Spectroelectrochemical analyses of electroactive microbial biofilms

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Abstract
Understanding the mechanism of ET (electron transfer) through electroactive microbial biofilms is a challenge in the field of fundamental and applied life sciences. To date, electrochemical techniques such as CV (cyclic voltammetry) have been applied successfully to study the ET process in intact microbial biofilms on electrodes, providing important insight into their redox properties. However, CV as such does not provide any structural information about the species involved in the redox process. This shortcoming may limit the understanding of the ET process in microbial biofilms. To overcome this restriction, spectroelectrochemical techniques have been designed consisting of a spectroscopic technique performed in combination with electrochemical methods on the same electrode sample. These analytical approaches allow in vivo measurements of microbial biofilms under physiologically relevant conditions and controlled applied potential. The present review describes these spectroelectrochemical methodologies and critically addresses their impact on the understanding of the ET through biofilms.

Introduction
BESs (bioelectrochemical systems) use living microorganisms as catalysts in electrochemical reactions [1]. The best known BES is the MFC (microbial fuel cell), a device using metal-respiring bacteria to convert the chemical energy of microbial substrates dissolved in wastewater into electrical energy [2]. The bacteria, forming a multilayered aggregation (i.e. a microbial biofilm) attached to the electrode, transfer electrons derived from substrate oxidation to the electrode (anode).

The ability of metal-respiring bacteria to reduce insoluble electron acceptors which cannot enter the cell (e.g. electrodes) requires an efficient ET (electron transfer) apparatus shuttling electrons across the outer membrane of the microbial cell to the electrode. To overcome the physical barrier of the outer membrane, metal-respiring bacteria have evolved several ET strategies, including MET (mediated ET) and DET (direct ET). In MET, soluble redox mediators are used for shuttling electrons between the bacteria and the electrode. In DET, bacteria establish a direct electrical contact with the electrode. This contact has been proposed to occur either through conducting proteinaceous nanowires or through multihem redox proteins denoted as OMCs (outer membrane cytochromes) located on the outer membrane of the micro-organisms [3,4] (Figure 1).

Despite the importance of these proteins to promote the ET that regulates the microbial physiology in BESs, little is known about their structural and redox properties. This is probably due to the lack of an experimental method able to provide structural information about these proteins in situ (i.e. on catalytically active biofilms grown on electrodes).

CV (cyclic voltammetry) is probably the most widely used analytical technique to investigate the ET process in electroactive microbial biofilms. The voltammetric signal of an intact biofilm can be recorded under turnover and non-turnover conditions (i.e. in the presence and absence of the substrate respectively). The former experiment affords the sigmoid-shaped trace typical of a catalytic process (i.e. substrate oxidation), whereas the latter probes the oxidation and reduction reactions of the redox centres embedded within the biofilm. The analysis of CV traces has brought substantial information about the ET process, such as the formal potentials of the redox couples involved in the ET [5]. Moreover, the analysis of the shape of the CV trace under turnover conditions has allowed a model describing the nature and the rate of the ET within microbial biofilms to be elaborated [6]. Unfortunately, CV as such cannot provide any insight into the molecular structure of the species involved in the redox process. However, coupling CV with spectroscopic techniques overcomes this limitation. This analytical approach, which has been often applied to study isolated proteins and enzymes attached on electrodes [7], is also applicable to microbial biofilms.

The present review is aimed at introducing the reader to the spectroelectrochemical methodologies used to study electroactive microbial biofilms, and shows how these approaches have provided important information complementary to those obtained by electrochemical methods.

Key words: cyclic voltammetry (CV), microbial biofilm, spectroelectrochemistry, surface-enhanced IR-absorption spectroscopy, UV-visible spectroscopy.

Abbreviations used: 
ADG, six-co-ordinated low-spin ligation; ATR, attenuated total reflection; BES, bioelectrochemical system; CV, cyclic voltammetry; DET, direct electron transfer; ET, electron transfer; EW, evanescent wave; F/O, indium tin oxide; MET, mediated ET; OMC, outer membrane cytochrome; RR, resonance Raman; SERR, surface-enhanced resonance Raman; UV-visible spectroscopy.

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Methodology
To date, spectroelectrochemical analyses of microbial biofilms have focused on the redox and structural properties of the OMCs. Accordingly, the description of each spectroscopic technique focuses on the relevant information that can be obtained for haem proteins.

Vibrational spectroscopies: SERR and SEIRA
SERR (surface-enhanced resonance Raman) spectroscopy exploits the combination of the SER (surface-enhanced Raman) and the RR (resonance Raman) effects. The former is responsible for the enormous enhancement of the Raman scattering achieved if a molecule (e.g. a haem protein) is close to an appropriately nanostructured metal surface; the latter applies when the frequency of the incident light is close to an electronic transition of a chromophore (e.g. the haem group). SERR spectroscopy probes almost exclusively the chromophore of the adsorbed molecules, since the non-resonant contributions of the apoprotein are very weak. This approach has been successfully applied to haem proteins adsorbed on silver electrodes in the submonolayer concentration range [7], and recently also to BESs, to probe the surface-confined OMCs embedded in microbial biofilms [8] (Figure 2A). Importantly, owing to the dramatic distance-dependence of the surface enhancement, SERR spectroscopy probes solely the proteins adjacent to the electrode (i.e. within 7 nm of the electrode surface) [9].

The structural information readily available from a SERR spectrum of a haem protein is the oxidation state, the coordination state and the spin state of the central iron ion. Detailed spectra analysis reveals also the nature of the axial ligands (water, histidine, methionine, lysine, etc.) and the protonation state of the haem propionates.

Similarly to SERR spectroscopy, SEIRA (surface-enhanced IR-absorption) spectroscopy exploits the surface enhancement of a nanostructured metal surface deposited on to an optical element (e.g. a gold film on a silicon prism). In a set-up operating in the ATR (attenuated total reflection) configuration, the IR radiation is focused on one side of the optical element (Figure 2B). Above a critical angle, the IR radiation is totally reflected. However, at the silicon–gold interface, the IR radiation penetrates to some extent through the gold layer, generating an EW (evanescent wave) that is absorbed by the molecules in contact with the gold film. SEIRA probes the vibrational modes that involve changes of the dipole moment component perpendicular to the gold surface. The surface enhancement is smaller than in the case of SERR spectroscopy (10^3 compared with 10^6); however, the attenuation of the enhancement with increasing distance from the surface is less pronounced than in the case of SERR spectroscopy, allowing for the detection of molecules placed at longer distances from the electrode surface (up to 10 nm) [10,11].

In the case of a protein adjacent to the gold film, the most prominent bands in a SEIRA spectrum are the amide I and II modes, primarily originating from the C=O and the C–N stretching of the peptide bond respectively. In contrast with SERR spectroscopy, where the spectrum of the chromophore can be selectively enhanced over all of the other vibrational modes (e.g. the protein backbone), SEIRA spectroscopy is a less sensitive and selective technique [10,11]. However, the lack of selectivity may be advantageous, since SEIRA is also suitable to study systems that do not include a chromophore, and is therefore applicable to a larger variety of target molecules (see below).

SEIRA spectroscopy is often performed in the difference mode, i.e. by subtracting the spectrum obtained at a certain applied potential from a reference spectrum obtained at a different potential. In this way, the bands in the difference spectrum can be readily ascribed to potential-induced conformational changes (see Figure 2 in [12]). This strategy allows the removal of the contribution of water to the spectrum and is furthermore sufficiently sensitive to probe...
potential-induced conformational changes at the level of a single peptide bond [13].

Absorption spectroscopy: UV–visible
UV–visible spectroscopy probes transitions between electronic states of a molecule. The large molar absorption of haem groups renders OMCs an ideal target molecule for UV–visible spectroscopy. As in the case of RR and SERR spectroscopy, the UV–visible spectrum of the OMCs is largely dominated by the haem contribution. The strong UV–visible absorption bands of the haem that originate from the $\pi \rightarrow \pi^*$ transitions yield information about the type of haem, the oxidation and the spin state of the central iron ion. In combination with electrochemical techniques, one can either grow a microbial biofilm on to an optically transparent electrode [e.g. ITO (indium tin oxide)], or operate in the EW mode (Figures 2C and 2D respectively). The former case probes all the OMCs present in the biofilm, without distinction between surface-confined (i.e. adjacent to the electrode) and species remote from the electrode. Moreover, also contributions arising from cytochromes in the periplasm or in the inner membrane cannot be safely ruled out [14]. In the latter case (i.e. the EW mode), the biofilm placed on an ITO-coated optical waveguide is irradiated by the incident light. The penetration depth of the evanescent electric field is wavelength-dependent and it has been estimated to be 110 nm for a decay of the electric field to $1/e$ at a wavelength of 400 nm [15]. This experimental approach can reveal the presence of surface-confined species at submonolayer coverage [16], and, in the case of microbial biofilms, it has been used to probe the UV–visible spectrum of the surface-confined OMCs [15,17]. However, owing to the large penetration depth of the UV–visible radiation, also in this case, other cytochromes inside the cell, as well as more remote OMCs, may contribute to the spectrum.

Importantly, all of the methodologies described above are non-invasive and, if performed under controlled experimental conditions that do not damage any component of the biofilm, are suitable for in vivo measurements of biofilms under physiologically relevant conditions.

Structural properties of the OMCs
Several UV–visible spectroscopic studies have shown that the haem groups of the OMCs are low-spin $c$-type cytochromes. This configuration is maintained in both oxidation states detected under potential control (i.e. Fe$^{2+}$ and Fe$^{3+}$), and it has been observed for both bulk [14,15,17,18] and surface-confined OMCs [15,17]. Importantly, this ligation pattern seems to be a common feature for different bacterial
Despite substantial research efforts, the OMCs promoting the ET across the bacteria–electrode interface have not yet been unambiguously identified. However, recent findings aiming to identify the spatial disposition of OMCs within biofilms of *G. sulfurreducens*, have shown that OmcZ accumulates in the vicinity (<0.5 μm) of the electrode surface, suggesting a possible role for this protein in the heterogeneous ET [19]. Despite the lack of a comprehensive structural characterization of OMCs, the spectroelectrochemical data obtained so far are in agreement with recent NMR and crystallographic data obtained for the decahaem conduit MtrF of *Shewanella oneidensis*, featuring c-type haems with a bis-histidine axial ligation [20]. In the case of mixed cultures dominated by *Geobacter* species, SERR spectroscopic measurements can exclude the presence of the soluble part of OmcF in the vicinity of the electrode surface as well as its involvement in the heterogeneous ET process. In fact, the soluble part of OmcF exhibits a histidine/methionine axial ligation that is not compatible with SERR spectroscopic data [21].

**Redox properties of the OMCs**

Spectroelectrochemical experiments of electroactive microbial biofilms have clearly shown variations of the oxidation state of the OMCs in response to the applied potential [8,12,14,15,17,18,22]. Accordingly, the redox changes detected by CV have been complemented by direct spectroscopic evidence acquired from probing the concomitant change in the redox state of the cytochromes. To date, this is the most convincing evidence for the involvement of OMCs in transporting electrons through microbial biofilms [14,18] and across the biofilm–electrode interface [8,12,15]. This finding allows redox titrations of the OMCs to be performed. Such experiments are based on recording the spectrum of the biofilm at a constant poised potential. Spectral analysis affords the relative amount of reduced and oxidized species present on the electrode at each applied potential. By plotting a quantity proportional to the amount of the oxidized and/or the reduced species as a function of the potential, a sigmoid-shaped curve is obtained (Figures 3, S2, 7B, 2B and 6 in [8], [12], [14], [15] and [18] respectively). The analyses on such plots can be carried out by fitting the curve with one [8,18] or a linear combination of two [8] Nernst equations to extract the contributions of individual redox species. These contributions are probably the macroscopic potentials of OMCs rather than the formal potentials of individual haem groups [8]. This approach provides the formal potential(s) ($E_{1/2}$) and the stoichiometric number of electrons transferred ($n$). Since the redox reaction is the one-electron reduction of Fe$^{3+}$ to Fe$^{2+}$, an $n$ value close to 1 is a good indication of the accuracy of the fitting procedure [8]. An alternative method to obtain the formal potential(s) from the sigmoid-shaped trace of a redox titration is based on calculating its first derivative [14,18]. The maximum in the derivative plot corresponds to the inflection point of the sigmoid-shaped curve, and therefore to the formal potential. Admittedly, lacking direct

![Figure 3](image.png)

**Figure 3 | Correlation between the CV trace and the spectroelectrochemical redox titration**


strains, since similar results have been obtained for biofilms of *Geobacter sulfurreducens* grown on ITO electrodes [14,18] and cell suspensions of *Shewanella loihica* PV-4 deposited on gold and ITO electrodes [15,17].

SERR spectroscopy has provided more detailed information by identifying also the nature of the axial ligands of the haem groups of surface-confined OMCs in biofilms dominated by *Geobacter* species grown on electrodes. SERR data have proven that the haem groups of surface-confined OMCs are c-type haems, exhibiting a 6cLS (six-co-ordinated low-spin ligation) with two histidine residues acting as axial ligands. No other species with different co-ordination patterns or spin states have been detected in the entire potential range that has been studied [8].
Comparison between the formal potentials obtained by CV and spectroscopic methods in different combined spectroelectrochemical approaches

$E_{1/2}$ CV and $E_{1/2}$ spectroscopy represent the midpoint potentials obtained from CV and spectroscopic measurements respectively in the same work. All potentials refer to the SHE (standard hydrogen electrode). When the potentials reported in the original papers are referred against a different reference electrode, the conversion into the SHE has been made by adding 244 and 210 mV to the potential expressed compared with the SCE (saturated calomel electrode) and the Ag/AgCl (3.0 M KCl) electrode respectively. The numbers in parentheses in the $E_{1/2}$ spectroscopy column represent the $n$ values (when available). $G_s$ and $S_l$ denote $G$. sulfurreducens and $S. lohia$ PV-4 respectively. $G_s'$ denotes a microbial biofilm obtained with wastewater as inoculum and whose electrochemical behaviour closely resembles that of $G_s$. Grown and suspended indicate whether the biofilm has been electrochemically grown on the electrode or whether it was obtained by washed and resuspended cells deposited on the electrode respectively.

<table>
<thead>
<tr>
<th>Spectroscopy</th>
<th>$E_{1/2}$ CV</th>
<th>$E_{1/2}$ spectroscopy</th>
<th>Biofilm</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERRS</td>
<td>−72</td>
<td>−85 (0.9)</td>
<td>$G_s'$, grown</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>−153</td>
<td>−157 (0.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEIRA</td>
<td>370</td>
<td>380</td>
<td>$G_s$, deposited</td>
<td>[12]</td>
</tr>
<tr>
<td>EW UV–visible</td>
<td>145*</td>
<td>145*</td>
<td>$S_l$, deposited</td>
<td>[15]</td>
</tr>
<tr>
<td>UV–visible</td>
<td>−60†</td>
<td>−150</td>
<td>$G_s$, grown</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>−110†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV–visible</td>
<td>−114‡</td>
<td>−176 (7.5)‡</td>
<td>$G_s$, grown</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>−169‡</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*The authors estimated $E_{1/2}$ from the redox titration and CV experiments. Therefore they did not provide the individual $E_{1/2}$ values obtained with different techniques.

†These values have been estimated from the CV plot in Figure 7(A) in [14].

‡Although Jain et al. [18] reported more values, the Table includes only those redox centres obtained from the non-turnover CV that contribute to the catalytic ET at the electrode interface.

Jan et al. [18] interpreted this value in terms of the level of co-operativity between different haem entities within the cytochromes $c_{552}$ rather than as the number of electrons involved.

Table 1 | Comparison between the formal potentials obtained by CV and spectroscopic methods in different combined spectroelectrochemical approaches

Information on the $n$ value, this method is less informative than the former approach. However, in both cases, good practice suggests to compare the formal potentials obtained by CV with those obtained by a spectroelectrochemical redox titration (Table 1).

SERR experiments on biofilms dominated by Geobacter species have shown that the heterogeneous ET across the bacteria–electrode interface involves two different haem species having identical spectral signature (i.e. the bis-histidine 6cLS), but different formal potentials. These observations are in full agreement with CV measurements, featuring two redox couples centred at the same formal potentials as found in the potential-controlled SERR experiment (Table 1) [8]. Analogous experiments performed by UV–visible spectroscopy displayed a similar behaviour, although the accurate identification of the individual redox transitions observed in the CV trace has not been as unambiguous as in the case of the SERR measurements. In fact, in two independent studies, the first derivative of the sigmoid plot obtained by the UV–visible redox titration under non-turnover conditions has produced a broad curve with one maximum only [14,18]. This result differs from the plot obtained by CV, revealing two prominent voltammetric couples (Figure 3). Since these CV couples have been proven to belong to one or more surface-confined OMCs [8], these discrepancies between UV–visible and CV measurements may reflect the different type of information provided by the two techniques. In fact, whereas the UV–visible signal at low scan rates arises from the OMCs inside the biofilm [14], CV probes primarily the surface-confined OMCs [8,18]. Thus being aware of the type of information accessible by the various techniques (i.e. the surface-confined, the periplasm or the bulk OMCs) is essential for comparing results by different methodological approaches (e.g. CV and spectroscopy) and, furthermore, a prerequisite to gain a deeper insight into the spatial organization of OMCs inside the biofilm.

Perspectives

Spectroelectrochemical analyses have enlarged our understanding of the ET in microbial biofilms. However, despite significant progress in this field, these analytical methodologies applied to electroactive microbial biofilms are still in their infancy. In fact, the spectroelectrochemical approaches have been used to probe mainly the thermodynamics (i.e. the formal potentials) of OMCs [8,12,14,15,18]. Admittedly, this is the first step to test the applicability of these analytical methodologies to microbial biofilms. The next step will be to extend these methodologies to the time-resolved domain in order to study the kinetics of the ET. Time-resolved spectroscopies can monitor an electrochemical reaction (triggered, for instance, by a potential jump) in the sub-millisecond timescale [23]. To date, the only example of a spectroelectrochemical time-resolved study on
microbial biofilms has been done by using EW–UV–visible spectroscopy [24]. Along with the kinetic insight provided by time-resolved experiments, further information may arise by combining surface-enhanced with non-surface-enhanced spectroscopies (e.g. SERR and RR). This combination may help to discriminate between processes of surface-confined and bulk OMCs, providing a more detailed picture about the ET within microbial biofilms.

Besides the OMCs, the spectroelectrochemical approaches can also probe other molecules participating in the ET process, such as the soluble redox mediators in biofilms of *Shewanella* species and the conducting nanowires. In fact, many molecules proposed to act as redox mediators, such as flavins, phenazines and anthraquinone, are UV-visible, Raman- and RR-active, and thus can be studied by these spectroscopies or by fluorescence spectroscopy in combination with electrochemistry. The conducting nanowires may possibly also be monitored by SEIRA spectroscopy, if they are in sufficiently close vicinity of the electrode.

In addition to molecular structure and dynamics information provided by static and time-resolved spectroelectrochemical techniques, insight into the spatial organization of the redox components in biofilms is highly desirable, which would require implementation of spectroelectrochemical techniques in imaging approaches. So far, established bioimaging approaches are not sensitive enough for molecular structure information and, furthermore, have usually not yet been applied under potential control in an electrochemical environment. In this respect, the recent study by Virdis et al. [25] is of particular interest since the work represents the first Raman imaging characterization of a microbial biofilm under controlled potentials.

In conclusion, spectroelectrochemistry of electroactive microbial biofilms has provided unprecedented structural and thermodynamic information on the species involved in the ET in microbial biofilms. Owing to the outstanding complexity of this process, such analytical approaches are strongly recommended to support the electrochemical observations as they provide important complementary insights. These spectroelectrochemical approaches will substantially increase our understanding of the ET in microbial biofilms.

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**References**


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