Microbial Electron Transfer

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In Situ Spectroelectrochemical Investigation of Electrocatalytic Microbial Biofilms by Surface-Enhanced Resonance Raman Spectroscopy**

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Metal-reducing bacteria not only play a key role in geochemical redox cycles,[1] but also attract increasing attention in view of their relevance for microbial bioelectrochemical systems, a seminal sustainable technology.[2] This growing research interest is triggered by the bacteria's capability to oxidize substrates such as acetate and to transfer the released electrons to an insoluble terminal electron acceptor, for example, iron-containing minerals in nature or a fuel cell anode in bioelectrochemical applications. The underlying electron-transfer (ET) mechanisms between the bacteria and the terminal electron acceptor may occur by different mechanisms, including direct and mediated electron transfer denoted as DET and MET, respectively.[3] In the case of DET, the electrons are transferred from the respiratory chain in the cell to extracellular inorganic material via a complex architecture involving several outer membrane cytochromes (OMCs).[4] These cytochromes are multiheme proteins whose function and number of heme groups may vary largely within the same family.[4] Although several studies investigated the behavior of these proteins embedded in microbial biofilms of wild-type and mutant *Geobacter sulfurreducens*,[5–7] the archetype bacteria family employing DET,[8,9] the role of these cytochromes in the heterogeneous ET across the biofilm/electrode interface is far from clearly understood. This is particularly true since structural data are currently only known for two OMCs, namely, OmcF and OmcZ.[10,11] In this respect, spectroscopic techniques that can be applied to biofilms in situ may provide important structural information about the OMCs involved in the DET.

To date, only two spectroscopic studies were devoted to the investigation of OMCs embedded in the cellular membrane.[12,13] The spectroscopic measurements of these works were carried out with washed and re-suspended cells, but did not refer to intact biofilms grown on an electrode.

Herein, we present for the first time in situ spectroscopic characterization of OMCs in a catalytically active microbial biofilm. By measuring the electrochemical and spectroscopic properties of microbial cells embedded in their natural biofilm habitat, a more realistic picture on the natural electron transfer will be provided. Therefore, we have employed surface-enhanced resonance Raman (SERR) spectroscopy in combination with cyclic voltammetry (CV). SERR spectroscopy exploits the combination of the molecular resonance Raman (RR) and the surface-enhanced Raman (SER) effect to probe selectively the heme groups solely of the proteins in proximity of the electrode surface.[13,14] This powerful technique, in our case performed under strict electrochemical control, reveals the redox, coordination and spin states of the heme iron as well as the nature of its axial ligand, thereby providing important structural information that may complement the interpretation of electrochemical data obtained by CV.[15,16]

The biofilms were grown at a constant potential on roughened (i.e. SER-active) silver electrodes using 10 mM acetate as substrate (see the Supporting Information for experimental details). These biofilms produced a maximum chronoamperometric current density of 600 $\mu A\,cm^{-2}$ (Figure SI2 in the Supporting Information), which is in good agreement with previous studies using graphite anodes.[17] The voltammetric behavior of the biofilms was monitored under turnover (Figure SI3) and nonturnover conditions [that is, with and without the substrate (e.g. acetate), respectively].

Figure 1 shows the CV behavior of such a biofilm for nonturnover conditions. The two redox couples that are proposed to be involved in the DET, $E_{f,1}$ and $E_{f,2}$, are centered at formal potentials of −282 mV and −363 mV, respectively (all potentials are reported versus the Ag/AgCl (3.0 M KCl) reference electrode).[18] The main overall shape and peak positions of the cyclic voltammogram shown in Figure 1 are very similar to those obtained on graphite electrodes in parallel experiments and in previous studies,[19] showing that biofilm formation is not affected by the nature of the electrode material. The similarity between these CV traces and those obtained solely from biofilms of *Geobacter sulfurreducens* indicates that the biofilm is highly dominated

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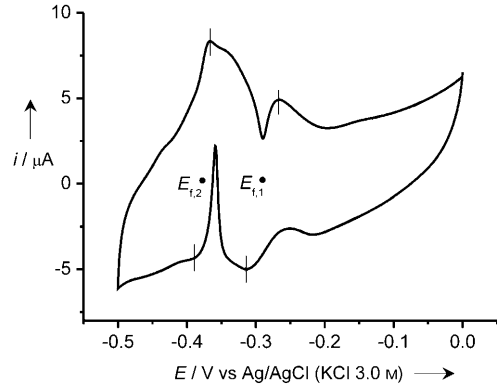

Figure 1. Cyclic voltammogram of a microbial biofilm formed at a silver electrode under nonturnover (i.e. substrate deprived) conditions. Experiments were performed in 100 mM mineral solution lacking acetate at pH 7.0. The scan rate was 1 $mV s^{-1}$.

by these bacteria.[20] This conclusion is supported by a parallel flow cytometric characterization of biofilms grown on graphite electrodes under identical conditions (i.e. identical bacterial source, substrate, medium composition, temperature etc.) described elsewhere.[21] Furthermore, this finding reveals that the nanostructured silver electrode and specifically the inevitable traces of Ag^{I} cations do not provide a toxic environment for *Geobacter sulfurreducens*, confirming recent observations by Law et al., who were able to grow *G. sulfurreducens* cells in the presence of nanoscale silver precipitate.[22] This high toxicity resistance of these biofilms is furthermore well in line with a recent study by Patil et al.[23]

Figure 2 shows the SERR spectra of a microbial biofilm grown on a roughened Ag electrode and measured at different applied potentials in the absence of metabolic substrate. The prominent bands at 1375, 1506, 1588, and 1639 cm^{-1} (Figure 2, bottom), and at 1361, 1495, and 1592 cm^{-1} (Figure 2, upper spectrum) are ascribed to a *c*-type heme in the oxidized and reduced state, respectively.[24] The SERR spectra are thus ascribed to the prosthetic groups of one or more OMCs wiring the bacteria to the electrode.

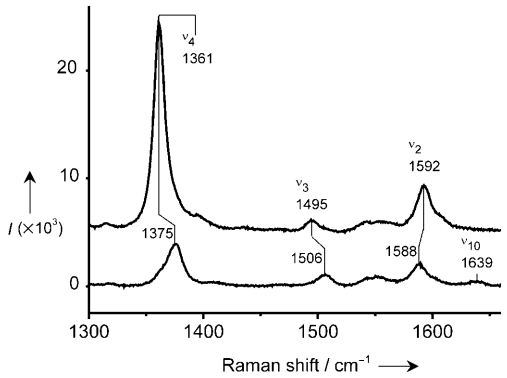

Figure 2. SERR spectra of the reduced (upper spectrum) and oxidized (lower spectrum) OMCs, obtained at −425 and 0 mV, respectively. The spectra were obtained with excitation at $\lambda = 413$ nm, laser power of 1 mW, and an acquisition time of 90 s. Potentials refer to the Ag/AgCl (KCl 3.0 M) reference electrode (210 mV vs. SHE).

Both the band positions and the relative intensities are indicative of a six-coordinated low-spin heme with two histidine groups serving as axial ligands.[24] SERR spectra obtained at constant applied potentials between −425 and 0 mV were simulated by the superposition of the component spectra of solely one reduced and one oxidized form to obtain the relative concentrations of the two oxidation states.[14,24,25] As shown in Figure 3, decreasing the electrode potential

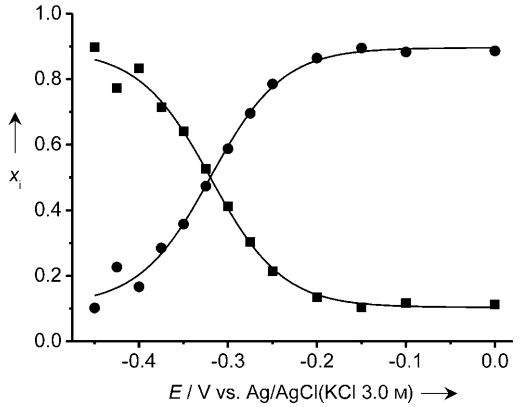

Figure 3. Fit of the Nernst equation for a one-electron couple to the relative concentrations (expressed in molar fraction x_i) of the oxidized (●) and reduced heme (■) as a function of the electrode potential. The data were derived from the component analysis of the experimental SERR spectra.

increases the contribution of the reduced form (■) at the expense of the oxidized species (●). This redox titration was performed from positive to negative potentials and vice versa without observing significant hysteresis (Figure SI4). However, a small fraction of the heme groups (ca. 10%) was redox-inactive, as indicated by the residual amount of oxidized and reduced hemes at the most negative and positive potentials, respectively. These findings clearly demonstrate the reversibility and the excellent electrochemical control of the redox processes of the cytochromes in the biofilm. Notably, the biofilms possess catalytic activity for acetate oxidation after the redox titration, revealing that exposing the biofilm to the laser did not affect its functional properties (not shown). A fit of the Nernst equation for a one-electron redox couple to the data in Figure 3 afforded an apparent formal potential E_{app} of (-317 ± 4) mV and a number of electrons exchanged in the redox reaction of $n = 0.6 \pm 0.1$. This n value, which is lower than 1 (the expected value for the reduction and oxidation of *c*-type cytochromes), suggests a dispersion of interfacial redox potentials. This hypothesis is supported by CV data indicating the presence of two redox couples having different formal potentials $E_{f,1}$ and $E_{f,2}$, whose arithmetic mean coincides with E_{app}. Although the SERRS spectra of these two couples are identical (only the bis(histidine) low-spin species are observed), they can be distinguished on the basis of their different formal potential. In fact, an equally good fit to the SERR data is achieved by using a Nernst equation for two independent redox couples (Figure SI5). The found values $E_{f,1} = (-295 \pm 2)$ mV $(n = 0.9 \pm 0.1)$ and

$E_{f,2} = (-367 \pm 7)$ mV ($n = 0.8 \pm 0.2$) are in very good agreement with the CV results obtained for low scan rates.[18]

In the first in situ SERR spectroelectrochemical analysis on a catalytically active biofilm grown on silver electrodes, we have demonstrated that two bis(histidine) coordinated heme cytochrome redox couples are involved in the DET between the bacteria and the electrode. The fact that both redox couples are spectroscopically indistinguishable implies that subtle structural differences in the heme pocket,[26,27] not reflected by the range of the SERR spectra analyzed in this work, are responsible for the different formal potentials $E_{f,1}$ and $E_{f,2}$. The lack of further structural information about the OMCs of these bacteria prohibits the a priori assignment of $E_{f,1}$ and $E_{f,2}$ to redox transitions of specific heme groups. In fact, $E_{f,1}$ and $E_{f,2}$ may either refer to individual or to macroscopic formal potentials. However, since the intramolecular ET in cytochromes including multiheme clusters is very fast,[28,29] it is reasonable to assign $E_{f,1}$ and $E_{f,2}$ to macroscopic redox potentials of two different OMCs.

The weighting of the two OMCs in the SERR spectra is very similar (Figure SI5), implying that the respective cofactors experience essentially the same surface enhancement of the RR scattering. In view of the pronounced distance-dependent attenuation of the SER enhancement,[30] it is likely that in the biofilm both cytochromes are located at similar distances with respect to the electrode. This conclusion suggests that each of the two OMCs undergoes direct electron exchange with the electrode, pointing to a DET with two parallel ET pathways or to one electron relay with two parallel electron exit sites.

Clearly, further studies and specifically more structural data on the OMCs are required for a comprehensive understanding of the DET of these biofilms. In this respect, the present combined SERR spectroelectrochemical and electrochemical approach is capable to provide further valuable contributions as it is the only in situ methodology that allows probing of the structure and function of OMCs in intact biofilms.

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