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Electrochemical insight into the mechanism of electron transport in biofilms of *Geobacter sulfurreducens*

Germán D. Schrott[a], P. Sebastian Bonanni[a], Luciana Robuschi[a], Abraham Esteve-Nuñez[b,c], Juan Pablo Busalmen[a,*]

[a] *Laboratorio de Bioelectroquímica, División Corrosión, INTEMA (CONICET), Juan B. Justo 4302, B7608FDQ Mar del Plata, Argentina*
[b] *Departamento de Química Analítica e Ingeniería Química, Universidad de Alcalá (28871) Alcalá de Henares, Madrid, Spain*
[c] *IMDEA Agua, Parque Tecnológico de Alcalá, Alcalá de Henares, Madrid, Spain*

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ABSTRACT

Electroactive bacterial biofilms can be produced on a polarized electrode by forcing its use as the final electron acceptor for bacterial respiration. This strategy offers the researcher the unique possibility to control the respiration process with extreme precision. The production of current, the accumulation of charge and the conducting properties of electroactive biofilms has been interrogated in this work through very basic electrochemical techniques including chronopotentiometry, chronoamperometry and cyclic voltammetry. Presented results indicate that charge can be accumulated in the biofilm conductive network, that network conductivity does not represent a limit for current production and that both the steady state current and the amount of accumulated charge depend on the redox state of cytochromes wiring the cells to the electrode. A model of biofilm conduction is presented as well.

1. Introduction

Since the discovery of electroactive microorganisms researchers have been able to study exocellular electron transfer with living bacteria without dealing with the isolating barrier imposed by biological membranes [1]. This is possible thanks to the evolution of extracytoplasmic electron transport in these organisms [2]. Although it has been found to be unspecific in relation to the external electron acceptor [2], this amazing line of evolution has been driven by the advantage of respiring insoluble oxidized compounds as iron and manganese oxides, which are ubiquitous in most sedimentary environments.

Replacing the natural electron acceptors by a polarized electrode has opened the way to an efficient bacteria/electrode communication and enabled the application of most common electrochemical techniques for the study of electroactive microorganisms, including biofilm formation, growth kinetics, degradation capabilities and most important, their electron transport properties and mechanisms [3–9].

Following the proposal previously made for planktonic cells [10] and focusing on the work with *Geobacter sulfurreducens* biofilms, the central issue of this work was the analysis of their charge storage capacity and electron transport properties. Presented results come to confirm some previous indications presented by others [11,12] about the possibility for these biofilms to accumulate charge, allow to confirm that network conductivity do not represent a limit for current production, highlight the role of interfacial cythocromes as regulatory elements and demonstrate that *Geobacter* biofilms are able to charge the cytochrome network for a period of time that is 3-fold higher than that shown for planktonic cells.

2. Experimental

2.1. Cell setup

All the experiments were performed in a three electrodes electrochemical cell using two 0.4 cm diameter graphite bars (XTG-15, Carbograf, Argentina) as the working electrode, a platinum wire as a counter electrode and a Ag/AgCl–3 M NaCl as a reference electrode (+0.209 V vs. the standard hydrogen electrode (SHE)). The surface of the working electrode was renewed before each experiment by polishing to grade 1000 with carbon paper, sonicating by 3 pulses of 5 s to remove debris and washing with deionized water. The exposed area of the working electrode was 8 cm^2. All potentials are here reported as relative to the SHE.

* Corresponding author at: Laboratorio de Bioelectroquímica, INTEMA (CONICET), Division Corrosion, Universidad Nacional de Mar del Plata (UNMdP), Juan B. Justo 4302, B7608FDQ Mar del Plata, Argentina. Tel.: +54 223 4816600x248; fax: +54 223 4810046.
E-mail address: jbusalme@fi.mdp.edu.ar (J.P. Busalmen).

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2.2. Electrochemical assays

All the experiments were performed using a Voltalab PGZ402 potentiostat controlled by the Voltamaster 4 dedicated software. For cyclic voltammetry the potential was scanned between 0.8 and −0.4 V starting anodically from 0.4 V. The scan rate was 0.01 V s^{-1}. Chronopotentiometry was performed at the open circuit potential acquiring 10 points per second. Chronoamperometry was performed at the selected applied potential and acquiring 10 points per second. Presented results are representative of those obtained from five independent biofilm grown under the same experimental conditions. Every measurement was repeated at least 10 times.

2.3. Culture of microorganisms

G. sulfurreducens was anaerobically cultured at 28 °C on a culture medium slightly modified from that described elsewhere [13] and containing 30 mM KCl, 50 mM $NaHCO_3$, 20 mM CH_3COONa, 9.3 mM NH_4Cl, 2.5 mM NaH_2PO_4 anhydrous, plus vitamins and trace minerals dissolved in distilled water. Acetate 20 mM was used as the carbon source and the electron donor, while fumarate 40 mM was the electron acceptor [13].

For biofilm production 10 mL of an early stationary phase batch culture were inoculated into a biofilm reactor containing about 90 mL of deoxygenated culture medium lacking the electron acceptor. After 24 h in batch a peristaltic pump was connected to continuously supply medium at a dilution rate of 0.02 h^{-1}. The reactor and all the liquid reservoirs in the continuous culture system were permanently flashed with a gas mix of N_2:CO_2 (80:20) to adjust the pH of the medium at 7.4 and to prevent the contamination with oxygen. All the experiments were performed under permanent magnetic stirring.

3. Results and discussion

3.1. Biofilm growth

After inoculation, the growth of electrogenic biofilm was evidenced by an exponential increase in the current output that reached a nearly stable value of about 1.2 A m^{-2} at day 7. As current in these systems is known to be the product of bacterial oxidative metabolism, the obtained value was taken as a reference for the steady state metabolic activity of biofilms under the imposed conditions.

3.2. Charge storage in biofilms

One of the advantages of using a polarized electrode as the electron acceptor is that bacterial respiration can be readily controlled through the external polarization. As potential can be varied in a continuous scale, the strategy offers the possibility to study bacterial respiration in detail. In this context, interrupting polarization, i.e. the electrochemical equivalent of instantaneously deleting the electron accepting compounds, is expected to induce a biological response corresponding to the lack of electronic acceptor. At the same time, the electrode potential is expected to change following the oxidized/reduced ratio of electro-active components at the electrode/electrolyte interface [14]. As shown in Fig. 1, upon the interruption of polarization to a biofilmed electrode, its open circuit potential (OCP) decreased rapidly to about −0.14 V (SHE). This change is interpreted as the consequence of the accumulation of reduced electro-active species at the electrode surface, which according to previous results [6] points to external cytochromes in the layer of cells that is contacting the electrode as the potential determining species.

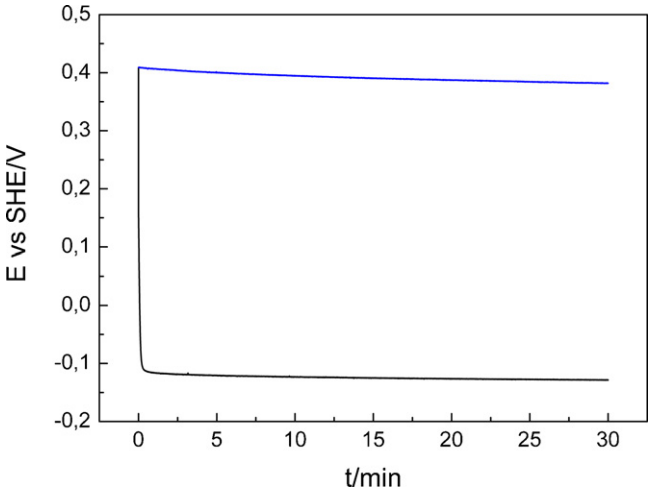

Fig. 1. Evolution of the open circuit potential after the interruption of the polarization applied to a graphite electrode covered by a biofilm of *Geobacter sulfurreducens* (black line). The grey line corresponds to the response of a bare electrode and was obtained after 24 h of polarization at 0.4 V, before inoculation.

The presence of external cytochromes at the interface is supported by results of cyclic voltammetry in Fig. S1, showing a redox process centered at about −0.14 V that controls a catalytic process associated to current production [11,15,16]. As has been modelled by Ritcher et al. [15], the CV response can be accounted by considering a rate-determining step dependent on the redox-status of the catalyst. Starting from negative potentials electrons conduction is null because wiring cytochromes are fully reduced do to the lack of a suitable electron acceptor; as the potential increases over the half wave potential of the catalysts, the net amount of oxidized catalyst increases and current can flow. It is analogous to the response of an electric diode, in which the number of charge carriers depends on the applied potential resulting in a threshold potential for current conduction [17,18]. At high potential on the other side, current output is thought to be much more probably limited by a metabolic constrain than by an interfacial limitation [19]. On this regard Marsili et al. [8] have demonstrated that the limiting current becomes dependent on the electron donor availability below a concentration of 3 mM, while Ritcher et al. [15] have proposed that the limit would be at the acetate income rate.

The identity of cytochromes wiring *Geobacter* cells to the electrode remains unknown, but available data point to *OmcZ* [20], an octaheme c-type cytochrome preferentially localized at the electrode interface [20] and that was found to be crucial for electricity production in biofilms [21], and also to *OmcS* [22], an hexaheme cytochrome associated to conductive pili [22] that seems to be essential for the conduction to electrodes under some conditions. Since these are two of the most abundant cytochromes in the cell exterior, they are expected to contact the electrode to some extend influencing potential. Inoue et al. [21] recently reported that the reduction of *OmcZ* expands over the range of −0.06 to −0.420 V, while Qian [23] has found that the reduction range for *OmcS* is from −0.04 to −0.360 V. Comparison of this biophysical information with more negative OCP value in Fig. 1 evidence a lack of agreement, leading to the proposal of at least two hypothesis: (1) the more negative heme groups in *OmcZ* and *OmcS* do not participate in electron conduction to the electrode or (2) they are far enough from the interface to influence the electrode potential. Intriguingly, the potential of NADH ($E'_0 = -0.32$ V) as the electron donor at the negative end of the respiratory chain do not match with the possibility for these hemes to be reduced metabolically, while the absence of reduction peaks at potentials below −0.2 V in the cyclic voltammetry (data not shown) suggests that they are located far enough

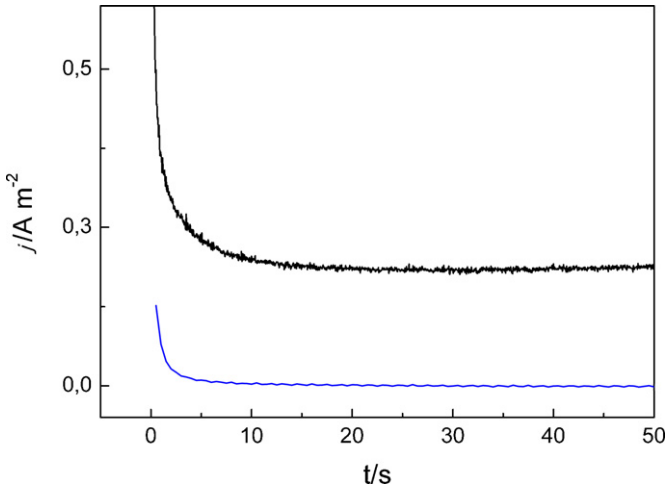

Fig. 2. Current obtained upon repolarization of a graphite electrode covered by a biofilm of *Geobacter sulfurreducens* after a period of 30 min at the open circuit potential (black line). Current obtained from a control experiment without biofilm is included for comparison (grey line). Applied potential: 0.4 V.

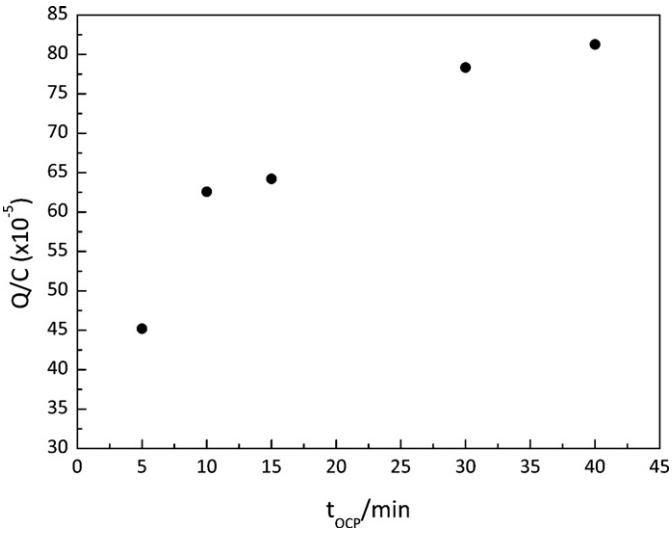

Fig. 3. Dependence of the accumulated charge on the time at the open circuit potential.

from the electrode to prevent their electrochemical reduction. In any case, the function of very negative hemes in the cell exterior does not seem to be related to electrons discharge. On the contrary, Summer et al. [24] have recently communicated about the possibility of electron exchange between co-cultured biofilms of *G. sulfurreducens* and *Geobacter metallireducens* and showed that *OmcS* is related to the process, giving support to the possibility for these cytochromes to act as receptors for electrons coming *from* the environment. In this sense, *Geobacter* sp. has been previously shown to accept electrons from negative polarized electrodes [25,26] although no redox elements have been identified so far.

The accumulation of electrons in the reduced form of heme groups has been proposed as an adaptive response of bacterial cells to cope with the depletion of electron acceptor [10]. Indeed, after measuring the cytochrome content of planktonic cells and the electron transfer rate, the authors have estimated in 8 min the time that maintenance requirements would be satisfied by draining respiratory electrons to extracytoplasmic cytochromes. In the present case, the OCP mostly decay during the first 2 min of disconnection following the reduction of cytochromes at the interface, but the question arise about the time course of reduction in upper levels of the biofilm, which could provide additional time for maintaining cell respiration. This point is explored in the following sections.

3.3. Biofilm discharging

After the time at OCP the electrodes were polarized again and chronoamperometric assays were performed. Fig. 2 shows the evolution of current upon re-polarization. A relevant feature of this curve is the occurrence of a transient peak whose maximal current is several folds higher than the steady state current produced by the biofilm. The excess current is thought to be due to the discharge of electrons accumulated in the biofilm during the disconnection time. It is in agreement to data presented by Marsili et al. [11] and showing an increased limiting current in cyclic voltammetry excursions performed after negative polarization steps.

To corroborate the hypothesis, biofilms were disconnected during increasing time intervals aiming to give more time for charge storing in the biofilm, and the current obtained upon every reconnection was registered. The data presented in Fig. 3 are the result of integrating every current plot (after subtracting the steady state current) and demonstrate that the excess of charge transferred to the electrode upon re-polarization increases with the time at the OCP until reaching a maximum after 30 min of disconnection. This indicates that charge storing an else activity of cells may continue for a very large period of time (e.g. 25–30 min).

From the analysis of results in Figs. 1–3 it can be concluded that biofilms can store charge, that cytochromes at the interface are charged before other cytochromes in the biofilm matrix and that biofilm cells outperform the planktonic cells in their ability to oxidize acetate in the absence of any electron acceptor, lasting for more than 30 min in this condition. This longer period of time is reasonable considering the higher content in cytochromes for current-producing biofilm [4,27]. A simple calculation from data presented in Fig. 3 shows that the amount of charge accumulated in the biofilm increased from 10^{-10} to 10^{-9} mol electrons cm^{-2}, clearly exceeding the amount of charge that can be stored in a cytochrome monolayer if a maximum cytochromes density of 10^{-12} mol cm^{-2} and 10 e^- per protein are taken as a reference [28]. The stabilization current in Fig. 2 corresponds to that produced by the biofilm before the polarization interruption, indicating that the entire population is conserved. At longer times at the OCP on the other hand, the current was observed to decrease (data not shown) suggesting both, the initiation of biofilm dispersion or some cell death, as a response to the lack of electronic acceptor. This information is relevant in showing an advantage of living in biofilms that has never been considered in the past because it offers the possibility to store charge beyond the limit for individual cells. Other important implication of results in Fig. 2 is that the fast discharge at high current clearly evidence that biofilm conductivity is high, and would not represent a limitation for current production. The stabilization at the steady state current on the other side, points to the metabolic rate of electrons production as the limiting step for electricity generation. Both conclusions are in line with results obtained by others from the modelling of voltammetric results [15].

3.4. Charge storage dependence on the applied potential

To further analyze the charge storing properties of *Geobacter* biofilms, discharge curves were collected after re-polarizing at different potentials. As can be observed in Fig. S2 both the stabilization current and the transient current peak increased with the applied potential. After subtracting the steady state current, integrating the current and normalizing to the value obtained at the highest potential, the results in Fig. 4 were obtained to show the dependence of stored charge with the discharge potential. It is evident from these

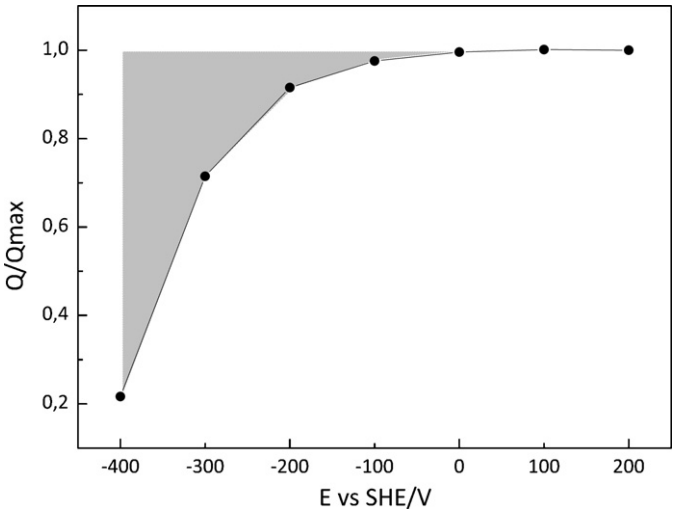

Fig. 4. Evolution of charge (Q) drained with the applied potential as related to the maximal obtained charge (Q_{max}).

results that not only the current but also the amount of charge remaining in the biofilm will change with the applied potential due to the influence of this parameter on the redox state of interfacial cytochromes.

3.5. Biofilm conduction model

Based on the above-presented information, a model for biofilm conduction as the one shown in Fig. 5 can be depicted, including charge storage. In this model, a single cell is represented for simplicity connected to the electrode through a conductive pathways composed by cytochromes. These molecules are here considered to be main constituents of the external network that held all the cells together in association to the electrode and are assumed to be responsible for the transport of electrons produced by each cell to the collecting electrode (Fig. 5). The network structure may include external cytochromes as *OmcZ* and/or *OmcS* that are in electric contact to the electrode, in addition to polysaccharides [29] and (putatively) conductive pili giving the structural support for both, charge accumulation and high current discharge.

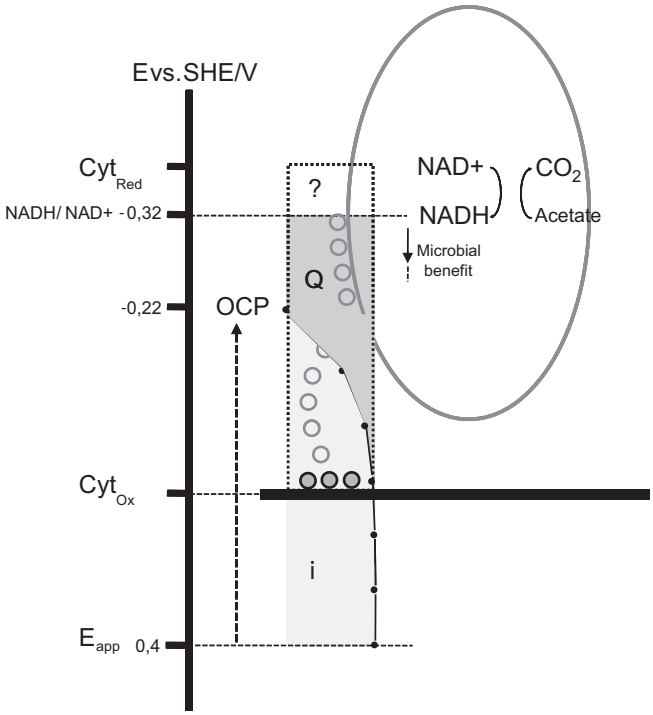

Fig. 5. Schematic conduction model of the *Geobacter* biofilm/electrode interface. Cells are interconnected and connected to the electrode through an external network of cytochromes supported by exopolysaccharides and (putatively conductive) pili. The dotted-line box indicates the redox range of extracytoplasmic cytochromes, including those of wide range as OmcS and OmcZ. The dark grey region corresponds to the amount of stored charge as related to potential (see text), while the light grey region indicates the production of current. Both, charge storage and current output are controlled at the interface *via* the wiring cytochromes redox state (see text). The large dotted line arrow indicates the OCP change reported in Fig. 1.

According to this model, during interruption of polarization electrons transferred outside the cell will distribute over the network until virtually every heme group in the external cytochromes is reduced. The possibility for charge accumulation is represented by the dark grey region notated as Q, limited on the lower-potential side by the formal potential value of electrons donors (NADH; acetate), and showing the dependence of accumulated charge on the applied potential (see Fig. 4) on the higher-potential side. As the driving force for the distribution of electrons is thought to be the local oxidized/reduced ratio of cytochromes across the network, electrons may flow from the mostly-reduced end located at cell bodies, to the mostly-oxidized end located at the electrode surface. In the absence of charge transfer to the electrode (i.e. when the circuit is open or at very negative potentials), electron accumulation initiates at the electrode-contacting cytochromes giving support to the sudden observed change of the open circuit potential reported in Fig. 1. This change is also indicated in Fig. 5. In a parallel plate electric capacitor the amount of stored charge is directly related to the potential difference between the plates. Analogously, the amount of stored charge in the biofilm capacitor is here proposed to increase with the difference between the instantaneous mean redox potential of the biofilm and the potential of the fully oxidized cytochromes. When the charge storage capacity of the exocytoplasmic cytochromes is fulfilled the potential difference across the biofilm capacitor is maximal and the network potential equals that of the electron donors, which is thought to represent the real stop signal for the cell respiration process. On this regard it is relevant to recall that the lower end of the redox transition for already studied external cytochromes exceeds the potential of the electron donors (see above), raising questions about the functionality of involved hemes. This fact is indicated by the question symbol in the model (Fig. 5).

As polarization is again connected, cytochromes touching the electrode are readily oxidized generating a local oxidation gradient that provides the driving force for biofilm discharge. It is important to note that while the kinetics of biofilm charging process is limited by the metabolic production of electrons, biofilm discharge is much faster because it depends on the availability of interfacial electron transporters (i.e. oxidized cytochromes), which in turn depends on the applied potential as related to the half wave potential of the cytochromes (Fig. S2). Exceeding this half wave potential current becomes progressively independent of the applied polarization (overpotential in Fig. 5).

4. Conclusions

In this work it has been demonstrated that well developed *Geobacter* biofilms can store charge for over 25–30 min upon polarization interruption, implying that cells can satisfy the energy maintenance requirement [13] by oxidizing acetate under the occasional absence of an electron acceptor by periods of time that are 3-fold higher than those previously shown for planktonic cells [10]. This ability has ecological significance because biofilm cells can clearly outperform their planktonic counterparts. It has been also

demonstrated that the amount of stored charge is dependent on the applied potential, highlighting the role of interfacial cytochromes in the regulation of the process. Finally, discharge current measured here were 5-fold higher than the steady state current confirming that conductivity of the biofilm matrix do not represent a limit for current production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.electacta.2011.07.001.

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