 V and a reduction wave at about 0.11 V from which an estimated half-cell potential[22] of 0.16 V was determined (Figure S1a in the Supporting Information). Since it is slightly negative to the applied polarization potential, it is thermodynamically favorable for the redox pair to mediate the electron transfer to the polarized electrode at the imposed potential (0.2 V) yielding an acceptable energy yield for bacterial metabolism and current production.[23] Differential IR spectra at increasing 0.05 V potential steps from a starting reference potential at −0.1 V, show a number of positive and negative bands increasing in relation to the oxidation process (Figure 2). Importantly, the intensity of the main signal at about 1680 cm^{-1} changed in close agreement with the evolution of transferred charge calculated from voltammetric results (Figure S2 in the Supporting Information). The midpoint

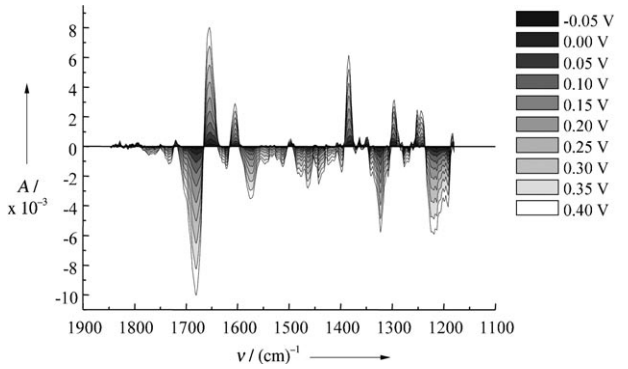

Figure 2. ATR-SEIRAS spectra of *G. sulfurreducens* acquired during the oxidation process in the voltammetric analysis of cells on a thin-film gold electrode.

potential derived from intensity data (Figure S2 in the Supporting Information) can be calculated to be 0.17 V, in excellent agreement with the value estimated from voltammetry.

To precisely define the steady-state profile and band positions in the fully oxidized state of bacterial cells, we performed a more detailed analysis by SNIFTIRS.[24] In this technique, interferograms are collected at both a reference and a sample potential selected to flank the electrochemical process. The potential step is repeated until a desired signal-to-noise ratio is reached so the relative change in absorbance can be calculated with high precision. SNIFTIRS spectra of attached *G. sulfurreducens* cells were acquired using −0.1 and 0.4 V as the reference and sample potentials, respectively (the results are shown in Figure S1b in the Supporting Information). Spectral changes were concentrated in the fingerprint region between 1100 and 1800 cm^{-1} (inset Figure S1b in the Supporting Information). The most prominent features were those in the amide I region, which notably agrees with those usually observed upon the redox transition of cytochrome C (Cyt-C) from different organisms.[13,21,25] A negative band at 1684 cm^{-1} (Figure S1b in the Supporting Information), previously assigned to the amide I mode of a β-turn segment in the reduced form of Cyt-C,[19] rapidly intensifies upon cell oxidation (Figure S1b in the Supporting Information). It was accompanied by the development of a positive band at 1662 cm^{-1} (Figure S1b in the Supporting Information), ascribed to an α-helical segment of Cyt-C.[19] Furthermore, these bands were found to be almost fully reversible upon voltammetric cycling (Figure S3 in the Supporting Information), strongly indicating the occurrence of the cytochrome's redox transition at the interface.

In addition to the amide I related bands, a number of differential bands were detected upon full oxidation of the cell molecules (Figure S1b in the Supporting Information). The assignment of some of them remains unknown, but major ones at 1602, 1573, 1385, and 1322 cm^{-1} and minor ones at 1554, 1479, 1406, 1398, 1243, and 1148 cm^{-1} have been reported in relation to Cyt-C[13,19] or Microperoxidase 8, the octapeptide containing the heme group in Cyt-C.[20] Most of these cytochrome-related bands were also observed to reverse during the redox transition (Figure S3 in the Supporting Information). Some assigned to different vibrational modes of the heme ring (1602, 1573, 1385, 1479, 1406, 1243, and 1148 cm^{-1}),[20] probably reflect the high number of heme moieties borne by cytochromes interacting with the gold surface. The assignation of bands in the IR spectra of *G. sulfurreducens* and Cyt-C are shown in Table S1 in the Supporting Information.

Herein, we have shown that upon recognition of the polarized electrode as an electron acceptor, *G. sulfurreducens* establish the electrical wiring to the conducting surface by mobilizing and/or producing outermost membrane cytochromes that directly interact with the electrode. This discovery is an example of using spectro-electrochemical measurements to help isolate the molecular mechanism operating in vivo under relevant technical conditions. In addition, besides improving our fundamental understanding of the cell/electrode interface, the recognition of specific interactions could lead to advances in nanotechnology. For example, elegant ways to manipulate materials at the nanometer scale may now be exploited to tailor the delicate biological structure in an improved connection, thus increasing the possibility of having better microbial fuel cells, biosensors, or bioreactors.

Experimental Section

ATR-SEIRAS: Spectro-electrochemical experiments were carried out in a glass cell at room temperature (ca. 20 °C) as described elsewhere.[12] Spectra were collected with *p*-polarized light with a resolution of 4 cm^{-1} (unless otherwise indicated) and are presented as the ratio −log(R2/R1), where R2 and R1 are the reflectance values of single beam spectra at the sample and reference condition, respectively. Interferograms were acquired every 1 s to calculate each one of these single beam spectra.

Culture of microorganisms and spectroscopic analysis: *G. sulfurreducens* were anaerobically cultured in chemostats as described elsewhere.[26] Acetate was used as the carbon source and electron donor under conditions in which the electron acceptor fumarate was the growth-limiting factor. Steady-state cells were harvested by centrifugation at 6000 rpm during 10 min, washed and concentrated by a factor of 10 in an anoxic solution containing KCl (30 mM), $NaHCO_3$ (30 mM), and acetate (5 mM). The bacterial suspension was anaerobically transferred to the ATR cell to perform the spectro-electrochemical analysis. To ensure that cells were using the gold electrode as the electron acceptor, no other acceptor was added to the solution.

Cyclic voltammetry: voltammetry was performed using a typical three electrodes configuration using an EA-161 potentiostat controlled by a universal programmer connected to a PC through an e-corder 401 unit (E-DAQ Pty Ltd.). The counter electrode was a coiled gold wire and the reference was an Ag/AgCl–3 M NaCl electrode. The potential was scanned between −0.1 and 0.4 V starting positively from −0.1 V. The scan rate was 0.005 $V s^{-1}$. All the experiments were performed using a sodium bicarbonate solution (30 mM) supplemented with KCl (30 mM) and acetate (5 mM) as the electrolyte. It was equilibrated at pH 7 under a N_2:CO_2 (80:20) atmosphere (L'Air liquide).

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[1] B. E. Logan, B. Hamelers, R. Rozendal, U. Schröder, J. Keller, S. Freguia, P. Aelterman, W. Verstraete, K. Rabaey, *Environ. Sci. Technol.* **2006**, *40*, 5181.
[2] K. Rabaey, P. Clauwaert, P. Aelterman, W. Verstraete, *Environ. Sci. Technol.* **2005**, *39*, 8077.
[3] B. R. Ringeisen, E. Henderson, P. K. Wu, J. Pietron, R. Ray, B. Little, J. C. Biffinger, J. M. Jones-Meehan, *Environ. Sci. Technol.* **2006**, *40*, 2629.
[4] P. Aelterman, K. Rabaey, H. T. Pham, N. Boon, W. Verstraete, *Environ. Sci. Technol.* **2006**, *40*, 3388.
[5] D. R. Lovley, *Nat. Rev. Microbiol.* **2006**, *4*, 497.
[6] K. A. Weber, L. A. Achenbach, J. D. Coates, *Nat. Rev. Microbiol.* **2006**, *4*, 752.
[7] D. R. Bond, D. R. Lovley, *Appl. Environ. Microbiol.* **2003**, *69*, 1548.
[8] K. B. Gregory, D. R. Bond, D. R. Lovley, *Environ. Microbiol.* **2004**, *6*, 596.
[9] S. Srikanth, E. Marsili, M. C. Flickinger, D. R. Bond, *Biotechnol. Bioeng.* **2007**, *99*, 1065.
[10] D. E. Holmes, S. K. Chaudhuri, K. P. Nevin, T. Mehta, B. A. Methe, A. Liu, J. E. Ward, T. L. Woodard, J. Webster, D. R. Lovley, *Environ. Microbiol.* **2006**, *8*, 1805.
[11] J. P. Busalmen, A. Esteve-Nuñez, J. M. Feliu, *Environ. Sci. Technol.* **2008**, *42*, 2445.
[12] J. P. Busalmen, A. Berna, J. M. Feliu, *Langmuir* **2007**, *23*, 6459.
[13] K. Ataka, J. Heberle, *J. Am. Chem. Soc.* **2003**, *125*, 4986.
[14] V. Biju, D. Pan, Y. A. Gorby, J. Fredrickson, J. McLean, D. Saffarini, H. P. Lu, *Langmuir* **2007**, *23*, 1333.
[15] J. Wei, A. Saxena, B. Song, B. B. Ward, T. J. Beveridge, S. C. B. Myneni, *Langmuir* **2004**, *20*, 11433.
[16] A. Barth, *Biochim. Biophys. Acta Bioenerg.* **2007**, *1767*, 1073.
[17] B. A. Methe, K. E. Nelson, J. A. Eisen, I. T. Paulsen, W. Nelson, J. F. Heidelberg, D. Wu, M. Wu, N. Ward, M. J. Beanan, R. J. Dodson, R. Madupu, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, M. Gwinn, J. F. Kolonay, S. A. Sullivan, D. H. Haft, J. Selengut, T. M. Davidsen, N. Zafar, O. White, B. Tran, C. Romero, H. A. Forberger, J. Weidman, H. Khouri, T. V. Feldblyum, T. R. Utterback, S. E. Van Aken, D. R. Lovley, C. M. Fraser, *Science* **2003**, *302*, 1967.
[18] A. Esteve-Núñez, J. Sosnik, P. Visconti, D. R. Lovley, *Environ. Microbiol.* **2008**, *10*, 497.
[19] K. Ataka, J. Heberle, *J. Am. Chem. Soc.* **2004**, *126*, 9445.
[20] L. Marboutin, A. Boussac, C. Berthomieu, *J. Biol. Inorg. Chem.* **2006**, *11*, 811.
[21] D. Moss, E. Nabedryk, J. Breton, W. Mantele, *Eur. J. Biochem.* **1990**, *187*, 565.
[22] A. J. Bard, L. R. Faulkner, *Electrochemical methods: Fundamental and applications*, 2nd ed., Wiley, Toronto, **1980**.
[23] U. Schröder, *Phys. Chem. Chem. Phys.* **2007**, *9*, 2619.
[24] S. Pons, T. Davidson, A. Bewick, *J. Electroanal. Chem.* **1984**, *160*, 63.
[25] J. F. Calvert, J. L. Hill, A. Dong, *Arch. Biochem. Biophys.* **1997**, *346*, 287.
[26] A. Esteve-Nunez, M. Rothermich, M. Sharma, D. Lovley, *Environ. Microbiol.* **2005**, *7*, 641.

