Journal of Electroanalytical Chemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

journal homepage: www.elsevier.com/locate/jelechem

Proving *Geobacter* biofilm connectivity with confocal Raman microscopy

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ARTICLE INFO

Article history:
Received 22 July 2016
Received in revised form 24 October 2016
Accepted 2 November 2016
Available online xxxx

Keywords:
Confocal Raman microscopy
Geobacter sulfurreducens
Biofilm
Redox state

ABSTRACT

We report here an *in-vivo*, non-invasive, depth profiling study on an electrically-active biofilm by Confocal Raman Microscopy. Films based on *Geobacter sulfurreducens* bacteria were grown onto an indium tin oxide (ITO) conducting glass, where the ITO piece plays a double role: it acts as a supporting polarized electrode for the bacteria population but also as a transparent window that gives direct access to the biofilm body. A confocal Raman microscope was used to examine the biofilm redox state at increasing distances from the ITO surface, upon variable polarization and under the presence or not of a chemical electron donor or acceptor. Resonant Raman effect allowed to selectively probe the redox response of biofilm cytochromes to the applied potential and chemicals, revealing internal connectivity details of the bacterial population. Full methodological details for this application case are given, while the importance of confocal Raman for the analysis of biological or electrochemically active systems in non-invasive way is highlighted.

1. Introduction

Gram negative bacteria from the *Geobacteraceae* family can use extracellular compounds as final electron acceptors. In particular, *Geobacter sulfurreducens* is able to oxidize acetate to CO_2 while reducing for example, extracellular insoluble Fe (III) compounds by transferring electrons directly to the extracellular medium [1]. It has also been demonstrated that extracellular electrons can be transferred to a polarized electrode, thus yielding an electric current. When grown in electrochemical reactors, *G. sulfurreducens* cells can form electro-conductive biofilms [2] and produce the higher current ever reported [3]. A remarkable feature of these biofilms is that cells located at tens of micrometers from the electrode are still able to contribute to the current, evidencing their electric connection to the collector electrode [2,4,5]. Conductivity in this biological material is nevertheless limited as shown by physiological stratification of the population as the distance to the electrode increases [6]. Understanding the physiology of electro-active biofilms is essential for the design of efficient bio-electrochemical devices but the task has been elusive for researchers in consequence of intrinsic complexity of these sessile communities. Combining spectroscopic with electrochemical techniques has helped to overcome this limitation by providing chemical information in a non-invasive way [7–10]. Raman spectroscopy has been particularly significant at this respect, as the highly localized nature of the Raman enhancement effect made possible the selective detection of the response of outer membrane cytochromes (OMCs), which are molecules called to play fundamental roles in the definition of bacterial physiology [11].

One of the actual challenges in electro-active biofilm research is obtaining a whole picture of biofilm physiology *in-vivo*, that is, with the microbial cells in their habitat of growth, in order to obtain realistic data on the process of electron transfer. One possible approach to this problem is the use of the *optical sectioning* capability of confocal microscopes, a major tool widely used in biology and medical research to reveal the internal structure of transparent media, without the need of physically sectioning the specimen. Unlike Surface-Enhanced Raman Spectroscopy (SERS), Confocal Raman Microscopy (CRM) allows the characterization of whole biofilms as spectra acquisition is not limited to the close proximity to the electrode, with evident benefits for studying for example, electron conduction along microbial networks.

In this line, our group has recently shown how confocal optical sectioning in tandem with Raman spectroscopy can be successfully applied to investigate electricity-producing *G. sulfurreducens* biofilms grown on transparent Indium Tin Oxide (ITO) electrodes, adding valuable chemical information to the structural and electrochemical characterization data obtained by standard phase contrast optical microscopy and electrochemical techniques [11]. In the same way, recent work by Virdis et al. [12] also made use of CRM combined with electrochemical measurements to investigate the electron transfer process *in-vivo*, but working on mixed community biofilms [12]. Except for this two works, spectroscopic measurements in the majority of reports published elsewhere about microbial electro-active biofilms were carried out *ex-situ*, *i.e.* by removing biofilms from the anaerobic electrochemical reactor and exposing them to the air in some extent [13–15]. Recognizing the difficulty

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http://dx.doi.org/10.1016/j.jelechem.2016.11.005
1572-6657/

and the importance of performing *in-situ* observations, we give here detailed information about the implementation of the experimental setup we recently developed. By using this setup, we indeed report on the results of performing measurements of the redox state of *G. sulfurreducens* biofilms, under a variety of conditions including the presence of oxygen, the presence of a chemical reductant, the availability of an electron donor or not and the polarization to different potentials. Results show that electrochemically induced redox states are not as deep as those induced chemically, evidencing that only a fraction of biofilm cytochromes is electrochemically accessible. Upon z measurements we demonstrated that accessible cytochromes distribute all along the biofilm thickness and that their redox state can be controlled from the electrode even at those cell layers located tens of microns away. The minor fraction of cytochromes not influenced by the electrode potential is proposed to be at the cell interior.

2. Materials and methods

2.1. Biofilm growth

G. sulfurreducens biofilms were anaerobically grown on electrodes in continuous culture at an applied potential of −0.15 V using a custom-made thin-film electrochemical cell previously reported [11]. Acetate was added as the carbon source and electron donor to a modified fresh water media described elsewhere [16]. To ensure that cells were using the electrode as the electron acceptor no other acceptor was added to the culture media.

For essays in absence of electron donor, biofilms were feed with culture media without acetate until current reached values below 1% of the catalytic current.

2.2. Electrochemical measurements

Chronoamperometric measurements were performed with an Autolab PGSTAT 101 potentiostat controlled by the NOVA 1.6 dedicated software. Soda lime glasses (500 μm thick) coated with a thin (10–15 nm) tin-doped indium oxide (ITO) layer were purchased from NANOCS™ and used as received. Glass surface resistivity was 5–12 Ω/sq while optical transmittance was above 90% at the laser excitation wavelength. The ITO glass was used as the working electrode while the counter electrode was a platinum wire. The reference electrode was a BASi RE6 Ag/AgCl-3M NaCl. The exposed working electrode area was 0.32 cm^2.

2.3. Confocal Raman microscopy

Raman spectra were recorded with a Renishaw in *Via* Reflex spectrometer system equipped with charge-coupled device (CCD) detector of 1040 × 256 pixels and coupled to a Leica microscope with a computer-controlled *xyz* stage. An Ar laser line (514 nm, 50 mW) was used as excitation source in combination with a grating of 2400 grooves/mm. Depth profiling experiments were carried out through the ITO electrode of the electrochemical cell. The cell was mounted on the stage of the confocal microscope remaining fully operative during Raman measurements. As the thickness of the ITO glass was about 500 μm, a long working distance (WD) objective was necessary to microscopically analyze the whole biofilm thickness. A dry metallurgical objective Nikon 0.7 NA (60×), 5 mm WD, with variable correction ring was employed in the excitation and collection path. Confocality is achieved by tuning the pixel binning of the spatial dimension of the CCD and the aperture of the spectrograph entrance slit. The regular configuration used (6 pixels of the CCD, 65 μm slit opening) delivers a depth resolution below 15 μm, as determined by z-scanning of a polished silicon wafer. Instrumental filters were used to reduce the incident laser power. Overall, the biofilms tolerated nominal laser powers up to about 50% without signatures of modifications in structure or activity associated to thermal or chemical degradation, as verified by a nearly invariant spectral and voltammetric response before and after depth-profiling experiments (data not shown). Raman results obtained under these conditions were virtually identical to those reported with lower laser powers (10%) [11]. Spectral post-processing was carried out using the analysis method previously reported [11], which considers the entire spectral information and solves the contribution of reduced and oxidized forms. Biofilms at open circuit potential in presence of acetate and polarized at −0.15 V in absence of acetate were used as reduced and oxidized limits, respectively, for relative reduce weight fraction calculation. The z-scale in results of z-profiling is corrected by a factor of 1.5 to account for the foreshortening of the depth scale due to refraction effects, see details in Section 3.2.

3. Results and discussion

The experiments shown in this section correspond to fully developed *G. sulfurreducens* biofilms grown at a constant potential beyond the point they attain a constant current production level (*i.e.* beyond 150 h of growth), using acetate as the electron donor and the polarized ITO glass as the electron acceptor. The thickness of the biofilm at the time we carried out the Raman experiments, as determined by optical microscopy in phase contrast mode through the ITO glass, was found to be about 100 μm (Fig. S1). The voltammetric profile of grown biofilms was the same as that described by others, which includes non-catalytic signals superimposed on a prominent turnover process that corresponds to the catalysis of acetate oxidation (Fig. S2).

3.1. Spectral features of Geobacter sulfurreducens biofilms

3.1.1. Spectral profiles in completely reduced or oxidized conditions

The characteristic Raman spectra of *G. sulfurreducens* biofilms at the basal plane in totally oxidized and totally reduced conditions (black lines) are given in Fig. 1. It has to be noted that these spectra have not been taken in identical conditions so they do not correspond to the same mass of biofilm. The spectral region shown is that where we found the most intense Raman bands for both oxidation states. The spectrum of the fully oxidized biofilm form was obtained by exposing it to air, thus forcing the oxidation of cytochromes. A small amount of the biofilm was transferred from the cell to a microscope slide and then spectroscopically characterized. The reduced spectrum was obtained *in-vivo* by subjecting the system to chemical reduction with sodium dithionite, a well-known chemical reducer typically used in biological research. Exposing the biofilm to air or to the treatment with dithionite is expected to fully oxidize/reduce virtually all redox molecules in the biofilm, including cytochromes, so the results presented reflect a *complete* redox change (Fig. 1); we will return to this point in Section 3.1.2.

The spectral profiles of both, oxidized and reduced states reveal characteristic features associated with *c*-type cytochrome structures. For instance, many of the prominent peaks observed in the oxidized biofilm (1171, 1315, 1373, 1405, 1503, 1564, 1587 and 1638 cm^{-1}) have also been found in earlier normal Raman studies of isolated *ferricytochromes c*, carried out with the same excitation line [17]. These bands have been primary assigned to stretching of C—C and C—N bonds of the porphyrin ring of the cytochrome structure [17]. The same applies to many of the bands observed in the reduced biofilm (1173, 1312, 1360, 1398, 1492, 1586, 1621 cm^{-1}), found to be essentially the same than those reported for *ferrocytochromes c* [17]. The peak observed around 1540 cm^{-1} in both forms, not previously found in cytochromes, can be well ascribed to amide groups as found in other proteins, by considering that several *Geobacter* cytochromes are much more complex and bigger than cytochrome *c* from horse heart typically used in spectroscopic studies.

The spectral contrast between oxidized and reduced forms is very clear. The reduction of the oxidized form leads to perceptible changes

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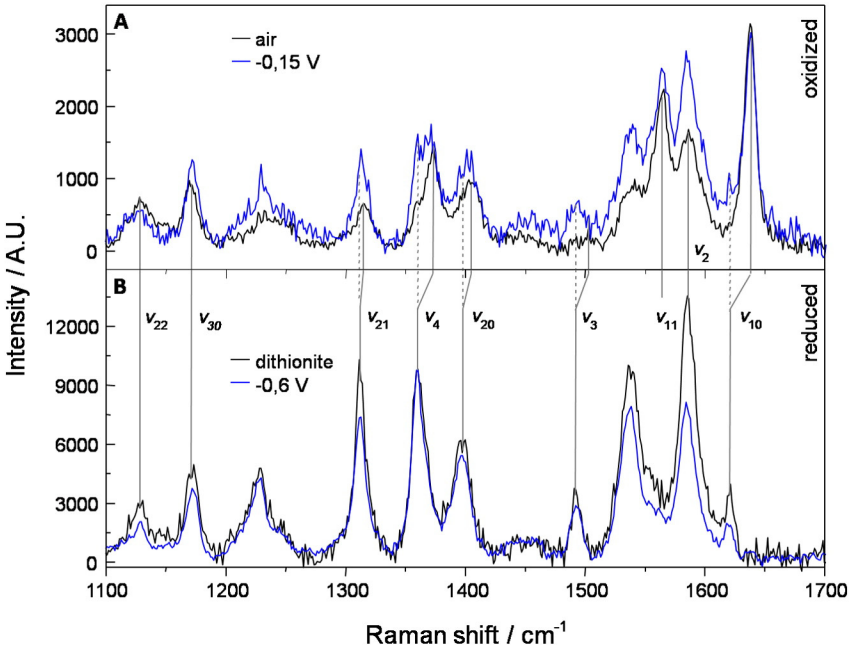

Fig. 1. Raman spectra at the basal plane, in the 1100–1700 region, of *G. sulfurreducens* biofilms electrochemically grown on an ITO electrode. The biofilm was chemically (black lines) or electrochemically (blue lines) oxidized (A) or reduced (B) by exposition to air, sodium dithionite or electrochemical polarization in absence of acetate at the indicated potentials, respectively. Notice the different scales of y axis. See Table S1 for peak assignment of bands indicated in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in relevant band parameters as intensity and frequency (Table S1). For instance, peaks at 1315, 1373, 1405 and 1587 in the oxidized form appear shifted at lower frequencies in the reduced counterpart (1312, 1360, 1398 and 1586), confirming the sensibility of those peaks to changes of the oxidation state of the iron atom of heme groups [17]. In the same line, using SERS, Ly et al. [18] found an increase of the band at 1375 cm^{-1} at the expense of the 1361 cm^{-1} band after electrochemical oxidation [18].

The dramatic change in peak intensity upon oxidation of bands at 1564 and 1638 cm^{-1} and the fact that those bands eventually disappear in the reduced form, make them very suitable as oxidation markers. Similarly, bands at 1492 and 1360 cm^{-1}, may serve to monitor the degree of reduction in the biofilm, although they present much modest changes compared with those useful to track oxidation. At this respect, recent works by Kuzume et al. [19] and Virdis et al. [14] have highlighted a band at 750 cm^{-1} as one of the most prominent of the reduced biofilm spectrum, which experiences an upward frequency shift and a marked intensity decrease upon biofilm reduction. The reason we did not observe this particular spectral feature in our data (in fact, we excluded this low frequency spectral range from our analysis due to the lack of prominent peaks) can be explained by the large dependency of the relative Raman band intensities on laser wavelength in the context of resonant Raman scattering. While the excitation line in the case of Virdis or Kuzume was 532 nm, we employed a 514 nm laser. Previous work of Adar and Erecińska [20] shows clearly how the band at 750 cm^{-1}, one of the most prominent peak in the reduced *c*-type cytochrome spectrum when excited at 568 nm, becomes a minor peak when the laser line is changed to 514 nm. This minor peak is also observed in spectra presented by Lebedev et al. [13] with a 514 nm laser.

The fact that the Raman scattering of cytochrome structures overwhelms those of other cellular components can be explained in terms of the coupling between the excitation line used (514 nm) and the electronic transitions of the heme chromophore (α and β bands in the 500–550 nm region) [17], giving rise to a resonant Raman effect. Excitation with a 514 nm laser line near the α β bands explains the rather straightforward detection of changes in cytochromes redox state, in some way boosted by the unprecedented content of this type of molecular structures bared by *Geobacter* cells [21]. It also explains why we obtained a virtually featureless biofilm spectrum using a 785 nm laser line as excitation source (data not shown). This selective enhancement constitutes an invaluable tool to individually probe the response of porphyrin-containing systems to redox changes.

3.1.2. In-vivo spectral profiles in extreme electrochemical conditions

To monitor *in-vivo* the redox state of *G. sulfurreducens* cytochromes under extreme electrochemical polarization, *i.e.* at completely oxidizing (−0.15 V) or reducing (−0.6 V) potentials (Fig. S2), the whole fully operational incubation cell was mounted onto the confocal microscope stage and the anodic potential was set at the selected values, allowing 10 min for equilibration. Prior to this analysis, biofilms were feed overnight with a culture media lacking electron donor (acetate), in order to prevent any influence on the redox state from metabolic contributions. Raman spectra obtained at the basal plane of electrochemically reduced or oxidized biofilms are reported in Fig. 1 (blue lines).

Peak patterns obtained under electrochemical conditioning are similar to those observed after treatment with chemicals (Fig. 1), but changes in features of individual bands are not so deep. Simultaneous signals corresponding to oxidized/reduced ν_4, ν_{20} and ν_{10} modes at 1360/1373, 1397/1405, and 1621/1638 after electrochemical oxidation (Fig. 1A, blue line), strongly suggests the occurrence of at least two coexisting cytochrome pools, one of which seems not to respond to the electrochemical perturbation (*i.e.* it may be isolated from the electrode). Results obtained upon electrochemical reduction on the other hand, also evidence a partial redox change (Fig. 1B, blue line). We interpret this apparently *incomplete* overall redox change by considering that in the particular case of *Geobacter* biofilms, confocal Raman senses a convoluted response over a variety of *c*-type cytochromes that may or may not be influenced by the electrode potential for at least three reasons: 1) they are not actually involved in the electron transport process, 2) the applied potential is not oxidative/reductive enough, or 3) they are partially isolated from the external signal (*e.g.* by cell membranes) [22]. According to actual models interpreting electron conduction across *Geobacter* biofilms, electrons move in the direction given by the most favourable redox gradient to any point in the matrix, provided it has

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the most oxidative potential [23]. In this scenario, virtually all redox centres are presumed to be "connected", even if they do not participate in the conduction pathway, strongly contending against reason 1. In the particular case of reduction and taking into account the −0.2 V difference between the formal potential of sodium dithionite (−0.86 V *vs.* Ag/Ag/Cl) and the applied potential (−0.65 V), partial response could be assigned to insufficient polarization, but it has to be noted that after leaving the electrode at the open circuit potential in the presence of acetate, the degree of reduction promoted by cell metabolism from the cell interior was virtually the same observed with sodium dithionite (Fig. S3). As cells cannot produce electrons to a potential lower than that of the electron donor (*i.e.* lower than −0.3 V for the $NAD^+ + e^-$ → NADH reaction), which is much higher than the applied potential, reason 2 is neither valid. After considering all the above evidence, we are inclined to think that partial electrochemical reduction shown in Fig. 1B is a consequence of electric isolation of the refractory pool of cytochromes in the cell interior. This interpretation is in line with genomic analysis showing that within the 111 different *c*-type cytochromes predicted to be produced by *Geobacter*, only about 30 are located in the extracellular space, presumably available for conditioning from the polarized electrode [24]. It should be noted indeed, that according to recent results periplasmic cytochromes seem to be associated to those at the external membrane and even to those at the external cell surface forming supramolecular complexes [23,25]. If this would be the case, only a low fraction of cytochromes at the cell interior may be electrically isolated, as suggested by results in Fig. 1B.

3.2. In-vivo z measurements of the redox state of G. sulfurreducens biofilms

The translation of variations observed in spectral features as a function of z, applied potential, or other relevant variables (as time or culture conditions), to a quantitative fraction of oxidized/reduced cytochromes, constitutes the basis for the interpretation of changes in physiology of *Geobacter* cells and has been one of the major concerns of our analysis. That calculation is somewhat challenging as we are detecting an overlapping contributions of several cytochromes forms. Despite of this, the spectral contrast was enough to resolve the biofilm redox state and to put on a quantitative basis experimental observations. Thus, we took advantage of variations in overall spectrum intensity upon reduction/oxidation and calculated the fraction of reduced species as previously described [11] in a variety of situations. The values presented are relative to the signal obtained from biofilms at open circuit potential in presence of acetate and polarized at −0.15 V in absence of the electron donor, taken as reduced and oxidized references, respectively (See Section 3.1.2 for details).

The redox state of the electricity producing biofilm was evaluated in the z dimension at extreme electrochemical conditions in presence or absence of the electron source acetate (Fig. 2). Each sequence of the optical slicing by confocal Raman corresponds to acquisitions started nearby the ITO/biofilm interface, followed by sequential movements of the microscope stage in 5 μm intervals, at a fixed polarization potential. Values of z has been zeroed at the ITO/biofilm interface and corrected with respect to the values read from the microscope stage by a factor of 1.5, to account for the foreshortening of the depth scale due to laser refraction [26]. That distortion is unavoidable and arises from the large mismatch in refractive index experienced by the laser beam along the optical path, as it travels through air, then passes through the ITO window, to end at the biofilm.

As we have previously shown [11], upon polarization at an oxidizing electrode potential (here −0.15 V) and in presence of acetate, a redox gradient can be observed across the biofilm thickness, denoted by the increase in the fraction of reduced species with the distance to the electrode surface. It is thought to be the result of the balance between the metabolic reduction of cytochromes by cells and their oxidation by the polarized electrode [11]. In accordance with this interpretation, when the system was evaluated at the same oxidation potential but in the absence of acetate, the fraction of reduced cytochromes was negligible across the entire biofilm (Fig. 2). It can be explained by considering the low metabolic rate exhibited by cells in the imposed conditions (see Section 2.1). On the other hand, when the applied potential was of −0.65 V, the fraction of reduced cytochromes was about 0.9 in the absence of acetate, and slightly higher when the electron donor was present (Fig. 2). The above results demonstrate that in the case of *Geobacter*, it is possible to electrochemically influence the whole biofilm and even reach cells located tens of microns away from the electrode, as previously observed by Virdis et al. [15] on a mixed population biofilm. It constitutes from our point of view, a direct probe of the full electric connexion exhibited by the biological population, with the exception of a minor pool of cytochromes presumably located at the cell interior, which cannot be accessed from the electrode at least within polarization times implemented in this study.

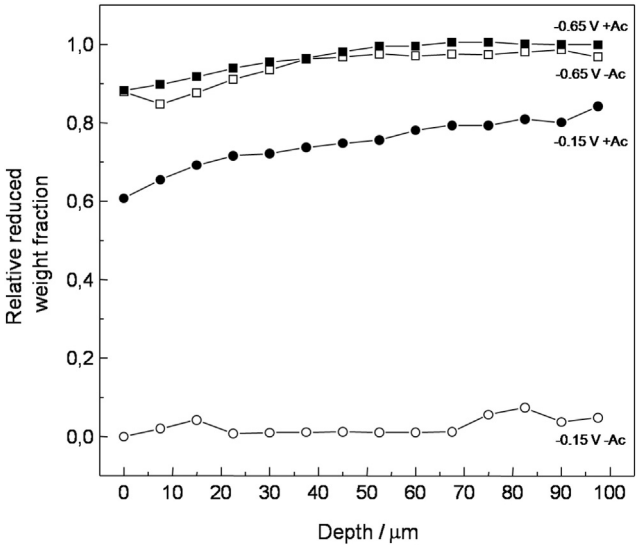

Fig. 2. Evolution of the fraction of reduced species with the distance to the electrode in biofilms polarized at −0.65 (squares) and −0.15 (circles) V in presence (full symbols) or absence (empty symbols) of the electron donor acetate (Ac), as calculated from Raman spectra by component analysis (see text). Raman spectra of each condition are presented in Fig. S4.

4. Conclusions

The confocal Raman-based strategy reported here made possible to prove *in-vivo* the connectivity in electricity-producing biofilms. The ability of *G. sulfurreducens* to develop onto transparent conducting glasses allowed the assessment of the physiological state of cells at any location within the biofilm, with the culture cell fully operational. Although a depth resolution in measurement condition of about 15 μm may appear a bit large when compared with diffraction-limited resolutions, typically below 2 μm, it is enough to resolve biofilms features that develop over distances of tens of microns.

The biofilm spectral profile is mostly dominated by the resonant Raman scattering of cytochrome structures. Only a part of these forms in the biofilm is accessible for electrochemical conditioning, presumably because of its cell external localization. Despite of this, the spectral contrast was enough to resolve the biofilm redox state and to put on a quantitative basis this observation, a fact that will definitively stimulate further investigation on the influence of factors such as, pH, temperature, ionic strength and availability of soluble electron acceptor, among others, on biofilm physiology and electrogenic activity.

The redox state of cytochromes was analysed in different levels of the biofilm and with electrode potentials ranging from oxidizing to reducing values and in presence or absence of electron donor. The redox changes observed all along the biofilm thickness upon changes in

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polarization potential are almost as deep as those chemically induced (complete oxidation and reduction) and confirm the connection of almost all cytochromes in the biofilm network to the electrode. Total oxidation or reduction by electrochemical conditioning indicates that electron flow across the external matrix of a *Geobacter* biofilm is not unidirectional, but depends on the relative potentials of the biological material and the electrode.

In a broader context, the confocal Raman technique appears very well suited to selectively and non-invasively monitor conformational transition induced electrochemically through the applied potential, providing relevant information of biological systems, which is definitively helpful to improve our understanding on mechanistic aspects that control electron conduction in these systems. In a wider sense, the experimental platform emerges as a versatile tool in analyzing time and polarization-dependent processes, which may open the avenue for new implementations in various fields, including biology, medicine or the investigation of conducting materials.

Acknowledgements

This work was prepared having in mind the curiosity and creativity aspects that defined the work by Dr. Antonio Aldaz, to whom we identify as a leading mind and a superlative advisor for our work. The work was sponsored by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jelechem.2016.11.005.

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Please cite this article as: L. Robuschi, et al., Proving *Geobacter* biofilm connectivity with confocal Raman microscopy, Journal of Electroanalytical Chemistry (2016), http://dx.doi.org/10.1016/j.jelechem.2016.11.005