NANYANG TECHNOLOGICAL UNIVERSITY

Understanding and Improving the Microbial Fuel Cell Anodic Electron Transfer Process

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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Summary

The interaction between microbes and solid electron acceptors is of particular interest for geobiology and microbial physiology. The electron transfer (ET) from microorganisms to electrodes opened opportunities for developing whole-cell bioelectrochemical systems (BES) like microbial fuel cells (MFCs).

In order to further the understandings on microbial extracellular ET (EET), this study investigated the exoelectrogenic process of a model anode reducer: *Shewanella oneidensis*. This bacterium is assumed to conduct EET through outer membrane *c*-type cytochromes (OM *c*-Cyts) or diffusive redox shuttles. However, the effectiveness of the proposed EET pathways is still in debate; it is also less discussed how the environmental conditions affect these pathways.

In this study, the *S. oneidensis* OM *c*-Cyts (OmcA/MtrC) demonstrated irreversible electrochemistry, where the electron flow from cytochromes to electrode appeared to be obstructed. The electrode-attached *S. oneidensis* cells expressed OmcA/MtrC at the bacteria-electrode interface when the electrode was poised at 0 V vs. saturated calomel electrode, but not when the poised potential was – 0.24 V. Cytochromes that had already been deposited on the interface could be eliminated under the poised potential of – 0.24 V. Unexpectedly, current density under the higher poised potential was very low, while the low current density was associated with accumulation of OmcA/MtrC at the *Shewanella*-electrode interface. Although OmcA/MtrC were widely believed to be electrode reductases, their electrocatalytic activity was found to be weak; due to the rectification behavior and sluggish ET kinetics. An additional EET pathway through diffusive electron shuttle flavins was found to be more efficient. The electrocatalytically inactive OmcA/MtrC could probably insulate the electrode surface and hinder the contact between flavins and electrode, which inhibited current generation.

The present study also investigated the electrochemistry and power-generating performance of *Ferrimonas balearica*, which represents the first exoelectrogenic species from the *Ferrimonadaceae* family. Purpose of this investigation was not only to identify novel exoelectrogens, it also aimed to evaluate whether the findings on *S. oneidensis* EET were specific. Similar to *S. oneidensis*, electrochemical analyses suggested that *F. balearica* was likely to synthesize both cell surface redox proteins and diffusive redox shuttles for EET. The cell surface electron conduits also exhibited rectification behavior. Moreover, same as observed with *S. oneidensis*, the accumulation of hemeproteins at the *Ferrimonas*-electrode interface seemed to be controlled by the electrode potential. An MFC using graphite granules as the anode material and *F. balearica* as the biocatalyst generated maximum power density of 2.55 mW/m^2, comparable to that generated by some *Shewanella* strains under similar experimental conditions. The Coulombic efficiency (CE) of the *F. balearica* fuel cell was measured as 42.2±3.9%. The study confirmed *F. balearica* as a biocatalyst for MFCs; it was also suggested that the EET behaviors of *S. oneidensis* might be representative for a large class of exoelectrogens.

The EET through microbial OM *c*-Cyts has a great significance for practical applications. To enhance such ET process of *S. oneidensis*, carbon nanotubes (CNTs) were applied as an electrode modifier. After being poised at 0 V for 15 hours, a bare glassy carbon electrode in an electrochemical cell inoculated with *S. oneidensis* delivered a low current density of 0.117±0.006 $\mu A/cm^2$. Modification of the working electrode with CNTs transformed the rectification behavior of OM *c*-Cyts. Additionally, the ET kinetics between cytochromes and electrode was enhanced. For the OM *c*-Cyts, the peak separation in the voltammograms was reduced from over 0.3 V to about 0.17 V (scan rate 0.01 V/s). The promoting effect of CNTs directly raised current density to 9.70±0.40 $\mu A/cm^2$, a level 82 times greater than that of the original. The CNTs had similar promoting effects towards the EET of *F. balearica*.

To probe a more general promoting effect of CNTs towards the anodic process of MFCs, CNT modified electrodes were evaluated in electrochemical cells or MFCs inoculated with mixed microbial consortium. An MFC with the CNT modified graphite foil anode delivered a maximum power density of 566 mW/m^2; 72% higher than an MFC with the same configurations but with unmodified anode. The MFC with modified anode had a higher cell voltage regardless of the external resistance; furthermore, it was observed that when the external resistance was low, the gap in cell voltage was larger. The MFC with the modified anode did not have higher CE when compared with the MFC with unmodified anode. Anodic cyclic voltammetry suggested that the modified anode could achieve limiting current at a lower electrode potential. However, the value of the limiting current was similar for the modified and unmodified electrodes, reflecting similar amount of exoelectrogens enriched at both electrodes, which was confirmed by microscopic study and biomass quantification. In summary, CNTs optimized the MFC performance by enhancing the anodic ET kinetics.

Publications

Papers published on peer-reviewed journals:

Peng, L., You, S.-J., Wang, J.-Y. 2010. Carbon nanotubes as electrode modifier promoting direct electron transfer from *Shewanella oneidensis*. Biosens. Bioelectron. 25 (5), 1248-1251.

Peng, L., You, S.-J., Wang, J.-Y. 2010. Electrode potential regulates cytochrome accumulation on *Shewanella oneidensis* cell surface and the consequence to bioelectrocatalytic current generation. Biosens. Bioelectron. 25 (11), 2530-2533.

Manuscript submitted to peer-reviewed journals:

Peng, L., Wang, J.-Y. Evaluating the exoelectrogenic process of *Ferrimonas balearica*, submitted to Biochem. Eng. J.

Paper accepted for conference oral presentation:

Peng, L., Wang, J.-Y. Evaluating carbon nanotubes as the anode modifiers in microbial fuel cells with mixed anodic consortium, the 227th Young Scientists' Forum of China Association for Science and Technology: "Microbial Fuel Cell Techniques", 3-5 December 2010, Guangzhou, China.

List of Figures and Tables

List of Important Symbols

a	Activity (mol/L)
c	Concentration (mol/L)
E	Potential (V)
$E°$	Standard Potential (V)
E_p	Peak potential (V)
ΔE_p	Separation between anodic and cathodic peak potentials (V)
F	Faraday constant (s.A/mol)
$\Delta_r H$	Reaction enthalpy (J)
I, i	Current (A)
n	Number of electron transferred
R	Ideal gas constant (J/mol.K); Resistance (Ω)
T	Absolute temperature (K)
t	Time (s)
V	Volume (L)
v	Potential scan rate (V/s)
α	Transfer coefficient
γ	Activity coefficient
ϵ	Energy efficiency
η	Overpotential (V)

Subscripts:

a	Anodic
c	Cathodic
O	Oxidized species
R	Reduced species

List of Abbreviations

AEM	Anion exchange membrane
AQDS	Anthraquinone-2,6-disulphonic disodium salt
BES	Bioelectrochemical systems
BOD	Biochemical oxygen demand
c-Cyts	Cytochromes, *c*-type
CE	Coulombic efficiency
CEM	Cation exchange membrane
CNT	Carbon nanotube
COD	Chemical oxygen demand
DET	Direct electron transfer
DI	De-ionized
DMRB	Dissimilatory metal reducing bacteria
EAB	Electrochemically active bacteria
EET	Extracellular electron transfer
EPS	Extracellular polymeric substance
ET	Electron transfer
GCE	Glassy carbon electrode
GFE	Graphite foil electrode
HPLC	High Performance liquid chromatography
LB	Luria-Bertani
MET	Mediated electron transfer
MFC	Microbial fuel cell
MWNT	Carbon nanotube, multi walled
OD	Optical density
OM	Outer membrane
PANI	Polyaniline
PEM	Proton exchange membrane
PTFE	Polytetrafluoroethylene

SAM	Self-assembled monolayer
SCE	Saturated calomel electrode
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SHE	Standard hydrogen electrode
SPE	Solid phase extraction

Chapter 1 Introduction

1.1 Background

Organic compounds are highly reductive substances. Under neutral pH, glucose has a standard electrode potential of - 0.43 V vs. standard hydrogen electrode (SHE) (Schröder 2007); other organic chemicals generally have standard potential well below 0 V. Thus, abundant free energy can be unleashed from the oxidation of organics. Large amount of waste biomass are supplied from various sectors, chemical energy can even be extracted from sources like aquatic sediment (Lovley 2006). Fuel cell is a promising option for harnessing energy from organic wastes. The advantages are obvious: firstly, due to high moisture, the organic matters are not suitable for energy conversion processes like combustion; secondly, a fuel cell has an energy efficiency which is not restrained by Carnot cycle (Rabaey et al. 2010).

Nevertheless, for conventional fuel cells, the fuels are mostly restricted among hydrogen, methane and methanol (Larminie and Dicks 2003). For more complex organic compounds, the ordinary electrocatalysts are not capable to break the chemical bonds and oxidize the compound. For instance, even with platinum, gold or alloy catalysts, direct electrochemical oxidation of glucose cannot be realized without high overpotentials; and such oxidation is generally incomplete (Ye et al. 2004). This prohibits the application of organic compounds to drive fuel cells.

Microbes are very effective catalysts towards the degradation of organic matters; microbial fuel cell (MFC) is a device that converts chemical energy to electrical energy based on the catabolism of microorganisms. The phenomenon of microbial electricity had been revealed in 1911 (Potter), but practical applications of MFCs had been proposed only in recent years, largely due to the discovery of electrochemically active microorganisms (Kim et al. 1999b). MFCs have a biomass growth yield lower than that of the aerobic treatments, thus produce less sludge (Rabaey and Verstraete 2005).

MFCs are generally operated at room temperature, distinguished from many bio-processes which require heating. In total, MFCs have unmatchable unique traits as a waste-to-energy strategy.

1.2 Motivations

Although the MFC technique is endowed with unique advantages, the application of MFCs is restrained by their low power densities (Schröder et al. 2003; Kim et al. 2007). One of the major limitations for MFC power output is the heterogeneous electron transfer (ET) process at the anode (Schröder 2007). The ET between microbes and electrode not only plays a central role in MFCs, but also in other promising bioelectrochemical systems (BES) for energy conversion and environmental remediation (refer to section 2.6). One estimation indicated that the maximum current density controlled by the diffusion of substrate can be 50 mA/cm^2 in a bacterial anode (Rabaey et al. 2010). However, the actual anodic current density is only 1-2% of this value at a maximum, suggesting a great potential for enhancing the bioactivities and extracellular ET (EET) processes. Previous studies suggested anode modifications (Park and Zeikus 2002; Park and Zeikus 2003) to be an effective approach to enhance the microbial EET. Up to 100-1000 folds increments in current densities were reported with these modifications.

Good understanding of EET processes is a prerequisite for its improvements. However, the significance in studying microbial EET is not confined to paving a way for optimized engineering systems. The ET between microbes and minerals is involved in biogeochemical cycles of Fe, Mn and other metals (Shi et al. 2009). More generally, such ET is an interested topic for microbial physiology. Numerous efforts had been carried out to elucidate the mechanisms in EET (Weber et al. 2006). The discovery on microbe-electrode interaction implicated a new approach in the quest. Compared with the conventional biological methodologies, the electrochemical approach is facile, real-time, and in-situ. Microbial respiration rate can be directly monitored through chronoamperometry. The presence of electron conduits at the microbe-electrode

interface can be conveniently and instantly detected by non-turnover voltammetries. The electrocatalytic activities of these conduits can be revealed in turnover voltammetries (Rabaey et al. 2010). The electrochemical investigations can be whole-cell scale or protein scale, the later gives details on the candidate electrode reductases (Hartshorne et al. 2009). Some important discoveries on microbial EET in recent years had been made through electrochemical characterizations (Marsili et al. 2008a).

1.3 Objectives and scopes

This work aimed to further the understanding on microbial EET processes and find a proper electrocatalyst to enhance this process.

It is very difficult to analyze the ET processes in a mixed exoelectrogenic community. This work adopted a model exoelectrogen, *Shewanella oneidensis*, as an object of study. There had been considerable works done on EET of *S. oneidensis*. It is known that *S. oneidensis* may use both outer membrane *c*-type cytochromes (OM *c*-Cyts) for direct ET (DET) and redox shuttles for mediated ET (MET) (Baron et al. 2009). Nonetheless, it is still in debate which EET pathway is actually effective (Jiang et al. 2010; Liu et al. 2010a). Moreover, the impact of environmental conditions on EET physiologies requires further discussions.

Compared with MET, microbial DET may be more suitable for practical applications. The mediators might be washed away in continuous-mode BES. Therefore, promoters for *S. oneidensis* OM *c*-Cyts are desirable.

It should also be questioned whether the results obtained with a model bacterium is representative. Based on these research questions, the specific objectives and scopes of this thesis included:

- To evaluate *S. oneidensis* EET through electrochemical assays aided with chromatographic and microscopic analyses.

- To identity novel exoelectrogen genetically distant from *S. oneidensis* and compare the EET behaviors.
- To evaluate carbon nanotubes (CNTs) as the promoter for DET from *S. oneidensis*.
- To validate the universality of the catalytic effect of CNTs through full cell and half cell experiments with mixed cultures

1.4 Structure of the thesis

The thesis consists of seven chapters:

i. Chapter 1 introduces the background of MFCs, as well as the challenges and issues that motivated the present works

ii. Chapter 2 thoroughly reviews the status quo of MFC researches, with emphases on the anodic ET mechanisms, known exoelectrogens as well as the methods to enhance the anodic process.

iii. Chapter 3 reports a potential-dependent physiology of *S. oneidensis*, where the presence of OM *c*-Cyts near electrode is controlled by electrode potential. This chapter also contains information on the electrocatalytic effects of redox shuttles and cell surface proteins.

iv. Chapter 4 presents a novel exoelectrogen, *Ferrimonas balearica*. The power-generating performance and possible EET mechanisms of this bacterium are discussed.

v. Chapter 5 reports the promoting effects of CNTs towards the ET through the OM *c*-Cyts of *S. oneidensis* or *F. balearica*.

vi. Chapter 6 reports the promoting effects of CNTs for ET from a mixed anodic consortium.

vii. Chapter 7 lists the main conclusions derived from this study and recommendations for future works.

Chapter 2 Literature Review

2.1 General aspects of MFCs

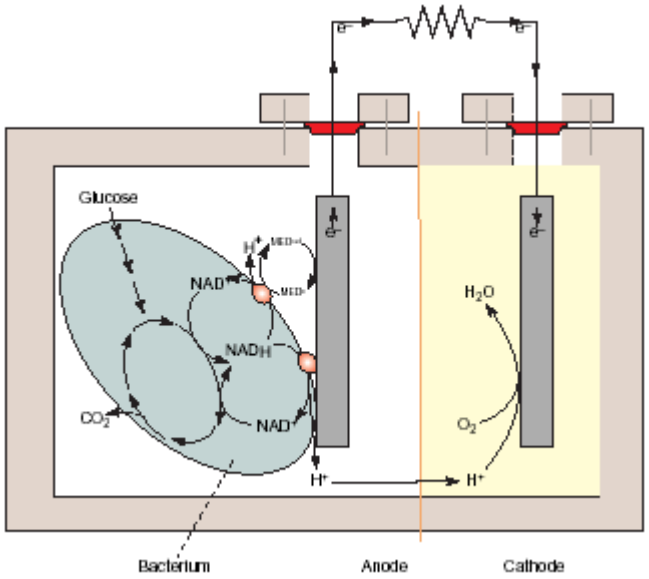


Figure 2.1 Sketch of a dual-chamber MFC with immersed air cathode (Rabaey and Verstraete 2005)

A typical dual-chamber MFC with immersed air cathode is shown in Figure 2.1. It consists of an anodic and a cathodic compartment separated by a separator, most often a proton exchange membrane (PEM). In the anodic compartment, fuel is oxidized by microorganisms, releasing electrons and protons. Electrons are transferred to the cathodic compartment through an external electric circuit, and the protons migrate to the cathodic compartment through the membrane. Electrons and protons are consumed in the cathodic compartment, combining with oxygen to form water. Despite of these basic concepts, the exact configuration of MFCs can be very diverse. Most notably, it was shown that the separator in MFC is not indispensable, membrane-less systems can steadily output bioelectricity (Liu and Logan 2004).

2.1.1 Thermodynamics and kinetics of MFCs

The electromotive force of a fuel cell is the difference between the anodic and cathodic equilibrium potential. The equilibrium potential of an electrode can be derived from the Nernst equation, which was established by correlating the electrical work in an electrochemical process with the change of Gibbs energy. Consider a half reaction: $O + ne \rightleftarrows R$, the Nernst equation writes:

$$E = E° + \frac{RT}{nF} ln \frac{a_O}{a_R} \tag{1.1}$$

Where E is the equilibrium potential of the half reaction. $E°$ is the standard equilibrium potential (when all reactants and products are under standard conditions). The real reaction condition is usually different from the standard condition that the concentrations of species are different. For such case, E deviates from $E°$. In the equation, R is the universal gas constant; T is the thermodynamic temperature; n is the number of electrons transferred; F is the Faraday constant. "a_i" refers to the activity of a chemical species, it can be expressed as activity coefficient multiply by concentration "$\gamma_i c_i$". In an infinitely dilute solution, the activity coefficient γ approaches one. The "standard biological electrode potentials" (pH=7) of some important redox pairs are shown in Figure 2.2.

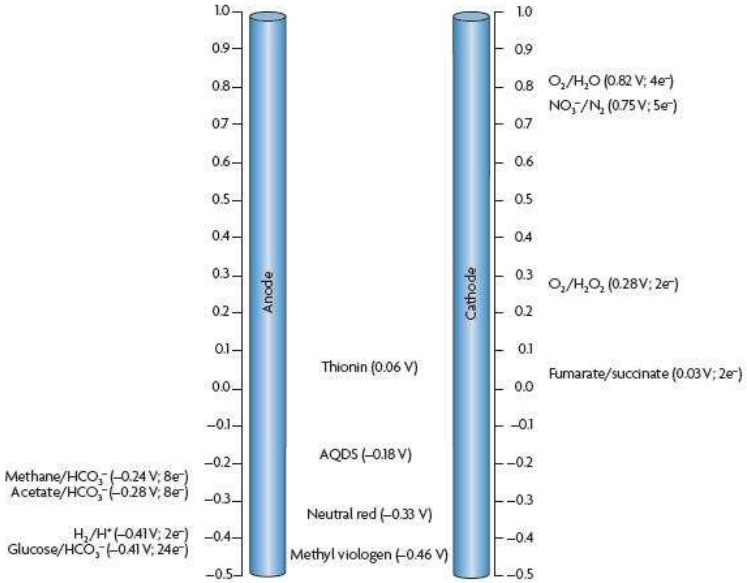


Figure 2.2 Standard electrode potential (pH=7) of some import redox pairs in microbial BES (Rabaey and Rozendal 2010)

However, in a real MFC system, there are various potential losses. Most of the losses are associated with current densities (reaction kinetics) in MFCs, which in turn regulate the system kinetics.

i. At the MFC anode, the chemical species engaged in the electrode processes might be cell surface proteins or redox shuttles; the original substrates usually do not participate the electrode reaction directly. The standard potentials of those redox substances (diffusive mediators, membrane-bounded cytochromes) are doubtlessly higher than that of the substrate. This can be explained as microorganisms consume part of the free energy for their own biological activities. The microbes obtain electrons from the oxidation of the substrates, only minimal amount of free energy can be acquired from this process. To capture more free energy, and to keep an electron balance, the microbes must transport the electrons across their inner membrane. ET through the inner membrane may establish a proton motive force, and this can be utilized by microbes to synthesize ATP for bioactivities (Unden and Bongaerts 1997). In the electron relay across the inner membrane, the electrons are always passed from a carrier with lower redox potential to a carrier with higher potential. Therefore, the final electron carrier in the ET chain has a redox potential much higher than that of the initial carrier (possibly NADH). Furthermore, for anode reducers, they need to continue the ET, from the inner membrane to the electrode reductases locate at the OM, or to some redox shuttles. Significant part of the chemical energy might be lost in these processes.

ii. No matter the final electron donor is a redox protein or diffusive mediator, activation losses will be incurred in the heterogeneous ET step. To enable a nonzero net rate of an electrochemical reaction, the electrode potential must deviate from the equilibrium potential. This potential deviation is coined as “activation overpotential”. The activation overpotential is the driving force for the electrode processes. Butler-Volmer model is one of the most widely accepted theories on electrode kinetics (Bard and Faulkner 2001). For a single-electron process, a simplified Butler-Volmer equation is given below:

$$j = j_0\left[e^{-\alpha f\eta} - e^{(1-\alpha)f\eta}\right] \quad (1.2)$$

Where, j stands for the current density; both α and f are constants, where α is named as transfer coefficient, its value depends on the nature of the electrode process. Obviously, current increases exponentially with the overpotential η; but it is also associated with the "exchange current density" j_0. j_0 largely determines the kinetic rate of an electrochemical reaction, especially when the overpotential is relatively low. Among the known heterogeneous ET processes, the exchange current density can differ for 10 orders of magnitudes (Bard and Faulkner 2001). j_0 relies on many factors, including the properties of reactants, material and surface condition of electrodes, as well as the interaction between reactants and electrodes. Activation losses occur in both anodic and cathodic processes in an MFC.

iii. Concentration overpotential: reactants near electrode surface are continuously consumed, accompanied by generation of products. Concentration of reactants and products adjacent to electrode can differ considerably from bulky solution, especially when the electrode process is fast. The activity term in the Nernst equation is the origin of concentration potential loss.

iv. Potential losses in electrolyte and separators, this can be simply described by Ohm's law. This kind of potential losses can be significant in MFCs, Fan et al. (2008) developed a model and described that the electrolyte resistance had a share of 47.3% in total internal resistance of their systems. Electrolyte resistance can be lowered by either reduced electrode spacing or excessive supporting electrolyte (Liu et al. 2005a).

2.1.2 Evaluating the performance of MFCs

To assess a performance of an MFC, there're several important parameters for comparison (Logan et al. 2006):

Power density is probably the most important index for comparison between fuel cells.

It is largely determined by cell electromotive force and various internal resistances. MFCs have power densities at least 3 orders of magnitudes lower than those of hydrogen fuel cells (Wang et al. 2005). Equation 1.3 describes the calculation of power density normalized to electrode area (either anode or cathode). In the equation, R is the external resistance; A refers to electrode area which can be either projected area, apparent geometric area, or the "true surface area" determined by gas adsorption methods. Some literatures report a volumetric power density, while the volume used for calculation can be of the whole reactor or of one chamber (anodic or cathodic).

$$P_A = \frac{{E_{cell}}^2}{RA} \tag{1.3}$$

Coulombic efficiency (CE) refers to the ratio of electrons passed through external circuit over theoretically collectable electrons from substrate degradation, expressed as:

$$CE = \frac{\int_0^\tau I(t)dt}{FnV\Delta COD} \tag{1.4}$$

COD is the abbreviation for chemical oxygen demand; where $FnV\Delta COD$ describes total amount of electrons unleashed from COD removal. For MFCs, CE can be influenced by operational conditions; satisfactory anaerobic condition at the anode is generally required for higher CE.

Energy efficiency compares the captured electrical energy with theoretical reaction energy:

$$\epsilon = \frac{\int_0^\tau I(t)^2 Rdt}{\Delta_r H} \tag{1.5}$$

In equation 1.5, $\Delta_r H$ is the reaction enthalpy of substrate oxidation. If complex substrate is used, the $\Delta_r H$ is generally unknown, and needs to be determined experimentally. Most literatures do not report the energy efficiency.

Besides these, COD removals rates are reported in some literatures, although such removal may not be solely associated with the electrochemical process.

2.2 MFC anodic ET mechanisms

The ET from microbes to electrodes is the key point for MFCs and other whole-cell BES. Since the bacterial cell walls are electrically nonconductive, special pathways must be employed to wire the microbial catabolic activities with the anode reduction. Many research activities were dedicated to mechanisms of microbial EET. To date, academia has identified DET and MET. DET can be achieved by microbial OM-bounded cytochromes and conductive nanowires. MET resorts to soluble redox shuttles, either exogenous or endogenous (Lovley 2008b; Logan 2009). Although microbial fermentation products can also act as the electron carriers (Schröder 2007), discussions in this section focus on microbial DET and MET.

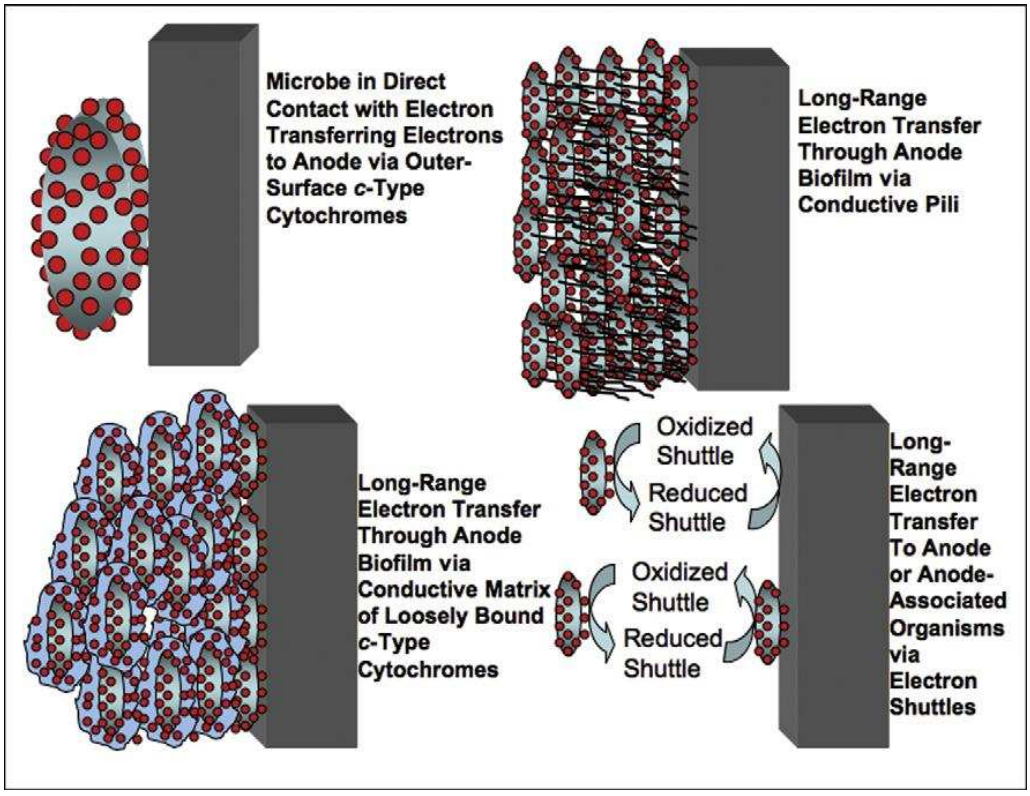


Figure 2.3 Anodic ET mechanisms (Lovley 2008b)

2.2.1 DET

2.2.1.1 Cell surface cytochromes

For the dissimilatory metal reducing bacteria (DMRB), the minerals cannot penetrate their cell wall, and they have to develop some approaches to access the external oxidants. These approaches may also enable them to use the anode as electron acceptor. The redox proteins localized at bacterial OM are widely assumed to be electron conduits (Shi et al. 2009).

S. oneidensis is one of the most intensively studied DMRB. *S. oneidensis* is known to adapt to great diversity of electron acceptors, including Mn(IV), Fe(III), Cr(VI), U(VI), Tc(VII) (Myers and Myers 2003b). It was observed that, when *S. oneidensis* were grown with minerals, they were exclusively found on the surface of the minerals, inferring that physical contact is important for metal reduction (Beliaev et al. 2001). Like other DMRB, *S. oneidensis* is known to possess numerous *c*-Cyts, 42 different *c*-Cyts had been predicted from its genome, comparing to only 7 of *Escherichia coli* (Shi et al. 2007).

More importantly, *S. oneidensis* is able to localized some *c*-Cyts to its cell surface. Myers and Myers (1992) discovered that *c*-Cyts were largely concentrated at the bacterial OM, when *S. oneidensis* were cultured under anaerobic conditions. The genome of *S. oneidensis* suggested 5 possible OM *c*-Cyts (Meyer et al. 2004), two decaheme *c*-Cyts had been confirmed and isolated, namely OmcA and MtrC (Myers and Myers 1998; Beliaev et al. 2001). These cytochromes were further shown to be localized at the cell surface. Myers and Myers (2003a) applied proteinase to degrade the possibly exposed *c*-Cyts. They found that, 71% of OmcA and 31% of MtrC were degraded. Lower et al. (2009) mapped the distribution of OM *c*-Cyts with antibody recognition force microscopy. They found that OmcA and MtrC were not only localized at the cell surface, but also the extracellular polymeric substances (EPS). OmcA were more concentrated at the cell-mineral interface, while the distribution of

MtrC was more uniform. Reardon et al. (2010) also studied the distribution of antibody-labeled OM *c*-Cyts with ferrihydrite-grown *S. oneidensis*. They confirmed localization of OmcA/MtrC at the cell surface, EPS and cytoplasm. However, in contrast to the finding by Lower et al. (2009), MtrC were found to be closely associated with iron precipitates, while the distribution of OmcA was more diffusive. The extracellular translocation of OmcA/MtrC was associated with type II secretion system, deletion of the corresponding gene reduced the cell surface exposure of these *c*-Cyts (Shi et al. 2008).

Various studies had confirmed the specific binding between *Shewanella* OM *c*-Cyts and mineral surface, which is a prerequisite for DET (Xiong et al. 2006; Lower et al. 2007). The ET rates from OM *c*-Cyts to the minerals had been evaluated. Xiong et al. (2006) estimated that the heterogeneous ET rate from OmcA to hematite was in the 0.1 s^{-1} range. Concurrently, electrochemical studies had been carried out with purified OmcA/MtrC. However, the results reported by different authors did not seem to be in agreement. Hartshorne et al. (2007) immobilized purified MtrC on basal plane graphite electrode, the electrochemistry of MtrC was chemically reversible, the kinetic rate was estimated to be around 100 s^{-1}. The redox peaks of MtrC spanned over 500 mV, possibly due to the multi-heme nature of MtrC. Firer-Sherwood et al. (2008) studied OmcA, MtrC and several other *Shewanella c*-Cyts. They found reversible electrode process for all cytochromes. The heterogeneous rate constant of MtrC was determined to be 276 s^{-1}. There were multiple redox centers in OmcA/MtrC voltammograms, probably represented hemes with different redox potentials. Eggleston et al. (2008) obtained totally different results: the ET process of OmcA appeared to be chemically irreversible, where the oxidation process was obstructed. In a further study by Meitl et al. (2009), both OmcA and MtrC showed irreversible electrochemistry, the voltammograms did not show recognizable "multi-heme" pattern. Using Laviron's theory, the rate constant was estimated to be around 0.045 s^{-1}, close to the estimation by Xiong et al. (2006).

The function of OM c-Ctys towards mineral or electrode reduction had been evaluated with knockout mutants. When either *omcA* or *mtrC* gene was deleted, the reduction rates towards manganese dioxide was reduced (Myers and Myers 2001); whereas, the deficiency in OmcA could be compensated by over-expression of MtrC (Myers and Myers 2003b). Bretschger et al. (2007) reported that, a mutant of *S. oneidensis* MR-1 deficient in both OmcA and MtrC lost over 80% of current-generating capability in MFC. However, the authors also described that: this mutant appeared to have a weakened ability to colonize graphite fiber anodes. Therein, it was not conclusive whether the loss in current generation was due to the inability of DET through OM *c*-Cyts.

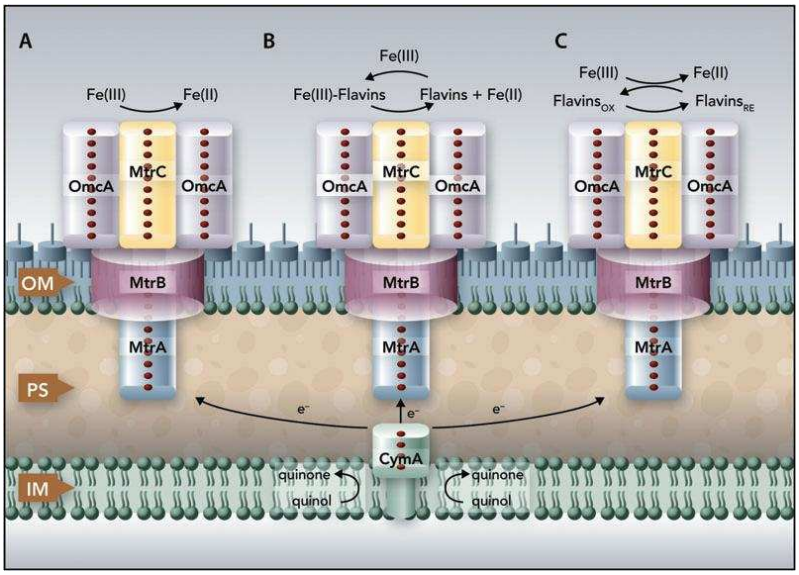


Figure 2.4 Proposed EET pathways of *S. oneidensis* (Shi et al. 2009)

Geobacter sulfurreducens possesses even more abundant *c*-Cyts, 111 different *c*-Cyts were predicted by the genome (Methé et al. 2003). Some *c*-Cyts were known to be located at the OM of *G. sulfurreducens*. Mehta et al. (2005) identified two *c*-Cyts from the cell surface, namely OmcS and OmcE. Qian et al. (2007) found that a multicopper protein OmpB is exposed on the cell surface, while *c*-Cyt OmcB is partially exposed. Another c-Cyt, OmcZ was found to be contained in loosely bound OM fraction of *G.*

sulfurreducens (Inoue et al. 2010).

Nevin et al. (2009) found that, *G. sulfurreducens* mutants deficient in OmcE, OmcB or OmcS were not significantly hampered in current generation; while the mutant lacking OmcZ lost over 90% of current generation compared with the wild strain. Another study reported similar results (Richter et al. 2009). This suggested that, among the known OM *c*-Cyts, only OmcZ is indispensible for exoelectrogenesis.

2.2.1.2 Conductive nanowires

Some DMRB were observed to develop conductive pili to enable long range ET to electrodes. The first evidences of EET through microbial pili came from observations with *G. sulfurreducens* (Reguera et al. 2005). Such pili were developed when *Geobacter* cells were grown with insoluble electron acceptors. These pili were highly conductive and were formed on one side of the cells. The pili were coined as "conductive nanowires". The nanowires were soon found to be relevant to current generation. Within *G. sulfurreducens* biofilm formed in an MFC, pili as long as 20 μm had been developed (Reguera et al. 2006). Meanwhile, a biofilm of 40-50 μm thickness had been observed. There were linear increases in the amount of anode-attached biomass when the current increased. This suggested the cells distant from anode were metabolically active and were involved in ET to anode. It remained questionable whether the cells on the top layers communicated the electrode directly with pili or through the cells lying beneath. In order to further evaluate whether the nanowires were indispensible for *Geobacter* EET, *G. sulfurreducens* was genetically engineered to produce mutant strains where the synthesis of pili was disrupted. The first finding was that there was less biomass attached to the iron oxides or electrodes, when a *ΔpilA* (a gene assumed to be involved in pili production) mutant was compared with wild strain (Reguera et al. 2005; Richter et al. 2009). This indicated that the pili were necessary for colonization of solid surface as well as long distance ET. It was also found that, the gene transcript level of *pilA* was greatly increased in exoelectrogenic *G. sulfurreducens*, compared to fumarate grown cells (Nevin et al. 2009). Current generation of various *G.*

sulfurreducens mutants including the *ΔpilA* strain was directly compared in an electrochemical cell (Richter et al. 2009). The wild strain generated current 2 times higher than that generated by *ΔpilA* strain; this was consistent with the fact that the wild strain biofilm was 20 μm thick, compared to 6 μm biofilm of mutant strain. For the mutant strain, there was also a longer lag time in current generation. Later finding showed that, *G. sulfurreducens* could still produce pili when the *pilA* gene was deleted (Klimes et al. 2010), which explained the fact that *ΔpilA* strain could still form multi-layer, exoelectrogenic biofilms. Although a number of studies emphasized the importance of nanowires, it was not yet clear how the nanowires donate electrons to the electron acceptors. One of the latest findings on *G. sulfurreducens* nanowires was that: cytochrome OmcS align along the naowires (Leang et al. 2010). There was obvious spacing between the cytochromes, thus, OmcS were not supposed to enhance the conductivity of pili, but probably acted as the final electron donors. In summary, for *G. sulfurreducens*, the microbial nanowaires are likely to be involved in DET. Nevertheless, such nanowires are far from being well understood, it should be answered: 1.how are the nanowires engaged in the EET; 2. which genes regulate the expression of nanowires.

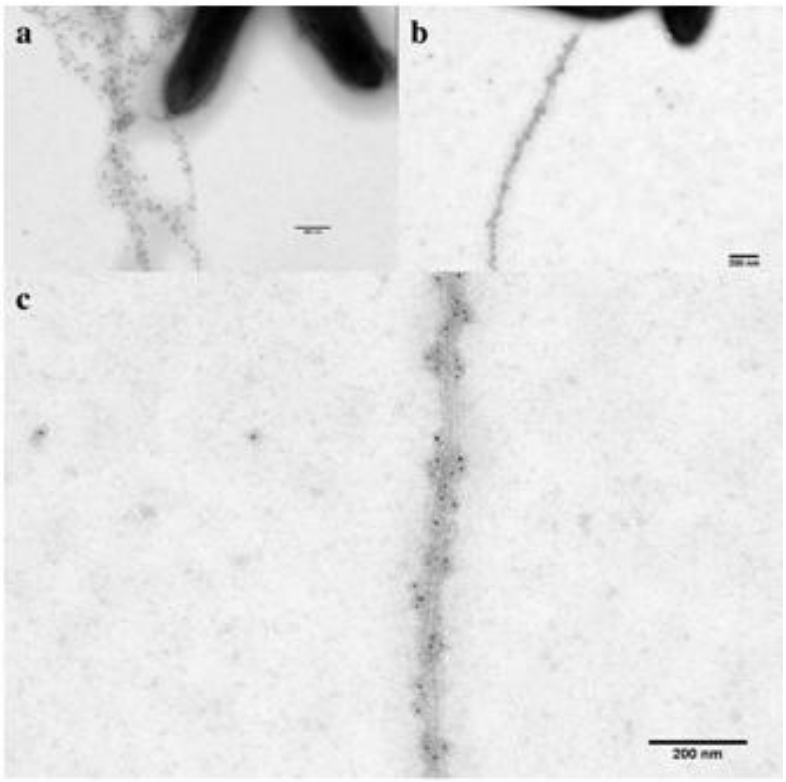


Figure 2.5 Labeled cytochrome OmcS (black dots) aligned on the *G. sulfurreducens* nanowires (Leang et al. 2010)

Nanowires were also observed with *S. oneidensis* (Gorby et al. 2006). Conductive pili were developed by *S. oneidensis* under anoxic conditions. Mutants of *S. oneidensis* deficient in OM *c*-Cyts possessed pili as well, but such pili were nonconductive. Nonetheless, there had not been sufficient evidence showing that the nanowires of *S. oneidensis* are actually engaged in exoelectrogenesis. More recent literatures regarding *Shewanella* EET had dropped out the nanowire concept (Leang et al. 2010). A new study found that, a mutant of *S. oneidensis* incapable of pili production unexpectedly delivered much higher current level than the wild strain did (Bouhenni et al. 2010). This result further questioned the role of nanowire in *Shewanella* EET.

2.2.2 MET

Artificial mediators were once widely applied in MFC systems (Bennetto et al. 1983). With the well known drawbacks, they were gradually abolished and gave rise to MFCs free from exogenous mediators. Microbial secondary metabolites (endogenous electron mediators), captured the attention of researchers in recent years (Watanabe et al. 2009).

For a long time it had been known: diverse bacteria, including *E. coli* and *Pseudomonas* species, can reduce iron but cannot be grown coupled with that. A study concluded that phenazines and other redox-active antibiotics released by *Pseudomonas* species could enhance mineral reduction (Hernandez et al. 2004). These antibiotics can serve as electron shuttles in MFCs. A research group at Ghent University (Belgium) observed *Pseudomonas aeruginosa* strain KRP1 to produce pyocyanin, phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN). Presence of an anode spurred the pyocyanin yield. When the produced pyocyanin was introduced to MFCs containing other bacterial species, exoelectrogenesis was stimulated. This manifested that *P. aeruginosa* can produce electron shuttles utilizable by other bacteria (Rabaey et al. 2003; Rabaey et al. 2004).

E. coli cells could be "electrochemically evolved" in the fuel cell environments, which in turn improved the fuel cell performance (Zhang et al. 2006). This phenomenon was later explained by detection of self-produced electron shuttling compounds in electrolyte (Zhang et al. 2008b). It was not totally clear which self-secreted chemical species mediated *E. coli* EET. Qiao et al. (2008b) suggested that *E. coli* releases hydroquinone to reduce the electrodes, while Zhang et al. (2008b) proposed several other redox compounds.

Newman and Kolter (2000) argued that *S. oneidensis* secreted quinone-like molecules for EET. The authors first found that a mutant of *S. oneidensis* incapable of menaquinone synthesis was inhibited in reduction of humic substances. Menaquinone was assumed to pass electrons to the terminal reductases for humic substances. While the mutant strain was grown in a petridish together with the wild strain, the reduction of

humic substances was restored, especially for the mutant cells in the proximity of wild-type cells. The authors thus concluded that the wild cells could release a diffusive factor utilizable by the mutant cells as mediators; they further analyzed the extracellular medium and found this diffusive factor could be some quinone-like molecules. However, this conclusion was challenged. Other researchers found that the diffusive factors released by the wild strain restored the menaquinone synthesis in the mutant cells, but might not act as electron mediators (Myers and Myers 2004). Some observations indirectly suggested that *S. oneidensis* could use mediators for EET. Rosso et al. (2003) observed that, hematite-attached *S. oneidensis* could conduct nonlocal ET. Lies et al. (2005) reported that *S. oneidensis* could reduce Fe(III) entrapped in nanoporous glass beads. Lanthier et al. (2008) found that there was high planktonic biomass in a *S. oneidensis* fuel cell, while such phenomenon had not been observed with *Geobacter* species.

Direct evidences on the MET of *S. oneidensis* were presented by Marsili et al. (2008a) and von Canstein et al. (2008). These authors determined flavin species, specifically riboflavin and flavin mononucleotide, as the extracellular mediators. The current density delivered by *S. oneidensis* could drop by over 70% when the medium in the electrochemical cell was replaced. When the original medium (with planktonic cells removed) was returned to the electrochemical cell, the current density immediately recovered. These findings confirmed the importance of diffusive shuttles. Later studies suggested that flavins were much more efficient towards the reduction of minerals when compared with *Shewanella* OM *c*-Cyts (Ross et al. 2009). Although the OM *c*-Cyts had higher rates towards the reduction of soluble electron acceptors, the kinetic rates towards reduction of geothite were lower than that of bacterial membrane fraction for three orders of magnitudes. Flavins were closely affiliated with the membrane fraction and could explain the higher kinetic rate. Hence, the flavins were more likely to be directly responsible for the reduction of electrodes.

Recent researches demonstrated that, the co-existence of microbes and redox shuttles (exogenous or endogenous) does not guarantee effective MET. The MET requires a complicated interaction between electroactive substances and the bacterial ET systems. Each redox compound may require a certain reductase. Baron et al. (2009) and Coursolle et al. (2010) showed that, when OM *c*-Cyts OmcA and MtrC were deleted from *S. oneidensis*, the bioelectricity generation was largely inhibited. Their results also indicated: the inhibition might not be due to the loss of bacterial DET; instead, the electrocatalytic activity of flavins appeared to be hindered. Thereafter, OmcA/MtrC either acted as the flavin reductases or played important roles in flavin respiration.

In general, it has been proven that certain microbes can release redox shuttle to the extracellular medium and utilize them to establish a connection with the electrodes. Nevertheless, it should be answered how the microbes reduce these redox compounds. Another issue worth further investigation is how can the endogenous mediators remain within anodic biofilm, which will otherwise dissipate to bulky fluid (Rabaey et al. 2007).

2.3 Exoelectrogens

Exoelectrogens can be roughly defined as bacteria capable of transporting electrons to electrodes without exogenous redox mediators (Schaetzle et al. 2008). Alternative terms include: “anodophiles”, “electrochemically active bacteria” (EAB) and “anode-respiring bacteria” (Lovley 2008b).

One of the earliest reported mediator-less MFC was operated with *S. oneidensis* MR-1 and several strains of *Shewanella putrefaciens* (Kim et al. 2002). Cyclic voltammograms with these microorganisms showed a redox pair centered at - 0.2 V vs. Ag/AgCl reference electrode. It was also reported that only anaerobically grown *Shewanella* cells manifested electrochemical activity. More *Shewanella* species were shown to be suitable biocatalysts for MFCs in the following years. Nakamura et al. (2009) studied the exoelectrogenesis of *Shewanella loihica* PV-4. Bretschger et al.

(2010) evaluated 8 different *Shewanella* strains for power generation, including *Shewanella amazonensis* SB2B. Huang et al. (2010) applied a newly isolated *Shewanella marisflavi* strain for MFC, and achieved power density of 9.6 mW/m^2 under high ionic strength. Feng et al. (2010a) investigated the interaction between *Shewanella decolorationis* S12 and an anode modified with polymers and redox mediators, they attained a power density of 1303 mW/m^2. These results strongly suggested: exoelectrogenesis might be ubiquitous among the *Shewanella* species. There are currently over 50 *Shewanella* species, new species are being continuously isolated from the environment or identified from known strains (Hau and Gralnick 2007; Fredrickson et al. 2008). It is reasonable to believe that the *Shewanella* genus contains a huge number of current-generating species.

Pham et al. (2003) isolated an *Aeromonas hydrophila* strain from an MFC, and named it as PA3. Both PA3 and the type culture of *A. hydrophila* showed electrochemical activity. The voltammogram of *Aeromonas* was similar to that of *Shewanella* (Kim et al. 2002), where a redox pair centered at - 0.2 V vs. Ag/AgCl was found, and the reductive peak was sharper than the oxidative peak. Furthermore, similar to the observations with *Shewanella* (Kim et al. 1999a), oxygen exposure diminished the signal of this redox pair. The MFC could immediately reach its maximum current density when *A. hydrophila* was introduced. *A. hydrophila* exhibited good ability to colonize the electrode. Chung and Okabe (2009) isolated *Aeromonas* ISO2-3 from a glucose fed MFC, and studied it in electrochemical cells and MFCs. Both *Shewanella* and *Aeromonas* are under the class of *γ-proteobacteria*.

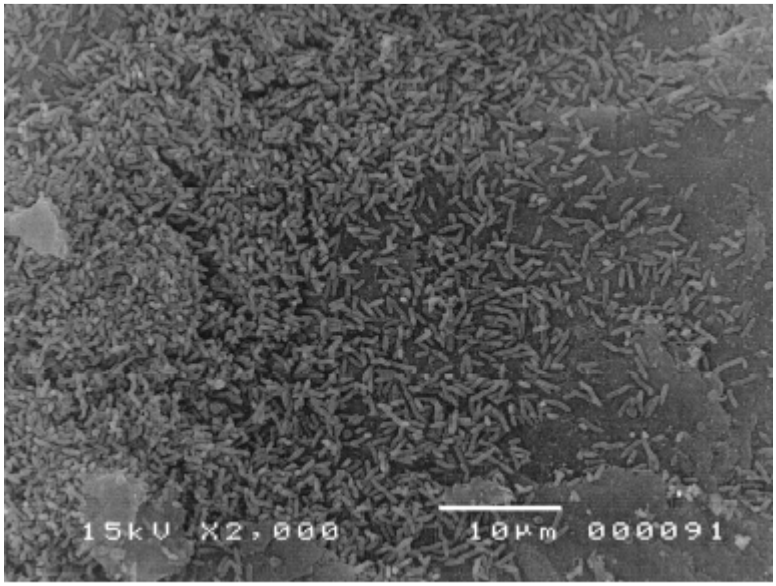


Figure 2.6 *G. sulfurreducens* on the surface of electrode (Bond and Lovley 2003)

Geobacter species were well known for their ability to reduce minerals (Lovley and Phillips 1988). Bond et al. (2002) showed that *Geobacter metallireducens* transferred electrons to a poised electrode, coupled with the degradation of benzoate. The ability to degrade aromatic hydrocarbon for power generation could be used for soil remediation (Zhang et al. 2010b). *G. sulfurreducens* strain PCA delivered a current density of 1143 mW/m^2 with a graphite stick poised at 200 mV vs. Ag/AgCl (Bond and Lovley 2003). In the initial fuel cell test, the power density was only about 13 mW/m^2, which was likely to be limited by abiotic factors in the MFC. Under exoelectrogenic condition, *G. sulfurreducens* completely oxidized acetate to carbon dioxide, while *S. oneidensis* do not utilize acetate under anaerobic conditions. Like many other exoelectrogens, *G. sulfurreducens* is facultative anaerobe (Lin et al. 2004), this makes it unnecessary to maintain a strict anaerobic condition in MFCs. Strycharz et al. (2008) found that *Geobacter lovleyi* coupled the acetate oxidation with electrode reduction, thus was qualified as an anode reducer. Very likely, the generation of bioelectricity is common in the *Geobacter* genus, and probably extend to the whole *Geobacteraceae* family (Bond et al. 2002).

Bond et al. (2002) also found *Desulfuromonas acetoxidans* to grow with acetate as the electron donor and poised electrodes as the electron acceptor. *D. acetoxidans* catalyzed an MFC without any exogenous mediators, addition of mediator only slightly enhanced the current output. Thus, this bacterium could probably conduct DET. Shortly after, *Rhodoferax ferrireducens* was reported for complete oxidation of glucose in MFC with a CE as high as 80% (Chaudhuri and Lovley 2003). Holmes et al. (2004a) reported that *Desulfobulbus propionicus* utilized pyruvate or lactate for current generation. The maximum current density was about 30 mA/m^2 with a graphite stick poised at 520 V vs. SHE. Unlike *Geobacter* which took days to attain its maximum current level (Bond and Lovley 2003), *D. propionicus* delivered its maximum current immediately after the inoculation. This was likely to be a result of different ET strategies. In the same year, these researchers isolated *Geopsychrobacter electrodiphilus* from the anode of a sediment MFC. This isolation was shown to generate current, and *Geopsychrobacter* represented a new genus under the *Geobacteraceae* family (Holmes et al. 2004b). Bond and Lovley (2005) verified *Geothrix fermentans* as another exoelectrogen. This species was likely to secret a redox shuttle for EET. The most recent exoelectrogen reported by Lovley's research group was *Anaeromyxobacter dehalogenans* (Strycharz et al. 2010). With a graphite electrode poised at 500 mV vs. SHE, *A. dehalogenans* delivered a current density around 40 mA/m^2.

Kodama and Watanabe (2008) isolated an *α-proteobacteria* strain from a cellulose fed MFC. 16S rRNA gene sequences suggested that this strain was not associated with any known EAB. It utilized various substrates and electron acceptors (including electrode) for growth. Fedorovich et al. (2009) found *Arcobacter* (*ε-proteobacteria*) to be dominant in their MFC community, while the MFC was generating a high power density at 296 W/m^3. They isolated *Arcobacter butzleri* ED-1 which could catalyze the MFC anodic process. Karthikeyan et al. (2009) used *Acetobacter aceti* and *Gluconobacter roseus* as MFC biocatalysts. Although bioelectricity was observed, the CEs were extremely low in their experiments. Kiely et al. (2010) isolated *Paracoccus denitrificans* PS-1 from a formic acid fed MFC. The *P. denitrificans* fuel cell with a

graphite fiber brush anode had a power density of 5.6 mW/m^2

Some anode reducers can generate electricity from complex organics. *Klebsiella pneumonia* could use starch as the fuel in MFCs (Zhang et al. 2008a). *K. pneumonia* L17 delivered a maximum power density of 218 mW/m^2 with starch. An isolation genetically related to *Klebsiella oxytoca* was known for exoelectrogenesis as well (Kim et al. 2006). Rezaei et al. (2009) isolated *Enterobacter cloacae* FR from their MFC enrichment. This bacterium used many organic compounds for current generation, including cellulose. The power density was 5.4 mW/m^2 when fueled with cellulose. The type strain of *E. cloacae* had a similar performance.

It had been considered that gram-positive bacteria play only supportive roles in MFCs and are not directly engaged in the current generation, based on the assumption that ET through their thick cell walls is more difficult (Ehrlich 2008). Park et al. (2001) found that a *Clostridium* strain was electrochemically active. Prasad et al. (2006) isolated a *Clostridium* strain from industrial effluent. They utilized this strain as the anodic biocatalyst in a mediator-less MFC. A thermophilic *Thermincola* strain was shown to generate electricity with a very high CE of 91% (Wrighton et al. 2008). Marshall and May (2009) further studied the EET process of *Thermincola ferriacetica*. With a solid graphite block anode, they achieved a current density around 400 mA/m^2 (1000 Ω load), while the maximum power density was 146 mW/m^2. The authors found that after each replacement of medium the electricity generation could be quickly recovered, suggesting that gram-positive bacteria can possibly reduce the electrode in a direct way. First derivative analysis of the turnover voltammogram revealed several redox centers with mid-point potentials ranged from – 0.45 to – 0.25 V vs. Ag/AgCl. *Bacillus* are very common gram-positive bacteria, *Bacillus subtilis* BBK006 was shown to form thick biofilm on carbon cloth, and donate electrons to it (Nimje et al. 2009). However, the power density was not particularly high, which was about 10.5 mW/m^2.

Malki et al. (2008) isolated an *Acidiphilium* strain from acidic river sediment. In a very acidic medium (pH=2.5), *Acidiphilium* sp. strain 3.2 delivered current density of 3 A/m^2 with a electrode poised at 0.15 V vs. saturated calomel electrode (SCE). It was not known whether the high current density could be attributed to the intrinsic properties of the microbe, since the electrode material was carbon felt with high specific surface area. Oxygen exposure did not negatively affect the exoelectrogenesis of this bacterium. *Acidiphilium* sp. strain 3.2 might represent the first extremophilic exoelectrogen. On another hand, Liu et al. (2010b) evaluated a gram-positive *Corynebacterium* strain under alkaline conditions. *Corynebacterium* sp. strain MFC03 could adapt to a variety of substrates. Under a pH of 9, it delivered a power density around 20 mW/m^2 when fed with acetate. However, the reported CE was relatively low: 5.9% with glucose as the fuel.

It was once considered that microbes capable of EET were confined within the scope of DMRB. However, this is challenged by some later discoveries. Yeast cells of *Hansenula anomala* were observed to transport electrons to electrode directly, very likely through the redox enzymes ferricyanide reductase and lactate dehydrogenase associated with cell OM (Prasad et al. 2007). Zuo et al. (2008) isolated a strain of *Ochrobactrum anthropi* from a U-tube MFC, this microorganism could utilize diverse substrates for power generation, the maximum power density with acetate was 89 mW/m^2. However, this bacterium could not be grown with iron oxides. Similarly, Xing et al. (2010) identified a denitrifying bacterium, *Comamonas denitrificans*, to be exoelectrogenic. This bacterium produced a power density of 35 mW/m^2 with a carbon paper anode, but did not respire with hydrous Fe (III) oxides. Contrarily, the type strain of *Pelobacter carbinolicus* steadily reduces iron oxides, but did not output electricity (Richter et al. 2007). These findings demonstrated that, the ET pathways for iron reduction and electrode reduction are not totally overlapping.

It is difficult to compare current generating capability of exoelectrogens, since the bioelectrochemical tests were conducted under very different conditions from literature

to literature. However, some data indeed suggested that anode reducers deliver very different level of current. Under quite similar experimental conditions, *G. sulfurreducens* generated a current density about 40 times higher than that by *S. oneidensis* (Marsili et al. 2008a; Marsili et al. 2008b). It is worth noting that: although *Shewanella* are reported to output high power densities by some researchers (Ringeisen et al. 2006; Feng et al. 2010a), these results were all obtained with very special reactor or anode designs. With usual reactors and commonly used anode materials, *Shewanella* generally delivered power density lower than 10 mW/m^2. Contrarily, *G. sulfurreducens* outperformed a mixed microbial consortium in term of power generation and CE (Nevin et al. 2008). Recent study estimated that *G. sulfurreducens* can respire with the electrode at a rate comparable to their respiration rate with soluble electron acceptors (Marsili et al. 2010). The gap in power generation might be induced by different ET mechanisms (Torres et al. 2010). The conductive matrix established by *Geobacter* nanowires and extracellular redox proteins has a greater potential in current generation compared to MET or ET through OM proteins which in principle allow only one layer of microbes to deliver electricity.

Other high-power-generating microbes may include a strain of *Rhodopseudomonas palustris* (Xing et al. 2008). *R. palustris* XD-1 delivered a power density comparable to a mixed consortium enriched from wastewater inoculum. This bacterium was attested to use a number of organics for electricity generation. Replacement of anodic electrolyte did not severely affect power delivered from the fuel cell, suggesting the anodic ET was directly from microbes to electrode. However, detailed studies on EET mechanisms of *R. palustris* XD-1 had not been initiated. Notably, the type strain of *R. palustris* could hardly deliver any electricity, indicating the exoelectrogenic capability can vary greatly even among strains of the same species.

2.4 The approaches to improve ET from microbes to the electrode

The MFCs distinguish from chemical fuel cells by a unique anode process involving the ET from microorganisms. This process is generally considered as sluggish, and can

be a rate-limiting step (Schröder 2007). The improvement of microbial EET is not only necessary for the enhancement of MFC power density, but also essential for the development of other whole-cell BES. To date, researchers had attacked this problem using different strategies.

2.4.1 Modifications of anodes

2.4.1.1 Modifications of anodes for microbial DET

DET, which resort to electron exchange between metalloproteins and electrode is usually hindered by the peptide chain adjoining the active center of the protein: even if physical contact between redox protein and electrode can be established, the ET still progress at low rate or is totally impossible. It is thus necessary to improve the communication between cytochrome redox cofactors and electrode surface. This can be realized by functionalization of electrode with redox (mediators) or non-redox (promoters) electrocatalysts.

Promoted electrode processes of redox proteins, especially cytochromes, are interested because of scientific values to biochemistry and bioelectrochemistry, as well as realistic needs for enzymatic biosensors. Initially, an indium oxide electrode (Yeh and Kuwana 1977) and an electrode adsorbed with 4,4'-bipyridyl (Eddowes and Hill 1977) were announced to obtain DET and fast kinetics of diffusive horse heart *c*-Cyts. In following years, many organic compounds were attested to be promoters for electrode process of cytochromes (Allen et al. 1984). It was later known that DET of *c*-Cyts can be achieved by electrodes modified with carboxylic acid terminated alkanethiol monolayer (Tarlov and Bowden 1991). This strategy was applied to improve the cytochrome-mediated ET of *Shewanella* (Crittenden et al. 2006). A gold electrode was modified by a self-assembled monolayer (SAM) with carboxylic acid termini. However, the modified electrode delivered a bioelectrocatalytic current only moderately higher than that of a bare glassy carbon electrode (GCE); the current was not stable, it declined 3 h after the initial inoculation. Additionally, there was no evidence showing the enhanced current

output was indeed a result of promoted DET.

CNTs, consist of only sp^2 hybridized carbon atoms, are cylindrical nanostructures with a diameter ranging from one to several nanometers and a length of tens of micrometers. They are made of graphene sheets wrapped into a hollow cylinder and capped by fullerene-like structures. There are two typical types of CNTs, single-walled CNT and multi-walled CNT (MWNT). CNTs have captured the imagination of researchers worldwide since they were first observed by Iijima (1991). CNTs have been widely applied in electrochemical sensors and fuel cells for electrocatalysis (Zhao et al. 2002; Sherigara et al. 2003; Wang et al. 2005). A number of literatures asserted direct and reversible electrochemistry of metalloproteins with the presence of CNTs (Wang et al. 2002; Yin et al. 2007). It was suggested that the redox cofactor of immobilized metalloproteins can directly communicate with CNT π systems (Davis et al. 2003). This feature potentially makes CNTs promising electrode modifiers for the DET from microbes.

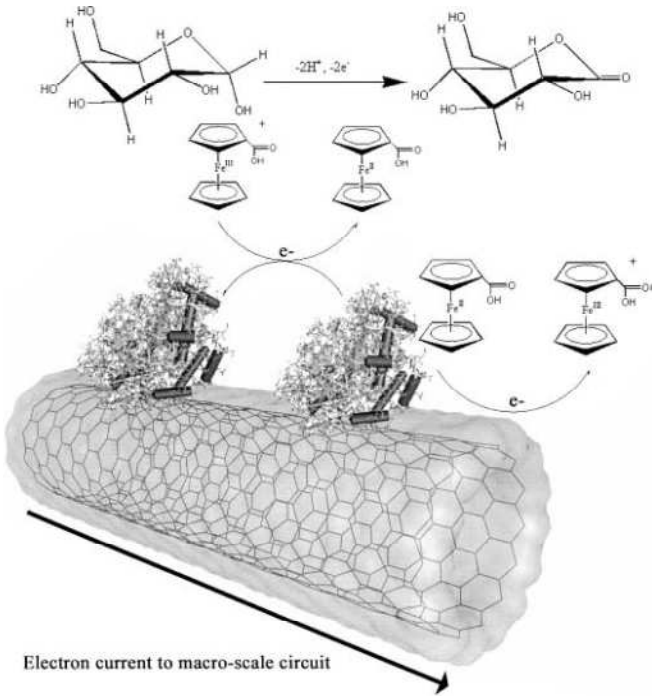


Figure 2.7 Interaction between CNT and hemoprotein (Davis et al. 2003)

On another hand, redox mediators are often used to facilitate the heterogeneous ET of redox proteins. A number of studies showed that electrode-immobilized organic dyes can still act as the mediators between proteins and electrode (Xu et al. 2003; Yan et al. 2006). Park and Zeikus (2003) tried to chemically immobilize an organic mediator, natural red, to a graphite felt electrode, and operated MFCs with *E. coli* or sewage sludge microbes. The modifiers had relatively little impact on *E. coli* MFCs, since unlike DMRB, *E. coli* does not possess OM bounded cytochromes to communicate with the modifiers. MFCs inoculated with sludge or pure culture of *S. putrefaciens* had their power density promoted for 100-1000 folds (Park and Zeikus 2002; Park and Zeikus 2003). Such results indicated that the DET is more sensitive to the composition/surface condition of electrodes. Mediator modified electrodes had been applied in sediment MFCs, the current densities in these MFCs could be 5 times greater than the previous sediment MFCs (Lowy et al. 2006). In these studies, the mediators were adsorbed on the electrodes, and the binding expired with time. The authors later studied other types of mediators as anode modifiers in the sediment MFCs (Lowy and Tender 2008). To promote the stability of the mediator-electrode binding, Adachi et al. (2008) chemically linked anthraquinone-2,6-disulphonic disodium salt (AQDS) with polymer layer formed on top of a graphite felt electrode. This design elevated the catalytic current by *G. sulfurreducens* for 60 times, and did not lose activity during operation for more than 1 month. Similarly, Feng et al. (2010a) immobilized AQDS through electropolymerizing of pyrrole using AQDS as the dopant. In their modified MFC with *S. decolorationis* S12 as the biocatalyst, the power density was found to be 13 times higher than a MFC with unmodified carbon felt.

The mediators can be modified to the electrodes as polymers as well, the osmium redox polymer directly wired metabolically enhanced *Bacillus subtilis* with the electrode (Coman et al. 2009).

2.4.1.2 Modifications of anodes for microbial MET

Anode modifications are proved to be effective for mediated systems as well. Current of 100-110 μA was recorded when a CNT/polyaniline (PANI) modified nickel foam electrode (1 cm × 1 cm × 0.1 cm) was poised at 0.1 V vs. SCE (Qiao et al. 2007). The electrolyte was inoculated with *E. coli* and artificially mediated by 2-hydroxy-1,4-naphthoquinone. Sharma et al. (2008) used CNTs functionalized with noble metal as the electrocatlysts in a *E. coli* fuel cells mediated by methylene blue or neutral red. The maximum power density reached 2470 mW/m^2, one of the highest power densities from mediated MFCs. Zou et al. (2008) applied polypyrrole coated CNT composite as the anode material in a self-mediated *E. coli* fuel cell and attained a power density of 228 mW/m^2.

In the aforementioned modifications, conductive polymers played an important role. Nano-structured TiO_2/PANI composite anode showed catalytic effects towards MET as well; maximum power density of 1495 mW/m^2 was delivered with artificially mediated *E. coli* (Qiao et al. 2008a). Electrodes coated by carbon black/Polypyrrole composite effectively enhanced power generation by *Proteus vulgaris* in an artificially mediated MFC (Yuan and Kim 2008a; Yuan and Kim 2008b). Zhao et al. (2010) synthesized PANI film with nanosized pores. Due to the grossly enlarged effective surface area, the MET by *Shewanella* was considerably enhanced. When the indium tin oxide conductive glass was modified with nano-structured PANI, the current density increased from 1 $\mu A/cm^2$ to 45 $\mu A/cm^2$.

Zhang et al. (2007) found that mixing graphite powders with polytetrafluoroethylene (PTFE) emulsion can enhance the anode performance in a self-mediated *E. coli* fuel cell. A PTFE content of 24-36% appeared to be optimal.

2.4.1.3 Other Modifications of anodes in MFCs

Researchers at Greifswald University (Germany) developed a unique strategy for bioelectricity by exploiting microbial fermentation products (Schröder et al. 2003). Platinized carbon cloth covered with conductive polymers was applied to oxidize hydrogen (from *E. coli* fermentation process) in-situ. Platinum is good catalysis for hydrogen oxidation, and platinum black has very high active surface area. Nonetheless, it was detected that in bacterial electrolyte, the poisoning of catalysis happened quickly. In their study, two measures had been carried out to counteract this: the platinum black was applied with a protective PANI overlay; a periodical potential pulse was applied on working electrode when it worked in half-cell mode. Better protection of platinum particle from deactivation could be attained by replacing PANI with fluorinated PANI (Niessen et al. 2004). Rosenbaum et al. (2006) found a noble-metal-free catalyst for in-situ degradation of microbial fermentation products. Tungsten carbide powders were applied to a carbon substrate electrode, this modified electrode exhibited good electrocatalysis towards hydrogen and formate produced by fermentative bacteria. The resistance against poisoning was also improved.

2.4.2 Biological approaches

2.4.2.1 Inoculation strategies

Despite the successes in isolation of EABs, most MFCs studies are conducted with mixed microbial communities. The mixed community usually has an exoelectrogenic capability comparable to the higher-power-generating species, such as *Geobacter* species (Call et al. 2009). Therefore, the structure of the anode-affiliated consortium is important, and such structure may be influenced by the MFC inoculation procedures.

Rabaey et al. (2004) reported an MFC with one of the highest power densities so far. The enrichment process distinguished from others by a sequential transfer of scratched biofilm from the anode to a new reactor. For this, anode-attached bacteria were scraped off the electrodes and suspended in sterile nutrient broth, then brought into a new sterile reactor. Five consecutive transfers had been carried out. The reactor had simple plain

graphite electrodes, and 100 mM potassium ferricyanide at cathode as electron acceptors. Maximum power density 3.6 W/m^2 was achieved, showing a great potential to increase power generation through the enrichment process.

Hydrogen and methane productions had been observed in MFCs. These processes will compete with exoelectrogenesis for nutrients. In a study on the acclamation process (Kim et al. 2005), three methods have been attempted to suppress the methanogens or to facilitate the growth of DMRB: 1. use of 2-bromoethanesulfonate (methanogen inhibitor) 2. use of a ferric oxide coated electrode 3. serial transfer in a medium containing ferric citrate and acetate for 25 times. All three ways were shown to effectively oppress activity of methanogens. However, the cell voltage established by the serially enriched iron-reducing bacteria was much lower than that by the unacclimated anaerobic sludge. This finding suggested that enrichment out of MFC environment may not be effective.

2.4.2.2 Genetically engineered microbes

Genetic engineering may enhance the EET rate of the anode reducers. This can be achieved by over expression of proteins essential for exoelectrogenesis or deletion of genes that are potentially disadvantageous for current generation. However, the ET systems of anode-respiring microorganisms are not fully understood. The role of individual component is yet to be confirmed.

Bretschger et al. (2007) created over 40 different mutants of *S. oneidensis* MR-1, each of the mutant had a deleted gene associated with cytochrome synthesis or secretion. Some strains were severely hampered in current generation, but over half of the mutants showed enhanced current generation. Kouzuma et al. (2010) found that disruption of cell surface polysaccharide biosynthesis gene could enhance the cell attachment to electrode and promote current generation of *S. oneidensis* MR-1. Johnson et al. (2010) expressed proteorhodopsin with *S. oneidensis* MR-1, which could enable light-dependent proton pumps, and thus enhanced cell survival and current generation.

Fishilevich et al. (2009) expressed glucose oxidase at the surface of *Saccharomyces cerevisiae*. *S. cerevisiae* continuously synthesized glucose oxidase at the cell surface, which coupled glucose oxidation with electrode reduction. However, this enzyme was not a part of the cell ET system; thus this design might be viewed as a special enzymatic biofuel cell.

2.4.2.3 Evolution of microbes in the MFC environment

It was an exciting discovery that the MFC environment can induce physiology changes of the biocatalysts. Microbes may optimize themselves for exoelectrogenesis in a relatively short period of time. Some researchers proposed the Darwinian natural selection as an explanation for this observation (Zhang et al. 2006).

Zhang et al. (2006) used *E .coli* cells from an MFC to inoculate a new MFC and termed them as "generation I" *E coli*. They repeated this procedure for 3 times, and found the "generation III" *E .coli* had significantly stronger electrocatalytic activities compared with the original *E .coli*. The authors finally achieved a power density of 600 mW/m^2 with the evolved *E .coli* in the absence of any artificial mediators. This was the first time that *E .coli* were shown to be suitable for mediator-less MFCs. Qiao et al. (2008b) further studied the "electrochemical evolution" of *E .coli*. They found that the surface of the evolved cells were much rougher than that of the original cells, probably allowed the cells to release redox compounds to the extracellular medium.

Adaptive evolutions under electrochemical selection were also observed with *Geobacter* species. Yi et al. (2009) incubated the type strain of *G. sulfurreducens* with an electrode poised at – 400 mV vs. Ag/AgCl for five months. They recovered the cells attached to the electrode and considered them as new strain named as *G. sulfurreducens* KN400. With a working electrode poised at – 400 mV, *G. sulfurreducens* KN400 delivered current density over 2.5 A/m^2 within about 30 h, while the original *G. sulfurreducens* DL1 took more than 400 h to reach 1.5 A/m^2. In a fuel cell with 560 Ω

load, strain KN400 attained a current density of 7.4 A/m^2 after 100 h of operation, compared to 1.4 A/m^2 by the DL1 strain. The maximum power density was enhanced for almost 7 times with the evolved strain. Surprisingly, it was found that the evolved strain contained less cell surface proteins, while such proteins were assumed to be essential for EET. Possibly some redox proteins irrelevant to the exoelectrogenesis were no longer expressed. Further, the thickness of KN400 biofilm was about 45% thinner than that of DL1 biofilm. A thicker biofilm presumably allowed more *Geobacter* to be involved in current generation. These findings again demonstrated that, the microbial EET processes were not yet fully understood.

2.4.3 Other approaches

Several researchers attempted very different measures to facilitate the MFC anodic ET. Nakamura et al. (2009) constructed a conductive network with iron oxides colloids. *S. loihica* entrapped in the network could access the electrode through electron hopping from colloid to colloid. The conductive network enabled a multiple layers of *Shewanella* to communicate with the underlying electrode, the current density was elevated for 100 folds. It was later discovered, given proper conditions, *Shewanella* can form such conductive network spontaneously (Nakamura et al. 2010). In a similar way, Yuan et al. (2009) immobilized *Proteus vulgaris* in a carbon nanoparticle paste, the mediated MFC achieved power density of 705 mW/m^2.

To enhance the electrode-attached biomass, Luckarift et al. (2010) immobilized *S. oneidensis* on the graphite felt through vapor-deposition of silica. Due to the immobilization, the cell power density was increased for over 50%.

2.5 Cathodes in MFCs

2.5.1 Electron acceptors in MFCs

A number of chemicals had been applied as the electron sink at MFC cathodes. Potassium ferricyanide had been used by some researchers (Rismani-Yazdi et al. 2008).

The ferricyanide/ferrocyanide redox couple has a standard potential of 0.36 V vs. SHE (irrelevant to pH value), significantly lower than that of O_2/H_2O couple under neutral pH (Logan et al. 2006), which means lower cell electromotive force. However, ferricyanide is favored due to the fast electrode kinetics. Ferrocyanide is known to be re-oxidized by oxygen, but such process is sluggish thus ferricyanide actually act as the final electron acceptor, rather than mediator (Pham et al. 2004). You et al. (2006) attempted permanganate in the MFC cathode, which provided higher oxidative potential, especially at lower pH. The power density was 4.5 folds higher than that from an MFC with the same configuration but using ferricyanide as the electron acceptors. The permanganate cathode shares a common limitation with the ferricyanide cathode: they require the replenishment of oxidant. Li et al. (2009) used persulfate as the oxidant, which resulted in cathodic working potential higher than 1 V vs. Ag/AgCl (when cathode pH was lower than 4.0). The initial power density using persulfate was lower than that using ferricyanide, however, after 2 days of operation, the MFC with persulfate outputted higher power. The exact reason was not clear, the authors described this as "self-activation" of electron acceptor, which might be related to hydrolysis of persulfate. The above listed alternative oxidants are consumable, thus the application is limited. Li et al. (2010) proposed I_3^- as "solar regenerable" electron acceptor. I_3^- can be electrochemically reduced to I^-; while under light, I^- can be oxidized by O_2 to I_2, I_2 further reacts with I^- to form I_3^-. During the operations, the authors did not detect depletion of I_3^-, indicating it acted as electron mediators, rather than the final electron acceptors. I_3^- also had faster electrode kinetics than ferricyanide, the reductive current under controlled potential was twice as the later.

With the microbes as the cathodic catalysts, electron acceptors for MFCs can be further diversified. Relevant discussions can be found in section 2.5.4.2.

2.5.2 MFC cathode structures

The developments on MFC cathode structures mostly aim to improve the air cathode process. The structure should facilitate a three-phase reaction involving O_2, H^+ and

electron.

In an early stage, the air cathode used in MFCs was mostly immersed (Oh et al. 2004). Since the O_2 solubility is only 8 mg/l under normal oxygen partial pressure, the cathode had limited access to the O_2; such system required purge of air. Liu and Logan (2004) constructed an MFC where the carbon paper cathode was directly exposed to air. The oxygen diffusion limitation was vastly reduced in this way. However, this design allowed excessive air penetration to the electrolyte. When the MFC was without any membrane, the exoelectrogenesis process at anode might be affected. Loss of liquid through the exposed cathode may also be a problem. Cheng et al. (2006a) modified the structure of the air-breathing cathode. They used wet-proofed carbon cloth as the substrate electrode. At one side of the electrode, they applied a carbon/PTFE gas diffusion layer plus four layers of PTFE coating. The other side of carbon cloth was loaded with Pt/C catalyst using Nafion as the binder. This design limited gas diffusion to the electrolyte and enhanced the CE. Additionally, the modification might improve the three-phase interface and elevated the overall power generation. This design had been widely accepted for constructing air-cathode in MFCs. Lately, Zhang et al. (2010a) used cheap metal mesh as the current collector replacing the wet-proofed carbon cloth. The MFC with the metal-mesh-based cathode had an improved CE. The authors also applied poly(dimethylsiloxane) to substitute the PTFE for constructing the diffusion layers.

2.5.3 Operational conditions for MFC cathode

The MFC air cathode is usually operated under neutral pH and atmospheric pressure, which is not an optimal condition for oxygen reduction reaction. The best electrocatalysis towards oxygen reduction only occurs in acidic or alkaline conditions, due to steady availability of reactant species of H^+ or OH^- (Zhou et al. 2007).

A straightforward idea is to operate air cathode under low pH, not only the kinetics will be promoted, the thermodynamic cathodic potential is also boosted which can be

predicted by the Nernst equation. Erable et al. (2009b) found that, when the electrolyte pH decreased from 7.5 to 1.0, the open circuit potential of air-breathing cathode positively shifted for over 100 mV, the MFC power density was increased for 2.5 folds. The MFC anode generally demands a neutral pH; in this study the pH difference was maintained by PEM. However, since the membrane is non-ideal, protons diffused to anode and the non-proton cations migrated to the cathode resulted in loss of acidity in the cathode and acidification of the anode. The power density of the MFC with acidic cathode dropped to 66% of the original value after continuous operation of 9 days.

On the other hand, the thermodynamic cathodic potential can be increased with higher O_2 partial pressure. For this, Fornero et al. (2008) pressurized the MFC cathode chamber, and claimed an improvement in power generation.

2.5.4 MFC cathode catalysts

2.5.4.1 Chemical catalysts

Pt is probably the most commonly used electrocatalyst towards the oxygen reduction. Pt embedded in a porous carbon cathode is a typical configuration for MFCs (Liu and Logan 2004) and other types of fuel cells (Winther-Jensen et al. 2008). However, the high cost and vulnerability to poisoning of Pt urged researchers to seek for alternatives. Zhao et al. (2005) applied iron(II) phthalocyanine and cobalt tetramethoxyphenylporphyrin as MFC cathodic catalysts, under the experimental conditions, these catalysts had a performance similar to that of Pt. Cheng et al. (2006b) also confirmed that the electrocatalytic effect of cobalt tetramethoxyphenylporphyrin under MFC condition was only slightly inferior to that of Pt. HaoYu et al. (2007) studied several non-Pt catalysts for MFC air cathodes, where the MFC with Fe phthalocyanine catalyst outperformed a MFC with Pt catalyst in term of power density. Harnisch et al. (2009) further suggested that plasma-treated Fe phthalocyanine had stronger oxygen reduction capability compared with pyrolyzed Fe phthalocyanine. Morris et al. (2007) claimed PbO_2 to be suitable catalyst for MFC air cathode. In their study, a MFC with PbO_2 catalyst had power density 73% higher than that of MFC with

Pt catalyst; whereas, the loading rate of PbO_2 in this study was many times higher than that of Pt. Zhang et al. (2009) evaluated 3 MnO_2 materials, α-MnO_2, β-MnO_2 and γ-MnO_2 as the catalyst for MFC air cathode. They found that β-MnO_2 could be a promising alternative for Pt. Yuan et al. (2010) used polypyrrole/carbon black composite to catalyze the oxygen reduction process, the authors had used the same configuration to catalyze the anodic process (Yuan and Kim 2008a).

Apart from catalytic chemicals, treatment of the carbon electrode may also improve their oxygen reduction activity. Erable et al. (2009a) activated graphite granules in nitric acid, and used this material for oxygen reduction in MFC. The MFC with the treated granules had a power density over one fold higher than that of an MFC with untreated granules. The open circuit cathodic potential positively shifted for 400 mV after the treatement. Duteanu et al. (2010) attempted to activate XC-72R vulcan through HNO_3, H_3PO_4, KOH or H_2O_2 treatment; and found that the HNO_3 treatment could obviously enhance the oxygen reduction activity.

2.5.4.2 Biocathodes

In contrast to the anode reducers, some microbes had been shown to accept electrons from the cathode and donate them to a final electron acceptor which can be oxygen or other chemical species (Huang et al. 2011; Rosenbaum et al. 2011). These microbes thus act as the biocatalysts for the oxidation of cathode.

Gregory et al. (2004) found that *G. metallireducens* can reduce nitrate to nitrite, coupled with the oxidations of electrode; *G. sulfurreducens* can accept electrons from an electrode using fumarate as the electron acceptor, which is reduced to succinate. This might be one of the first reports on biocathodes. In this study, the electrode was poised at – 500 mV vs. Ag/AgCl. On another hand, *G. sulfurreducens* could use electrode as the electron donor to reduce U(VI) to U(IV) (Gregory and Lovley 2005). Since *Geobacter* species are not known to secrete any redox shuttle (Lanthier et al. 2008), the cathode oxidation can be assumed as a result of DET. The discovery of

cathodic nitrogen reduction inspired some researchers to incorporate the denitrification process into MFC cathode. Clauwaert et al. (2007a) reported complete denitrification in MFC cathode. Significant denitrification activity was only observed when the cathode potential was below 0 V vs. SHE, the processing rate of the whole system was limited by cathodic microorganisms.

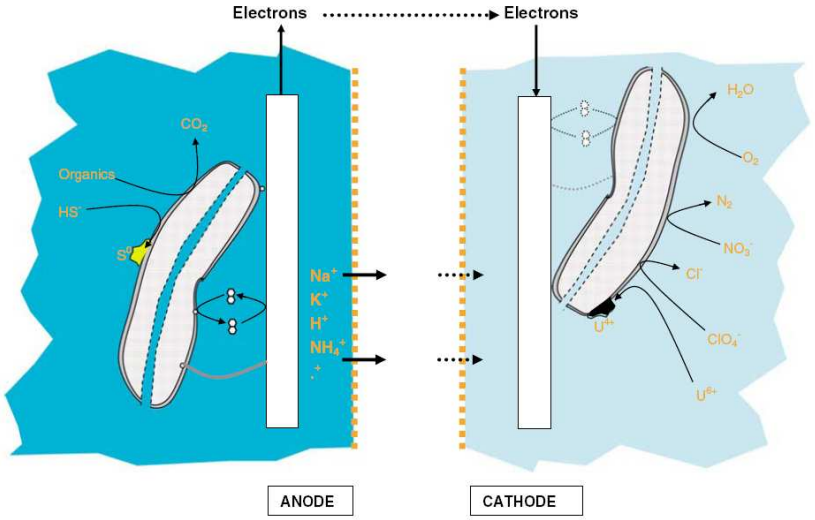


Figure 2.8 Illustration of MFCs with biocathode (Rabaey et al. 2007)

Biocathodes are shown to be suitable for reductive degradation of groundwater contaminant tetrachlorethene (Strycharz et al. 2008). *G. lovleyi* reduced tetrachlorethene to cis-dichloroethene, the rate for such reduction using electrode as the electron donor was similar to that using acetate. This again showed the effectiveness of *Geobacter* species in respiration with electrodes. A similar contaminant, trichlorethene, was reported to be degradable by biocathode of mixed cutltures (Aulenta et al. 2010). Butler et al. (2010) attempted biocatalyzed cathodic perchlorate reduction. The cathodic biofilm was acclimated from a denitrifying biocathode, through gradual supplementing of perchlorate in NO_3^- influent, and finally totally switched to perchlorate. The perchlorate reducing biocathode microbial community was composed mostly of putative iron oxidizing bacteria.

A surprising electron acceptor for biocathode is CO_2, which is generally the end product of biodegradation. Cao et al. (2009a) inoculated an bicarbonate-containing electrochemical cell with sludge from photoautotrophic reactor, and poised the electrode at 0 V vs. SCE. The electrochemical cell was illuminated by incandescent lamps during operation. After operation for 1 month, stable current generation was observed, accompanied with consumption of bicarbonate. When the bicarbonate was depleted, current generation was inhibited. Combined with an acetate-fed microbial anode, the MFC with CO_2 biocathode had power density of 750 mW/m^2. Since the reduction of CO_2 with acetate is thermodynamically unfavorable, photo-driven processes should play a role in this study. However, the details are yet to be explored.

Apart from all the aforementioned studies, most of the biocathode researches had been dedicated to O_2 reduction. Pham et al. (2004) used "critical dissolved oxygen concentration" as an index to evaluate the electrocatalysis towards O_2 reduction. When the dissolved O_2 level is beyond of a critical value, it will not restrain the cathode performance. Lower critical value is considered to be associated with better electrocatalysis. This value was 6.6 mg/l and 2.0 mg/l for bare graphite and platinized graphite respectively; while aerobic bacteria have much lower values that are lower than 1.0 mg/l. Bergel et al. (2005) formed aerobic biofilm on stainless steel, and used the biofilm-modified stainless steel as cathode in a PEM fuel cell fueled by hydrogen. With biofilm, the fuel cell could reach a short circuit current density of 1.8 A/m^2; while after the removal of the cathodic biofilm, the current density dropped to 0.2 A/m^2; highlighting the effect of the cathodic bacteria. Rhoads et al. (2005) applied *Leptothrix discophora* to facilitate the manganese mediated oxygen reduction. As described by the authors, with *L. discophora*, Mn^{2+} can be oxidized to MnO_2 by O_2, while MnO_2 can be electrochemically reduced to MnOOH, and futher reduced back to Mn^{2+}. Clauwaert et al. (2007b) enriched a mixed cathodic consortium for open air biocathode. They evaluated the effect of MnO_2 deposited on cathode, and found that although the deposition could shorten the acclamation time, the final performance of the biocathode was not improved. MFC with a biocathode (without MnO_2 deposition) had a power

density of approximately 1.75 W/m^2, after the cathode had been autoclaved, the power generation became negligible. Ter Heijne et al. (2007) applied an iron oxidizing bacterium, *Acidithiobacillus ferrooxidans*, for O_2 biocathode, using Fe^{2+}/Fe^{3+} as the mediator. In their system, *A. ferrooxidans* was immobilized in an aerated bioreactor supplied with $Fe^{2+,}$ where Fe was oxidized by O_2 with biocatalyst. The produced Fe^{3+} was pumped to an MFC cathode and was electrochemically reduced to Fe^{2+} which was pumped back to the bioreactor containing *A. ferrooxidans*. Lately, Carbajosa et al. (2010) suggested that *A. ferrooxidans* can directly catalyze oxygen reduction process, without any artificial mediators. The authors cultivated the chemoautotrophic *A. ferrooxidans* in acidic medium with electrode as the sole electron source. After polarizing the electrode at 0 V vs. SCE for 20 days, reductive current was observed. The biocathode ultimately reached a current density of 5 A/m^2 in oxygen saturated medium. The onset of catalytic current positively shifted for over 300 mV when compared with bare cathode. The cyclic voltammogram revealed multiple well defined redox couples, where a couple centered at 0.045 V appeared to be associated with metabolic activities (delivered catalytic current). The natures of these redox couples were unknown, the authors suggested a possibility of cell surface cytochromes. Another pure culture shown to catalyze O_2 reduction was *Pseudomonas aeruginosa* (Cournet et al. 2010a). When *P. aeruginosa* was introduced to an electrochemical cell containing O_2 saturated phosphate buffer, O_2 reduction activity, which occurred at – 0.4 to – 0.2 V vs. SCE, was significantly enhanced. The authors found that the O_2 reduction catalysis was associated with colonization of working electrode. Also, the catalysis persisted after replacement of phosphate buffer. Thereafter, the authors argued that the *P. aeruginosa* biocathode was based on DET, in spite of the fact that this bacterium was well known for self-secrete redox shuttles. Cournet et al. (2010b) further evaluated 20 microbial species, including gram-positive species, for catalyzing electrode reduction of O_2. 70% of tested microbes showed activity towards O_2 reduction. *Burkholderia cepacia* and *Branhamella catarrhalis* had the most positive onset potential towards O_2 reduction; the most positive O_2 reduction peak potential; and the highest peak current.

It would be interesting to know which bacteria are dominant in the mix-culture O_2 biocathode. Rabaey et al. (2008) analyzed the structure of their acclimated cathodic biofilm, and found *Sphingobacterium multivorum*, *Bacteroides* species to be heavily enriched. When some cathodic isolations were inoculated to a new sterile cathode, improved power generation compared with abiotic cathode was observed. However, the power density was way below that with the mixed community. Microscopic studies found that the cathode was barely colonized by the consortium, which disfavored a DET hypothesis.

Although large number of literatures had confirmed the microbial enhancement towards O_2 reduction, the exact mechanisms for such enhancement is yet to be elucidated. Besides the widely assumed DET or MET, Freguia et al. (2010) proposed a mechanism that the microbial metabolism is not involved. It was found that, when 2-Amino-3-dicarboxy-1,4-naphthoquinone or hemin presented in electrolyte with a concentration of 1 μM, significant oxygen reduction activity could be observed with an abiotic electrode. In a biocathode, these compounds can be generated from microbial secretion.

Another class of biocathode was based on enzymes. Schaetzle et al. (2009) applied laccase as the catalysts for oxygen reduction. The laccase was wired with the cathode by artificial redox mediators. The same design had been used by Fishilevich et al. (2009). The MFCs with enzymatic biocathode might be viewed as microbial-enzymatic hybrid biofuel cells.

2.6 Future applications of MFC techniques

There are already reports on applying MFCs as power sources for small appliances at remote area (Tender et al. 2008; Donovan et al. 2011). The major limitation for applying MFCs in large-scale wastewater treatment is still the low power density and high capital cost (Rozendal et al. 2008). It was estimated that the capital costs of

current lab-scale MFC treatment is 800 times higher than that of anaerobic digestion in term of per Kg COD treatment capability. However, continuous efforts had been made to increase the power generation or to lower the costs (Rabaey et al. 2010). Although it might be too early to assert the feasibility to apply MFCs for large-scale waste-to-energy processes; promising niche areas for MFC, and other MFC-based techniques had been proposed.

2.6.1 Novel cathodic processes

There had been increasing interest in developing nonconventional cathodic processes in MFCs. These processes are designed to degrade hazardous wastes, recover resources, and generate value-added products.

Liu et al. (2009) fed azo dye to the MFC cathode, these dyes can serve as cathodic electron sinks and are degraded in the process. Mu et al. (2009) performed nitrobenzene reduction in MFC cathode, the end product was aniline. Ter Heijne et al. (2010) supplied Cu^{2+} to the MFC cathode. They found that Cu^{2+} could be reduced to the elemental form and thus recovered. Wang et al. (2008) applied Cr(VI) as the cathodic electron acceptor, which can be reduced to the less hazardous Cr(III). Tandukar et al. (2009) demonstrated cathodic reduction of Cr(VI) to $Cr(OH)_3$ precipitate. This process was catalyzed by cathodic bioflim dominant by *Trichococcus pasteurii* and *P. aeruginosa*. More examples of cathodic reduction of hazardous wastes with biocatalysts had been discussed in section 2.5.4.2.

Two electron reduction of O_2 will result in production of H_2O_2, this usually takes place in cathodes without catalysts. Based on this, Rozendal et al. (2009) reported that, under a controlled cell voltage of 0.5 V, per m^3 of their system could generate 1.9 Kg H_2O_2 from acetate each day. You et al. (2010) had attempted H_2O_2 production in a solid polymer electrolyte MFC.

2.6.2 Variants of MFCs

While the MFCs were designed to capture electrical power, modification on MFC operational mode or MFC structure resulted in novel BES suitable for other purposes.

Microbial electrolysis cell (MEC) was the first and probably the most important variant of MFC so far. In the MECs, protons act as the electron acceptors. The H^+/H_2 couple has a biological standard potential of – 0.41 V, significantly lower than that of anode working potential in MFCs (Geelhoed et al. 2010). To overcome this thermodynamic barrier, Liu et al. (2005b) found that when applied an additional voltage higher than 0.25 V, cathodic hydrogen production became possible. 8-9 mol of hydrogen could be potentially produced from 1 mol of glucose, while the input energy was just equivalent to 1.2 mol of hydrogen. Comparatively, fermentative method could maximally produce 4 mol of hydrogen from 1 mol of glucose, where the acetate could not be further fermented. Cheng and Logan (2007b) further reported that the hydrogen production rate was 1.1 m^3 from each cubic meter of reactor per day. When the applied voltage was 0.8 V, 99% of theoretically recoverable hydrogen had been collected. More recent literature indicated that, the hydrogen production rate in MECs already approached or surpassed that of fermentative biohydrogen production (Call et al. 2009).

Cao et al. (2009b) installed one anion exchange membrane (AEM) near the anode and one cation exchange membrane (CEM) near the cathode in their MFC-based bioelectrochemical reactor. Under the electrical field, the ions in between of the two membranes migrated. The cations traveled through the CEM to the cathode, while the anions passed through the AEM to the anode. The ionic strength in between of the two membranes was lowered in this way. The authors named their device as “microbial desalination cell”. 90% salt removal was observed in a single operational cycle.

2.6.3 Microbial biosensors

The correlation between substrate concentration and microbial current suggested a possibility to develop biosensors. Currently, there are two types of microbial biosensors, the “MFC type”, and the “half cell type”.

Kim et al. (1999c) found that, when *Shewanella* were injected into their MFC with lactate, stable current could be achieved in 10 min, the rate of current increment was linear with the lactate concentration in the range between 2 and 25 mM. Chang et al. (2004) developed a MFC type biosensor for continuous monitoring of biochemical oxygen demand (BOD). At a hydraulic retention time of 1.05 h and BOD lower than 100 mg/l, the current level had a linear correlation with the BOD. Kumlanghan et al. (2007) reported that, the maximum cell voltage in their MFC increased linearly with glucose concentration, up to the concentration of 25 g/l.

"Half cell type" microbial biosensor generally works under controlled potential or controlled current conditions. Prasad et al. (2009) coated *Shewanella* on screen-printed carbon electrode. The fabricated biosensor had good response to As^{3+}, H_2O_2 and NO_2^-.

2.6.4 Microbial electrosynthesis

The above discussions suggested a great potential to develop variations from the prototype MFCs. Nonetheless, all these techniques are based on degradation of substrates and extraction of electrons. Very recently, a new concept termed as "electrosynthesis" emerged, which may represent a more radical innovation to the microbial BES (Rabaey and Rozendal 2010). The term "electrosynthesis" is analogous to photosynthesis, it refers to the electricity-driven reduction of CO_2. Biofuels, drugs, and other value-added products may be produced from this process.

Cheng et al. (2009) directly converted CO_2 to methane with a electrical current. The authors termed this as "electromethanogenesis". When the poised potential was – 1.0 V vs. Ag/AgCl, the conversion efficiency was 96%. It was shown that abiotic cathode could only produce a small amount of hydrogen under negative potential, thus the electromethanogenesis was a result of bioactivity. Nevin et al. (2010) showed that more complex organics can be derived from electrosynthesis. When the electrode was poised at – 400 mV vs. SHE, *Sporomusa ovate* consumed electrons supplied by electrode,

synthesized acetate and 2-oxobutyrate from CO_2.

Chapter 3 Electrode Potential Regulates Cytochrome Accumulation on *S. oneidensis* Cell Surface and the Consequence to Bioelectrocatalytic Current Generation

3.1 Introduction

EAB are endowed with the capability to exchange electrons with electrodes without exogenous redox mediators. The discovery of EAB triggered rapid developments in MFCs, MECs and other whole-cell based BES like microbial biosensors (Prasad et al. 2009). EAB play a central role in microbial BES, thus, to understand and optimize these systems, it is very important to comprehend the EET physiology of EAB. It is now known that the exoelectrogenic ability of EAB is largely attributed to their unique ET systems: some EAB conduct DET with cell surface redox proteins; while some perform MET through diffusive electron shuttles (Schröder 2007). Nevertheless, EET processes of EAB are still obscure in detail (Lovley 2008a). Moreover, it is less discussed whether the EAB's respiratory systems are immutable or variable with environmental conditions.

It had been previously identified that the presence of soluble electron acceptors may impact the cell surface localization of *Shewanella* OM *c*-Cyts (Myers and Myers 1992; Kim et al. 1999a; Biju et al. 2007). Recently, there has been an argument that the electrode potential exerts an influence on the ET system of electrode-attached microbes (Busalmen et al. 2008). While studying the voltammetric behavior of *G. sulfurreducens,* researchers found a redox pair centered at – 0.08 V vs. Ag/AgCl after poising the working electrode at 0.1 V for 18 h, but a different redox pair centered at 0.48 V after polarization at 0.6 V. These researchers thus proposed that the EAB could sense the poised potential, and alter their ET pathway accordingly to maximize energy gain. Nevertheless, the voltammograms recorded by Busalmen et al. were very different from those reported by subsequent studies (Fricke et al. 2008; Marsili et al. 2008b; Srikanth

et al. 2008; Liu et al. 2010d). These subsequent studies found two redox systems with formal potentials in between of – 0.4 and – 0.3 V vs. Ag/AgCl to be responsible for current generation; but did not detect the redox peaks reported by Busalmen et al.. Marsili et al. (2010) and Katuri et al. (2010) further investigated the effect of poised potential on *Geobacter* EET process. They varied the applied potential from – 0.46 V to 0.4 V vs. Ag/AgCl, but did not find any noticeable change in voltammetric patterns. Hence, more solid and unambiguous evidences should be presented, to shed some light on the impact of electrode potential on the physiology of EAB, and its effect on the performance of BES.

Apart from *G. sulfurreducens*, *S. oneidensis* is another intensively studied EAB. The first whole-cell voltammetry of *S. oneidensis* showed a notable asymmetric redox pair, this electrochemical activity was hypothetically linked to OM *c*-Cyts (Kim et al. 2002). The redox wave has been lately confirmed as signals of OM *c*-Cyts OmcA/MtrC (Meitl et al. 2009), a mutant of *S. oneidensis* lacking OmcA/MtrC did not display this redox pair. On the other hand, *S. oneidensis* is also known to secrete flavins for EET (Marsili et al. 2008a). This study intended to compare biological current generation by *S. oneidensis* under different poised potentials, and monitored microbial physiological alterations induced by the change of electrode potential through electrochemical assays.

3.2 Experimental section

3.2.1 Electrochemical instrument and set-up

Electrochemical measurements were performed with an LK-6200 electrochemical workstation (Lanlike, Tianjin, China). A GCE of 3 mm diameter was used as the working electrode. Before use, the GCE had been sequentially polished with 1 μm and 50 nm alumina paste, and subsequently ultrasonically cleaned in de-ionized water for 5 min. A platinum plate counter electrode and an SCE were employed. The electrochemical cell was a 7.5 ml capacity glass cylinder mounted with silicone elastomer cap (Figure 3.1). All electrochemical assays were carried out at 31°C (±1°C)

with an independent replication. For cyclic voltammetries, the scan rate was 0.01 V/s.

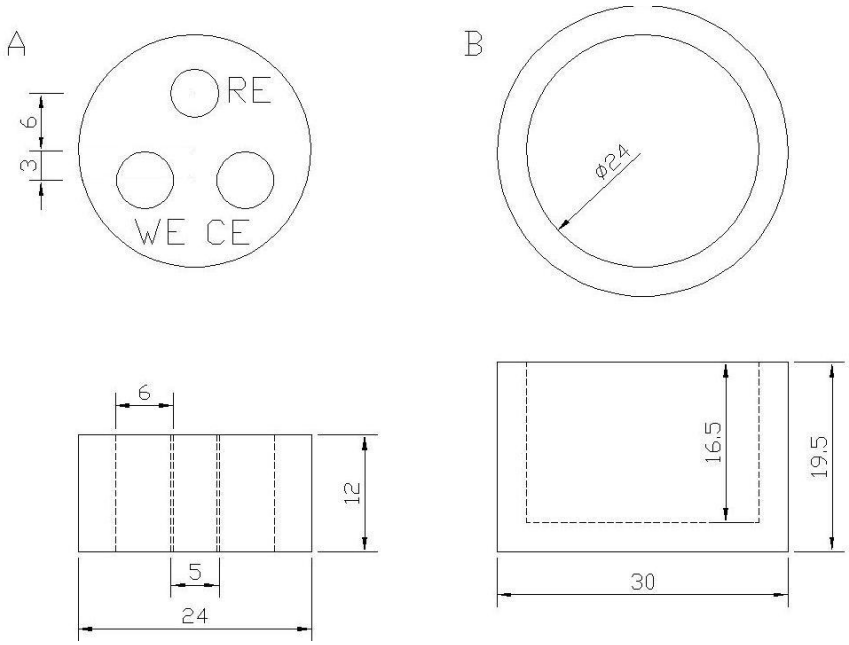


Figure 3.1 Drawings of the electrochemical cell (B) and its cap (A), where WE refers to the working electrode, CE refers to the counter electrode and RE refers to the reference electrode. The scales are in mm.

3.2.2 Bacterial growth and inoculation

S. oneidensis MR-1 was procured from the Belgian Coordinated Collections of Microorganisms (LMG 19005). Luria-Bertani (LB) broth (Difco Laboratories, MI, USA) prepared in 50 mM sodium phosphate buffer (pH 7.2) was used as growth medium for *S. oneidensis* and also functioned as electrolyte in the electrochemical cell. The bacterium was grown aerobically in a shaking flask (30°C, 150 rpm) for approximately 18 h until the optical density (OD_{600}) reached 2. 100 µl of the bacterial culture was inoculated into 4.9 ml of fresh medium in a sterilized electrochemical cell. Anaerobic condition was maintained during all bioelectrochemical characterizations.

3.2.3 High Performance liquid chromatography (HPLC) for flavin detection

After bioelectrochemical assays, bacterial culture from the electrochemical cell was

filtrated through a 0.45 μm cellulose acetate membrane. Before HPLC analysis, the filtrate was pretreated and concentrated by a solid phase extraction (SPE) process. For SPE, 4 ml of the filtrate was passed through a preconditioned Bond Elut Env SPE cartridge (1 g/6 ml, Varian, CA, USA), then eluted with methanol. The eluate was evaporated under a nitrogen stream and the dry residue was reconstituted in 0.2 ml of water.

The HPLC test was performed by a Prominence LC-20A ultra fast liquid chromatography system (Shimadzu, Japan) equipped with a photodiode array detector. A Spherisorb ODS2 (150 mm × 4.6 mm, 5 μm support) chromatography column (Waters, MA, USA) was used. The mobile phase was water: methanol (50:50, 1 ml/min).

3.3 Results and discussion

When polarized at 0 V, the oxidative current increased rapidly at first, but reached a peak within 1 h, it then declined dramatically and remained stagnant. The current density after 15 h of incubation was as low as 0.486 ± 0.007 μA/cm^2 (n=2, Figure 3.2A). While the working electrode was poised at – 0.24 V, the current grew with a lower rate at the beginning, however, the growing trend lasted for 9-10 h. The current density reached roughly 10 μA/cm^2 and then it fluctuated slightly; no sharp decline was observed within 15 h (Figure 3.2A). Strikingly, *S. oneidensis* generated a drastically lower oxidative current while the electrode was poised at a higher potential. It is worth mentioning that the poised potential of 0 V was not so high as to cause inactivation of attached bacteria. Many bioelectrochemical researches involving *Shewanella* were conducted under a poised potential higher than 0 V vs. SCE (Nakamura et al. 2009).

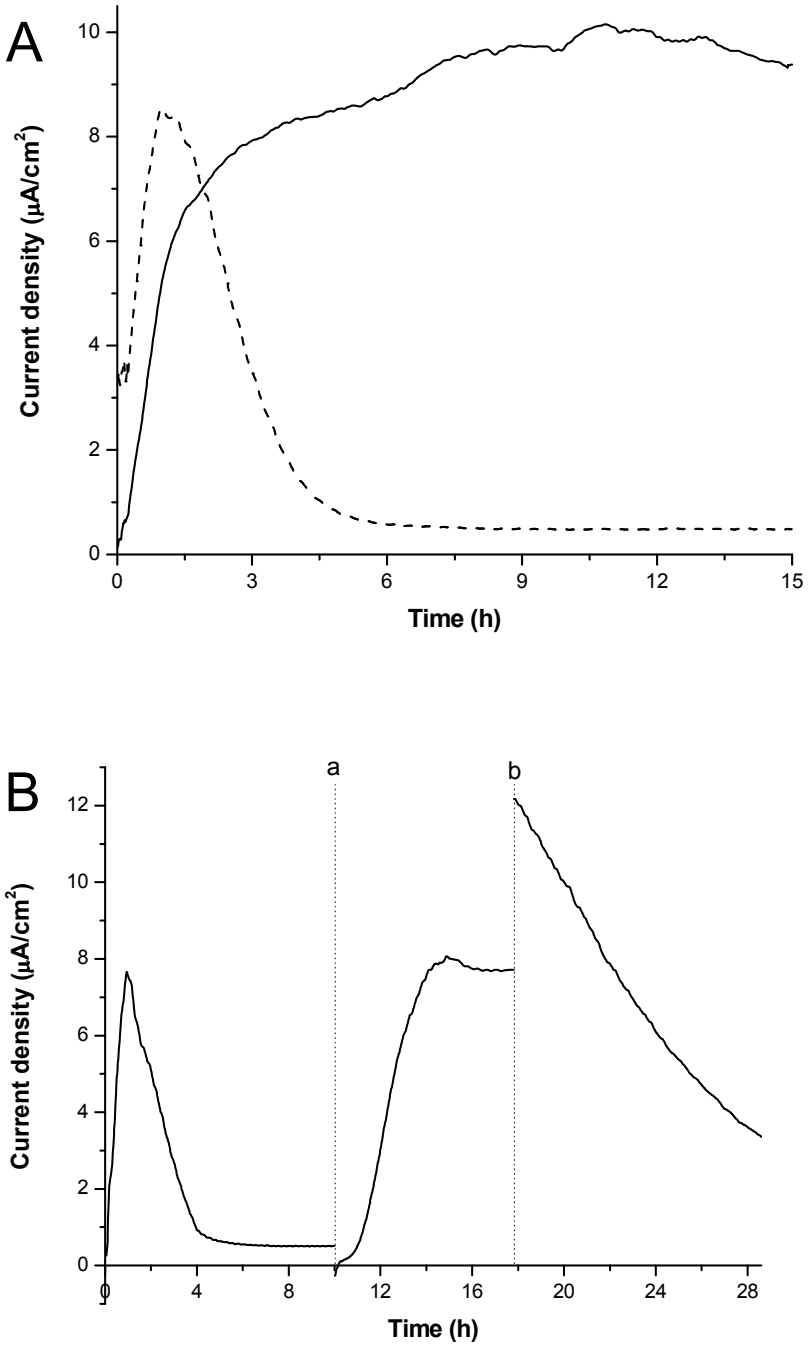
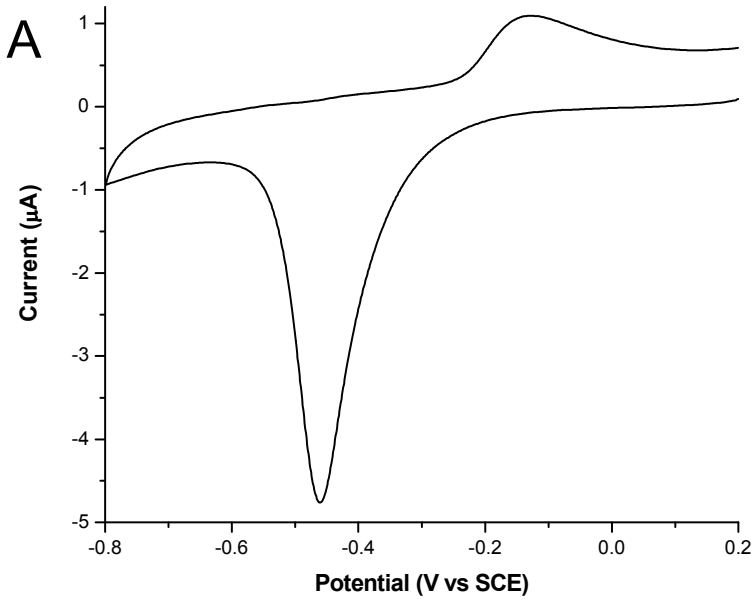
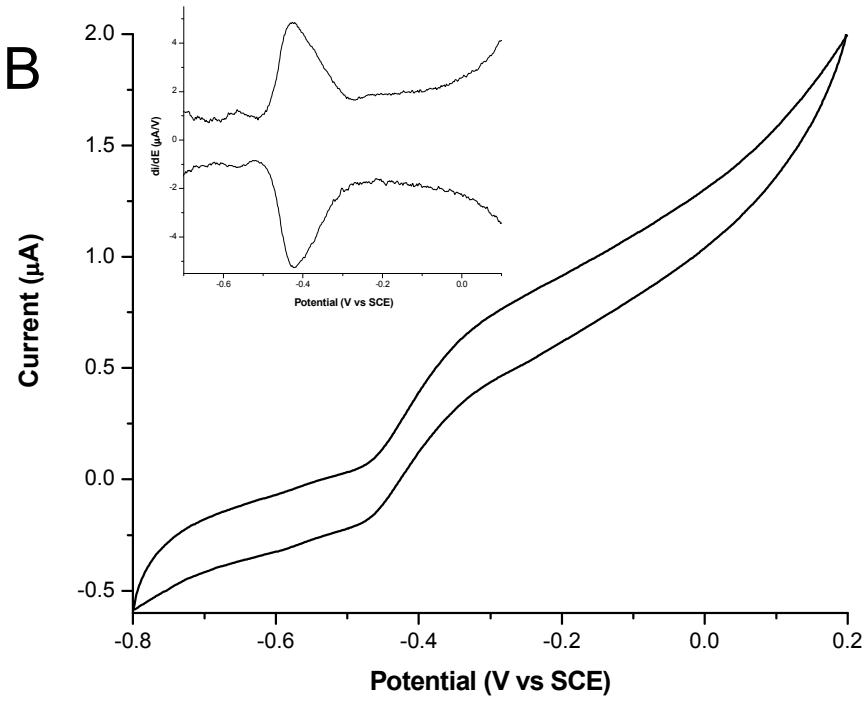


Figure 3.2 Chronoamperometric plots. (A) Single-step chronoamperometries of GCE poised at – 0.24 V (solid) and 0 V (dashed). (B) Multi-step chronoamperometry, a: poised potential switched from initial 0 V to – 0.24 V; b: poised potential was restored to 0 V.

The voltammogram after polarizing at 0 V for 15 hours revealed a sharp cathodic peak at - 0.46 V, with a broad and flat anodic peak at roughly - 0.12 V (Figure 3.3A). Such an asymmetric profile suggested irreversible electrochemistry of the redox element, where the electro-oxidation was obstructed. This voltammogram corresponded the first report on electrochemical activity of *S. oneidensis* (Kim et al. 2002). Furthermore, the recorded profile bore close resemblance to the voltammogram of OM *c*-Cyts OmcA/MtrC (Eggleston et al. 2008; Meitl et al. 2009). Therefore, this voltammetric signal can be attributed to *Shewanella* OM *c*-Cyts. If these cytochromes were actively transporting electrons to the electrode, the cyclic voltammogram would show a sharp rise of current over the midpoint potential of the proteins, this was known as a catalytic wave (Fricke et al. 2008; Srikanth et al. 2008). Nonetheless, the registered voltammogram did not show such catalytic feature (Figure 3.3A). OmcA/MtrC are electrocatalytically less active because: (1) these cytochromes demonstrate irreversible electrochemistry; (2) the heterogeneous ET of OmcA/MtrC is sluggish, characterized by widely separated redox peaks in voltammograms.

OM *c*-Cyts of *S. oneidensis* are widely considered terminal reductases for solid electron acceptors. However, only recently has there been a kinetic study to examine this consideration (Ross et al. 2009). The study showed that, *S. oneidensis* whole cells have a kinetic constant for the reduction of goethite 100-1000-fold higher than that of purified OmcA/MtrC. It was suggested that flavins, rather than OmcA/MtrC, were directly responsible for the reduction of minerals. OmcA/MtrC may mediate the ET from the cell interior to flavins (Ross et al. 2009); however, this process does not require the localization of OmcA/MtrC to the cell surface.


A
Current (μA)
Potential (V vs SCE)
1
0
-1
-2
-3
-4
-5
-0.8
-0.6
-0.4
-0.2
0.0
0.2


B
Current (μA)
Potential (V vs SCE)
2.0
1.5
1.0
0.5
0.0
-0.5
-0.8
-0.6
-0.4
-0.2
0.0
0.2
di/dE (μA/V)
Potential (V vs SCE)

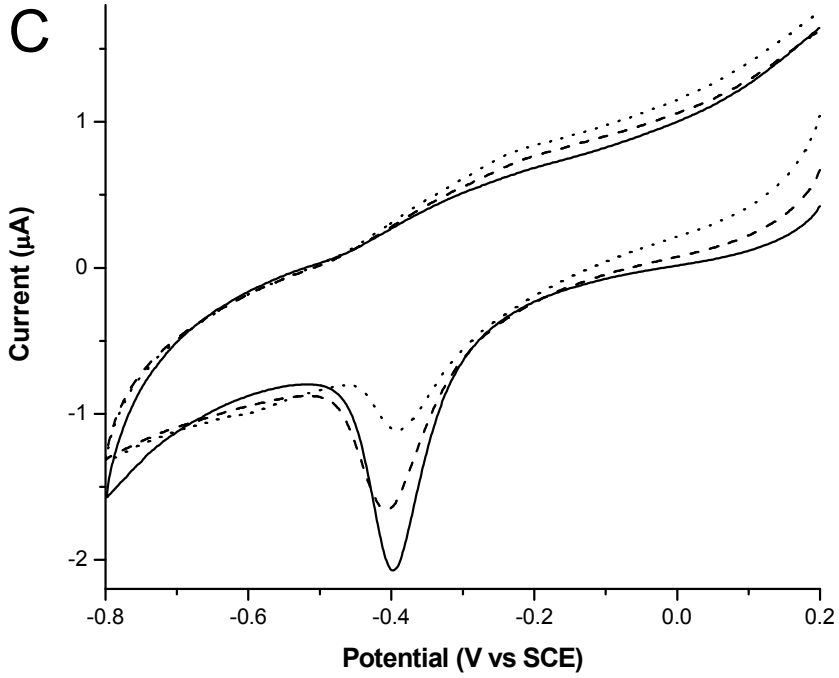


Figure 3.3 Cyclic voltammetric characterizations. (A) Cyclic voltammogram after poised at 0 V for 15 h (B) Cyclic voltammogram after poised at – 0.24 V for 15 h, inset: first derivatives of the voltammetric curve (C) Cyclic voltammograms during multi-step chronoamperometry, when the poised potential had been restored to 0 V for 2 h (dotted line), 4 h (dashed line) and 6 h (solid line) respectively.

The voltammogram recorded after polarization at – 0.24 V for 15 h (Figure 3.3B) was entirely different. The characteristic redox peaks of OM *c*-Cyts no longer existed, suggesting that they were absent from the bacteria-electrode interface. Nevertheless, there was a clear catalytic wave: the current rose sharply between – 0.47 V and – 0.35 V. A first derivative analysis of the voltammogram revealed a redox pair centered at – 0.44 V (Figure 3.3B), which corresponded to the redox potential of flavins under neutral pH (Marsili et al. 2008a).

The presence of endogenous flavins was further confirmed by HPLC. For HPLC, the

target analyte was the dominant flavin species released to the extracellular medium by *S. oneidensis*: riboflavin (Marsili et al. 2008a). Chromatography with 8 μM standard riboflavin solution indicated that this substance eluted at 2.92 min (Figure 3.4). The SPE-treated culture supernatant from the electrochemical cell eluted a substance at the same time (Figure 3.4). The UV-vis spectrum of the peak at 2.92 min was in accordance with that of riboflavin (Biffinger et al. 2008), proving the existence of this compound.

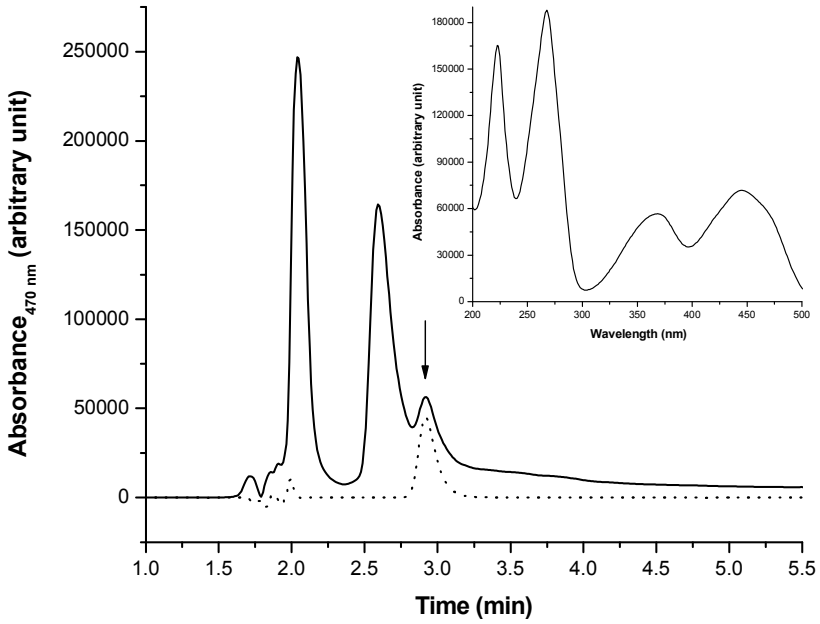


Figure 3.4 HPLC chromatogram of culture supernatant (after SPE pretreatment) from the electrochemical cell (solid line) and 8 μM riboflavin aquatic solution (dotted line). Inset: UV-Vis spectra of the HPLC peak at 2.92 min.

It was perplexing as to why flavins did not show electrocatalytic activity when the electrode was poised at a higher potential. To further analyze the effect of poised potential on microbial physiology and current generation, a multi-step chronoamperometry was initiated. For this, the working electrode was first polarized at 0 V, for 10 h. Within a few hours, as expected, the current dropped to a very low level

after an initial increase (Figure 3.2B). The poised potential was then instantly switched to – 0.24 V. After this switch, a negative current was observed at the beginning. However, the current steadily grew thereafter, and saturated at 7.86 ± 0.16 μA/cm^2 after 4-5 h and remained stable (Figure 3.2B). A cyclic voltammogram recorded at this time was similar to that shown in Figure 3.3B, suggesting that OmcA/MtrC were not expressed at the cell surface in contact with the electrode under – 0.24 V. Moreover, the cytochromes accumulated during the first step chronoamperometry vanished during polarization at – 0.24 V. Then the working electrode was poised at 0 V again. The current density instantly increased to 12.56 ± 0.39 μA/cm^2, as a result of larger overpotential for electro-oxidation of flavins (Figure 3.2B). Nonetheless, the current immediately began to decline. After 10 h, the current density diminished to 30% of the initial level (Figure 3.2B). In order to evaluate whether the drop in current was associated with the physiological changes in *S. oneidensis*, voltammetric curves were registered periodically. 2 h after the poised potential was restored to 0 V, there appeared a cathodic peak at – 0.4 V with an inconspicuous corresponding anodic peak, suggesting the presence of OM *c*-Cyts (Figure 3.3C). Meanwhile, the catalytic wave became weaker. After 4 h and 6 h, the cathodic peak at – 0.4 V continued to grow (Fig 3.3C), which reflected the accumulation of OM *c*-Cyts.

The present study demonstrated that the presence of OmcA/MtrC at the *Shewnella*-electrode interface reversibly adapted to the electrode potential. This kind of adaptive behavior had already been hypothesized, based on some preliminary observations (Finkelstein et al. 2006; Busalmen et al. 2008). As an explanation for this behavior, the anode respiring bacteria were assumed to upregulate and utilize terminal reductase with the highest redox potential (but lower than the anode potential), when alternative EET pathways are available. This strategy was believed to be energetically advantageous for anode reducers. However, the actual situation may not be so straightforward. Microbes conserve energy from ET and associated proton pumping across their inner membrane, but not the subsequent ET processes (Lovley 2008b). Thus, a higher redox potential of the terminal reductase does not necessarily mean a higher microbial energy gain.

Besides, under certain electrode potential, terminal reductase with a higher redox potential does not kinetically favor EET. There should be more in-depth researches to reveal the mechanism by which the electrode potential regulates cytochrome expression on the cell surface, as well as the significance of this potential-dependant physiology to energy conservation for EAB. Actually, the physiological response to the higher electrode potential probably has a negative impact on the anode respiration.

Concomitant with the accumulation of OM *c*-Cyts, the catalytic current was declining (Figure 3.2B, 3.3C). The hampered current output was therefore likely to be a result of the accumulation of OM *c*-Cyts at the bacteria-electrode interface. One study estimated that up to 34% of the *S. oneidensis* cell surface could be covered by OM *c*-Cyts under specific experimental conditions (Lower et al. 2009). If the electrode poised at positive potential could induce more intensive OmcA/MtrC expression at the *Shewanella*-electrode interface, the interface might be largely or near-fully occupied by proteins, disallowing flavins from accessing the electrode. This explains the vast decrease in current during chronoamperometries at 0 V.

It was also plausible that, a higher electrode potential induced physiological changes in the attached cells, hence they could no longer secrete flavins for EET. To evaluate this possibility, a chronoamperometry was performed at 0 V with the medium amended with 2 μM (final concentration) of riboflavin, seven times more concentrated than endogenous riboflavin in the solution (Marsili et al. 2008a; Ross et al. 2009). As expected, a high level of riboflavin spurred the current generation, which reached approximately 30 $\mu A/cm^2$ within 1.5 h (Figure 3.5). Nonetheless, the current level soon dropped to a near-background level, similar to that observed without exogenous flavins. After interrupted the chronoamperometry and conditioned the working electrode at –0.24 V for 10 min, during which OmcA/MtrC were largely removed from the cell surface, the current density immediately recovered. Thus, the inhibited current generation under 0 V could not be a result of the inactivation of bacteria, substrate depletion or flavin deficiency. The accumulation of cell surface *c*-Cyts seemed to be a

more likely explanation.

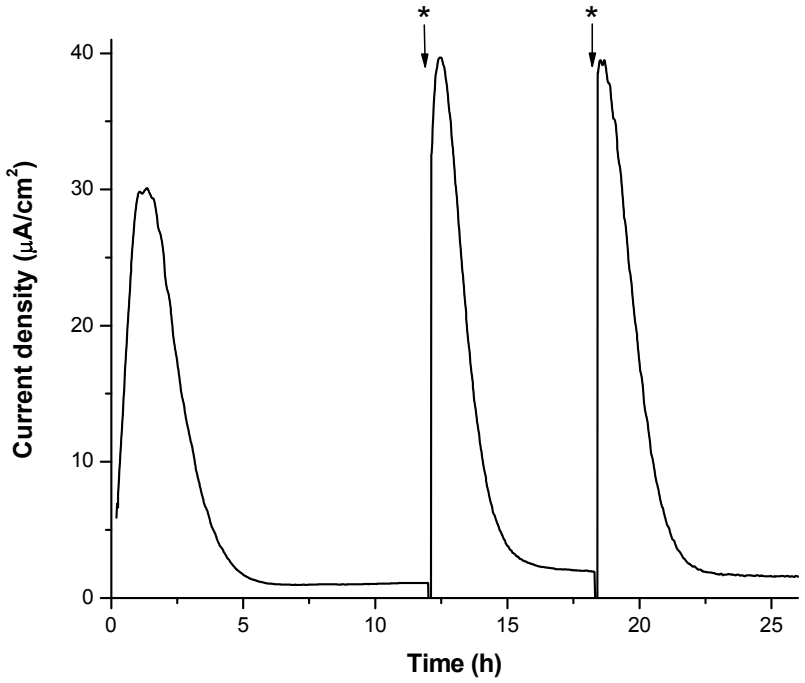


Figure 3.5 Evolution of current with time when the working electrode was poised at 0 V. "*" denotes interruption of chronoamperometry when the working electrode was briefly conditioned at – 0.24 V. The electrolyte was amended with riboflavin to a final concentration of 2 μM.

The observations have further implications on MET by *S. oneidensis* and similar bacteria. After 15 h of discharge under 0 V, the *S. oneidensis* biofilm was still sub-monolayer (refer to chapter 5). Therefore, only a small portion of the electrode surface was insulated by OM *c*-Cyts. One would assume that the MET by planktonic bacteria was not suppressed. While in fact, the current loss was close to 95% when the attached *S. oneidensis* expressed OmcA/MtrC at the interface. In this scenario, MET might be mostly carried out by attached bacteria and mostly through the bacteria-electrode interface. In the aforementioned iron mineral reduction study involving *S. oneidensis* (Ross et al. 2009), the cells had been centrifuged and washed prior to the iron reduction

assays. Therein no flavin existed in the extracellular medium, but activity towards iron reduction was still observed. This was due to a membrane-affiliation of flavins, they persisted in bacterial membrane fraction even after repeated washing (Ross et al. 2009). According to the prevailing views on MET (Schröder 2007), the flavins released by *S. oneidensis* travel across extracellular space to reduce electrodes. Possibly, this process can actually be accomplished by the attached cells through membrane-contained flavins. Nevertheless, the concentration of flavins in the extracellular medium still affects current generation, as it impacts the flavin level in the bacterial membrane fraction.

3.4 Conclusions

Through an unclear mechanism, electrode potential reversibly controls accumulation of OmcA/MtrC at the *Shewanella*-electrode interface. The accumulated OmcA/MtrC are ineffective towards EET to unmodified carbon electrode, but are possibly able to impede more efficient EET by membrane-affiliated flavins of attached bacteria. This induces an abnormal polarization behavior where *S. oneidensis* delivers a much lower current under a higher electrode potential.

These findings demonstrate the complicated natures of biocatalysts, which have profound impacts on microbial BES.

Chapter 4 Utilizing *F. balearica* as a Biocatalyst for MFCs

4.1 Introduction

One of the major topics in the field of BES is to isolate and identify current-generating microbes. Identification of new exoelectrogens may bring about practical impacts on the development of BES. Some anodophiles may enhance power/current densities of bioelectrochemical systems; examples include *Geobacter* species (Nevin et al. 2008; Yi et al. 2009) and a strain of *R. palustris* (Xing et al. 2008). On the other hand, some anode reducers can generate electricity from complex substrates (Rezaei et al. 2009). However, in a more general sense, the discoveries of novel electrode-respiring microbes help to understand the prevalence and ecological significance of exoelectrogenesis. Furthermore, the exoelectrogens are distinct in their EET physiologies, studies on novel exoelectrogens may contribute to the knowledge of cell-electrode interaction.

By the end of 2008, 21 microbial strains were known to catalyze the MFC process without exogenous redox mediators (Logan 2009). Nevertheless, some of these microorganisms were under the same genus (e.g. *S. putrefaciens* and *S. oneidensis*); some even belonged to the same species (e.g. *S. oneidensis* MR-1 and *S. oneidensis* DSP10). It is still a challenging issue to explore the microbial genera or families that are not known to contain exoelectrogens. This study confirms *F. balearica*, a member of the *Ferrimonadaceae* family (Rossello-Mora et al. 1995; Ivanova et al. 2004), as an anode-respiring bacterium. Electroanalytical methods were applied to investigate current generation and EET processes of *F. balearica*; the power output in an MFC with air cathode was also evaluated.

4.2 Experimental section

4.2.1 Electrochemical instrument and set-up

The electrochemical cell, electrodes, as well as the pretreatment of working electrode were identical with those described in chapter 3.

LB broth (Difco Laboratories, MI, USA) prepared in 50 mM sodium phosphate buffer (pH 7.2) was used as the medium for *F. balearica* in the electrochemical cell.

All bioelectrochemical assays were anaerobically performed at 30°C (±1°C) with an independent replication. For cyclic voltammetries, the scan rate was 0.01 V/s. For the chronoamperometry, the reported current density was normalized to the geometric area of GCE.

4.2.2 Construction of MFC

A single-chamber MFC was constructed as described by Liu & Logan (2004). The cylindrical chamber had a cross section area of 7.07 cm^2 and a length of 4 cm.

Graphite granules were employed as the anode material. The granules were rod shaped, with a diameter of 3 mm. Most granules had a length between 4 and 12 mm. A graphite rod (8 mm diameter) was inserted into the chamber as a current collector. After filling the chamber with graphite granules, the liquid volume of the MFC was 10 ml.

The air-breathing cathode was fabricated with wet-proofing carbon cloth (30% PTFE; Gashub, Singapore). A carbon/PTFE base layer and two layers of PTFE coatings were applied at one side of the carbon cloth, using methods described by Cheng et al. (2006a). Pt/C catalyst powders (Pt content 30%; Gashub, Singapore) blended with Nafion solution (5%; Sigma-Aldrich) were applied at the other side of the carbon cloth. 1 mg of the Pt/C powder was mixed with 6.67 μl of Nafion solution. The Pt loading on

the carbon cloth was 0.5 mg/cm^2. The cathode was hot pressed (135°C, 140 atm, 60 s) with a Nafion 117 membrane (Dupont, USA) to form a membrane-electrode-assembly. Before hot pressing, the membrane had been pretreated at 80°C in DI water, hydrogen peroxide (3%), sulfuric acid (1 M) and DI water again for 1 h each.

10 mM sodium acetate medium was used for the fuel cell test. Besides sodium acetate, 1 l of the medium also contained: NaH_2PO_4 0.96 g; Na_2HPO_4 5.96 g; NH_4Cl 0.3 g; $MgSO_4.7H_2O$ 0.12 g; $CaCl_2$ 0.1 g; KCl 0.13 g; 10 ml of vitamin mix and 10 ml of mineral mix (Baron et al. 2009). The MFC was operated at room temperature, 25°C (±2°C).

4.2.3 Bacterial growth and inoculation

The type culture of *F. balearica* was procured from the German Resource Centre for Biological Material (DSM 9799). Prior to inoculating the electrochemical cell or the MFC, *F. balearica* had been aerobically grown overnight in a 250 ml shaking flask (30°C, 150 rpm) containing 50 ml of LB broth.

For inoculating the electrochemical cell, 100 µl of the bacterial culture was added into a sterilized electrochemical cell containing 4.9 ml of fresh LB medium.

For inoculating the MFC, The bacterial culture was first centrifuged for 20 min (4°C, 4000 rpm). The resulting bacterial pellet was washed and resuspended in the sodium acetate medium. The bacterial suspension was diluted with the acetate medium to an OD_{600} of 0.05. Prior to inoculation, the MFC was filled with 70% ethanol for sterilization, followed by flushing thoroughly with fresh acetate medium. 10 ml of the diluted suspension was then added to the MFC.

4.2.4 MFC operation and data acquisition

After inoculation, the MFC was operated with a 2 kΩ external load. The cell voltage

was recorded every 10 min by a Keithley 2700 digital multimeter (Keithley, OH, USA) with data acquisition system.

After each feeding cycle, the medium from the MFC had been filtrated through syringe filters (0.45 μm, cellulose acetate membrane) for COD measurement. The COD was determined by the dichromate method (Liu et al. 2010b). The COD removal rate was used to calculate the CE with a method given in chapter 2.

4.2.5 Scanning Electron Microscopy (SEM)

Immediately after the fuel cell experiments, the graphite granules were transferred to a 2% glutaraldehyde solution and stayed for 2 h. The granules were washed in a 0.1 M sodium cacodylate buffer 3 times (30 min each time), then dehydrated by a graded ethanol series (50, 70, 85, 90, 95, 100%; 20 min each stage). Finally, the granule samples were critical point dried, and sputtered with gold. The SEM observations were made with a JSM-6390LA microscope (JOEL, Japan).

4.3 Results and discussion

4.3.1 Electrochemical analyses and possible EET mechanisms of *F. balearica*

After inoculation of the electrochemical cell, the working electrode was initially poised at 0 V. Within 1 h, the current density increased from the background level to roughly 0.4 μA/cm^2 (Figure 4.1). The current density then deceased from this peak value, to about 0.2 μA/cm^2 in the following hours (Figure 4.1).

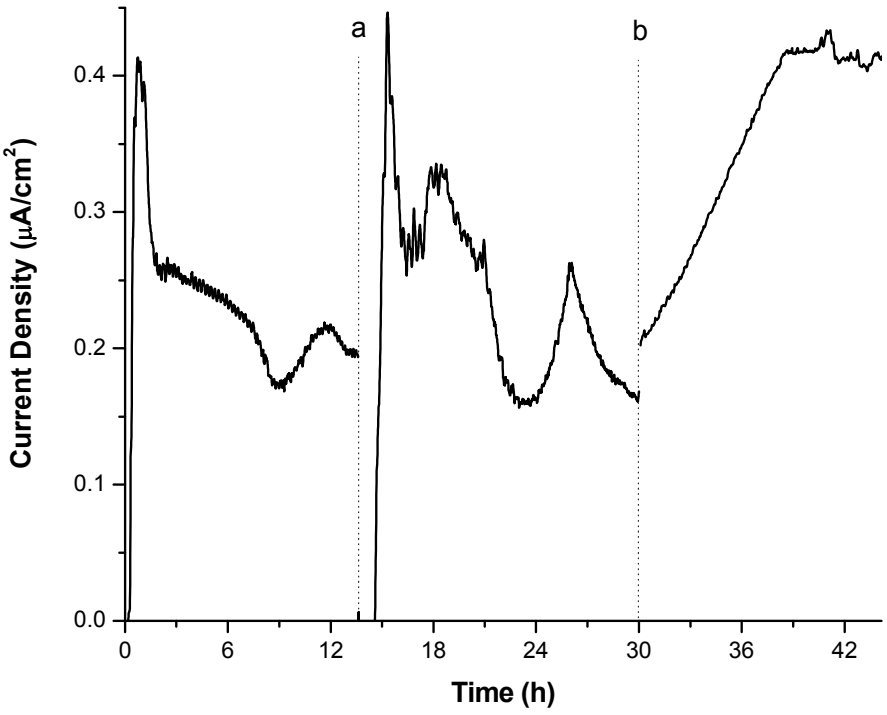


Figure 4.1 Chronoamperometric plot of a 3 mm GCE in the electrochemical cell inoculated with *F. balearica*; a: poised potential switched from the original 0 V to – 0.24 V; b: addition of exogenous riboflavin to the electrochemical cell to a final concentration of 2 μM.

The chronoamperometry paused after 13.6 h of incubation. A cyclic voltammogram registered at this time is shown in Figure 4.2. The voltammogram was characterized by an asymmetric redox pair. This feature was observed with *S. oneidensis* in chapter 3, it had been reported by other researchers as well (Kim et al. 2002). This irreversible electrochemistry was also found on purified cell surface cytochromes from *S. oneidensis* (Eggleston et al. 2008; Meitl et al. 2009). Therefore, such unique electrochemistry might be viewed as an indication for the presence of cell surface *c*-Cyts. The prominent cathodic peak and flat anodic peak suggested that the electrons may flow more readily from the electrode to the OM electron conduits than the reverse,

while the exact reason is still not clear. A recent study proposed that specific kinetic and thermodynamic properties of individual heme centers can result in directional ET by small tretaheme cytochromes in *Shewanella* species (Paquete et al. 2010). However, currently it is not clear whether similar reasons induced the rectification behaviors observed in the whole cell voltammograms of *S. oneidensis* and *F. balearica*.

After the voltammetry, the chronoamperometry resumed with the poised potential shifted from the original 0 V to – 0.24 V. Results presented in chapter 3 indicated that, current delivered by *S. oneidensis* MR-1 increased significantly when the poised potential was switched from 0 V to – 0.24 V. With the lower poised potential, OM *c*-Cyts vanished from the *Shewanella*-electrode interface, while a more effective EET pathway through diffusive redox shuttles (flavins) became active. After the poised potential had been switched to – 0.24 V, *F. balearica* could not immediately adapt to this low potential, and negative current was observed. After about 2 h, the oxidative current recovered and fluctuated between 0.2 and 0.3 μA/cm^2 (Figure 4.1). This current level was substantially lower than that generated by *S. oneidensis* under – 0.24 V.

A cyclic voltammogram was recorded when the chronoamperometry paused again, 30 h after the inoculation (Figure 4.2). A cathodic peak was observed at around – 0.23 V, with inconspicuous corresponding anodic peak. This was likely to be the voltammetric signal of OM *c*-Cyts. The height of the cathodic peak vastly reduced from 3.2 μA to less than 0.1 μA (Figure 4.2). The observation suggested that, the presence of redox proteins at the *Ferrimonas*-electrode interface can be regulated by the electrode potential; a lower poised potential may eliminate hemeproteins from the interface. Hence, the previous finding on the potential-dependent EET physiology was not restricted to *Shewanella* species. Besides this asymmetric redox pair, there was also a less noticeable redox couple at about – 0.4 V (Figure 4.2), which was close to the redox potential of flavins. The secretion of flavins is common among *γ-proteobacteria* (von Canstein et al. 2008), *F. balearica* may also secrete flavins for EET. To evaluate this possibility, the exhaust medium from the electrochemical cell had been analyzed after

bioelectrochemical assays. The presence of riboflavin was confirmed through a chromatographic analysis as described in chapter 3. Very likely, the redox couple near – 0.4 V represented flavin species and should be responsible for the catalytic current under the poised potential of – 0.24 V. Nonetheless, in contrast to the previous observation with *S. oneidensis* MR-1 where the current rose steeply over the midpoint potential of flavin, the *F. balearica* voltammogram did not show obvious catalytic feature (Figure 4.2).

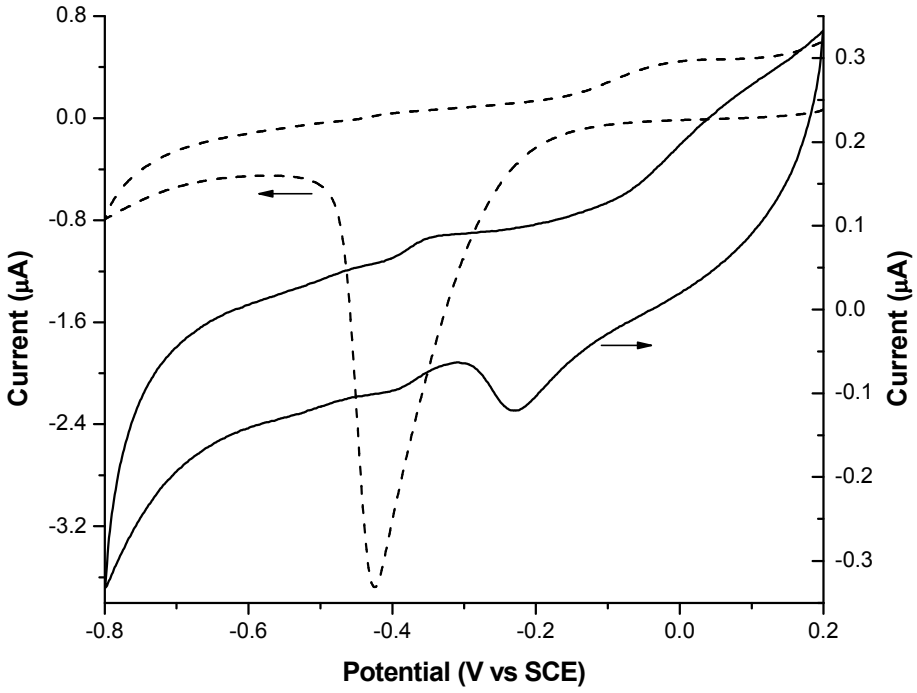


Figure 4.2 Cyclic voltammogram after polarization at 0 V (dashed line) and – 0.24 V (solid line).

In order to probe whether the mediated EET was limited by the insufficient flavin secretion, excessive riboflavin was supplied to the electrochemical cell before restarting the chronoamperometry. Exogenous riboflavin was added to a final concentration of 2 μM, seven times higher than the level of endogenous riboflavin in *S. oneidensis* culture (Marsili et al. 2008a). The current density gradually rose after the

addition of riboflavin (Figure 4.1), supporting the hypothesis that flavins can be utilized by *F. balearica* as redox shuttles for EET. However, the final stable current density was approximately 0.4 μA/cm^2, still significantly lower than that achieved by *S. oneidensis* without any exogenous flavin (chapter 3). Thus, the relatively low respiration rate with flavins might not be attributed to insufficient flavin secretion, but a less effective ET system towards flavin reduction.

The ability to secrete electroactive compounds had been considered as a key point for self-mediated EET (Rabaey et al. 2004). Nevertheless, more recent studies demonstrated such ability does not necessarily translate into the capability of exoelectrogenesis. Some *γ-proteobacteria* species secrete flavins, but cannot utilize them as redox shuttle for anaerobic respiration (von Canstein et al. 2008). It was also observed that mutants of *S. oneidensis* that lack OM *c*-Cyts MtrC or OmcA were hampered in current generation (Baron et al. 2009; Coursolle et al. 2010), where the electrocatalytic activity of flavins was suppressed (even when the medium was amended with excessive riboflavin). A comparative study between *S. oneidensis* and *F. balearica* ET systems may help to elucidate the mechanisms for efficient MET.

4.3.2 Fuel cell performance

Cell voltage over 100 mV was instantly observed after the inoculation. However, this voltage did not sustain, it dropped rapidly and stabilized at around 60 mV after 1.5 d (Figure 4.3). The voltage remained at 60±2 mV for about 2 d, and gradually decreased. After 8 d of operation, the medium was replaced. The peak voltage in the second and third feeding cycle was very close to that in the first cycle (60.3-60.6 mV, Figure 4.3). While *S. oneidensis* cannot use acetate as a fuel under anaerobic conditions (Lanthier et al. 2008), *F. balearica* can steadily couple the acetate oxidation with the anode reduction.

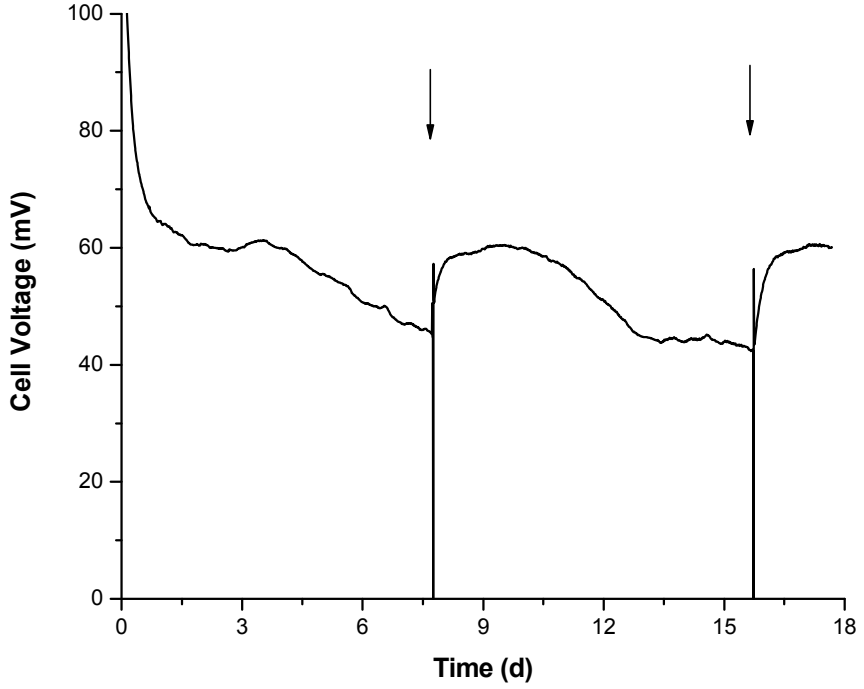


Figure 4.3 Evolution of cell voltage with time after the MFC had been inoculated with *F. balearica*, arrows indicate the replacements of medium.

The MFC polarization test was carried out in the third feeding cycle when the cell voltage reached 60 mV. The previous cycles showed that this peak voltage could be sustained for over 24 h, during which the system was almost steady. The test was performed by varying the external resistance. 0.5, 1, 5 and 10 kΩ resistors had been used. Once a new resistor was connected to the circuit, the system was allowed to stabilize for 3 h before the cell voltage was recorded. Polarization and power curve are shown in Figure 4.4, current densities and power densities reported here are normalized to the projected cathode area (7.07 cm^2). The maximum power density 2.55 mW/m^2 was achieved when the current density was 42.5 mA/m^2 (2 kΩ load). In an MFC evaluation involving eight *Shewanella* strains (Bretschger et al. 2010), five stains generated power density of 1.5-3 mW/m^2, while *S. oneidensis* MR-1 delivered a higher power density of about 4.5 mW/m^2. This is in agreement with the finding that *S.*

oneidensis MR-1 can more efficiently utilize endogenous flavins as the redox shuttle.

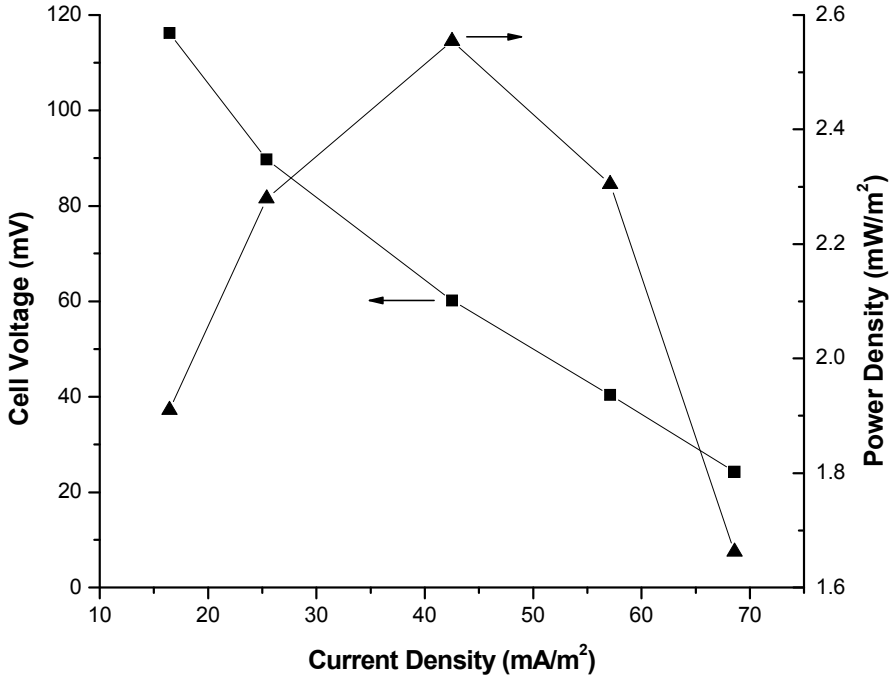


Figure 4.4 The polarization curve (squares) and power curve (triangles) of a MFC with *F. balearica* as the biocatalyst.

Even with a relatively high external resistance of 2 kΩ, the *F. balearica* MFC achieved a CE of 42.2±3.9% (average of the first and second feeding cycle). Comparatively, MFCs with *Shewanella* strains had CEs of 18-35% (Bretschger et al. 2010); an MFC using an electrochemically active *Corynebacterium* strain reported CE of 5.9% (Liu et al. 2010b); MFCs based on *Acetobacter aceti* or *Gluconobacter roseus* had CEs of 1-2% (Karthikeyan et al. 2009). One possible reason for the very low CEs was: the microbes may divert a vast majority of the electrons derived from the substrate to their growth, rather than current generation.

4.3.3 SEM characterization

After operation for 20 d, the graphite granules were still barely colonized (Figure 4.5). At a magnification of 5000, most view-fields did not include any bacterium. Therefore, the power generation in the *F. balearica* MFC might not be the effect of close physical association between bacteria and graphite. This was consistent with the observation that cell voltage could be instantly established when live bacteria were inoculated to the MFC. The poor electrode colonization may also be a reason for the limited current generation by *F. balearica*. The inset of Figure 4.5 shows the typical morphology of *F. balearica* attached to the graphite.

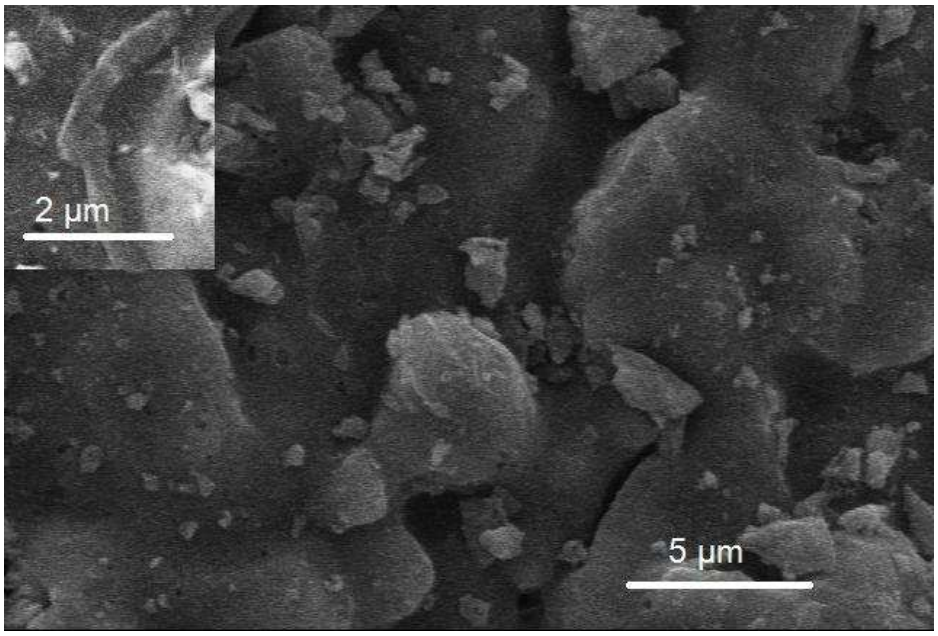


Figure 4.5 Typical SEM image of the graphite granules from the *F. balearica* MFC; inset: *F. balearica* cells attached to the graphite granule.

4.4 Conclusions

In this study, attempts had been made to evaluate the EET physiology and power-generating capability of *F. balearica*.

F. balearica was likely to express cell surface hemeproteins which exhibited irreversible electrochemistry. Under a lower poised potential, these redox proteins appeared to vanish from the *Ferrimonas*-electrode interface. It should be further evaluated whether they are homologs of OmcA/MtrC (OM *c*-Cyts of *S. oneidensis*). *F. balearica* also released flavins to the extracellular medium, and utilized them as redox shuttles for EET.

The fuel cell tests proved *F. balearica* to be a valid biocatalyst. *F. balearica* readily catalyzed MFC anodic reaction with a Coulombic yield higher than many other exoelectrogens.

Chapter 5 CNTs as Electrode Modifier Promoting DET from *S. oneidensis*

5.1 Introduction

Chapter 3 and 4 indicated that, many *γ-proteobacteria* exoelectrogens may possess OM *c*-Cyts for EET. Nonetheless, the electrochemistry of these proteins was irreversible; electron flow from bacteria to the electrode was hindered. Additionally, the heterogeneous ET rate constants of these OM *c*-Cyts were very low. Thereafter, in spite of the fact that these bacteria are endowed with abundant cell surface cytochromes, the DET may not be able to proceed at a considerable rate. A low current density of 0.6 $\mu A/cm^2$ was observed in the initial MFC evaluation with *S. oneidensis* as the biocatalyst; several other *Shewanella* strains yielded similar results (Kim et al. 2002).

Although it was found that *S. oneidensis* could conduct more effective EET through redox shuttles; it is not clear whether the shuttling compounds can be confined within biofilms (Rabaey et al. 2007), otherwise MET will not be applicable to continuous systems. For practical applications, the DET is of greater interest.

To overcome the sluggish DET, several attempts had been made to immobilize mediators on electrodes, such innovation successfully enhanced current delivered by microorganisms (Lowy et al. 2006; Adachi et al. 2008). However, mediator is effective only when its redox potential is higher than that of OM proteins and lower than the anode potential. In addition, the durability of mediator functionalized electrodes is a problem (Watanabe 2008). SAM with carboxylic acid termini is known to promote electrochemical response of horse heart *c*-Cyt (Tarlov and Bowden 1991). However, current density was still low (0.38 $\mu A/cm^2$) and unstable with SAM modified electrode and *Shewanella* as biocatalyst (Crittenden et al. 2006). This result suggested that

promoters for well-studied redox proteins like horse heart *c*-Cyt might not be equally effective for bacterial OM *c*-Cyts. Bacterial OM *c*-Cyts have distinct structures of their own (Hartshorne et al. 2007; Eggleston et al. 2008), and their behavior may not be analogous to archetype redox proteins, especially in the whole cell context. It is obscure how cell surface *c*-Cyts behave under MFC condition, and what mechanism inhibits electron flow to anode. Most importantly, it is necessary to find a way to promote heterogeneous ET of OM c-Ctys and consequently enhance power density of MFCs.

CNTs, due to various desirable properties, had been utilized as electrocatalyst in fuel cells and biosensors (Wang 2005). They have been reported to enable direct electrochemistry of some important biomolecules (Agüí et al. 2008). Recently, CNTs have been applied as electrode modifier in mediated MFCs (Qiao et al. 2007). Nonetheless, interaction between CNTs and cell surface *c*-Cyts had not been investigated. In order to evaluate possible promoting effect of CNTs towards DET in MFC systems, chronoamperometries and cyclic voltammetries were performed in an electrochemical cell inoculated with *S. oneidensis* using a bare GCE or a CNT-coated GCE.

5.2 Experimental section

5.2.1 Electrochemical instrument and set-up

Electrodes and electrochemical cells had been described in chapter 3. All electrochemical tests were conducted at room temperature of 25 °C (±1 °C). For cyclic voltammetries, the scan rate was 0.01 V/s unless otherwise indicated.

5.2.2 Electrode preparation

Prior to use, the GCE was polished sequentially with 1 μm and 50 nm alumina paste, followed by ultrasonic cleaning in de-ionized water for 5 minutes.

MWNTs (95% in purity, 8 nm diameter) were purchased from Chengdu Organic Chemicals Co. LTD. (Chengdu, China). MWNTs were purified and activated by refluxing pristine MWNTs in 4 M nitric acid at 150 °C for 48 hours. The sample obtained was filtrated through nylon membrane and dried in an oven for 16 hours at 105 °C. 10 mg of MWNTs were then added to 10 ml of ethanol and agitated with an ultrasonic homogenizer for 10 minutes to give a stable black solution. To coat the GCE with MWNTs, 6 µl of the suspension was dropped to glassy carbon plate, and allowed to dry in air. The electrode thus obtained was loaded with 84.5 µg/cm^2 MWNTs and designated as MWNT/GCE.

5.2.3 Bacterial growth and inoculation

S. oneidensis MR-1 was received from the Belgian Coordinated Collections of Microorganisms (LMG 19005). LB broth (Difco Laboratories, MI, USA) prepared in 50 mM sodium phosphate buffer (pH 7.4) was used as growth medium for *S. oneidensis* and also functioned as electrolyte in electrochemical cell. The bacterium was grown aerobically in a shaking flask (30 °C, 150 rpm) for approximately 18 hours until the OD_{600} reached 2. 100 µl of the bacterial culture was inoculated to the sterilized electrochemical cell with 4.9 ml fresh medium. After inoculation, electrolyte was aseptically purged with nitrogen gas and the working electrode was poised at 0 V.

5.3 Results and discussion

5.3.1 Chronoamperometric results

When a bare GCE was poised, oxidative current grew steadily at the beginning, indicating that *S. oneidensis* began to attach and proliferate at the electrode. Nevertheless, the current density peaked at 0.450±0.033 µA/cm^2 after 1-2 hours incubation (n=2), then dropped sharply and remained almost stagnant thereafter. Previous study with bare GCE and SAM modified gold electrode reported this perplexing decrease in current (Crittenden et al. 2006). As discussed in chapter 3, this

was possibly caused by the accumulation of OM *c*-Cyts at the electrode surface. Current density recorded 15 hours after the start of chronoamperometry was 0.117±0.006 μA/cm^2 (Figure 5.1A). Sustainable current delivered with bare GCE was very low at the poised potential of 0 V.

The polarized MWNT/GCE delivered 9.70±0.40 μA/cm^2 after 15 h (Figure 5.1A). Compared with the bare GCE, the MWNT modifier vastly boosted the current density by 82 times while other experimental conditions remained unchanged.

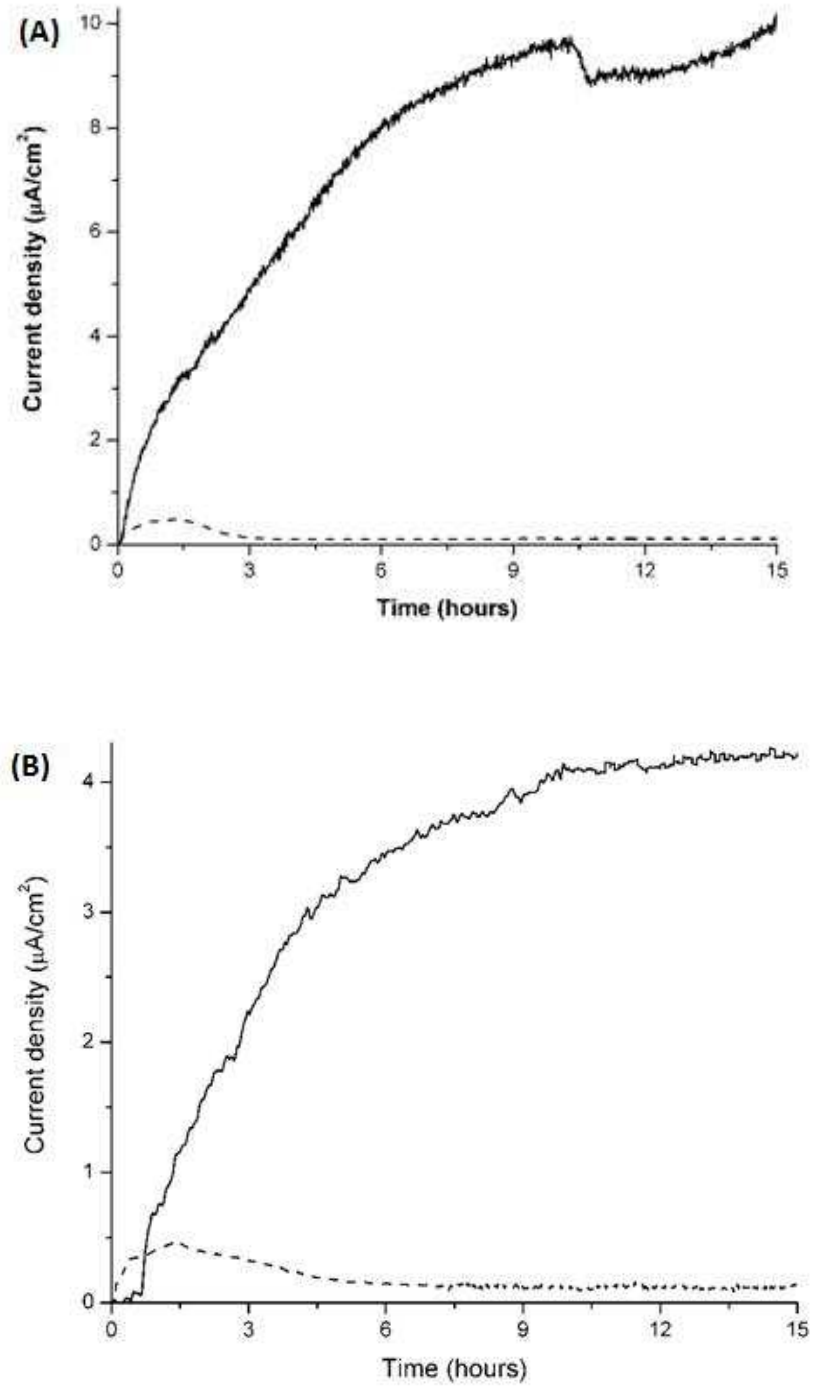


Figure 5.1 Evolution of current density with time, when MWNT/GCE (solid) and bare GCE (dashed) were poised at 0 V in electrochemical cells inoculated with *S. oneidensis* (A) or *F. balearica* (B).

5.3.2 Cyclic voltammetric results

Cyclic voltammetry was performed instantly after termination of chronoamperometry. The pH of electrolyte at the time of cyclic voltammetric characterization was 7.1±0.1. The voltammetry after polarizing the bare GCE for 15 hours revealed an asymmetric redox pair as had been observed in chapter 3 (Figure 5.2A), which was the voltammetric fingerprint of *S. oneidensis* OM *c*-Cyts (Meitl et al. 2009).

Contrarily, after conditioning at 0 V for 15 hours, the voltammogram of MWNT/GCE showed a well-defined redox couple with cathodic peak at - 0.35 V and anodic peak at -0.17 V which should be responses of OmcA/MtrC (Figure 5.2B). To exclude capacitive current and background signals, noises were discarded by subtracting the baseline, and the resulting profile is shown in Figure 5.2B. At MWNT/GCE, the anodic peak of OM *c*-Cyts was much more pronounced, and quite symmetrical to the corresponding cathodic peak. The ratio of anodic peak current to cathodic peak current (i_{pa}/i_{pc}) was determined to be 0.93, clearly showing that the electrode process of cytochtomes with the MWNT modifier was chemically reversible. This could largely explain how *S. oneidensis* delivered current almost 2 orders of magnitude higher with the MWNT/GCE. It was hypothesized that the irreversible electrode behavior of OM *c*-Cyts was related to flat-band potential of electrode (Eggleston et al. 2008), however this hypothesis is yet to be validated. Presumably, reversible electrochemistry with the modified electrode could be the result of either cytochrome's special conformation at CNTs, or certain electronic property of CNTs.

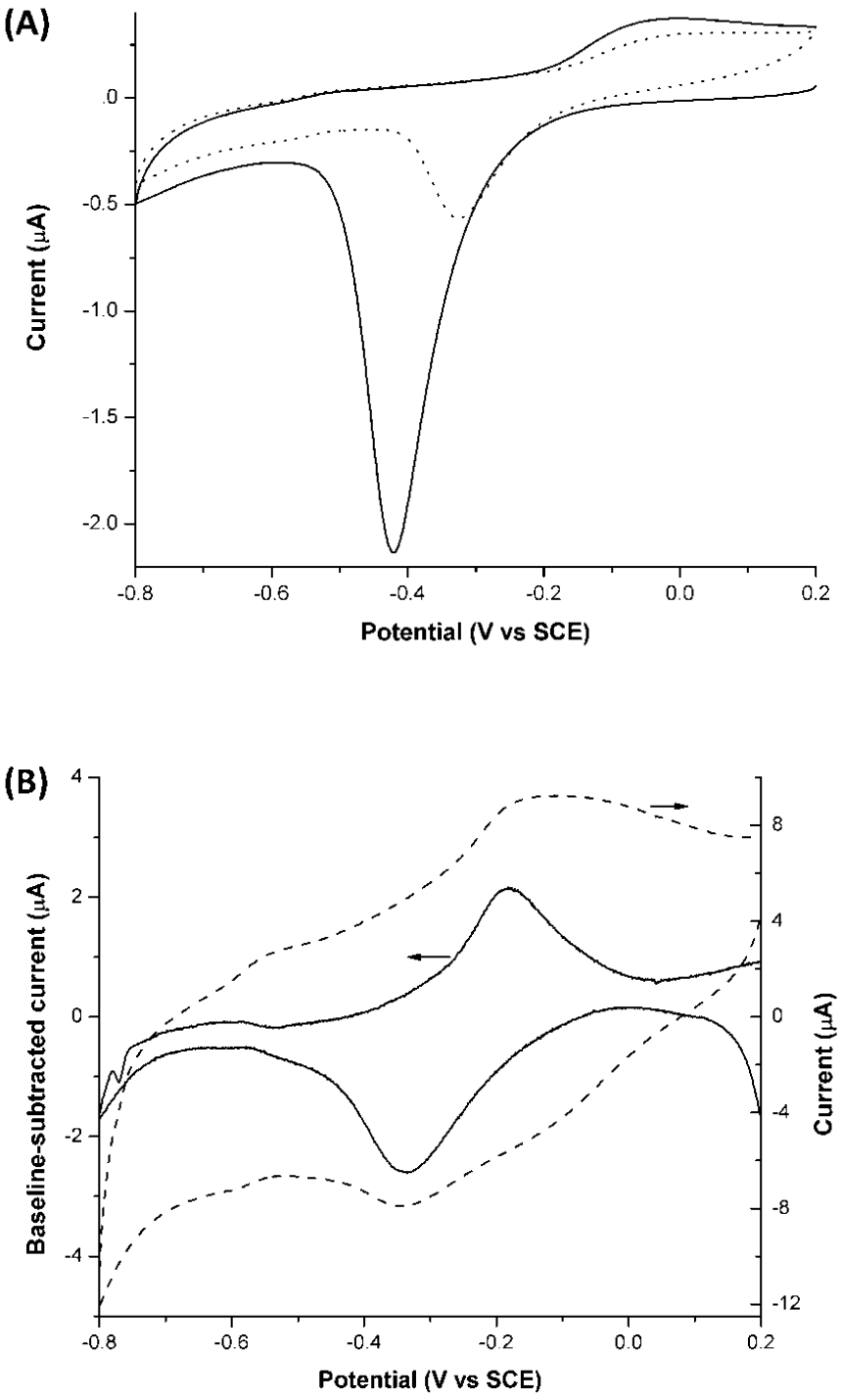


Figure 5.2 Cyclic voltammograms in an electrochemical cell inoculated with *S. oneidensis*. (A) Bare GCE after poised for 15 hours: first scan (solid) and second scan

(dotted). (B) MWNT/GCE after poised for 15 hours: original (dashed) and baseline-subtracted (solid).

Notably, many other *Shewanella* strains possess homologs of OmcA/MtrC (Fredrickson et al. 2008), while the *Shewanella* species can be dominant at the MFC anode (Logan et al. 2005). Furthermore, several other electrochemically active microbial strains also showed asymmetric redox waves with a sharp cathodic peak, a flatter and broader anodic peak (Schröder 2007; Zhang et al. 2008a), implying that such irreversibility is shared by many microorganisms in the exoelectrogenic consortium. In chapter 4, the supposed OM *c*-Cyts of *F. balearica* were found to behave in a similar way. Therefore, the electrochemistry of *F. balearica* was investigated with GCE and MWNT/GCE. The results were similar to those obtained with *S. oneidensis* (Figure 5.1B, Figure 5.3). It can be assumed that the irreversible electrochemistry of OM *c*-Cyts may be induced by a common mechanism, and can be transformed by CNT modifiers.

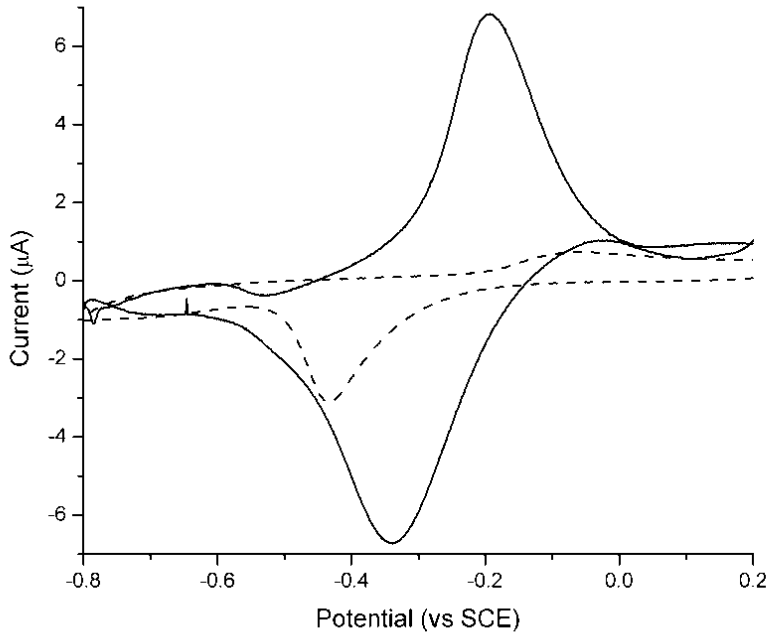


Figure 5.3 Cyclic voltammograms after 15 h of incubation (at poised potential of 0 V) with *F. balreaica*, using MWNT/GCE (solid line, baseline-subtracted) and bare GCE

(dashed line) as the working electrodes

To study the ET kinetics of *S. oneidensis* OM *c*-Cyts at MWNT/GCE, the change of oxidative/reductive peak potential with the scan rate v was monitored (Figure 5.4). At a scan rate of 0.01 V/s, the separation between the anodic and cathodic peaks (ΔE_p) was 0.17 V, significantly smaller than the ΔE_p recorded at bare GCE. The result suggested that the MWNT modifier concomitantly improved ET kinetics. The ΔE_p increased gradually with increasing scan rate, reached 0.43 V when the scan rate was up to 1 V/s. When the scan rate was higher than 0.3 V/s, cathodic peak shifted faster than the anodic peak. Based on the trumpet plot and Loviron's (1979) theory, the transfer coefficient was calculated as 0.27, while the heterogeneous ET rate constant was estimated to be 1.25 s^{-1} (Appendix). To analyze the dependence of the peak currents on scan rate, the peak currents were plotted against scan rates. The logarithmic peak current was found to be linearly ($R > 0.99$) related to logarithmic scan rate, with a slope of 0.62 for the anodic branch and 0.65 for the cathodic branch (Figure 5.4). These linear correlations suggest $i_{pa} \propto v^{0.62}$, $i_{pc} \propto v^{0.65}$ respectively, distinguished from the typical behavior of neither adsorbed nor diffusive redox substances (Bard and Faulkner 2001; Compton and Banks 2007). A voltammetric study with *G. sulfurreducens* (Fricke et al. 2008) reported similar results. The whole cell electrochemical behavior is thus complex and not fully understood with current state of knowledge, calling for further research in this aspect.

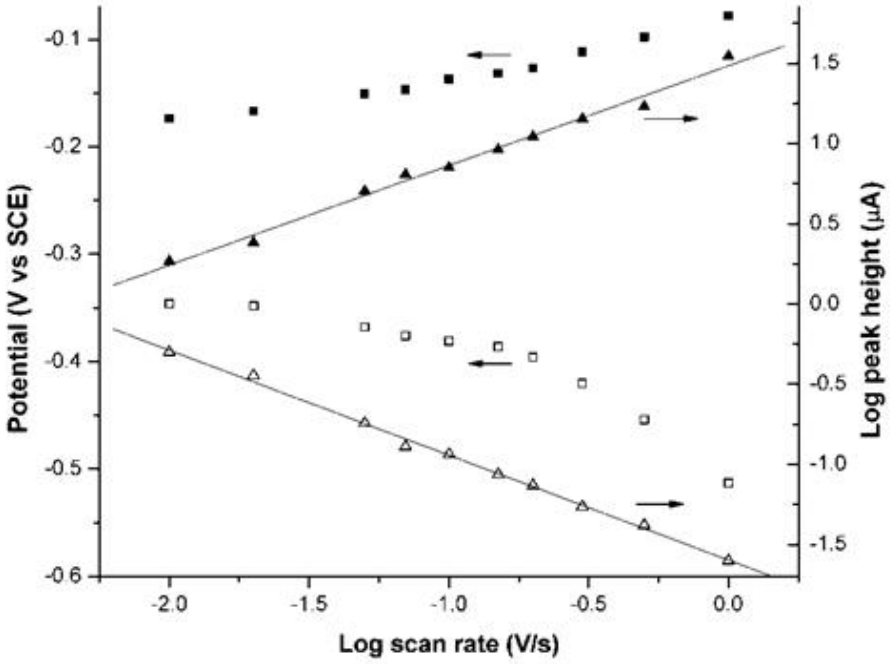


Figure 5.4 Influence of scan rate on *S. oneidensis* redox peaks, with MWNT/GCE as working electrode: shift of peak potential (squares) with scan rate and dependence of peak current (triangles) on scan rate, where closed symbols are for anodic branch and open symbols are for cathodic branch.

5.3.3 SEM

One may query whether the MWNT film has the structure to create more electrode area for microbes to colonize. Morphologies of the bare GCE and MWNT/GCE after chronoamperometric tests were examined with a Zeiss EVO-50 SEM. The MWNT/GCE (Figure 5.5B) apparently had a higher surface roughness than the bare GCE (Figure 5.5A). But this unevenness was inadequate to accommodate more bacteria, whose size is at the micrometer scale. Therefore, MWNT film did not increase electrochemically accessible area for DET. This confirmed that the increase in current was a result of the promoting effect of CNTs, rather than augmentation of surface area. After 15 hours of discharge, surface coverage by microbes was still low for both bare GCE and MWNT/GCE (roughly 5×10^4 cells/mm^2, or 6-8% electrode surface had been

covered). Considerably higher current density can be expected when mature biofilm is formed.

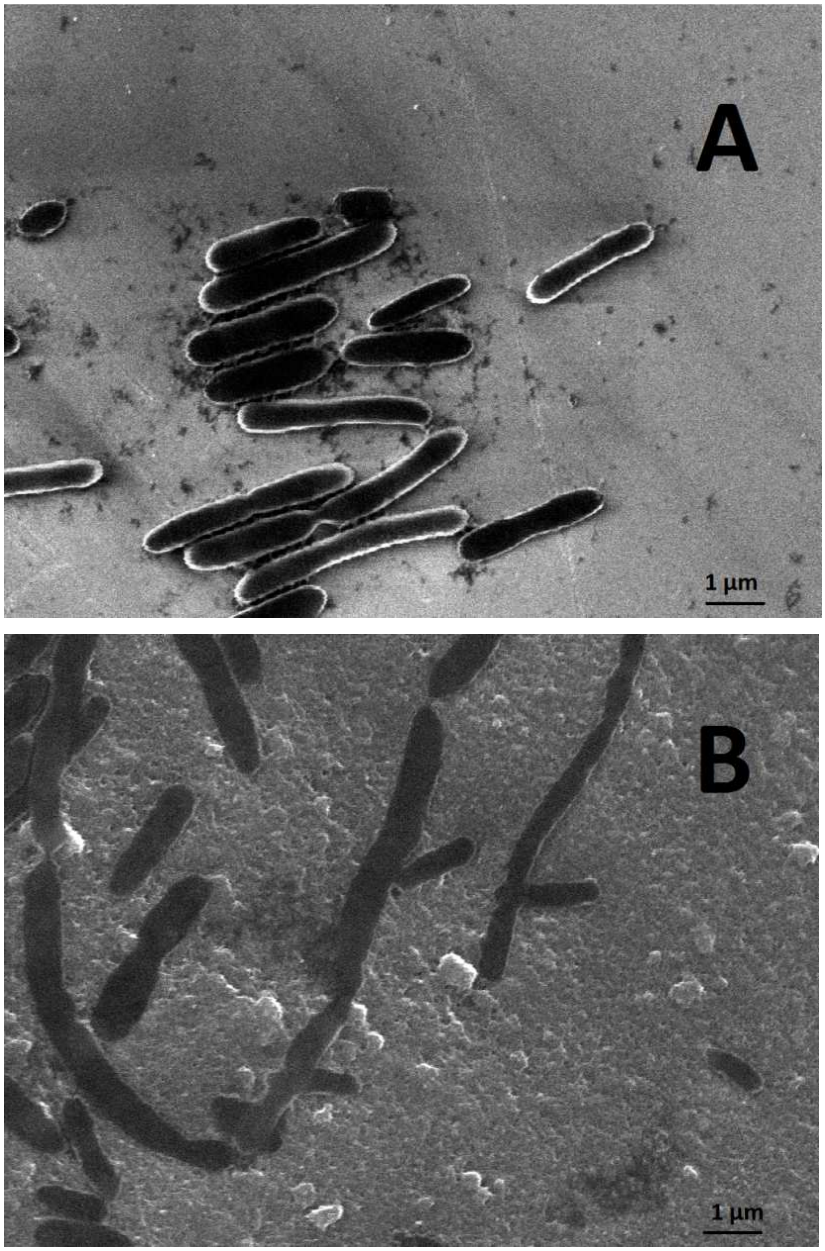


Figure 5.5 SEM images of (A) bare GCE and (B) MWNT/GCE after chronoamperometries with *S. oneidensis*

5.4 Conclusions

Discovery of DET between microorganisms and the electrode brought a fundamental

innovation to MFC, it may be introduced to other whole cell BES like microbial biosensors.

Bacterial OM *c*-Cyts are generally electrochemically active on commonly used electrodes, which is different from many well-studied redox proteins. However, irreversible electrochemistry and sluggish ET rate of these cytochromes, as unveiled in this study, severely hamper biological current generation. Instead of using immobilized mediators to facilitate ET from microorganisms to electrode; CNTs were observed to assist electro-oxidation of *S. oneidensis'*s OM *c*-Cyts, and enhanced their heterogeneous ET rate. By promoting electrochemistry of membrane protein, CNTs greatly improved the current generation. Such promoters will be in great demand for whole cell BES.

Chapter 6 Evaluating CNTs as the Anode Modifiers in MFCs with Mixed Anodic Consortium

6.1 Introduction

In chapter 3, the OM *c*-Cyts of *S. oneidensis* were found to display rectification behavior: the ET from cytochromes to electrodes was obstructed. Also, the ET rate appeared to be low, the heterogeneous rate constant was estimated to be below 0.1 s^{-1} (Meitl et al. 2009). In chapter 4, the exoelectrogenesis of *F. balearica* was studied, this bacterium seemed to synthesize OM *c*-Cyts as well. Irreversible electrochemistry similar to *S. oneidensis* OmcA/MtrC had been observed. These findings suggested that the EET behaviors of *S. oneidensis* might be representative for a large class of similar microbes. In chapter 5, CNTs were applied as the electrode modifiers. Reversible electrochemistry of *S. oneidensis* was obtained in the presence of CNTs, the heterogeneous rate constant of OM *c*-Cyts was promoted to the 1 s^{-1} range. These were accompanied with enhancement in current density for over 80 folds.

Nevertheless, some microbes showed very different electrochemical behavior from *S. oneidensis*. Fricke et al. (2008) recorded the turnover and non-turnover voltammograms of *G. sulfurreducens*, they found two redox systems actively engaged in microbial EET, and both displayed reversible redox waves. In addition, exoelectrogens may adopt EET pathways other than the cell surface metalloproteins. Therefore, it remained questionable whether CNTs can effectively catalyze the MFC anodic process with a mixed consortium.

Mixed microbial consortium is used in most of the MFC studies, due to high power-generating capability and convenience to obtain. A number of studies had been carried out to modify the anodes for facilitating the heterogeneous ET of mixed microbial

community. Many of such attempts were made through immobilization of redox mediators (Park and Zeikus 2003; Lowy et al. 2006; Wang et al. 2011). The limitations of the mediator functionalized anode had been discussed in chapter 5. Other approaches for improving the anodic process had been attempted. Cheng and Logan (2007a) treated carbon cloth with ammonia gas under high temperature. The carbon electrode was functionalized with nitrogen-containing functional groups, which could increase the positive charge. The treatment shortened the acclamation time, and enhanced the power density for about 20%. Feng et al. (2010b) pretreated carbon fiber brush in ammonium peroxydisulfate and concentrated sulfuric acid, which modified the electrode with various functional groups. The treatment brought about 25% increment in power density. When the acid treatment was in combination with heat treatment, power density increased for 34% compared with untreated control.

CNTs had also been applied as the anode modifiers in MFCs with mixed exoelectrogenic consortium. Tsai et al. (2009) modified carbon cloth with MWNTs, and used this modified electrode in a single-chamber MFC. Sun et al. (2010) adopted a layer-by-layer technique to modify carbon fibers with CNTs, and used the modified carbon paper as MFC anode. Both study confirmed the improvement in power generation by anode modifications. However, the authors in these studies did not show the improvement was indeed a result of better anode performance, and it was not known how the CNTs optimized the anode performance.

This study extended the investigation on CNTs' promoting effects, through full cell and half cell tests with mixed microbial cultures enriched from wastewater.

6.2 Experimental section

6.2.1 Preparation of electrodes

6.2.1.1 Anode preparation

Graphite foil (0.25 mm thickness, Alpha Aesar) served as the anode material. The foil

was cut into 2 cm x 2 cm pieces (4 cm^2 projected area), and then connected to a titanium wire with silver conductive paste. The obtained electrode was referred as GFE, it was either directly used for MFC or further modified with MWNTs.

MWNTs (95% in purity, 8 nm diameter) were purchased from Chengdu Organic Chemicals Co. LTD. (Chengdu, China). 1 mg of MWNTs were added into a 2% sodium dodecyl sulfate (SDS) solution. After 10 min of agitation with an ultrasonic homogenizer, a stable MWNT dispersion was formed. The GFE was gently dipped in the dispersion and allowed to dry in air; this procedure had been repeated for 5 times. SDS strongly adsorb on MWNTs (Richard et al. 2003), which may affect ET from microbes. To remove SDS, the modified GFE was aggressively rinsed with DI water, and then soaked in a 4 N nitric acid over night (Geng et al. 2008). The electrode was then rinsed and dried in an oven (60°C) for 24 h. The electrode thus obtained was designated as GFE/MWNT.

6.2.1.2 Cathode preparation

The cathode was fabricated as described in chapter 4; except that the cathode was directly used, without being hot pressed with a PEM.

6.2.2 Construction and operation of MFCs

A single-chamber MFC described in chapter 4 had been used for evaluating the anode performances. The MFCs with GFE and GFE/MWNT anode were named as MFC1 and MFC2 respectively. The medium in the MFCs was 10 mM sodium acetate medium, its formula had been given in chapter 4.

MFC1 and MFC2 were inoculated with the effluent from another MFC which had been operated for several months and was originally inoculated with domestic wastewater. For the startup, MFC1 and MFC2 were operated with a 500 Ω load. The cell voltage was recorded every 10 min by a Keithley 2700 digital multimeter (Keithley, OH, USA) equipped with data acquisition system. The medium in the MFCs were replaced every

two days. Once the variation of maximum cell voltage in 3 consecutive feeding cycles was less than 3 mV, the fuel cell was considered as stable. The fuel cell was then subjected to polarization test by varying the external resistance. 2000, 1000, 750, 500, 300 and 75 Ω resistors had been used. During the polarization test, only one resistor was used for each batch cycle, the maximum voltage in the cycle was used to plot the polarization curve. Current densities and power densities were normalized to the cathode projected area (7.07 cm^2).

The COD reduction rate of each feeding cycle had been measured, and the CE was calculated. Detailed methodologies have been presented in chapter 4.

6.2.3 Cyclic voltammetries

Cyclic voltammetries were carried out when the cell voltage reached its maximal value in a batch cycle. For the voltammetries, the anode was used as the working electrode, while the cathode acted as the counter electrode. An SCE reference electrode had been employed. The potentials reported in this chapter were converted to "vs. SHE", assuming a 0.242 V potential difference between SCE and SHE (Bard and Faulkner 2001). The scan rate was 0.005 V/s.

6.2.4 SEM

The morphology of GFE or GFE/MWNT was studied using a JSM-6700F field emission microscope (JOEL, Japan).

After the fuel cell experiments, the anodic biofilms were characterized with an EVO-50 microscope (Zeiss, Germany). Before the SEM characterization, the biofilms had been fixed and dried using methods described in chapter 4.

6.2.5 Biomass quantification

To understand whether the electrode modification could affect the accumulation of

anodophilic biomass, anode-attached biomass was measured. For this, the anodic biofilm was detached through ultrasonic treatment, the total protein content was quantified through well established method (Liang et al. 2008).

6.3 Results and discussion

6.3.1 Morphologies of the anodes

The surface of GFE was smooth (Figure 6.1A), it was clear that the graphite foil was aligned by pieces of graphene stacks. In contrast, the surface of graphite stick was much rougher, consisting of micrometer scale structures, which may accommodate more bacteria (Busalmen et al. 2008).

SEM showed that, the surface of GFE/MWNT was largely covered with CNTs (Figure 6.1B), however there was still some graphite surface exposed. The presence of MWNTs evidently increased the electrode roughness. Nonetheless, same as noticed in chapter 5, the cavities and fluctuations were of the nanometer scale, and might not increase effective surface area for DET.

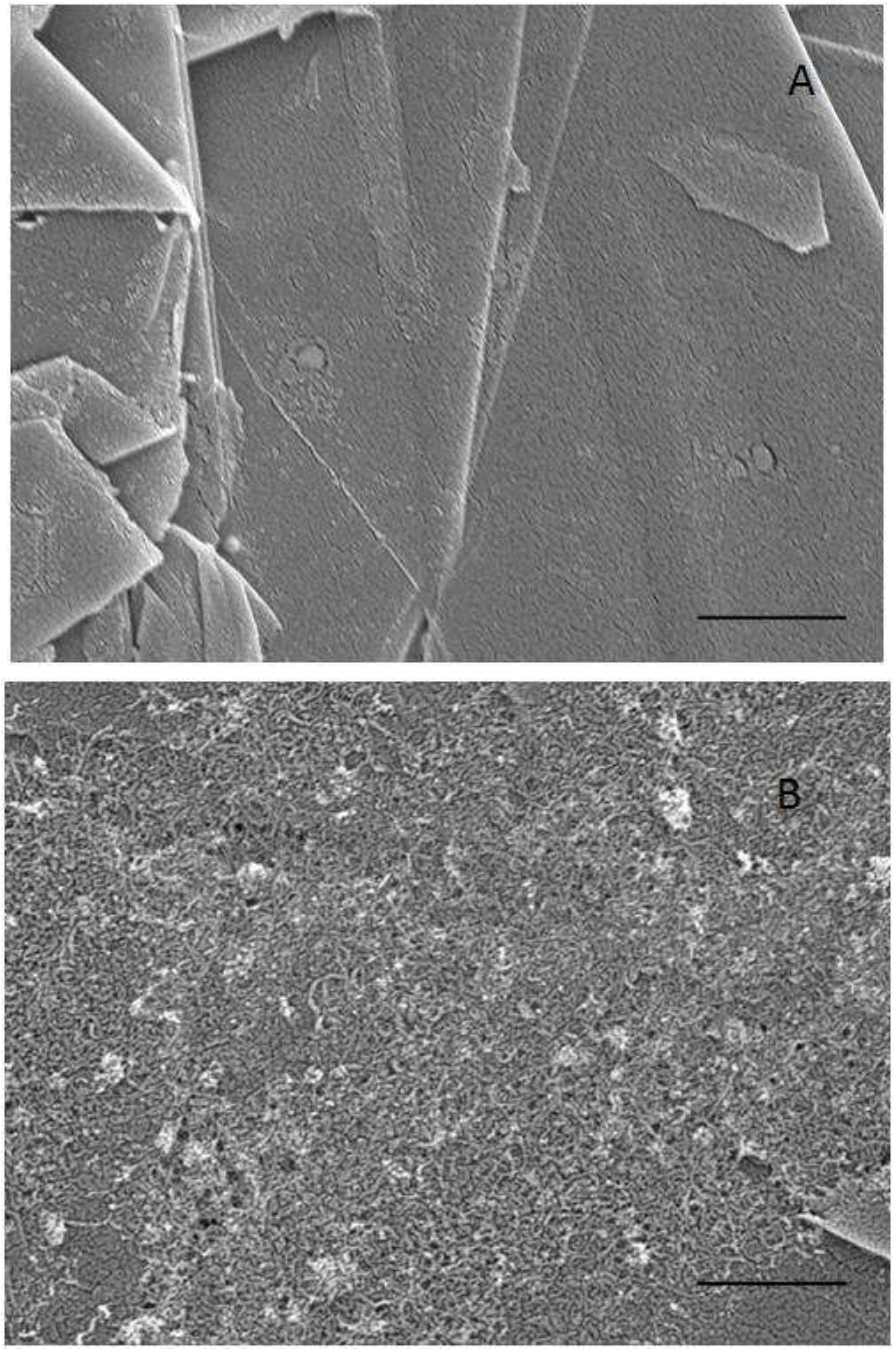


Figure 6.1 SEM images of bare GFE (A) and GFE/MWNT (B); bar length: 1 μm.

6.3.2 Fuel cell performances

6.3.2.1 Power generation

Both MFC1 and MFC2 steadily generated power after a startup period of about 1 month (Figure 6.2). A recent study suggested that electrochemically active biofilm cannot be properly formed on GFE, which generated only negligible current level with a mixed inoculum (Liu et al. 2010c). Currently, the reasons for the different results are not known.

Under all external loads, MFC2 generated higher peak voltage than MFC1. It was also noticed that, under lower external resistance, the difference in peak voltage was more pronounced. With the 2000 Ω resistor, MFC2 had a peak voltage 8% higher than that of MFC1. The peak voltage was 50% higher when a 75 Ω resistor was used. Possibly, with lower loads, the cell voltage depends more heavily on the anode performance. It was further observed, the cell voltage in MFC2 always dropped sooner than that in MFC1.

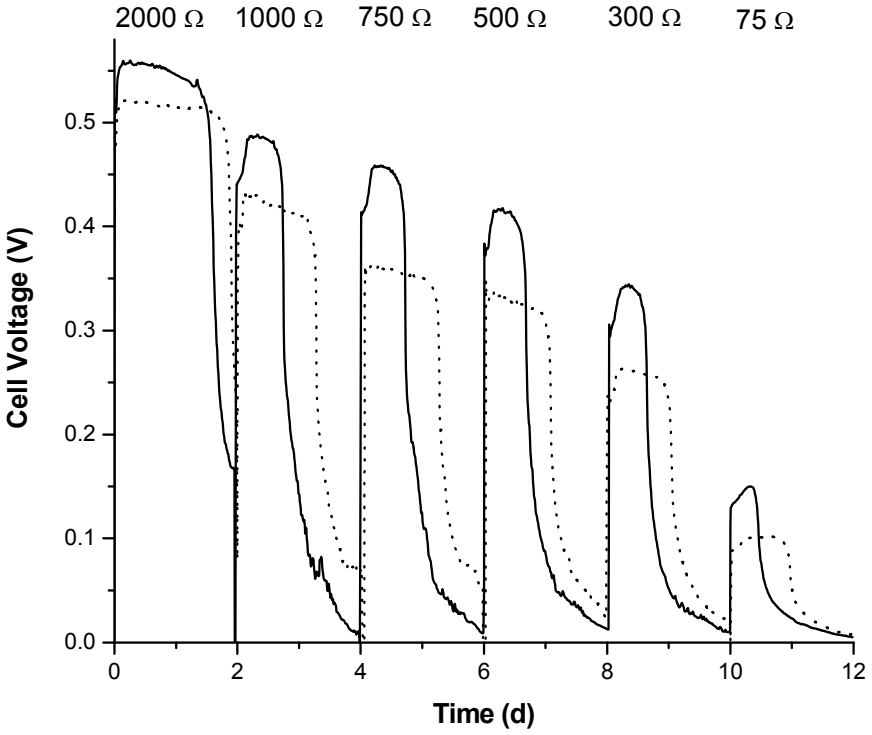


Figure 6.2 Cell voltage of MFC1 (dotted line) and MFC2 (solid line) with time, when they were subjected to polarization test

The power curve indicated that the maximum power density of MFC1 was 329 mW/m^2 (Figure 6.3), achieved at the current density of 1.25 A/m^2 (300 Ω load). MFC2 had a maximum power density of 566 mW/m^2, achieved at the current density of 1.64 A/m^2 (300 Ω load). Comparatively, MWNT modifiers increased the power density for 72%. Some researchers reported much higher power densities in their MFC evaluations (e.g. higher than 1 W/m^2). However, those power densities are usually achieved by anodes with high specific area, such as graphite felt or graphite fiber brush (Nevin et al. 2008), thus are not suitable to be directly compared with the results presented here.

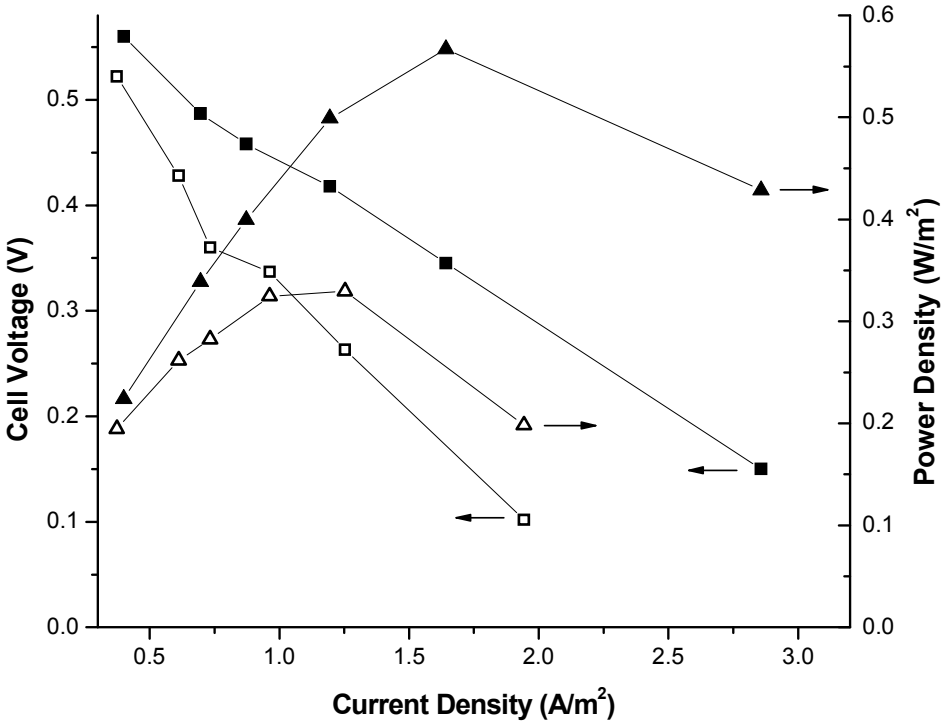


Figure 6.3 Polarization curve (squares) and power curve (triangles) of MFC1 (hollow symbols) and MFC2 (filled symbols)

6.3.2.2 COD removal rates and Coulombic yields

COD removal rates and CEs of MFC1 and MFC2 under different external loads are listed in Table 6.1. The COD removal rates did not show obvious trend with the change in external load. Generally, for both MF1 and MFC2, over 85% of fed COD had been degraded by microbes in each batch cycle.

It is well established that higher CEs can be achieved with lower loads (Gil et al. 2003). With small loads, the anode works at relatively higher potential, this can be kinetically favorable for microbial EET. Under such condition, anode reducers may gain an advantage to outcompete the electrochemically inert microbes. Overall, MFC2 did not have higher current efficiency compared with MFC1; instead, under most loading

levels, MFC1 had a higher CE. This suggested the CEs may be controlled by factors other than electrode kinetics, e.g. the reactor design and operation.

Table 6.1 COD removal rates and CEs of MFC1 and MFC2 under different loads.

MFC 1

External Resistance (Ω)	2000	1000	750	500	300	75
48 h COD Removal Rate (%)	93.4	89.9	95.7	98.0	88.5	92.1
CE (%)	23.9	30.4	30.9	36.9	53.1	73.9

MFC 2

External Resistance (Ω)	2000	1000	750	500	300	75
48 h COD Removal Rate (%)	93.7	88.8	86.5	88.5	89.8	89.8
CE (%)	23.5	24.6	29.7	37.6	45.7	69.1

6.3.3 Cyclic voltammetries

Both CFE and CFE/MWNT showed typical sigmoid voltammograms (Figure 6.4). No oxidative current could be observed when the electrode potential was lower than – 0.2 V. This observation is consistent with existing knowledge. When the anode potential approaches or goes below the redox potential of the operational electrode reductases (or redox shuttles), exoelectrogens cannot reduce the anode. Through the first derivative analysis of turnover voltammogram, Marsili et al. (2008b) revealed several redox systems of *G. sulfurreducens*, the dominant one centered at – 0.15 V. The redox potential of riboflavin, the endogenous redox shuttle of *Shewanella*, was – 0.21 V (Marsili et al. 2008a). Torres et al. (2009) also found that, the onset potential of their catalytic voltammogram (with a mixed electrochemically active biofilm) was around – 0.2 V. Comparatively, the standard electrode potential of the "acetate/CO_2" couple was – 0.28 V under neutral pH (Schröder 2007). The potential difference is necessary to

drive the ET systems of anode reducers.

Once the electrode potential was higher than – 0.2 V, the current rose sharply with the potential sweep (Figure 6.4). The Butler-Volmer model predicts the Faradic current to grow exponentially with the overpotential (Bard and Faulkner 2001). It was noticed that the current rose steeper for the GFE/MWNT than the bare GFE. Within the potential range between – 0.2 V and 0 V, the GFE/MWNT always delivered higher current than the bare electrode. When the overpotential passed certain level, current no longer increased with the potential. Beyond this potential level, the current generation was not controlled by the heterogeneous ET. For an abiotic electrochemical system, the current plateau indicates that the transportation of reactants/products reaches the maximum rate and becomes the rate-limiting step (Bard and Faulkner 2001). However, for a microbial anode, the "limiting current" is likely to be regulated by enzymatic activities (Richter et al. 2009). The GFE/MWNT and bare GFE reached their limiting current at about 0.05 V and 0.15 V respectively. In another word, the bare GFE required additional 0.1 V of overpotential to drive the process to the maximum rate limited by microbial catabolism and homogeneous ET. Although GFE demanded a higher overpotential to attain the limiting current, the ultimate current level was similar to that achieved with GFE/MWNT (about 3 mA, Figure 6.4). This limiting current level was determined by the total amount of anode reducers (Liu et al. 2010d). Therefore, there might be similar amount of anode-respiring microbes on both electrodes. This was supported by protein quantification of anode-attached biomass. For the modified anode, the protein density was 121.2 $\mu g/cm^2$, compared to 118.7 $\mu g/cm^2$ on the unmodified anode.

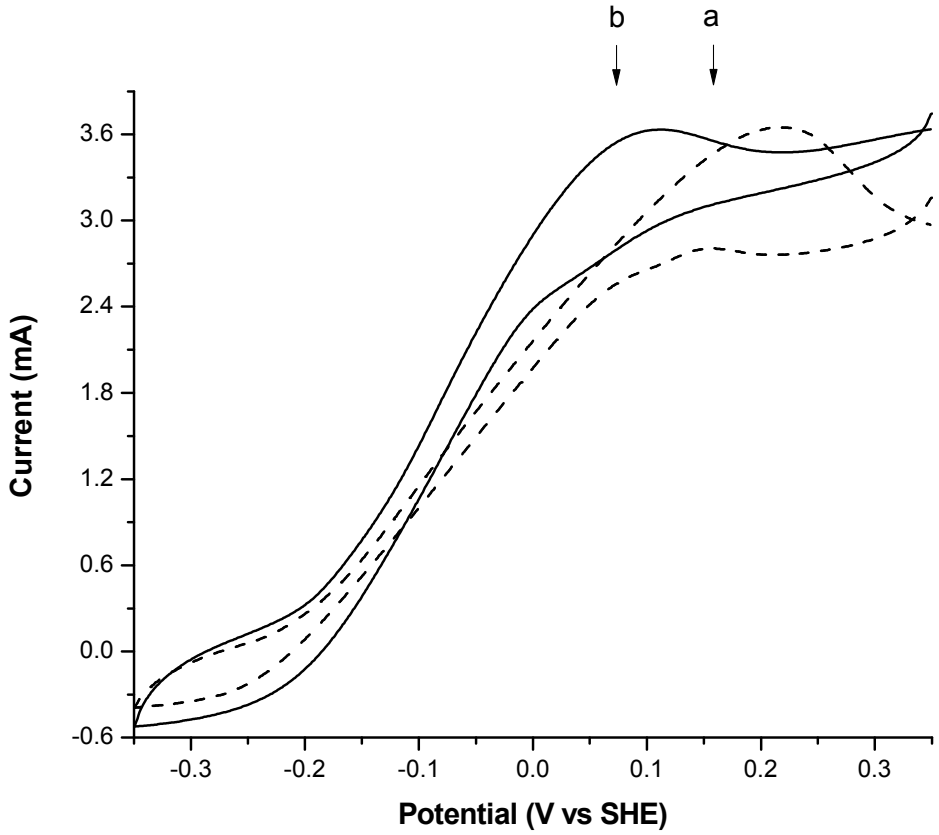


Figure 6.4 Turnover cyclic voltammograms of GFE (dashed line) and GFE/MWNT (solid line); a: the approximate electrode potential GFE reached limiting current, b: the approximate electrode potential GFE/MWNT reached limiting current.

6.3.4 Morphologies of the anodic biofilm

After the operation for approximately one and half months, the anodes were covered with thick layers of microbes. The anode showed orange color which can be an indication for the presence of anodophiles (Torres et al. 2009). These microbes are rich in OM *c*-Cyts thus can show orange or reddish colors. The biofilm on the modified electrode appeared to be more flat (Figure 6.5)., the CNT layer beneath probably acted as a flattening layer. Biofilm on GFE or GFE/MWNT had a thickness of roughly 8-10 μm.

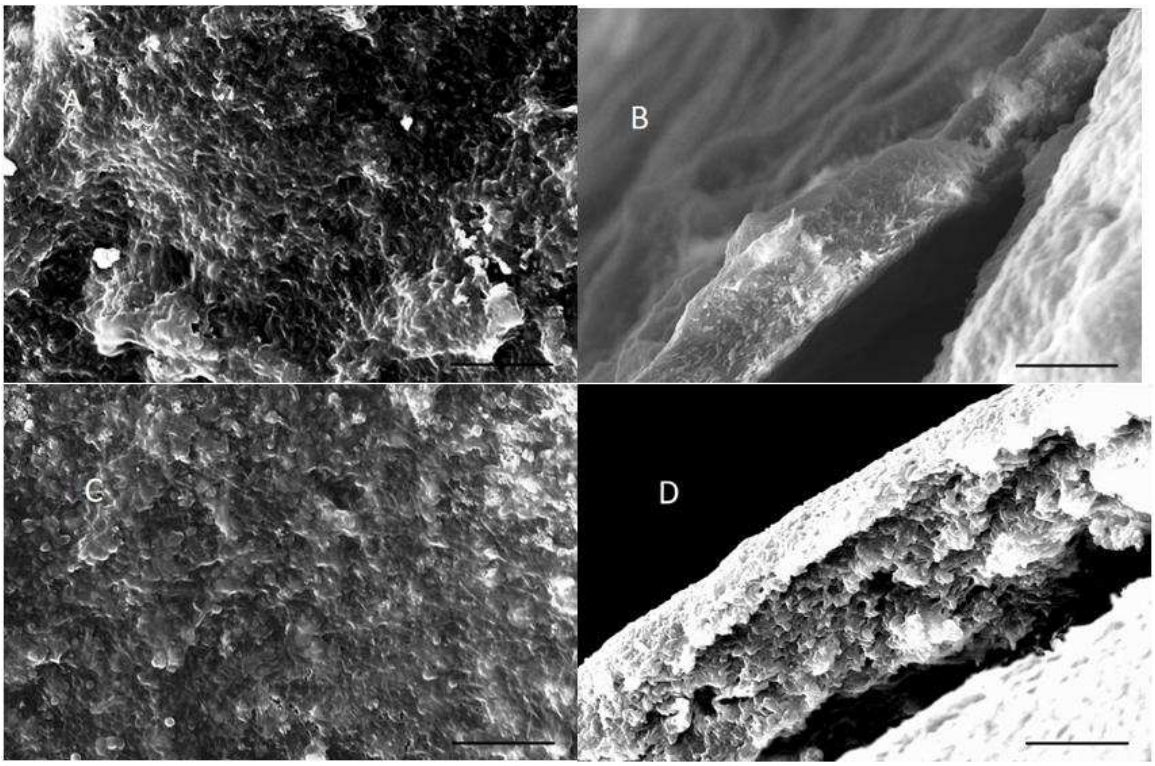


Figure 6.5 SEM images of anodic biofilms: A: top view of the GFE biofilm; B: side view of the GFE biofilm; C: top view of the GFE/MWNT biofim; D: side view of the GFE/MWNT biofilm. Bar length: 10 μm.

6.4 Conclusions

The study compared the power generation of MFCs with and without anode modification with CNTs, using mixed microbial community as biocatalysts. The MFC with a modified anode had a power density 72% higher. Nevertheless, the enhancement in power generation was not accompanied with enhancement in CE.

Anodic voltammetries revealed that, with the MWNT modifiers, the catalytic current rose steeper within the potential range of – 0.2 V to 0 V. Bare GFE and GFE/MWNT reached a similar level of limiting current. However, the modified anode reached the

limiting current at a lower electrode potential. These finding testified the faster anodic kinetics brought about by the MWNT modifiers, which could explain higher fuel cell power generation.

Chapter 7 Conclusions and Recommendations

7.1 Conclusions

Microbial EET is an emerging research area of great scientific and engineering significances. One insufficiently covered issue in this area is how the environmental conditions influence the EET strategies adopted by exoelectrogens. Also less discussed, is the representativeness of the model exoelectrogens. Enhanced microbial EET is demanded for practical applications of microbial BES. It was hypothesized that, suitable electrode modifiers may promote the electrochemistry of cell surface redox proteins and subsequently elevate the bioelectrocatalytic current generation. Nevertheless, evidences are needed to support this hypothesis. The above listed research questions motivated this study.

In a non-stirred electrochemical cell inoculated with *S. oneidensis*, an unmodified carbon electrode poised at 0 V vs. SCE could deliver very low current density (well below 1 $\mu A/cm^2$). After hours of polarization under this potential, the registered voltammogram revealed a redox pair exhibited directional ET, which could be attributed to the OM *c*-Cyts of *S. oneidensis*. Due to the irreversible electrochemistry and sluggish ET rate (characterized by widely separated redox peaks), the OM *c*-Cyts did not display obvious electrocatalytic activities. When the electrode potential shifted from 0 V to – 0.24 V, current density from the poised working electrode could be increased to about 10 $\mu A/cm^2$. Voltammetries suggested this unusual polarization behavior (higher oxidative current at lower poised potential) was caused by microbial physiological changes. When poised at lower potential, the OM *c*-Cyts appeared to be eliminated from the electrode surface, which was a microbial adaptive behavior. While the OM *c*-Cyts were absent from the electrode surface, the redox shuttle, flavins were actively mediating an electron flow towards the electrode. When the potential of the working electrode was restored to 0 V, characteristic redox waves of the OM *c*-Cyts were observed again, suggesting that the adaptive behavior was reversible. It was

further observed that the presence of OM *c*-Cyts at the bacteria-electrode interface was always accompanied with hindered current generation, even when the medium was amended with excessive riboflavin. These observations implied that the accumulation of OM *c*-Cyts could probably insulate the electrode surface from flavins. It also implicated that, in the quiescent electrolyte, the MET by *S. oneidensis* was mostly carried out by electrode-attached cells through the cell-electrode interface. The planktonic cells might play only minor roles.

When *F. balearica* was inoculated to an electrochemical cell, catalytic current could be observed when the electrode was poised at 0 V or – 0.24 V. After polarization at 0 V, *F. balearica* voltammogram displayed asymmetric redox peaks bore close resemblance to those of *S. oneidensis*. Presumably, these were the signals of OM *c*-Cyts similar to *Shewanella* OmcA/MtrC. After polarization at – 0.24 V, the signals of the asymmetric peaks were largely weakened. Therefore, the potential-depend expression of redox proteins at the bacteria-electrode interface is not specific to *S. oneidensis*. Evidences showed that *F. balearica* might be able to secrete flavins as redox mediators as well, further suggested that *γ-proteobacteria* exoelectrogens may possess a very similar ET system. However, different from the observations with *S. oneidensis*: after the assumed OM *c*-Cyts had vanished from the *Ferrimonas*-electrode interface, the oxidative current was still low (lower than 0.5 $\mu A/cm^2$). Comparative studies with *S. oneidensis* and *F. balearica* may shed some light on the mechanisms of efficient MET. The *F. balearica* fuel cell had a maximum power density of 2.55 mW/m^2 and a relatively high CE, justifying this bacterium as a valid biocatalyst for MFCs.

CNTs showed catalytic effect towards ET through *S. oneidensis* OM *c*-Cyts. When MWNT-modified electrode was poised at 0 V in an electrochemical cell inoculated with *S. oneidensis*, the oxidative current was over 80 folds higher than that registered with unmodified electrode. Voltammograms showed that, with CNTs, the electrode process of OM *c*-Cyts was a reversible one. Additionally, the ET rate was significantly improved. The heterogeneous ET constant of the OM *c*-Cyts with CNT modifiers was

higher than 1 s^{-1}; while literatures reported that to be below 0.1 s^{-1} with ordinary electrode materials. CNTs showed similar promoting effecting towards the EET process of *F. balearica*.

CNT modified anode improved power generation in MFCs inoculated with mixed cultures, showing a more general electrocatlysis. The MFC with the modified anode had power density 72% higher than that of an MFC with unmodified anode. Catalytic voltammetry with the anodes suggested that, the modified anode required less overpotential to achieve the limiting current, confirming an elevated ET kinetics. The limiting current was around 3 mA for both the modified and unmodified anodes, suggesting that there were similar amount of anodophiles on both electrodes. This was justified by measurement of electrode-attached total protein. Nevertheless, different from some previous reports, it was found that the improved anode performance did not concomitantly improve the fuel cell current efficiency.

7.2 Recommendations

Some discoveries presented in this study require further explanation. Notably, the origin of the rectification behavior of OM *c*-Cyts of *S. oneidensis* or similar bacteria is not yet understood. Eggleston et al. (2008) and Meitl et al. (2009) suggested that the oxidative potential of OM *c*-Cyts were significantly higher than the flatband potential of the hematite electrode used in their study, a Schottky barrier existed and inhibited the current flow. However, in the present study and the study by Kim et al. (2002), where GCE and graphite electrode were used, the directional ET was still observed. Therefore, the irreversible electrochemistry might be caused by the common denominator in these studies: the OM *c*-Cyts. Paquete et al. (2010) conducted kinetic experiments with small tetraheme cytochromes using non-physiological redox patterns. Combing the three dimensional structure as well as the known thermodynamic parameters, the authors could demonstrate the directional ET of these small proteins. Nonetheless, for the OM *c*-Cyts, the atomic structure is unknown, redox potentials of

individual hemes are not known. Thereafter, the structural assignment of potentials which is essential to show directional ET is unknown. Furthermore, the analysis on the decaheme cytochromes can be much more complicated than that on small cytochromes (personal communication with Dr. Paquete).

The significance of the potential-dependent physiology of *Shewanella* and similar exoelectrogens is unclear, and worth more careful considerations. As discussed in chapter 3, electrode reductases with higher redox potentials do not necessarily enhance the microbial energy conservation. Bacterial energy gain is derived from the ET through inner membrane, and depends on the exact ET process. Hunt et al. (2010) suggested that *Shewanella* primarily conserve energy from the substrate level, there is no additional energy gain in the ET process, which just maintains an electron balance. This implicated that any change in EET system is unlikely to affect the microbial energy gain. The exact mechanism that electrode potential regulated the presence of metalloproteins at the electrode surface may also be an interesting subject for further study. There could be a sensing system in the adjacency of bacteria-electrode interface, and is associated with gene regulation.

Another unanswered issue is the reason for CNTs' promoting effects. Although many studies confirmed the promoting effects of CNTs towards the electrode process of redox proteins (Wang et al. 2002; Zhang et al. 2004; Zhou et al. 2008), the mechanism for the promoted ET was largely unexplored. Davis et al. (2003) proposed that the π-π stacking between protein redox centers and the CNT π system might be a reason. This stacking may shorten the distance for ET and alter the orientation of the protein cofactors. Therefore, a higher apparent ET constant can be assumed. However, this hypothesis had not been rigorously examined. Actually, if this theory is correct, it should follow that: graphite electrode, comprised of graphene stacks, have similar promoting effects. However, this had not been reported by literatures.

Overall, results from this study demonstrated that electrochemical techniques can be

powerful tools in analyzing ET processes in biological systems. On the other hand, the limitations of electroanalyses were also noticed. The electroanalyses cannot provide exact information on the source of the electrochemical signals. For instance, in chapter 4, the nature of the asymmetric redox waves could not be determined solely with voltammetry. To confirm these peaks represented cell surface *c*-Cyts, and to determine which *c*-Cyts they were, there should be biological assays with the bacterium using gene knockout experiments.

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Appendix: Calculation of Heterogeneous Rate Constant from a Trumpet Plot

Transfer coefficient (α) and heterogeneous ET rate constant (k_s) were estimated from the variation of peak potentials with scan rate. When ΔE_p is larger than $0.2/n$ V, E_{pa} and E_{pc} shift linearly with logarithmic scan rate, yielding two straight lines. α was calculated using slopes (k_a, k_c) of the two straight lines:

$$log\left(\frac{k_a}{k_c}\right) = log\left(\frac{\alpha}{1-\alpha}\right) \quad (1)$$

Then k_s was calculated by the equation:

$$logk_s = \alpha log(1-\alpha) + (1-\alpha)log\alpha - log\left(\frac{RT}{nFv}\right) - \frac{\alpha(1-\alpha)\Delta E_p nF}{2.3RT} \quad (2)$$