Electrochemical Impedance Spectroscopy on biofilm electrodes – conclusive or euphonious?

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Abstract: Electrochemical impedance spectroscopy (EIS) is a versatile tool that is also exploited to study bioelectrochemical systems and biofilm electrodes. EIS can be used to examine characteristics of biofilm electrodes, which are not accessible by direct current measurements like biofilm resistance and biofilm capacitance. EIS in microbial electrochemistry is sometimes applied superficially or evaluation of presented data is not comprehensive due to misinterpretation or missing data validation. This hinders a more widespread application of this method, not only for determination of specific biofilm electrode parameters, but also from a more practical perspective, e.g. as tool for in situ condition monitoring of biofilm electrodes. We discuss how a careful choice of the experimental setup as well as extraordinary diligent EIS data interpretation using electrical equivalent circuit models can lead to conclusive data and meaningful insights. We illustrate the special challenges of studying biofilm electrodes on the example of graphite anodes. We provide an initial guidepost on how to use EIS on biofilm electrodes that requires several preconditions, careful choice of experimental parameters and, nearly mandatory for novices like us, the consultation of experienced operators of EIS.

Keywords

Microbial electrochemical technologies, Electroactive microorganisms, Exoelectrogens, Alternating current, Biofilm properties.

1. Why using EIS for the study of biofilm electrodes?

Microbial electrochemistry has developed from the periphery to the center of bioelectrochemistry. Microbial electrochemistry is the study, engineering and application of interactions of microbial cells with solid electron conductors (electrodes). For the versatile types of interaction please see Schröder et al 2015 [1]. Here we focus on the most-immediate interaction: biofilm electrodes. Biofilm electrodes are composed of electroactive microorganisms (EAM) embedded in (their self-produced) matrix of exopolymeric substances (EPS) on the surface of the electrode. The electrode serves either as terminal electron acceptor (biofilm anodes) or as electron donor (biofilm cathodes). Biofilm electrodes are the beating heart of primary microbial electrochemical technologies (MET). MET are a fascinating technology platform [1,2] departing from its archetype the microbial fuel cell (MFC) [3,4] and recently moving towards building electronic circuits based on microbial wires [5].Using biofilms electrodes in primary MET, e.g. biosensors [6,7], does not only require comprehensive knowledge of their electrochemical and metabolic performance as well as their ecology [8]. It also creates the need of in situ monitoring of physical integrity and functionality [9].

Biofilm electrodes are assessed at different hierarchical levels (subcellular – cellular – biofilm) using a whole arsenal of techniques [10]. These include electrochemical methods. Most popular are direct current (DC) methods, e.g. chronoamperometry (CA) and cyclic voltammetry (CV) [11]. Using DC methods always mixtures of non-faradaic current and faradaic current, including pseudo-capacitive currents, e.g. caused by excess electrons stored in the outer membrane cytochromes of *Geobacter* spp. biofilms, are recorded as well as only the sum of overpotentials. Distinction of different overpotentials and their causation, e.g. diffusion, activation or resistance, is highly tedious or even impossible. Electrochemical impedance spectroscopy (EIS) uses alternating current (AC) and thereby enables to obtain properties difficult to determine otherwise [12]. EIS allows to detangle ohmic as well as frequency dependent resistance that is the impedance Z. Furthermore, using EIS the capacitive (ZC) and inductive (ZI) nature as well as diffusion processes (e.g. Warburg impedance ZW) can be examined [13]. Thus, EIS is a helpful method that can provide unprecedented insights into electrode properties. EIS is not only applied, for instance on lithium-ion batteries or fuel cells, but also for biosensing and biofilm electrodes [14,15]. However, its application is not straightforward and needs an experienced operator, but especially knowledge on the electrochemical system under study. This combination seems to be rare when EIS is applied on biofilm electrodes. At the same time, highly valuable work exists that can be used as a foundation for the increased meaningful application of EIS on biofilm electrodes. Here we like to provide an overview on important basics of EIS in general as well as main aspects that specifically have to be considered when applying EIS on biofilm electrodes. Within this article we intend to provide a “guidepost” that helps interested researchers as well as newcomers in the field of EIS to find their way to conclusive application of EIS on biofilm electrodes.

2. A brief introduction into EIS and its application on biofilm electrodes

This article solely refers to EIS in the frequency range using potential perturbation. Therefore, a small ΔE is applied at a defined frequency range and the current response I_t is measured. The application of galvanostatic EIS, potential step functions, random signals or advanced EIS measurements in the time domain [16,17] are not subject of this article, as these have not yet been applied on biofilm electrodes to the best of our knowledge.

EIS is based on recording the current response I_t to a sinusoidal potential perturbation stimulus E_t (see also BOX1). The impedance spectrum is obtained by sweeping E_t at constant ΔE through a frequency range, being for biofilm electrodes usually in the mHz to MHz range. Two types of graphs are commonly used to visualise and interpret EIS data [18]. The Nyquist Plot (or Complex Plane Plot, see Figure 1a) shows the imaginary part ($Z_{Im} = i|Z|sin\varphi$) over the real part ($Z_{Re} = |Z|cos\varphi$) of the impedance, whereas the Bode plot (Figure 1b), shows the absolute magnitude of the impedance IZI, also called modulus, and the phase shift φ at two different Y-axes over the $\log_{10}$ of the applied frequencies.

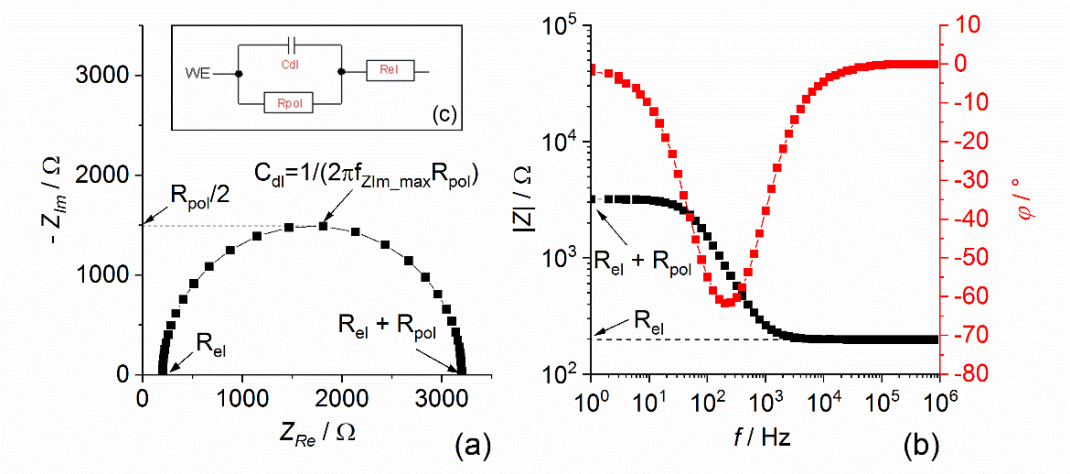


Figure 1: Idealised EIS data of an electrode immersed into an electrolyte with (a) Nyquist plot and (b) Bode Plot. The system is modelled with (c) an electrical equivalent circuit with two resistors in series and a capacitor in parallel, WE: Working electrode C_{dl}: Capacitance of the electrochemical double layer, R_{pol}: Polarisation resistance of the electrode, R_{el}: Electrolyte resistance (the value depends on the distance between working electrode and reference electrode), f_{ZIm_max}: frequency at maximum Z_{Im} (own data, J. Kretzschmar, PhD thesis, 2017).

In the following, we highlight the challenges especially associated to the experimental setup for EIS on biofilm electrodes. Specific data analysis such as model based evaluation of EIS data or data validation can be covered only limitedly and the reader is referred to textbooks [13,18,19] or specific literature, e.g. [20,21]. Evaluation of artefacts in EIS, e.g. caused by induction, are discussed, e.g. by Veal et al. [22].

BOX1 Basics of Electrochemical Impedance Spectroscopy

The Impedance Z can be described analogously to Ohm's law:

$$Z = \frac{E_t}{I_t} = \frac{\Delta E \sin(2\pi f t)}{\Delta I \sin(2\pi f t + \varphi)} \qquad \text{Eq. 1 [19]}$$

E: potential $[V]$, I: current $[A]$, $2\pi f = \omega =$ angular velocity $[rad\ s^{-1}]$, t: time $[s]$, φ: phase shift $[rad]$ or [°], E_t and I_t represent E and I as function of time. E_t is applied as AC potential perturbation at constant ΔE while I_t is measured.

The phase shift φ relates the sinusoidal wave of I_t and E_t. I_t can be leading (being ahead of) or lagging (being behind of) $E_t$in the phasor diagram. The phase shift φ is depending on the properties of the electrochemical system (leading corresponds to capacitance, lagging to inductance). To describe this

relationship for a sinusoidal AC signal over time, Z needs to be represented as a complex function and therefore, consists of a real part (Z_{Re}) and an imaginary part (Z_{Im}) described by $i = \sqrt{-1}$ or $i^2 = -1$.

$$Z = Z_{Re} - iZ_{Im} \qquad \text{Eq. 2 [19]}$$

Electrical circuit elements being purely imaginary such as capacitors or inductors, show $\varphi = \pm 90° \left(or \pm \frac{\pi}{2} rad\right)$ whereas purely ohmic resistors show $\varphi = 0$ (only Z_{Re}). For capacitive elements, e.g., the electrochemical double layer, the phase shift φ between E_t and I_t is related to charge storage. The most simple example is a capacitor with $\varphi = +90°$. Here, the capacitor stores charge during half the time of one oscillation period of E_t and releases charge during the other half. To conclude, depending on the properties of the electrochemical system, I_t resulting from E_t does not only differ in terms of the amplitude but also in terms of φ.

BOX2 Prerequisites for EIS on electroactive biofilms

Meaningful EIS requires **i) linearity, ii) time invariance (stability) with iii) guaranteed causality and iv) finiteness of Z** for the applied frequencies, f. Linearity means that the relation of the potential stimulus and the current answer of the biofilm electrode must be linear and is independent from the amplitude of the stimulus. For electrochemical systems that are usually highly non-linear this is only true for small potential perturbations of $\Delta E \approx 10\,mV$, as $\Delta E < 10\,mV$ worsen the signal to noise ratio, whereas $\Delta E > 10\,mV$ increases the risk to leave the pseudo-linear region. Time invariance means that the systems remains stable until perturbation and returns to its initial state after perturbation. Causality means that the current response of the system is strictly determined by the applied potential perturbation. Finiteness means that there is always a response (I_t) of the system to the applied stimulus [23]. Biofilm electrodes fulfil these preconditions to a limited extend, as especially time invariance is difficult to achieve over several hours.

3. Preconditions and consequences for experimentation and data evaluation

EIS on biofilm electrodes follows the same steps like in other fields, that are i) a setting up a suitable experimental system, ii) choice of parameters (e.g. ΔE and range of f), iii) data evaluation and iv) data validation [14,23]. In the following, we focus on challenges of EIS measurement being specific for biofilm electrodes. A comprehensive overview on using EIS for MET is given by Yoho et al. [23].

Experimental setup

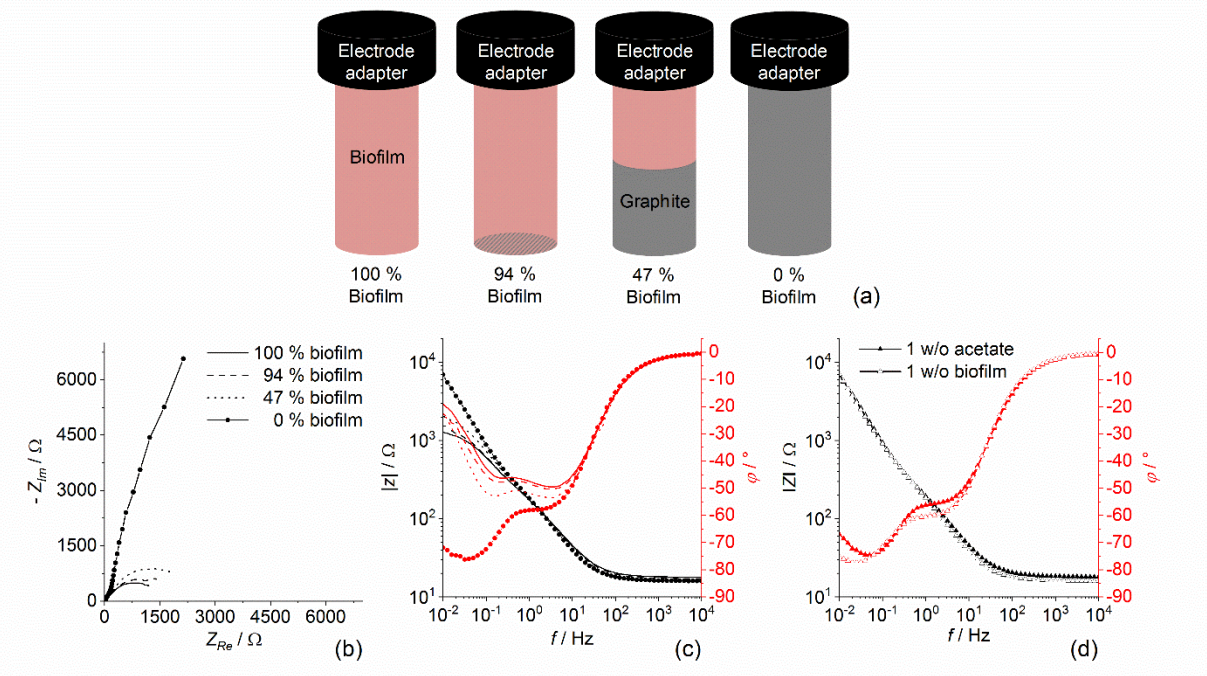
Electrode size, shape and material have a substantial impact on EIS data. For biofilm electrodes carbonaceous materials are very common due to their biocompatibility, chemical and microbial stability and low price [24]. Unfortunately, carbonaceous materials such as graphite felt or graphite rods possess a high capacitance (~1 mF cm^{-2}) as already shown by ter Heijne et al. 2015 [25] that influences the accurate determination of biofilm properties such as charge transfer, biofilm and diffusion resistances, and biofilm capacitance as a measure of biofilm thickness or mass [25,26]. The challenge of using graphite electrodes to determine specific biofilm properties is exemplarily shown in Figure 2 a-c. Here, fractions of mature *Geobacter* spp. dominated biofilm were successively removed from a monolithic graphite anode ($A = 3.34\ cm^{-2}$) implemented in a flow cell ($V = 100mL$) while all other parameters remained constant, i.e. temperature, substrate concentration ($c = 2mmol\ L^{-1}$ acetate), anode potential ($E_a = 0.399\ V\ vs.SHE$) and stirrer speed ($250\ rpm$). The Nyquist plot (Figure 2 b) and the Bode Plot (Figure 2 c) show results of EIS using $\Delta E = 10\ mV$ and a range of $f =$

$10\ \mathrm{mHz} - 10\ \mathrm{kHz}$. It is evident that successive removal of biofilm changes the impedance only to a limited extent. Total biofilm removal, however, may indicate a massive contribution of capacitance of the naked graphite electrode that is shown by the wide opened arc in Figure 2 b. Furthermore, when removing the biofilm partially, capacitance seems to increase due to the increasing diameter of the small arcs in Figure 2 b. This seems counterintuitive, as biofilm capacitance should decrease during biofilm removal. Another explanation might be that without biofilm, the data shows "infinite" charge transfer resistance of the naked graphite electrode. If a biofilm is present, a finite charge transfer resistance is introduced that decreases with increasing biofilm coverage and therefore, a semi-circle appears.

Figure 2 d shows the effect of removal of acetate (sole energy and carbon source) from the electrolyte solution for an electrode fully covered with biofilm compared to an electrode without biofilm. Here, it seems that limiting the metabolism of the biofilms has apparently a similar impact as total removal of the biofilm. This could be a consequence of the dominating capacitance of the graphite electrode, changed diffusion regimes or increased charge transfer resistance due to reduced metabolic activity.

One possible way to overcome, e.g. limitations induced by the capacitance of electrode backbones, is the use of materials with low capacitance but sufficient biocompatibility. Ter Heijne et al. 2018 [26] successfully used Fluorinated Tin Oxide as anode material to identify capacitance of biofilm anodes with up to 450 µF cm^{-2} as a measure of biofilm growth.

Mass transport and especially diffusion is also influencing. As often stirring is used to prevent mass transfer limitations in solution, diffusion at and in biofilm electrodes also plays a crucial role when interpreting EIS data [25,27,28]. Noteworthy, from a practical perspective stirring may interfere with the measurement by compromising the causality, e.g., due to induction caused by magnetic stirrers [23]. EIS data displays different types of diffusion: i) semi-infinite linear diffusion that appears as a 45° line in Nyquist plots (i.e. the Warburg impedance ZW), ii) finite space diffusion that appears as 45° line followed by vertical line in low frequency regions and iii) finite length diffusion that appears as 45°line followed by a half circle in low frequency regions of a Nyquist plot, for details please see, e.g. [23,29] (another convenient overview is: Matt Lacey – Battery Science and Electrochemistry, URL: http://lacey.se/science/eis/diffusion-impedance/). Using stirring, EIS data of biofilm electrodes may furthermore show transition from semi-infinite linear diffusion to finite length diffusion. However, using a rotating disc electrode Babauta and Beyenal [27] showed that diffusion in biofilms is only rarely affected by mass transport in solution. To conclude, EIS is very suitable for evaluating mass transfer effects at biofilm electrodes, in biofilms or porous electrodes whereby systematic studies on this topic are rare.



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173 **Figure2:** Effect of biofilm removal from graphite rods and change of substrate concentration for fully
174 intact biofilms on EIS data, (a) scheme of successive biofilm removal with pink parts showing biofilm
175 and grey parts showing the graphite electrode, (b) Nyquist Plot and (c) Bode Plot of successively
176 removing *Geobacter* spp. dominated biofilms at constant substrate concentration (2 mmol L^{-1} acetate),
177 (d) combination of complete biofilm removal and complete substrate (acetate) removal at fully intact
178 *Geobacter* spp. dominated biofilm electrodes, red data in (c) and (d) shows phase shift φ of I_t. (own
179 data, J. Kretzschmar, PhD thesis, 2017).

180 **3.1. Choice of parameters**

181 EIS on biofilm electrodes is mostly performed with a potential perturbation at a set electrode potential
182 to sustain metabolic activity of the EAM. EIS at open cell potential (OCP) is also possible, but bares the
183 risk of instability that is losing time invariance. It is very difficult and takes at least hours to achieve
184 steady state conditions at OCP for *Geobacter* spp. dominated biofilm anodes.

185 Generally, ΔE between 10 -20 mV has been shown as suitable [23]. The frequency range defines the
186 duration of the measurement and hence, has a direct influence on achieving time invariance. This is of
187 special notice for measurements at OCP as well as biofilm electrodes facing non-constant substrate
188 concentration or substrate limiting conditions, e.g. in batch operation. Consequently, EIS on biofilm
189 electrodes should be limited to small frequency ranges and frequencies higher than 10 mHz. Careful
190 data validation (see paragraph 3.3) is required to assure time invariance as well as linearity and
191 causality.

192 **3.2. Model based evaluation**

193 EIS is analysed by fitting the acquired data to a mathematical model of an electrical equivalent circuit
194 (EEC) describing electrochemical processes in the system under observation. EEC models consist of a
195 limited number of electric circuit elements including capacitors and resistors as well as diffusion
196 processes and others [19,21,23]. This is challenging, especially when examining porous biofilm
197 electrodes in stirred experimental setups. Increasing the number of EES elements may improve the fit
198 but not the understanding of the system and it also increases the challenge of model validation.

Therefore, we need to differentiate two cases where EIS on biofilm electrodes can be applied. The first case is application of EIS to improve mechanistic understanding of electrochemical processes in biofilm electrodes. The second case is to use EIS as a tool for rather simple condition monitoring of biofilm electrodes, e.g. for sensors.

In the first case the prime option is using "white box models" that rely on validated physical equations of the examined electrochemical system. This is challenging for biofilm electrodes due to unknown constants and manifold functional dependencies and needs careful design of the experimental setup, as exemplarily shown in [26]. Several EEC models have been proposed for analysis of EIS data of biofilm electrodes, see e.g. [15,21,26]. Yet, these differ significantly and there is no "gold standard" that is generally applicable due to the heterogeneities of the experimental setups and biofilms. Therefore, it is of high importance to always publish the used EEC model to enable the verification of presented data. The values derived from EEC elements with direct physical meaning, e.g. resistor ($Z = R = \frac{E}{I}$) or capacitor ($Z_c = \frac{1}{i\omega C}$) are relatively easy to interpret. Versatile EEC elements such as the CPE ($Z_{CPE} = \frac{1}{(i\omega)^{\alpha} A_{CPE}}$) may enable fitting but have to be used with great care. The CPE parameters A and α are frequency independent constants. If $\alpha = 1$, A represents a pure capacitor with $\varphi = -90°$. For $\alpha = 0$ the CPE represents a resistor with $\varphi = 0°$ and for $\alpha = -1$ an inductor with $\varphi = 90°$ [21]. Furthermore, semi-infinite linear diffusion can be described with a CPE using $\varphi = 45°$ or $\alpha = 0.5$. This makes the CPE a versatile element. However, fitting the CPE in an EEC without further information may not allow to unravel detailed physical-chemical characteristics of the underlying processes. This disadvantage turns into an advantage in the second case, where EIS is applied for condition monitoring of biofilm electrodes. Here, the intention is not to decipher specific electrochemical processes but changes of these processes and therefore allows to apply CPE in rather unspecific "grey box models". Grey box models are easier to develop and still allow determination of structural or functional changes of the biofilm electrode such as the biofilm integrity (see Figure 2). Nevertheless, they are only applicable to one specific setup and do not reveal specific numbers of, e.g. diffusion constants, resistance or capacitance.

3.3. Data validation

When using EIS it is crucial to prove linearity, causality and stability of the system under observation (see BOX 2). The Kramers-Kronig (K-K) relation is used by default for validation of EIS data [13,23,30,31]. The K-K relation is the mathematical proof that the studied system fulfils the four requirements of EIS by calculating Z_{Re} from Z_{Im} and vice versa. There exist several methods to carry out K-K transformation in practice, some are described in [18]. Usually, software for EIS also contains a K-K function for data validation. However, K–K transforms are only mathematical results that do not reflect the real physical properties of the system [13] and are also sensitive for stochastic errors [18].

A much simpler but more robust alternative to the K-K transform is to perform a forward as well as backward scan of the applied range of f, meaning performing EIS from high to low and from low to high frequencies. Validity is confirmed in first approximation, if the results of the both scans are identical. Unfortunately, K-K transforms, simple frequency scans and even simple replicates are rarely reported for EIS on biofilm electrodes making it difficult to verify the published data.

4. Conclusions

EIS can be a valuable tool to study biofilm electrodes providing advantages over common DC methods. Several pitfalls during application of EIS on biofilm electrodes and data evaluation define the need of careful design of the experimental setup in order to avoid interference, e.g. by the electrode material (capacitance) or stirring (diffusion). Furthermore, definition of measurement parameters such as frequency range and electrode potential need care to assure linearity and stability. Here, frequency range, amplitude of the perturbation signal and applied electrode potential are of specific interest, as e.g. measurements at OCP requires time and careful experimentation to achieve steady state. Finally, we highly recommend performing validation of EIS data of biofilm electrodes. It is a necessity to improve and enhance the meaningful application of EIS in microbial electrochemistry. EIS allows to derive parameters such as biofilm capacitance, charge transfer resistance or diffusion at biofilm electrodes or in-situ monitoring of the biofilm integrity. For the latter it is worth to evaluate the applicability of grey box models or the use of indirect or contactless EIS for biofilm characterization as shown by Turick et al. [32]. Finally, we encourage to consider using EIS in a meaningful way to evaluate biofilm electrodes or MET in general, e.g. for monitoring membrane fouling [33].

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