Probing electron transfer with *Escherichia coli*: A method to examine exoelectronics in microbial fuel cell type systems

Marc Sugnaux, Sophie Mermoud, Ana Ferreira da Costa, Manuel Happe, Fabian Fischer

*Institute of Life Technologies, University of Applied Sciences and Arts Western Switzerland, Valais, Route du Rawyl 64, 1950 Sion, Switzerland*

**Highlights**

- Step-by-step methodology to detect electron transfer in microbial fuel cells.
- Extracellular electron transfer driven unequally by biofilm and by host medium.
- Anode potential of physically separated *E. coli* biofilms synchronised during growth.
- Medium generated currents overshadowed electron transfer from *E. coli* biofilms.
- Cultivation media stored virtual hydrogen and power, respectively.

**Graphical abstract**

**Abstract**

*Escherichia coli* require mediators or composite anodes for substantial outward electron transfer, >8 A/m². To what extent non-mediated direct electron transfer from the outer cell envelope to the anode occurs with *E. coli* is a debated issue. To this end, the redox behaviour of non-exoelectrogenic *E. coli* K12 was investigated using a bi-cathodic microbial fuel cell. The electromotive force caused by *E. coli* biofilms mounted 0.2–0.3 V above the value with the surrounding medium. Surprisingly, biofilms that started forming at different times synchronised their EMF even when physically separated. Non-mediated electron transfer from *E. coli* biofilms increased above background currents passing through the cultivation medium. In some instances, currents were rather high because of a sudden discharge of the medium constituents. Mediated conditions provided similar but more pronounced effects. The combined step-by-step method used allowed a systematic analysis of exoelectronics as encountered in microbial fuel cells.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

The native outward electron transfer from non-exoelectrogenic microbes such as *Escherichia coli*, yeast and others is considered insignificant in microbial fuel cells (MFC). Their cell envelopes contain no outer surface cytochromes or nanowires such as *Shewanella oneidensis* MR-1 (Gorby et al., 2006) or *Geobacter sulfurreducens* (Yi et al., 2009). Furthermore quantum mechanical electron transfer through the outer cell lipid bilayer is unlikely according to the Marcus equation (Marcus, 1993), as the membrane is too thick and the free enthalpy change –ΔG too small to allow electron transfer (kET) through this barrier. The outward electron transfer has been broadly investigated for exoelectrogenic microbes (Jiang et al., 2010; Bond and Lovley, 2005). The question to be asked is whether outward electron transfer by microbes is a broader phenomenon than previously thought. In this paper *E. coli* is discussed and investigated in detail for outward electron transfer as a representative of a non-exoelectrogenic microbe. Most studies report no or negligible outward electron transfer for *E. coli* (Logan, 2012; Cao et al., 2009). *E. coli* biofilms were found to be isolators and recorded currents were not higher than in the absence of a biofilm (Malvankar et al., 2011). In contrast, significant direct mediator
free electron transfer from *E. coli* was observed (Zhang et al., 2007, 2008), but questioned (Logan, 2012). Some contested and contrasting electron transfer measurements may be explained by differences in the methodology used and by other effects. Methodology is the subject of this study.

One of the other effects comprises mediator excretion, which was examined by cyclic voltammetry, but it remained uncertain to what extent medium components participated in the electron transfer (Qiao et al., 2008). An important recent discovery is that co-cultivations improve currents beyond expected levels. This is attributed to the fact that mediator producing microbes supply their extracted mediators to non-exoelectrogenic neighbours. As a consequence, co-cultivations provide a higher power output than the sum of the pure cultivations would indicate (Raghavulu et al., 2011). Rabaey et al., 2005; Reiche and Kirkwood, 2012). In other studies electricity generation from glycerol digestion in *E. coli* improved substantially after metabolic engineering of the citric acid cycle (Liu et al., 2012). This is remarkable as the outer cell membrane remained unchanged and no mediators were used.

As mentioned before, the outer cell membrane of *E. coli* contains no conducting or redox membrane proteins (Ruiz et al., 2006). The only contact to the outside are porins, which allow small molecule exchanges and therefore ionic electron transport. When a porin is in contact with the electrode, a quasi-direct electron transfer occurs (Fig. 1). It has recently been shown that a higher porin content in the outer cell membrane enhances currents (Yong et al., 2013). Another effect is secondary metabolism oxidation on composite anodes that caused powers and currents in MFCs with *E. coli* that were among the highest reported. A Pt/C anode allowed a short circuit current of >11 A/m² (Wang et al., 2013). With a multi-walled nanotube coated with a tin–platinum mixture (Sn–Pt/MWNT) and neutral red >8 A/m² were recorded (Sharma et al., 2008). A metal-free polypyrrole carbon nanotubes (PPy-CNT) anode also improved currents to 1.8 A/m² using *E. coli* (Zou et al., 2008).

In this study, the use of a bi-cathodic MFC made it possible to distinguish electron transfers from the biofilm and from the medium, and to identify unexpected currents using *E. coli*. The bi-cathodic MFC comprises two anodes and two cathodes; it was constructed by joining two bi-chambered MFCs through their anode compartments (Ducommun et al., 2010). The two anodes are separated by a filter membrane that divides the reactor into a bio- and an abiotic cavity containing only the shared cultivation medium (Fig. 2).

In a microbial fuel cell the level of the anode potential determines the current that can be produced under the premise that the cathode remains unchanged (Aelterman et al., 2008). However, open circuit potentials (OCP) of biofilms, planktonic cells, and the host medium are different and currents vary under closed circuit conditions. The investigations with the bi-cathodic MFC also allowed the analysis of open circuit potentials with biofilms and their synchronisation during growth. The synchronisation of *E. coli* (Sauvageau et al., 2010), yeast cells (Palková and Forstová, 2000) and other microorganisms are a widely encountered phenomenon. In microbial fuel cells, large and interconnected anodes are envisioned for scaled-up operations. It is therefore important that no large variations occur, as current inversions would cause efficiency losses (Dewan et al., 2008).

In the following, various open circuit potentials with *E. coli* were examined first. Subsequently, related electron transfers were recorded and a cause for the occurrence of false currents was simulated. As a result, a set of methods was obtained and used to examine electric effects in an *E. coli* microbial fuel cell.

### 2. Methods

#### 2.1. Material

*E. coli* K12 ATC 23716 DSM 498 [Deutsche Sammlung für Mikroorganismen, Germany] were used in all experiments (Castellani and Chalmers, 1920; Blatter et al., 1997). The cultivation medium was prepared with yeast extract obtained from Lonza [Visp, Switzerland], tryptic soy broth from Biolife [Italy] and b-glucose monohydrate from Brenntag [Basel, Switzerland]. Sodium bicarbonate and sodium dihydrogen phosphate were bought from Fluka Analytical [Buchs, Switzerland], disodium hydrogen phosphate from Merck [Darmstadt, Germany] and potassium hexacyanoferrate(III), K₃[Fe(CN)]₆, as a 0.5 M solution, from Acros Organics [Geel, Belgium]. The mediator Methylene Blue was purchased from Fluka Chemika [Buchs, Switzerland].

Carbon felt for the electrodes and Nafion™ proton exchange membranes (PEM) were furnished by NCBE, The University of Reading [United Kingdom]. Pure nitrocellulose membrane
(0.45 \mu m) was bought from Bio-Rad Laboratories [Crissier, Switzerland]. Regenerated cellulose membrane 12–14k MWCO came from ZelluTrans Roth [Karlsruhe, Germany], polyethersulfone (PES) membrane pore size 0.2 \mu m from Sartorius Stedim [Tagelswangen, Switzerland], and Membrane Slide-A-Lyzer G2 2k MWCO 2k from Thermo Scientific [Zürich, Switzerland].

2.2. Microbial fuel cell design and construction

2.2.1. Bi-cathodic MFC

The microbial fuel cell was constructed from poly(methyl methacrylate) (PMMA) and polyvinylchloride (PVC) (Duc communism et al., 2010). It consisted of a 500 mL bioreactor cavity and a smaller abiotic 10 mL anode compartment, which was separated from the bioreactor by a dialysis or filter membrane. Two 10 mL cathodes were added on opposite sides of the MFC and separated from the anode by 12 cm² Nafion™ PEM. The electrodes were made from disposable carbon felt with a surface area of 15.3 cm². The construction was later modified to facilitate experimental work with reference electrodes. In the modified MFC the original 500 mL anode became a 350 mL bioreactor cavity, whereas the abiotic compartment was enlarged to 150 mL. The two anode sectors were divided by a 25 cm² permeable membrane and the anodes were sized according to the total anode volumes (55 and 21.3 cm³).

2.2.2. MFC for medium reduction experiments

A standard bi-chambered microbial fuel cell equipped with an additional intermediate third cavity (40 mL) was used for medium reduction experiments. The construction was based on the bi-cathodic MFC described above, in which the permeable membrane was replaced by a Nafion™ 114 PEM to allow selective proton migration into the intermediate storage cavity (Fischer et al., 2012). An external circuit was installed for electron transfer into the intermediate storage cavity. A 10 mL cathode was added on the other side of the storage cavity, also separated by a proton exchange membrane.

2.3. General procedure for E. coli cultivation

200 mL preculture were prepared from 30 g/L tryptic soy broth medium, inoculated with E. coli K12 ATCC 23716 DSM 498 and incubated at 37 °C/24 h. 15 mL of the preculture was then added to the left anode of the bi-cathodic MFC, which already contained cultivation medium made from 4 g/L yeast extract, 10 g/L glucose, 8 g/L NaHCO₃, and 6.67 g/L NaH₂PO₄. All media were sterilised for 20 min/121 °C and the plastic parts of the MFC were decontaminated in a 1% Korsolex solution for 12 h before being used. The catholyte was a 0.5 M potassium hexacyanoferrate(III) K₃Fe(CN)₆ solution in a 0.1 M buffer (pH = 7) made from NaH₂PO₄ and Na₂HPO₄.

2.4. Potential comparisons

Standing potentials were recorded with VC-960 digital multimeters, equipped with a data logger [Voltcraft Plus] [internal resistance 10 MΩ]. The potentials recorded using multimeters were validated with a reference electrode. For this purpose, the abiotic cavity of the bi-cathodic MFC was enlarged (second version of the bi-cathodic MFC).

2.5. Simultaneous recording of biofilm and medium potentials

The open circuit potentials of the biofilm and the surrounding medium were measured with a 500 mL bi-cathodic MFC (Fig. 2). The two anode compartments were filled with the same medium. The experiment was launched by adding 15 mL E. coli preculture to the larger anode cavity. The open circuit potentials generated by the biofilm and the medium were recorded simultaneously. The cathode contained in both cases a 0.5 M hexacyanoferrate(III) solution. All electrodes were made from carbon felt and disposed of after single use. The permeable membranes were dialysis membranes with 2 kDa and 12–14 kDa MWCO, as well as 0.2 \mu m and 0.45 \mu m pore size filter papers. The experiments were carried out in the presence and absence of 0.3 mM Methylene Blue.

2.6. Potential of planktonic E. coli and biofilm growth synchronisation

A bi-cathode MFC with a 500 mL anode (without anode separation) and two opposite cathodes was filled with medium. The E. coli preculture was added and a biofilm started to grow on the left anode, while the second anode (right side) was initially not inserted. One hour later the second anode was connected to the right cathode (Fig. 2) and immersed in the planktonic E. coli mixture for 1 min. This operation was repeated every hour using a new carbon felt electrode. Alternatively, the electrode was submerged for ~2 h, recording an upward potential drift. The measurement was stopped and a new carbon felt electrode was inserted as soon as the drifting potential equalized the potential of the older biofilm that was not exchanged during the experiment.

2.7. Power storage properties of fresh yeast extract

The purpose of this experiment was to determine whether freshly prepared medium can be reduced and store MFC energy (Fischer et al., 2012). Yeast extract, as obtained from the supplier, was dissolved in demineralised water (1.0, 5.0 and 10.0 g/L) and poured into the central chamber of a three chambered microbial fuel cell. The abiotic medium solutions were reduced by E. coli cultivations containing 0.3 mM Methylene Blue. When the open circuit potential reached 0.5–0.7 V, the reduced yeast extract was subjected to polarisation sweeps using a series of resistances (100 kΩ to 100 Ω). Polarisation curves and power-current plots were established to compare internal resistances and power maxima P_max.

2.8. Electron transfer monitoring methods

2.8.1. Electron transfer analysis with biofilms (Method A)

The electron transfer from E. coli to carbon felt anodes and related background currents were monitored with the bi-cathodic MFC (Fig. 2), the anode chamber was separated by a 0.2 \mu m filter membrane. A total of 500 mL cultivation medium was added to the biotic and abiotic anode cavities. The experiment was launched by adding 15 mL preculture to the larger anode cavity, and amperometric signals were recorded online for 20 h.

2.8.2. Mediated electron transfer observations

The experiments according to Method A were repeated as a control in the presence of 0.3 mM Methylene Blue.

2.8.3. Background current detection with fresh cultivation medium (Method B)

The purpose of this experiment was to determine to what extent electro-active compounds in the cultivation medium cause a background current (Duc communism et al., 2010). A 500 mL bi-cathodic MFC with a single anode was filled with fresh medium, as obtained from the supplier. It contained either pure yeast extract, a mixture of yeast extract and glucose, or yeast extract and Methylene Blue. The cell potential and the oxidation currents were recorded until they became asymptotic.
2.8.4. Medium-generated current simulation (as observed during the launching phase) (Method C)

The objective was to show that bioelectrochemically well reduced cultivation medium causes an elevated background current when added rapidly to an empty or medium containing microbial fuel cell. For this purpose the bi-cathodic MFC was prepared with cultivation medium in both anode compartments separated by a 0.45 μm filter membrane. The preculture was added to the larger anode compartment. The cultivation was kept electrically isolated for 24 h to prevent accidental discharging. The circuit of the abiotic cavity was then closed and the current caused by medium oxidation recorded in short circuit mode.

2.9. Methylene Blue absorption by E. coli

A 200 mL E. coli cultivation was incubated for 24 h at 37 °C. Three times 10 mL of the incubated cultivation were transferred to 15 mL falcon tubes and centrifuged at 7500g for 5 min. The supernatant solutions were decanted and the sedimented cells were suspended in 10 mL 0.3 mM Methylene Blue in 0.9% NaCl. The NaCl solution balanced the osmotic pressure of the cells and prevented cell lysis during the experiment. The sealed falcon tubes were gently mixed on a Stuart rotator SB3 and every 5 min the vented cell lysis during the experiment. The sealed falcon tubes were immersed in a planktonic E. coli cultivation and an abiotic zone. A filter paper with 0.2 μm pore size withheld E. coli in the biotic sector (Fig. 2). The biofilm potentials found correspond to reported values for the E. coli MFC with hexacyanoferrate(III) catholyte (Wang et al., 2010), but potentials of the medium in such systems have not been reported yet.

The same potentiometric analysis was also carried out under mediated conditions. The addition of 0.3 mM Methylene Blue to the cultivation quickly generated an electromotive force of 0.6 V (Fig. 2). This force was 0.1 V smaller than without mediator, which indicates that Methylene Blue was in contact with the carbon felt electrode and the biofilm. In contrast, the EMF in the mediator containing medium decreased only by 50 mV. This asymmetric EMF decrease is due to the absorption of Methylene Blue by E. coli. In any case, a reduced EMF has to be expected using a synthetic mediator. The redox potential of Methylene Blue at pH 7 is +0.011 V and as such more positive than the redox potential of NAD/NADH with −0.32 V.

The different potentials of the biofilm and the surrounding medium were confirmed by several experiments (Fig. 3 and Fig. S1). The contrasting biofilm and medium potentials directed the interest toward measuring the EMF with planktonic E. coli.

3.2. Planktonic E. coli, biofilm growth and synchronisation

Planktonic E. coli readily adsorbed on carbon felt electrodes as evidenced by an exponential rise in potential (Fig. 3). The initial EMF of planktonic E. coli corresponded to the values of the hosting medium, 0.35–0.4 V (Fig. S2). When a clean carbon felt electrode was immersed in a planktonic E. coli cultivation the signal drifted. This was attributed to continuous attachment of planktonic E. coli to the electrode. However, unstable electrochemical signals are

![Fig. 3](image-url)
possibly caused by redox species with reduced activity and a certain waiting period is necessary to record the correct potential. Nevertheless, the drift persisted and therefore short and prolonged potentiometric readings were compared: (i) a new clean carbon felt electrode was repeatedly immersed in the planktonic cultivation for only 1 min. This resulted in potentials of 0.35–0.4 V (Fig. S2) matching the values of medium potential during cultivation (Fig. 3 and Fig. S1). (ii) Next, the electrode was placed in the cultivation for 1–2 h and the potential rose to the level of the reference biofilm residing in the same cultivation (Fig. 3). Surprisingly, once the new biofilm potentials matched the reference biofilm potential, both continued to rise in parallel. This synchronisation of metabolic activity is remarkable as the biofilms were separated by several centimetres and were only connected by the cultivation medium. Moreover, it appeared that the new biofilm grew at a higher speed than the existing biofilm. This relates to the presence of more planktonic E. coli and secreted factors than at the outset of the experiment. It is also of interest to know that the accumulated biomass on the newly inserted anode showed a linear development with time, while the open circuit potential caused by this biomass rose exponentially. Finally, the open circuit potential of planktonic E. coli cultivations could not be determined as such, as biofilm development begins with the insertion of an electrode.

3.3. Currents from E. coli biofilms

The electron transfer from E. coli biofilms to carbon felt anodes (Fig. 4) was 10 times higher than the simultaneously recorded background current passing through the surrounding medium. At the beginning of an experiment the current in the biotic cavity often decreased, and then started to increase exponentially after 1 h, only to decline later, reaching a second minimum after 10 h. Afterwards electron transfer increased linearly (Fig. 4) but no increase in the abiotic part became visible. The different phases can be explained as follows: during the first hour the oxidation of the electroactive medium components of the added precursor is faster than the electron transfer due to metabolic activity, and therefore the combined signal declines. Glucose consumption in the newly formed biofilm on the anode then causes electron transfer, and the signal rises. Later on, the metabolism changes because of glucose depletion and the current density rises again.

The electron transfer from the E. coli biofilm to the anode is of central interest. The weak indirect electron transfer through the medium is based on the diffusion of reduced components of the electro active medium and metabolites. The background current through the cultivation medium remained small. Thus, if any mediator is excreted it is either apolar or, when soluble, excreted in traces. To establish a biofilm on a surface, E. coli secrete adhesins located on pili and fimbriae (Raghavulu et al., 2011). In consequence, some E. coli cells become closely connected with the electrode surface and transfer electrons. This transmission is also facilitated by the oxidative force of the cathodes, rendering this process more spontaneous than under native conditions. Nevertheless, the free enthalpy remains below the threshold of direct electron tunnelling through the lipid bilayer.

3.4. Medium reduction during MFC operation

Currents passing through the cultivation medium sometimes increased well above expected levels (Fig. S3). This happened in particular during the launching phase of the MFC experiments. These short circuit type currents originated from sudden oxidation of reduced preculture medium, but also reduced metabolites and planktonic E. coli, when added to a prepared microbial fuel cell. Their density declined rapidly and reached a minimum within an hour.

The assumption that electroactive components of the medium participated in extracellular electron transfer with E. coli was verified in three experiments: (i) pure yeast extract solutions spontaneously caused a small current when added to an empty 500 mL MFC. Three combinations of such medium mixtures were analysed: pure yeast extract, a yeast extract–glucose mixture and a yeast extract–Methylene Blue combination (Fig. 5A, Fig. S5). All three solutions became oxidized and the redox equilibrium was reached after 10 h with a constant background current of ~10 μA. (ii) Conversely, the same fresh yeast extract solution was further reduced. For this experiment a slightly altered bi-cathodic MFC was used. The central filter membrane in the anode was exchanged with a Nafion™ proton exchange membrane. This setup allowed only proton transfer into the abiotic storage cavity (Fig. 2). Fresh yeast medium solutions, as obtained from the supplier, were reduced with electrons and protons generated by the anodic E. coli cultivation. In this case the electrons passed through an external circuit into the storage cavity, whereas the protons migrated across the proton exchange membrane. Under these conditions, the yeast medium was reduced and the open circuit potential rose to 0.5 V. Polarisation experiments showed a redox battery-like behaviour of the yeast extract (Fig. 5B) as if it were storing virtually seen hydrogen, $\text{ne}^{-} + n\text{H}^{+} + \text{YE} \rightarrow \text{YE} - n\text{H}_{2}$ (iii) Finally, it was shown that medium reduction occurs in any cultivation. For this purpose, E. coli was cultivated under open circuit conditions in...
the bi-cathodic MFC for 1 day. The circuit of the abiotic cavity was then closed and a diminishing current was observed, as hypothesised. This experiment also confirmed that unusually high short circuit currents were caused by reduced electroactive mediators; solid lines = potential. (B) Power storage capacity of pure and fresh yeast extract solutions made from 1 g/L, 2 g/L (■) and 10 g/L (▲).

Fig. 5. (A) Redox properties of the cultivation medium as received from the supplier in a 500 mL bi-cathodic MFC. Oxidation of yeast extract-glucose. Dotted lines = current; solid lines = potential. (B) Power current plot of reduced yeast extract solutions made from 1 g/L, 2 g/L and 10 g/L.

the biofilm' electromotive force was consistently 0.2–0.3 V higher than the electromotive force of the surrounding medium. Potentiometry also showed that biofilm growth synchronisation occurred between two physically separated biofilms.

Amperometry indicated that electron transfer from non-exoelectrogenic E. coli to the anode was small but non negligible. It could be shown that fresh medium stored electrons and protons in a redox battery-like manner. This effect had also been observed within an E. coli cultivation. This is of practical significance when working with non-exoelectrogenic microbes, as currents based on medium effects can overlap direct electron transfer from biofilms at the beginning of an experiment.

4. Conclusions

The biofilm' electromotive force was consistently 0.2–0.3 V higher than the electromotive force of the surrounding medium. Potentiometry also showed that biofilm growth synchronisation occurred between two physically separated biofilms.

References

Qiao, Y., Li, C.M., Bao, S.-J., Lu, Z., Hong, Y., 2008. Direct electrochemistry and
electrocatalytic mechanism of evolved Escherichia coli cells in microbial fuel
Rabaey, K., Roon, N., Höfte, M., Verstraete, W., 2005. Microbial phenazine
39, 3401–3408.
analysis of Pseudomonas aeruginosa and Escherichia coli with anaerobic consortia
as anodic biocatalyst for biofuel cell application. J. Appl. Microbiol. 110, 666–
674.
consortia derived from compost as anodic biocatalysts in a glycerol-oxidizing
Ruiz, N., Kahne, D., Silhavy, T.J., 2006. Advances in understanding bacterial outer-
Sauvageau, D., Storms, Z., Cooper, D.G., 2010. Synchronized populations of
Escherichia coli using simplified self-cycling fermentation. J. Biotechnol. 149,
67–74.
carbon nanotubes and nanofluids based microbial fuel cell. Int. J. Hydrogen
Energy 33, 6749–6754.
Wang, C.-T., Chen, W.-J., Huan, R.-Y., 2010. Influence of growth curve phase on
electricity performance of microbial fuel cell by Escherichia coli. Int. J. Hydrogen
Wang, Y., Li, B., Zeng, L., Cui, D., Xiang, X., Li, W., 2013. Polyaniline/mesoporous
tungsten trioxide composite as anode electro catalyst for high-performance
Yi, H., Nevin, K.P., Kim, B.-C., Franks, A.E., Klimes, A., Tender, L.M., Lovley, D.R.,
2009. Selection of a variant of Geobacter sulfurreducens with enhanced capacity for
Yong, Y.-C., Yu, Y.-Y., Yang, Y., Liu, J., Wang, J.-Y., Song, H., 2013. Enhancement of
extracellular electron transfer and bioelectricity output by synthetic porin.
Zhang, T., Cui, C., Chen, S., Yang, H., Shen, P., 2008. The direct electrocatalysis of
Escherichia coli through electroactivated excretion in microbial fuel cell.
Zhang, T., Zeng, Y.L., Chen, S.L., Ai, X.P., Yang, H.X., 2007. Improved performances of
E. coli-catalyzed microbial fuel cells with composite graphite/PTFE anodes.
fuel cell using polypyrrole coated carbon nanotubes composite as anode