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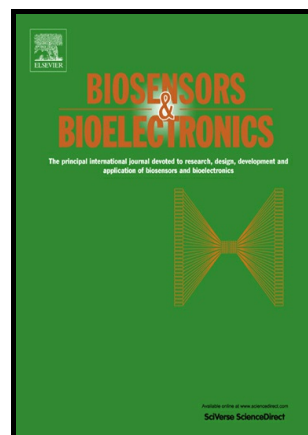
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New method for characterizing electron mediators in microbial systems using a thin-layer twin-working electrode cell

Md Mahamudul Hassan[1], Ka Yu Cheng[1,2*], Goen Ho[1], Ralf Cord-Ruwisch[1]

[1]School of Engineering and Information Technology, Murdoch University, WA 6150, Australia

[2]CSIRO Land and Water, Floreat, WA 6014, Australia

*Correspondence: Tel.: +61 8 9333 6158; fax: +61 8 933 6499. kayu.cheng@csiro.au

Abstract

Microbial biofilms are significant ecosystems where the existence of redox gradients drive electron transfer often via soluble electron mediators. This study describes the use of two interfacing working electrodes (WEs) to simulate redox gradients within close proximity (250 μm) for the detection and quantification of electron mediators. By using a common counter and reference electrode, the potentials of the two WEs were independently controlled to maintain a suitable "voltage window", which enabled simultaneous oxidation and reduction of electron mediators as evidenced by the concurrent anodic and cathodic currents, respectively. To validate the method, the electrochemical properties of different mediators (hexacyanoferrate, HCF, riboflavin, RF) were characterized by stepwise shifting the "voltage window" (ranging between 25 and 200 mV) within a range of potentials after steady equilibrium current of both WEs was established. The resulting differences in electrical currents between the two WEs were

1

recorded across a defined potential spectrum (between −1 V and +0.5 V vs. Ag/AgCl). Results indicated that the technique enabled identification (by the distinct peak locations at the potential scale) and quantification (by the peak of current) of the mediators for individual species as well as in an aqueous mixture. It enabled a precise determination of mid-potentials of the externally added mediators (HCF, RF) and mediators produced by pyocyanin-producing Pseudomonas aeruginosa (WACC 91) culture. The twin working electrode described is particularly suitable for studying mediator-dependent microbial electron transfer processes or simulating redox gradients as they exist in microbial biofilms.

Keywords

Twin working electrode; Electron mediator; Mid-point potential; Riboflavin; Pseudomonas aeruginosa; Redox gradient

1. Introduction

Electroactive bacteria can transfer electrons to insoluble electron acceptors via different mechanisms such as using exogenous or endogenous electron shuttles or mediators (Brutinel and Gralnick, 2012) direct electron transfer through outer membrane c-type cytochromes (OM c-Cyts) (Shi et al., 2007) and production of conductive nanowires (Bond et al., 2012 and Reguera et al., 2005). Arguably, mediator dependent electron transfer is the most common mode of electron transfer, where a wide range of bacteria accomplish extracellular electron transfer (EET) by producing various soluble electron shuttles or mediators (Rabaey et al., 2007). For instance, it is well known that the distal Fe (III) reduction capability of Shewanella species is dependent on their ability to excrete flavin molecules (Baron et al., 2009, Lies et al., 2005, Marsili et al., 2008 and von Canstein et al., 2008). Respiration of Geothrix on insoluble Fe(III)

2

oxides was also reported to be soluble electron shuttle dependent (Nevin and Lovley, 2002).

Recently, it has been reported that the direct electron transfer of Geobacter also required flavin

molecules to mediate electron transfer between the heme group of OM c-Cyts and the electrode

(Okamoto et al., 2014). In fact, the role of mediators in EET has been well-known in microbial

fuel cell (MFC) and microbially induced corrosion (MIC) research where the mediators were

often shown to enhance current production (Lin et al., 2014) and accelerate corrosion rate (Li et

al., 2015 and Zhang et al., 2015), respectively. Along with flavins, some other compounds such

as anthraquinone-2, 6-disulfonic acid (AQDS) (Sun et al., 2013), cysteine (Doong and Schink,

2002) and quinone (Freguia et al., 2009) have also proved their electron transfer capability.

Given their important role in EET, it is crucial to characterize and quantify soluble mediators in

the relevant microbial systems. Currently, various amperometric and voltammetric techniques

have been widely used to detect and evaluate electrochemical properties of redox active entities,

including soluble mediators (Dryhurst, 2012 and Roussel et al., 2008). These conventional

techniques are typically carried out by using three-electrode systems, whereby oxidation or

reduction of the target compound present in the boundary layer of the working electrode (WE)

can be precisely controlled and/ or monitored (Compton et al., 2013). In particular, cyclic

voltammetry (CV) has been the most common technique, as it enables determination of kinetic

and thermodynamic information of a redox system by varying the scan rate and the potential of

a single working electrode (Bai et al., 2010, Fricke et al., 2008, Marsili et al., 2010 and Sumathi

et al., 2015). However, the use of a single working electrode in these conventional settings only

enables the record of either anodic or cathodic electron flow at a particular time point during the

analysis. It also does not enable the evaluation of the electrochemical behavior of those

compounds present beyond the WE's boundary layer (Bard and Faulkner, 2001). Since both

electron accepting (anodic) and electron donating (cathodic) reactions may concurrently occur

3

within close proximities where EET prevails (Malkin et al., 2014), using a single WE for the evaluation of electron mediators in microbial systems may not be ideal.

In this study, we developed a new electrochemical method for evaluating electron mediators in small (µL)-scale aqueous systems. Such method involves the use of two closely mounted, but independently controllable WEs to facilitate simultaneous measurement of both electron accepting (anodic) and donating (cathodic) reactions within a thin-layer system (here 250 µm). As such, a redox gradient (potential difference) between the two WEs (here termed as "Twin-WE" system) can be precisely controlled over a range of electrode potentials, and the resulting currents independently recorded at the two WEs can be used to detect soluble electron mediators within the system. Compared with conventional CV, such approach may be more suitable for characterizing the redox behavior of a microbial system. The aim of this study was to evaluate and demonstrate the suitability of this method for qualitative and quantitative sensing of soluble biological electron mediators.

2. Materials and Methods

2.1 Construction of the Twin-WE system

Two electronically isolated potentiostats (manufactured by Murdoch University Electronic workshop) were combined to establish the electrochemical device used in this study. Each potentiostat enabled an independent control of one of the two juxta-positioned WEs (i.e. Twin-WE) located within a thin aqueous layer (250 µm). The material of the WEs was either gold-coated or indium tin oxide (ITO) coated glass slide (dimension 75 mm ×25 mm ×1.1 mm and resistance 8-12 Ω/sq; RileySupplies, Richmond Hill, Canada). The two potentiostats shared a common counter electrode (CE) (4 mm in diameter and 105 mm in length) and a silver-silver

chloride (Ag/AgCl) reference electrode (MF-2052, BASi, USA) (Figure 1). All potentials

mentioned in this article are quoted against Ag/AgCl reference electrode (+0.210V vs the

standard hydrogen electrode, SHE).

A WE chamber was constructed by placing a nonconductive rectangular reverse osmosis

membrane frame spacer (0.25 mm thick, 55 mm length, 25 mm width) between the two

conductive surfaces of the WE (mounted with sticky tape). This configuration allowed the

conductive side of both WEs to be positioned at a fixed distance (250 μm). As such, the

boundary layers of the two WEs could be housed in a commonly shared, thin-layer aqueous

environment with a maximum working liquid volume of 304 μL. The WE chamber, CE and the

reference electrode were immersed in a 50 mL bulk sample solution within a Perspex container

(length 8 cm, height 4 cm, width 2.5 cm) (Figure 1). The electrochemical system was automated

and monitored using a personal computer interfaced with a LabVIEW™ software program

(National Instrument™) and a data acquisition card (Labjack™ U12).

2.2 Working principle of the Twin-WE system for mediator detection

Mid-potential (MP) of an electron mediator is defined as the potential at which the oxidized and

reduced forms of the mediator are present at equal concentration (50:50) (Bard and Faulkner,

2001 and Bard et al., 1980). When the potential of the WE is higher (more positive) than the

MP, anodic current could be recorded as a result of an oxidation of the reduced mediators.

When the potential of the WE is lower (more negative) than the MP, cathodic current could be

recorded as a result of a reduction of the oxidized mediators.

5

In the Twin-WE system, both WEs can be poised at a fixed potential gradient (or "voltage window) across a range of defined potentials. When such "voltage window" is maintained at either beyond or below the mediator's MP, short-lasting current is expected as both WEs will only enable either oxidation or reduction, leading towards exhaustion of reactants (reduced or oxidized mediators). However, a continuous and stable current between the two WEs should be attainable when one of the WEs is poised at a potential beyond and the other one below the mediator's MP. As such, the former one will enable an oxidation (i.e. anodic current), whereas the latter one will enable a reduction (i.e. cathodic current). Hence, the maximal steady-state current between the WEs should occur when their potentials are oppositely (one at oxidizing and one at reducing) deviated from the mediator's MP by an equal value. Such electrical flow path is closed by the diffusion of mediator and the overall electroneutrality of the system is maintained by the diffusion of ions. In other words, as one electrode continuously oxidizes (WE1), while the other reduces (WE2) the mediator, there is also a net electron transfer from the reducing to the oxidizing electrode within the common bulk solution via diffusion of mediator and counter ion (Figure 1).

Based on this principle, the presence of electron mediators between the WEs and their respective MPs can be identified and quantified by maintaining a defined "voltage window" within a range of potential (e.g. ±1V). The resulting current difference between the two WEs can be plotted against the corresponding mid-point of the "voltage window". In this study, it was hypothesized that with this plot the presence of electron mediators could be characterized and quantified based on the peak position and signal magnitude of the current difference.

2.3 General operation and detailed experimentation with the Twin-WE system

To begin with an electrochemical analysis, the liquid sample was first loaded (via capillary suction created by the two closely packed WEs) into the WE chamber. This was done by immersing the chamber into a sample solution. Thereafter, all the edges of the WE chamber were immediately sealed with a molten agar (1.5%) (with a 1-mL disposable syringe). The agar was allowed to solidify under ambient condition (within approximately two minute). This step was to retain the mediators in the WE chamber, as well as to minimize air (oxygen) intrusion from the supporting bulk electrolyte into the WE chamber. It also enabled ionic flux between the WE and CE.

2.3.1. Electron mediators

Hexacyanoferrate (HCF, $Fe(CN)_6^{3-}$) and riboflavin (RF) were used as model electron mediators. The mediators were dissolved in phosphate buffer (100 mM, pH 7.3) to obtain their respective standard solutions with different concentrations. An active culture of Pseudomonas aeruginosa (WACC 91) known to produce pyocyanin (characteristic blue-greenish colored mediators) was used as a surrogate for a redox active microbial system. The culture was pre-grown in a Pseudomonas Minimal Medium (PMM), which contained (L^{-1} in deionized water): glycerol (15 mL), L glutamine (5 g), K_2HPO_4 (1.5 g), $MgSO_4$ (0.2 g) and was harvested 72 hours after sub-culturing. When testing the use of the Twin-WE system for mixed mediators detection, predefined amounts of HCF and RF standard solutions were mixed with the P. aeruginosa culture (25 mL) before being suction-loaded into the WE chamber as described above.

2.3.2. Cyclic voltammetry (CV) with the Twin-WE system

The electrochemical interaction between the ITO-coated glass slide and the model mediators was first validated by running a conventional CV using the Twin-WE system. Only one of the two WEs was potentiostatically controlled as per conventional practice. CV of HCF was performed at a scan rate of 0.02 Vs^{-1} between +0.7 V and -0.7 V, whereas CV for riboflavin (RF) was done at a lower scan rate of 0.001 Vs^{-1} between 0V and -0.95V. CV for the active P. aeruginosa culture and its mixture with HCF and RF was performed at a scan rate of 0.001 Vs^{-1} between +0.85 and -0.95V.

2.3.3. Shifting a fixed "voltage window" across a potential range with the Twin-WE system

The new method was first validated with a single mediator (HCF) system (1 mM). A series of "voltage window" (25, 50, 75, 100, 125, 150, 175 and 200 mV) was tested to select a suitable window for subsequent tests. Each voltage window was allowed to shift between -0.15V and +0.4V. The currents recorded at the two WEs were allowed to reach a steady state before the voltage window was shifted to the next set point. The time required to reach such steady state was typically within two minutes (data not shown). Hence, a sufficient duration of four minutes was given for each step. The voltage window was shifted (either downward or upward) to the next set point by one quarter of the window's width (e.g. 12.5 mV/step was given for a 50 mV window). The resulting current difference between the two WEs was plotted against the corresponding mid-point of each voltage window set point. The same approach was used with the selected voltage window for the validation of other mediator systems.

8

2.4 Use of the Twin-WE system as a coulomb counter

Since the aqueous system between the Twin-WE was concealed, the charge storing capacity of

the mediators in the WE chamber should in principle, be directly proportional to the amount of

mediators present and may thus be quantified electrochemically. To validate this, a sequential

charging (reduction)- discharging (oxidation) (i.e. coulomb counting) experiment was

conducted. Different concentrations of HCF were precisely loaded to the WE chamber. The

theoretical coulomb in the system was calculated based on equation 1.

Theoretical Coulomb $= (V \times C \times F \times n)/1000$ ………… (1)

Where, V= sample volume inside the WE chamber (mL), C= HCF concentration in the sample

(mmol/L), F= Faraday constant (96485 C/mole e⁻) and n= number of electrons involved in the

redox reaction (1 e⁻ for HCF) (reactions 1)

$Fe(CN)_6^{3-} + e^- + H^+ \rightarrow Fe(CN)_6^{4-}$ (reaction 1) (MP = +0.23V vs. Ag/AgCl) (Kwong, 2004)
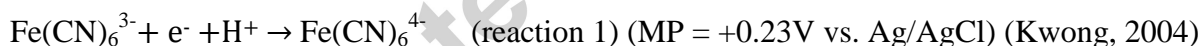
After that, complete oxidation and reduction of the mediators inside the WE chamber was

performed by maintaining both WEs at either an oxidizing (150 mV beyond the MP of HCF, i.e.

+0.3625V) or a reducing (150 mV below the MP of HCF, i.e. +0.0625V) potential. In each case,

the currents for both WEs were recorded until they approached zero. The total amount of

coulomb involved for the anodic or cathodic electron transfer was calculated according to

equation 2.

9

$$\text{Total Coulomb} = \int_0^t I dt \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(2)}$$

Where I = anodic or cathodic current recorded at both WEs (C/s) and t = time period of the current measurement (s)

Finally, the theoretical coulomb present in the sample volume was compared with that measured from charging (cathodic) and discharging (anodic) currents.

3. Results and Discussion

3.1 Standard cyclic voltammetry of hexacyanoferrate

Prior to the testing of the described Twin-WE system, the use of the ITO electrodes for oxidizing and reducing common electron mediators was evaluated using conventional CV technique. Different concentrations of HCF (7.8 - 1000 µM) were tested (Supplementary Figure S1). As expected, the typical electrochemical behavior of HCF was obtained with distinct oxidation and reduction peaks at approximately +0.322V and -0.058V, respectively. These peaks were reversible as reported in other similar aqueous systems (Garabagiu and Mihailescu, 2011). Also, the cyclic voltammograms obtained with different HCF concentrations were distinctive. This result confirms that the Twin-WE system could be effectively used to perform conventional CV for mediator detection.

3.2 A novel technique for mediator detection with two juxta-positioned working electrodes

In comparison to the conventional CV method using single-WE, the Twin-WE system consisted of two independently controllable juxta-positioned electrodes (distance of about 250 µm).

10

Instead of rapidly sweeping the potential of the single WE to oxidize or reduce the mediators within the diffusion layer (boundary layer) of the electrode, a constantly oxidizing electrode faces a reducing counterpart would result in two opposing currents, which could be recorded simultaneously. Moreover, instead of a bulk electrolyte solution, the diffusion layers of both electrodes overlap. The presence of a mediator between the two WEs would cause an anodic and cathodic current respectively at the same intensity, with the mediator enabling an electron transport via the diffusion of reduced mediator from the more reduced electrode to the more oxidized one.

3.2.1 The Twin-WE system could effectively detect hexacyanoferrate

Poising the juxta-positioned twin WEs, one at an oxidizing potential (e.g. +0.4V) and the other at a reducing potential (e.g. -0.4V) (i.e. "voltage window" of 0.8V across the HCF's MP of +220 mV) enabled a continuous current generation at both WEs (Supplementary Table S1). The currents were of similar values but with opposing directions and only observed in the presence of electron mediators. This result suggests that oxidation and reduction of the mediator (HCF) occurred simultaneously at a similar rate at the two WEs.

A more narrow "voltage window" of 0.2V was chosen by poising the two WEs at +0.1 and +0.3 V, respectively. These two potentials were respectively well below and beyond the MP of HCF and also resulted in a current each of which was similar but opposite to the other (Supplementary Table S1). As expected, poising the WE potentials both at above (e.g. +0.5V and +0.3V) or below (e.g. 0.1V and -0.1V) the MP of HCF resulted in much lower current differences. This result affirms the underlying principle of the proposed mediator detection method with the Twin-WE system.

3.2.2. Signal improvement for mediator detection by optimizing the "voltage window"

Positioning a "voltage window" of the two WEs close to the mediator's MP resulted in a current difference, which was maintained by the oxidative and reductive half reactions of the two working electrodes. By recording the current difference between the twin WEs at different window positions, distinct peaks (of current difference) were obtained that revealed the MP of HCF of +0.22V (Figure 2). Large voltage windows of 0.15-0.20 V gave relatively high current peaks of up to 0.1 mA (current density of 7 $\mu A/cm^2$), whereas smaller voltage windows showed more narrow peaks. This result clearly highlights the potential of using the Twin-WE system for detecting redox active compounds in aqueous samples.

Based on the above optimization trials with various voltage window widths, a "voltage window" of 0.05V was selected for subsequent tests. With the selected voltage window of 0.05V, the sensitivity of using Twin-WE system for HCF detection was explored. As above, the current difference between juxta-positioned WEs was recorded and plotted against the corresponding mid-point of the voltage window (i.e. average of the twin WE potentials) (Figure 3 A). The result indicated that with concentrations of less than 8 μM, the redox activity of HCF could still be detected, and a linear relationship between the current differences and the concentration of HCF was obtained (Figure 3 B). The current plots also reveal the MP potential of target mediator (HCF) at the current difference peak at 0.225V (Figure 3 A).

3.3 Using the Twin-WE system as coulomb counter for mediator quantification

The defined electrolyte volume between the Twin-WE system could theoretically be used for sequential oxidation/ reduction tests to determine the electron storage capacity of the mediators present. For these tests, both WEs were initially poised at the same reducing potential of +0.062

V (i.e. 0.150V lower than the MP of HCF as determined in this study) to charge up (reduce) the mediators (HCF). This generated a cathodic current until the mediator was completely reduced. Subsequently an anodic current was obtained upon oxidation of the HCF (at +0.36 V, i.e. 0.150V higher than the MP of HCF as determined in this study) in the confined volume between the two WEs. By integrating the current over the time interval, the total coulombs could be counted for the anodic and cathodic currents (Figure 4). The result indicated that the coulombs derived from both anodic and cathodic currents were well in line with the theoretically expected coulombs, indicating reliable electron balances for the system.

3.4 Comparison of Riboflavin monitoring by CV and the Twin-WE system

After analyzing a readily reactive chemical mediator (HCF), the electrochemical properties of RF, a well-known biologically important mediator was evaluated. CV for three different concentrations of RF was performed within the agar sealed WE chamber with only one functional WE (Supplementary Figure S2). Detection of oxidation and reduction peaks of RF was possible only at concentrations ≥ 0.1 mM, as below which no clear oxidation and reduction peaks could be identified.

However, with the same electrodes and RF concentrations the Twin-WE system could generate distinctive peak signals at a much lower RF concentration of 0.05 mM (Figure 5). The peaks were clearly identifiable at a potential of approximately -0.5 V (Figure 5). A linear correlation between RF concentration and peak current differences between the two WEs was established (see insert of Figure 5), suggesting that the Twin-WE system was more sensitive for quantification and more precise for midpoint potential identification than the traditional CV technique.

13

3.5. Multiple mediators detection in an active microbial culture

The above experiments confirmed that the Twin-WE system could effectively characterize and quantify both HCF and RF in a single-mediator containing system. Since microbial systems would typically contain more than one type of mediator, it is meaningful to test our system with a mixture of mediators. Hence, experiments were conducted to test whether our proposed method was effective for a real microbial system containing multiple mediators (Figure 6). The tested system was a mixture of RF (0.1 mM), HCF (0.03 mM) and an active Pseudomonas aeruginosa (WACC 91) broth culture. The Twin-WE system was operated as described above to establish the profile of current difference over a wide range of potentials (between -0.8V and +0.8V). The profile clearly revealed four distinct peaks at potentials of approximately -0.5, -0.35, -0.15 and +0.2V (Figure 6A). Based on the previous single-mediator experiments, the peaks at -0.5V and +0.2V were most likely indicative of RF and HCF, respectively. The extra peaks occurring at -0.35V and -0.15V were most likely symptomatic of the mediators excreted by the Pseudomonas aeruginosa culture. In comparison, the use of CV with only one functional WE under the same conditions generated multiple oxidation and reduction peaks that were less distinctive (Figure 6 B). Therefore, our new method with the Twin-WE system offers a better qualitative and quantitative evaluation than conventional CV method.

3.5. Implication of the findings

Bacterial biofilms are significant ecosystems in which strong redox gradients are known to exist and in which the use of soluble electron mediators is known to mediate electron transfer within the biofilm. This has been described in research work on microbial fuel cells, bio-electrochemical systems (Smith et al., 2015), biosensors (Chaubey and Malhotra, 2002) and

14

microbially induced corrosion (Zhang et al. 2015). The described Twin-WE system allows the analysis of electrochemically active species in very small volumes.   In comparison with CV using the same equipment the described scanning twin WE method enables lower detection limit and a distinctly higher resolution in the analysis of electron mediator mixtures.

Traditional electrochemistry methods rely on defined mass transfer conditions such as rotational speeds with rotating disc electrodes to establish reproducible boundary layer conditions (Bard and Faulkner 2001). In contrast the twin WE system described here consisted only of boundary layers that overlap between the two WEs, hence enabling a complete electron recovery in sequential oxidation /reduction tests (Figure 4). The use of such overlapping boundary layer leads to a controllable redox gradient between the two electrodes that can mimic redox gradients encountered in nature such as in biofilm, water sediment interfaces, the human intestine and may offer novel approaches of controlling and studying such biofilms. Further studies to exploit the use of this approach for studying microbial electron transfer in natural or engineered systems are warranted.

4. Conclusions

This study highlighted the suitability and potential of using the Twin-WE system for detecting and quantifying multiple electron mediators in a micro-scale microbial system. Contrary to the conventional CV technique, the Twin-WE system adopted a unique method of maintaining a defined potential gradient (i.e. voltage window) across the thin-layer over a range of potentials. Such approach enabled the generation of steady-state current signals, facilitating a more precise determination of the mid-potentials and concentrations of two model compounds (HCF and RF) within the thin-layer system. The method was also effective for detecting soluble mediators

excreted by an active Pseudomonas aeruginosa culture. Overall, this unique method of manipulating redox gradients across a thin-layer system could be further exploited to study extracellular electron transfer in important biofilm systems.

5. Acknowledgements

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17

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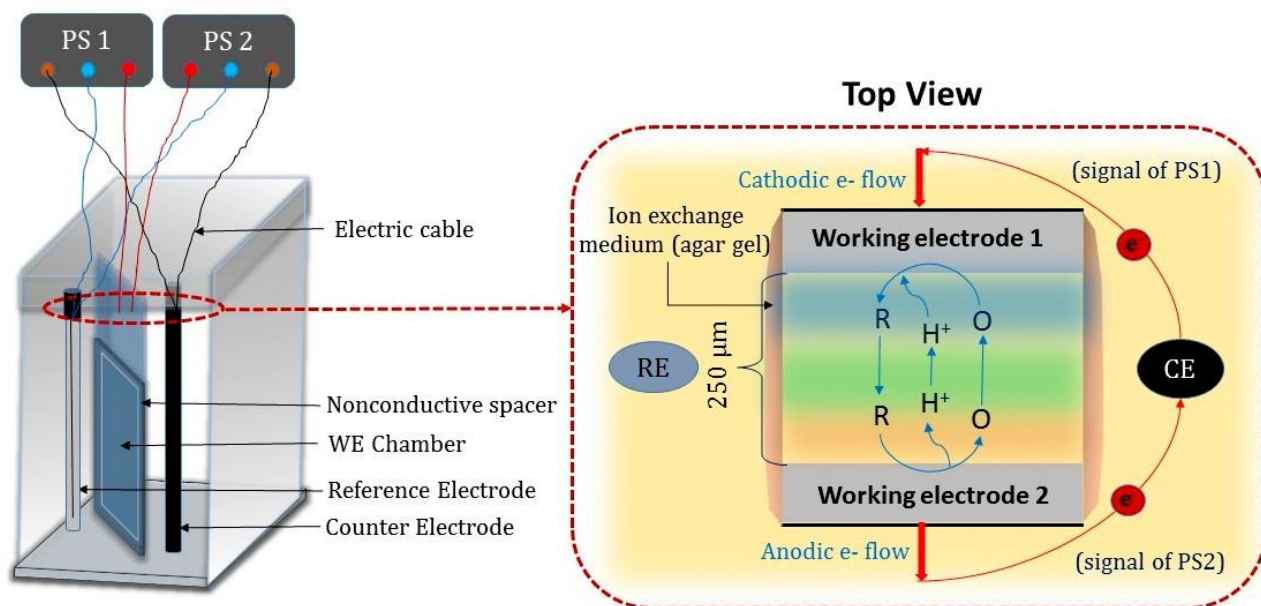Zhang, P., Xu, D., Li, Y., Yang, K., Gu, T., 2015. Bioelectrochemistry 101, 14-21.

Figure 1. Schematic of a Twin-WE system and an anticipated redox reaction mechanism.
(Legends: PS=Potentiostat, RE=Reference electrode, CE= Counter electrode, e⁻=electron, R=
reduced mediator, O= oxidized mediator. Both counter and reference electrodes were kept
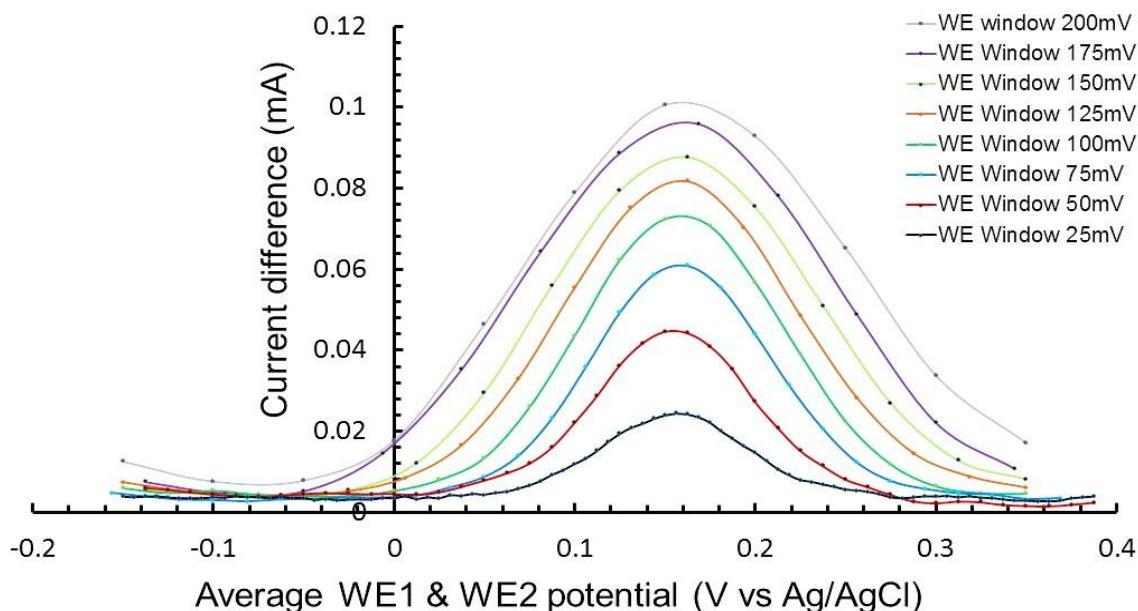1centimeter away from the two ITO glass slides serving as the working electrode.)

Figure 2. Current differences measured between the facing twin gold WE in a solution of 1mM
hexacyanoferrate for different "voltage windows" (difference between the oxidizing and the

reducing electrode given in V) used. The potential given as the X-axis represents the middle of the voltage window. For example, at a voltage window of 0.1V and a given potential of +0.2 V, the reducing and oxidizing electrodes are at +0.15 V and +0.25 V, respectively.
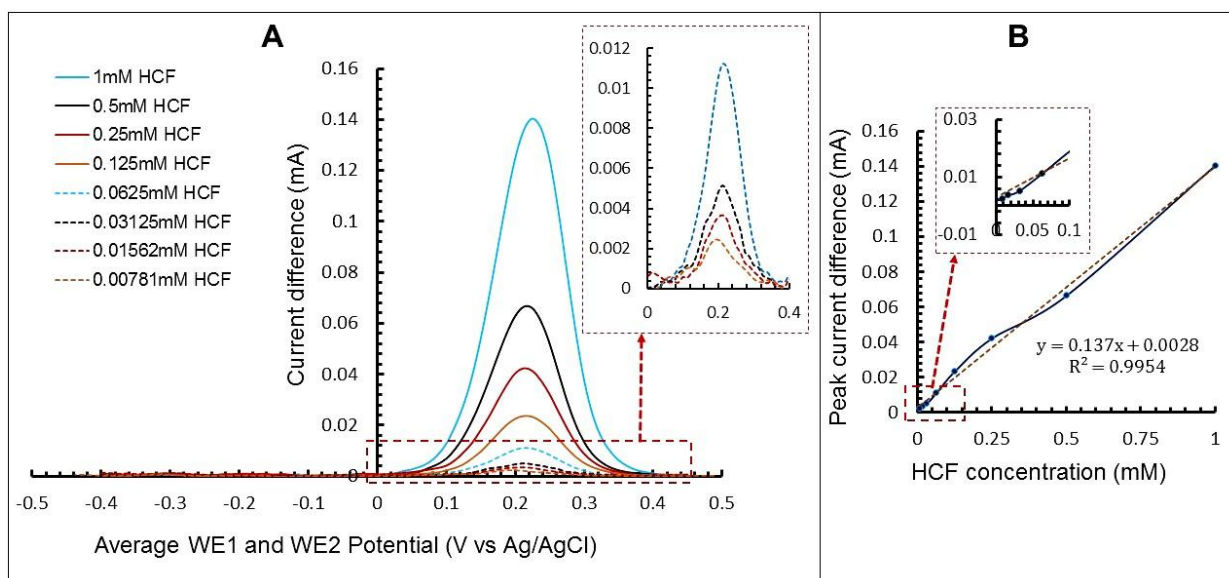

Figure 3. (A) Current difference peaks generated for various HCF concentrations by the ITO Twin-WE system. The "voltage window" between the twin WE was 0.05V. (B) Relationship between the current difference peak between the twin WEs with a voltage window of 0.05V and the corresponding HCF concentration.
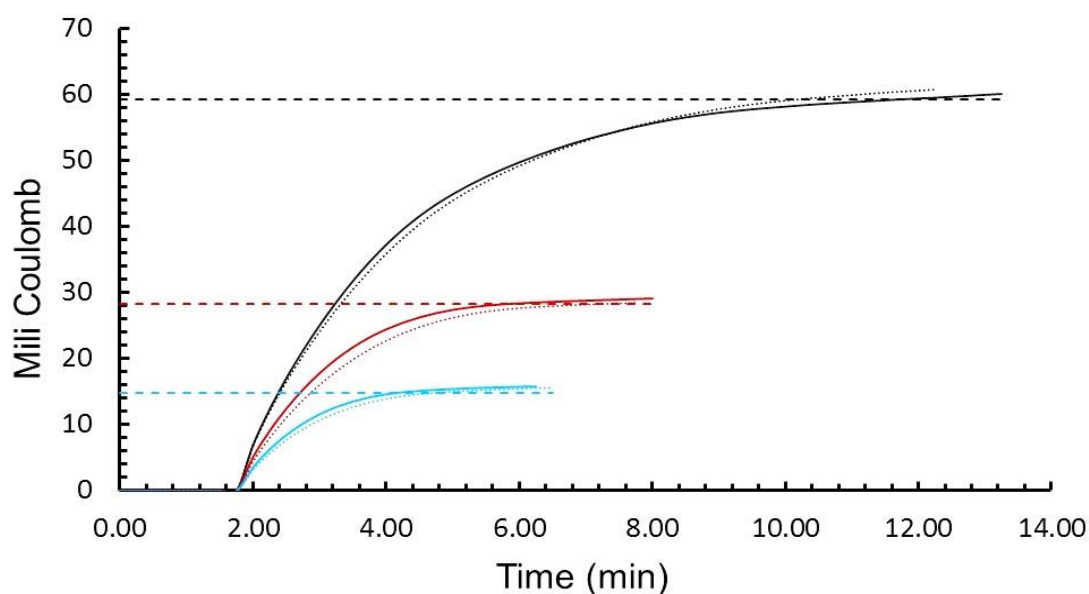
Figure 4. Integrated anodic (solid lines) and cathodic (dotted lines) currents (expressed as coulombs) obtained from the oxidation and reduction of 2 (black), 1 (red) and 0.5 mM (blue) of HCF. The expected theoretical coulombs for each HCF concentration is indicated by dashed lines.
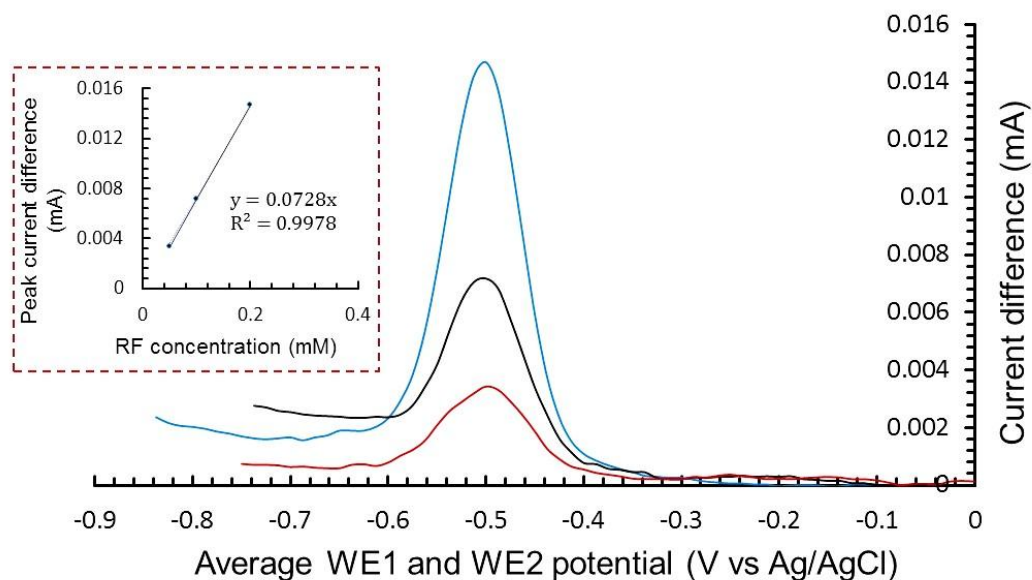

Figure 5. Current difference peaks generated for 0.2 (blue), 0.1 (black) and 0.05 mM (red) RF concentrations by Twin-WE system. Electrolyte solution within the WE chamber was separated from the counter electrode bulk solution by means of agar (see methods). Inset: Relationship of peak current with the corresponding RF concentration.
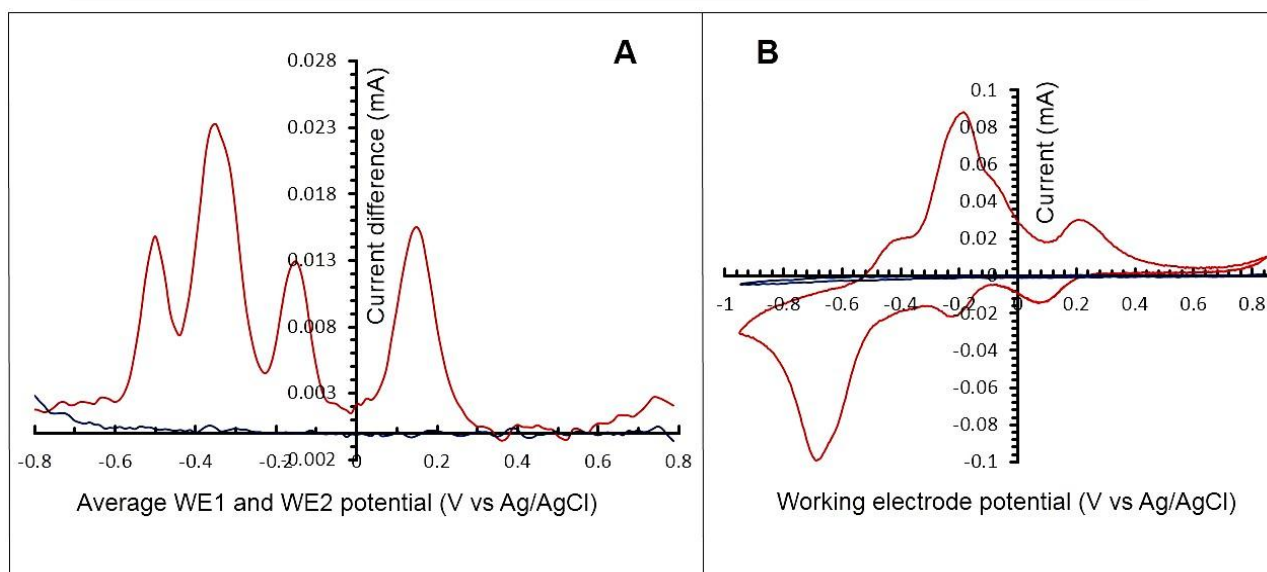
Figure 6. (A) Current difference peaks obtained with the Twin-WE system for the mixture of RF (0.1 mM), HCF (0.03 mM) and Pseudomonas aeruginosa culture (50% v/v) grown in PMM (red line). Current differences between the two WE were generated by maintaining a voltage difference ("voltage window") of 0.05 V between the twin electrodes. (B) Cyclic voltammogram for the same mixture with ITO electrode (scan rate 1 mVsec^{-1}) between +0.85V to -0.95V within Twin-WE chamber with only one functional WE. In both cases, control (blue line) was performed with PBS and PMM mixture (50:50).

Highlights

- A new method for characterizing electron mediators in thin-layer (μm-scale) systems

- It involved two working electrodes (WE), one counter and one reference electrode

- The thin-layer between the two WEs can be controlled at different redox gradients

- Concurrent oxidation and reduction of mediators occurred only at distinct gradients

- The method enabled precise determination of mid-potentials of different mediators