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Biochemical Capacitance of *Geobacter Sulfurreducens* Biofilms

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An electrical model able to decouple the electron pathway from microbial cell machinery impedance terms is introduced. In this context, capacitance characteristics of the biofilm are clearly resolved. In other words, the model allows separating, according to the advantage of frequency and spectroscopic response approach, the different terms controlling the performance of the microbial biofilm respiratory process and thus the directly related electricity production process. The model can be accurately fitted to voltammetry measurements obtained under steady-state conditions and also to biofilm discharge amperometric measurements. The implications of biological aspects of the electrochemical or redox capacitance are discussed theoretically in the context of current knowledge with regard to structure and physiological activity of microbial *Geobacter* biofilms.

Geobacter is a genus of the δ-proteobacteria characterized by its respiratory versatility and has attracted increased interest since the demonstration that it can oxidize organic compounds to produce electricity while respiring polarized electrodes.[1] Interestingly, *Geobacter* can form biofilms on electrodes, where bioenergetic processes of the colony can be viewed as a type of "natural battery" that renews organic wastes into electricity.[2] Respiratory activity of the so-called electrogenic biofilm has been shown to be modulated by the potential applied to the working electrode as cells are networked through external cytochromes that provide conducting pathways towards it (Figure 1a).[3] Usable charge generation, conduction and accumulation features found in *Geobacter* are unprecedented in the microbial world and, besides the number of creative applications that have prompted its use in energy production, wastewater treatment, biosensors technology and bioelectronics, these processes still represent a problem to solve in terms of the definition of their control steps and their electric properties.[3b,c,4]

Geobacter biofilms are typically grown on graphite working electrodes in three-electrode electrochemical cells that function as an electrochemical continuous-culture reactor.[5] Provided that optimal nutritional and redox conditions for bacterial growth are defined, while any other electron acceptors are excluded, biofilms develop on polarized electrodes as irregular films of up to 350 μm thickness that produce a constant current.[6] The biofilm/electrode interface obtained in this way can be represented by following the macro-homogeneous approach proposed by Paasch as a mixture of two phases:[7] the electron-conducting network[3b,8] connects each cell in the biofilm to the electron collector and the fuel or ionic chemical environment. These two phases are separated by the biochemical biofilm machinery, that is, the biochemical processes in the biofilm, which effectively mediates the conversion of chemical into electric energy (Figure 1a).

According to this approach the biofilm can be represented by a transmission line (derived in the Supporting Information and depicted in Figure 1), where the impedance of the individual microbial elements (ζ_C) consists of simple equivalent-circuit elements that can be detected externally. These elements include a redox capacitance (C_r), a charge-transfer resistance (R_{Ct}) and a shunt resistance R_t (details of the electric model are provided in the Supporting Information, revealing the usefulness of impedance modelling in separating kinetics parameters from those that dominate thermodynamics—herein C_r).

The detection of C_r (herein a capacitance per unit volume) denotes the existence of redox states distributed along the biofilm volume (see more details in the Supporting Information).[9] By fitting experimental data collected under different applied potentials (Figure 2a) to the presented model, C_r is confirmed to be an intrinsic property of the living colony that can be graphically quantified at low frequencies, as shown in Figure S1c, reaching a maximum (C_{max}) at the maximum respiration rate (Figure 2a; e.g., at the maximum respiration level, $C_r \approx 2800\ \mu F\,cm^{-2}$ or $0.56\ F\,cm^{-3}$ when considering an active biofilm thickness of 50 μm, see below and the Supporting Information for more details). Interestingly, C_r markedly decreases for negative applied potential, that is, on decreasing the respiration rate, evidencing that occupancy of cytochrome redox states is a function of this variable (Figure 2a). Notably, C_r depends on potential through a logistic functional, which is in contrast to the Gaussian dependence previously observed for molecular confined systems;[9] this corresponds to a behaviour expected for a system in which C_r is internally charged by the

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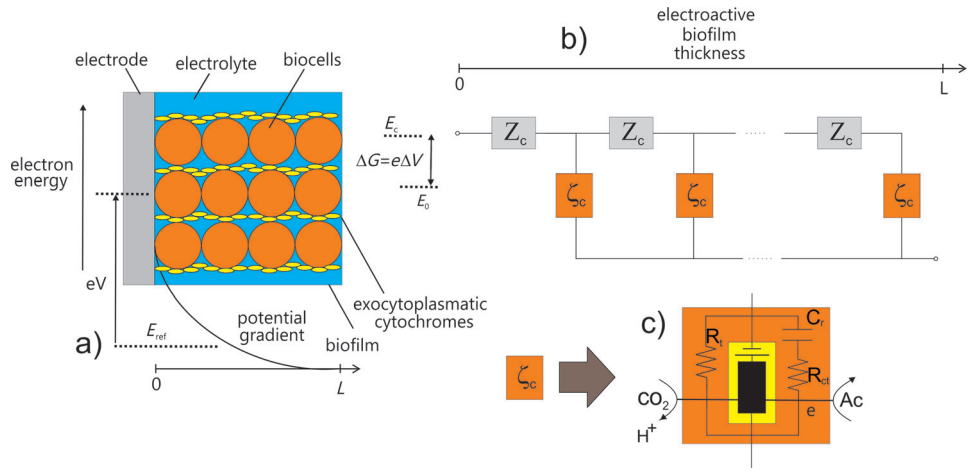

Figure 1. a) Schematic representation of the biofilm's microscopic structure consisting of individual microbial entities (in orange) interconnected with the electrode through cytochromes (in yellow). The existing gradient of electrochemical potential throughout the biofilm thickness is indicated. The energy level of the electrode probe modulates the access to an internal battery that drives the microbial respiratory activity. The reference potential is represented by E_{ref}. b) Depiction of the equivalent circuit for the microbial activity (electric model) comprising of a transmission line model able to shape the existing gradient of the electrochemical potential; Z_c is an impedance element that models the electron transport throughout the exocytoplasmic cytochrome chains along the biofilm thickness. c) Impedance provided by the individual microbial elements at a distance L from the electrode represented by ζ_C consisting of circuit elements described in the Supporting Information. Each of the black-yellow background elements in (c) represents the biological process of breaking acetate molecules (Ac) to release electrons into the conduction line, with the resulting chemical products being CO_2 plus H^+.

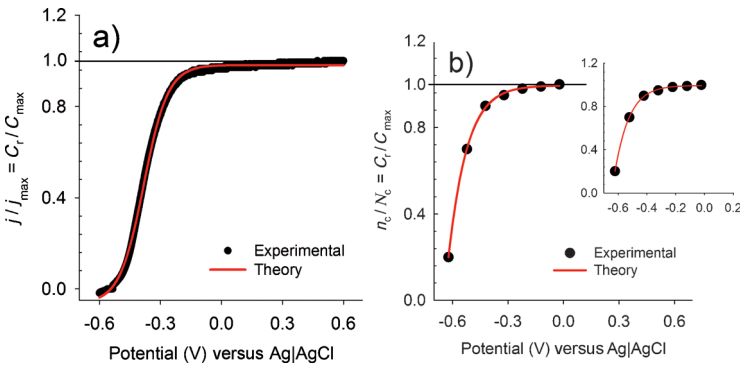

Figure 2. a) Normalized (per maximum value, j_{max}, as discussed in the main text) steady-state current density, j, versus probe electrode potential showing an excellent agreement between experimental data and introduced theory. The shape of the occupation function $f=n_C/N_C=C_r/C_{max}=j/j_{max}$ is compared to typical voltammetric results obtained from a *Geobacter* biofilm. b) Discharging curves obtained by chronoamperometric measurements[5a] after a period of 30 min at OCP (see the Supporting Information for additional information). The inset in (b) is the zoomed view of the data on the potential axis.

biological machinery and where redox states typically form a band (on a micrometer scale, in contrast to spatially confined states existing in films on the molecular scale[9c,10]). In a similar way, R_t changes according to the respiratory level from $R_t \rightarrow \infty$ (at the maximum respiratory level) to a quantifiable low and minimal value when respiration is suppressed (Figure S2b in the Supporting Information). It confirms that R_t acts as a shunt resistance to the energy-storage process (C_r), indicating that if respiration is fully suppressed, as upon interruption of polarization, the energy associated with C_r is completely lost through R_t instead of being delivered to the electrode; that implies that electrons are stored (up to a maximum limit) in the biofilm, as shown in Figure 2b and experimentally demonstrated previously.[5a,11] It indeed indicates that energy is stored[11a,b] through the redox density of states (DOS) of the biofilm up to a maximum value that can be theoretically defined, as shown in the following section. The following demonstrates the importance of C_r in modelling thermodynamic properties associated with the energetics of respiration in *Geobacter* biofilms and also shows that its behaviour as a function of respiration level can be accessed by means of steady-state DC-based current–voltage measurements.

Theoretically,[9c,10] C_r can be defined for a single electrochemical energy level as Equation (1):[12]

$$C_r = L\frac{e^2 N_C}{k_B T} f(1-f) \qquad (1)$$

where k_B is the Boltzmann constant; T is the absolute temperature; e is the elementary charge; L, as mentioned above, is the useful biofilm length (typically around 50 μm); and N_C is the volumetric DOS, that is, the number of exocytoplastic cytochromes per unit volume of biofilm (calculated to be $\sim 9.0\times10^{10}$ redox states per cm), introduced here to account for the (mesoscopic) redox states[13] distributed in the biofilm volume on a microscopic scale. According to this, the occupancy of redox states is controlled by the respiration level (proportional to C_r) and limited to N_C; thus, the origin of C_r is key to understand how the redox energy levels in the biofilm equilibrate with those in the electrode.[9] Indeed, as demonstrated below, the ratio $f=C_r/C_{max}$ equalizes to $f=j/j_{max}$, where j and j_{max} are the current density at any respiration level and the maximum current density (maximum respiration level), respectively. Therefore, f is maximum when the current density is at its maximum (physiologically representing the maximum respiration level). Note that f is a function that shows Boltzmann distribution with respect to energy (in accordance with the physical meaning of C_r and with the meaning of its maximum value, both in the context of electrogenic films; see below). It is important to note that although f has been extensively used to model these microorganisms,[3b,14] to our knowledge it was never related explicitly to a redox capacitance[11a,b] and its physical/biological meaning in biofilms.

Coupling the electrode with a C_r redox charging element has been very effectively used as a transduction of biological recognition in sensors based on electroactive molecular films;[15] in the same way, it can herein reveal the respiratory efficiency of *Geobacter* biofilms that can thus be measured or investigated under different external potentiometric conditions.

As previously shown for other systems,[9,15a,b,16] $f = F(E_0,\mu_e) = n_C/N_C$ in Equation (1) represents an occupancy function that follows the Fermi–Dirac statistics (and can be approximated to Boltzmann statistics at certain conditions) and that can be used to suitably predict changes in the occupancy of electrode-coupled redox states (driven by the potential of the probe, which in the case of *Geobacter* biofilms also controls respiratory activity). E_0 is here proposed to represent the energy associated with the electrochemical half-wave potential of the microbial colony, which is related to the biochemical capability of *Geobacter* to consume acetate ions (redox pair CH_3COO^-/H^+) and convert them into NAD^+/NADH to sustain an electrochemical potential difference between the inside and the outside of the cells. It can be measured as the open-circuit potential (OCP) when biofilms are in contact with a metallic probe due to the electrical connectivity across *Geobacter* membranes and the electrode, achieved through a pathway containing cytochromes with an unusually wide redox potential range.[17] On the other hand, n_C is the electron density occupancy associated with the effective redox occupancy (for each energy level of the electrode) that can be delivered as electricity to the external circuit; in the absence of a polarization this is limited to N_C.

Assuming μ_e is the chemical potential of the electrons (or the Fermi level, E_F) in the probe, it is interesting to note that this energy level can be driven into the probe by an electrical potential V (with respect to *Geobacter* internal electrochemical potential V^0, measured, for instance, with respect to the Ag|AgCl reference electrode). It is associated with Gibbs free energy through: $\Delta G = -e(V-V^0) = \mu_e - E_0$, where $-d\Delta G = -e\,dV = d\mu_e$. This indicates that, resembling what happens when bacteria switch between chemical electron acceptors with different energy yields (e.g., fumarate $< Fe^{III} < VO_2$),[18] a variation of the driving force of the electron transfer process governed by a change in the applied potential causes a change on the Gibbs free energy of the microbial cell system (assuming that the reference is invariant to any potential changes). Therefore, both the volumetric energy density and consequently the respiratory associated metabolism of *Geobacter* can be modelled by performing logistic Fermi–Dirac statistics on the probe by following a potential occupancy, $F(E_0,\mu_e) = n_C/N_C$, which is indeed governed by Equation (2):[12]

$$f = F(E_0,\mu_e) = \frac{1}{1+\exp[(E_0-\mu_e/k_BT)]} \quad (2)$$

The maximum occupancy of Equation (2) is found at $f = 1/2$, that is, at the half-wave potential where μ_e equates to E_0. But it is interesting to note that if the external cytochrome path is considered as an electronic band (equivalent to semiconductor band states[19]), then $f \ll 1$ (thus entirely dominated by Boltzmann statistics). By stating this, Equation (1) turns into $C_r = L e^2 N_C f/k_B T$ and Equation (2) changes into Equation (3):

$$f = F(E_0,\mu_e) = \exp[(\mu_e - E_0)/k_BT] \quad (3)$$

Now it is possible to note that the electron density in the conducting channels of cytochromes is governed by $n_C = N_C f$ [Eq. (4)]

$$n_C = N_C \exp[(\mu_e - E_0/k_BT)] \quad (4)$$

following a Boltzmann occupancy statistics with a maximum occupancy (when $\mu_e = E_0$ and $n_C = N_C$).

Accordingly, the electrochemical capacitance of the biological system is represented by Equation (5)

$$C_r = C_{max}\exp[(\mu_e - E_0)/k_BT] = C_m \exp(-\Delta G/k_BT) \quad (5)$$

where $C_{max} = L e^2 N_C/k_B T$ is the maximum electrochemical capacitance of the biofilm able to be delivered as electricity to the external circuit, provided that $C_r = C_m$ when $\Delta G = 0$zeroed, which is exactly the energy that *Geobacter* expends in its internal respiratory chain. Such internal energy source is exemplified in Figure 1c as an "internal battery" that pumps electrons from NAD^+/NADH to reduce the available cytochrome energy levels to balance the externally driven potential.

The above derivation states that the energy to drive $\mu_e = E_0$ ($-W_e$) externally, is exactly the energy spent internally (in the biochemical respiratory chain) by the microbial battery ($+W_e$) and represents the maximum electric energy the biofilm is able to deliver. It is in accordance with the energy conservation law, which states that it is not possible to extract more energy than the microbial colony metabolism can provide, or, from the other side, that steady-state changes are equilibrated by the metabolism of the microbial colony to the externally imposed potential levels.

According to previous work[9b] C_r is related to current through $j = C_r s$, where s is the scan rate in voltammetry experiments (consequently leading to $j/j_{max} = C_r/C_{max}$). As expected, C_r follows the same potential dependence as observed in electrochemical capacitance spectroscopy (ECS) experiments:[9b,10] at sufficiently low scan rates it is possible to move from one energy state to another close to equilibrium; the ECS model provided by Equation (5) can then be used to accurately predict experimental steady state voltammetry curves, as shown in Figure 2a. Additionally, it is possible to accurately predict using this model (with consistent values) the charge accumulated in exocytoplasmatic cytochrome levels, which were measured using amperometric experiments (Figure 2b).[11a]. The model demonstrates that N_C accurately represents one of the most interesting structural features of these biofilms with regard to technological applications, that is, the exocytoplasmic cytochrome density of states. C_{max} is, therefore, what was metaphorically identified as the iron lung of the biofilm and can be now directly measured non-invasively by using ECS, adding a tool to analyse these biofilms for research and applications.

Finally, by combining Equations (4) and (5) it is possible to demonstrate that Equation (4) can be rewritten as:

$$\Delta G = -k_B T \ln(n_c/N_c) = -k_B T \ln(C_r/C_{max}) = k_B T \ln(f) \quad (6)$$

so that $f = n_C/N_C = C_r/C_{max}$ provides a conservative relationship that takes into account the amount of energy per electron and the energy that can be delivered externally. Importantly, it allows to demonstrate that the energy produced by the microbial colony is thermodynamically limited by ΔG of the respiratory process, as evidenced by the limiting values shown in Figure 2a at oxidizing potentials.

Equation (6) summarizes the work present herein; as a final note, we would like to stress that it represents the expected Nernst thermodynamic relationship that drives the equilibrium between electrochemistry on the probe and microbial internal states in a colony,[20] which has not been previously described using impedance/capacitance modelling nor the redox capacitance concept. Equation (6) has implications for improving the properties of *Geobacter* biofilms for technological applications, not only because it elucidates the energy concept involved in converting biochemical energy into electricity, but also because it qualifies how this is quantitatively achieved from a physiological point of view. For instance, the concept states that the physiology of the microbial colony cannot be forced by the probe to go energetically beyond its electrochemical/biochemical internally energy level, that is, the efficiency of the biofilms is limited by the energetic alignment of the probe with the internal battery ($\mu_e = E_0$). In other words, according to Equation (6) it is explicit that the energy obtained from the biofilms is limited to ΔG no matter how the biofilm can be polarized externally and that the limit is at the intrinsic ΔG of *Geobacter* biochemical respiration process.

Accessing the redox capacitance from outside will surely be of help. However, *Geobacter* biofilms could be improved if engineered to maximize the number of redox states as quantified by the physical/physiological parameter N_c (associated with volumetric density of the exocytoplastic cytochromes) as stated in Equations (5) and (6).

The key contribution of the model presented here is that ΔG can be intrinsically associated with N_C and C_{max}, parameters that can be easily measured or calculated from steady-state current–voltage curves of *Geobacter* biofilms. We expect that accessing the redox capacitance noninvasively will thus be useful for researchers interested in modifying or studying the density of redox states in the bacterial colony as well as for those interested in dissecting the respiration process of Geobacter strains. In a wider context, since the analysis can be performed without any background knowledge of impedance spectroscopy, we expect our work can constitute an additional tool for refining future research and developments involving electrogenic biofilms.

Keywords: biofilms · capacitance · microbial respiration · spectroscopy · thermodynamics

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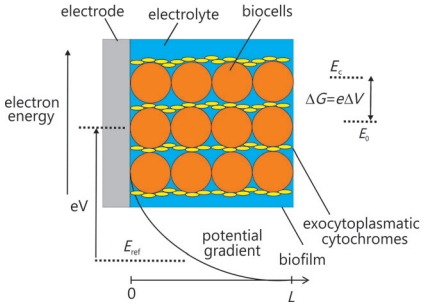
A tale of capacitance and biofilms: A capacitance-based model of *Geobacter sulfurreducens* biofilms is presented, where the respiratory gradient of the biological population is taken into account. Essentially, the model is able to decouple the electron conduction pathways from the impedance terms of microbial cell machinery. Thus, capacitance characteristics of the respiratory process of biofilms can be clearly resolved and matched with experimental results.



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