Electrocatalysis of new strain E. coli and nanostructured anode for high performance microbial fuel cells

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**Electrocatalysis of New Strain *E. coli* and
Nanostructured Anode for High Performance
Microbial Fuel Cells**

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Summary

The microbial fuel cell (MFC) is a promising clean energy source to recycle organic wastes while producing electricity. Since the MFC can borrow the matured cathode technology from conventional H_2 - O_2 based fuel cells, enhancing its anode electrocatalytic performance, which is greatly related to the microbe, electrode, and electron-transfer scheme between the microbes and the electrode, is very crucial to improving the power density and energy density for practical applications. This research focuses on the electrocatalysis of *Escherichia coli* (*E. coli*)/nanostructured anodes in MFCs. Novel nanostructured anodic materials, genetically engineered *E. coli* and electro-evolved new strain *E. coli* were explored to significantly improve the MFC performance, while the direct electrochemistry of *E. coli* in MFCs was investigated for the fundamental insights.

A carbon nanotube (CNT)/polyaniline (PANI) composite was evaluated as an anodic material for high-power MFCs. The results demonstrate that a CNT/PANI nanocomposite anode containing 20 wt % CNT yields higher electrochemical activity than other reported anodes used in the *E. coli*-MFC. To further enhance the electrocatalytic capability of the anode, a unique nanostructured PANI/mesoporous TiO_2 composite with uniform nanopore distribution and a large specific surface area was synthesized and explored as an anode in *E. coli* MFCs. Optimization of the anode indicated that the composite with 30 wt % PANI provided the best bio- and electrocatalytic performance, as

it yielded twice the power density of previously reported *E. coli* MFCs. These works may provide new approaches for improving different types of MFCs.

After long-term operation of MFC, the original *E. coli* K-12 cells were found to have been evolved into a new strain with an ability of direct electron communication with the anode. The results reveal that some hydroquinone derivatives excreted by evolved *E. coli* cells may be responsible for the direct electrochemical process. The evolved *E. coli* cell surface displayed a highly permeable outer membrane of evolved *E. coli* cells.

In addition, a new strain of *E. coli* was genetically engineered that possessed direct electrochemical abilities. The engineered *E. coli* was constructed with recombinant plasmid containing overexpressing *gldA* gene that encodes glycerol dehydrogenase. The engineered *E. coli* exhibited excellent performance in the MFC anode, yielding a higher power density than that of the evolved *E. coli* cells.

To further explore the cellular-based mechanisms behind the direct electrochemical behaviour of *E. coli*, a detailed investigation was conducted into the relationship between *E. coli* gene expression and electrochemical behaviour. It was found that 107 *E. coli* genes were significantly up- or down-regulated after 24 hours of MFC discharge. The operon analysis reveals that the outer membrane porin genes and terminal oxidase genes are crucial for the direct electrochemical process.

This research indicates the strong potential to improve MFC performance by the use of nanomaterials and new strains of bacteria. The investigation of the direct electrochemistry of both evolved and genetically engineered *E. coli* cells advances the fundamental knowledge regarding the electron-transfer process between cells and the electrode, providing scientific insight into the bioengineering of new strains for increased power output.

Chapter 1 Introduction

1.1. Introduction

In recent years, increased requirements for the use of sustainable alternative power sources has promoted the development of power sources using “green” energy, such as fuel cells and solar cells. The microbial fuel cell (MFC) is a new type of fuel cell that has high energy conversion efficiency, operates in mild operational conditions, and contains low-cost substrates. The MFC has attracted much attention from both the research community and industry. Although the existence of the first MFC was reported about 100 years ago¹, it has only been since the 1980s, when several technological breakthroughs occurred, that the MFC became a viable source of energy. Like a conventional hydrogen-based fuel cell, the MFC consists of an anode, cathode, and cation-exchange membrane. The main difference is that the MFC anode uses microbes rather than noble metals as catalysts for the conversion of chemical energy into electricity. The microbes work as multiple-enzyme complexes to catalyze the oxidation of organic substrates, such as glucose, and pass the obtained electrons to the electrode. MFCs can work under operational conditions milder than conventional fuel cells and require no additional energy source for heating. Since the MFC microbes can self-regenerate, the cost of this kind of catalyst is much lower than that of platinum. Furthermore, since no

pollutant is generated during the operation of MFCs, they are environmentally friendly.

In spite of the various merits of MFCs, they are not yet viable sources of energy due to their relatively low power output. To date, a single MFC can only produce a power density of less than 6 W m^{-2} , while a single direct methanol fuel cell, a type of conventional fuel cell², can deliver a power density of 800 W m^{-2} at atmospheric pressure at an operational temperature of 60°C . There are a number of factors affecting the power output of MFCs, such as the anode and cathode, the metabolism of microbial species, the chemical species present in the electrolyte, the ion-exchange membrane, the fuel cell configuration, and operational conditions. Extensive efforts have been made to increase the power density of MFCs by modifying the anode, adding artificial electron mediators, discovering electro-active bacteria, fabricating MFCs structure et al.

1.2. Motivations

The MFC is a promising power source, but it remains a great challenge to increase its low power density to make it a viable source of energy in practical applications. Among the various factors that affect MFC performance, anodic electrocatalysis is the most critical. The electrocatalytic capability of the MFC anode depends on the electrode material, microbes, and electron-transfer rate between the microbe and electrode.

Currently, most MFCs use conventional porous carbon materials, such

as carbon cloth, carbon paste, and graphite felt, which have reasonable conductivity, biocompatibility, and long-term stability but very poor electrocatalytic activity. Furthermore, the active surface area of these carbon materials is relatively small and their pore structures are easily clogged by bacterial cells, resulting in dead pores that cause nutritional deficits. Accordingly, there is a great need to develop novel anodic materials with high electrocatalytic activity, a large specific surface area, appropriate pore structures, and good biocompatibility.

Besides the anodic materials, the MFC biocatalysts need further improvement. Some unique strains of bacteria have been found to have direct electrochemical capacity for increased power density. However, some of these bacteria require complex and relatively strict culture conditions, and are difficult to scale up for practical applications. Consequently, it is necessary to explore novel easily handled bacteria with more rapid direct electron-transfer rates that can be produced at lower cost for further improvement of MFC power density.

Although the mechanism of the direct electron-transfer process of the bacterial cells is very important to further improvement of MFC performance, it remains unclear. The entire electron-transfer path should include a metabolic pathway inside bacterial cells and an extracellular electron-transport process. Understanding the mechanism of the direct electron-transfer process is not only

theoretically but also practically important, as it is important in exploring and bioengineering new strains to increase the direct-electron transfer rate.

1.3. Objective of study

The overall objective of this research is to significantly increase the MFC power density by improving MFC anodic electrocatalysis. This PhD research project focuses on developing novel nanostructure materials, exploring new electroactive strains of bacteria through both electrochemical and genetic engineering technologies, and investigating the mechanism of direct electron transfer in MFC bacteria. More specifically, the objective of this study can be divided into the three following sections.

1.3.1 Synthesis or/and fabrication of nanostructured materials for high electrocatalytic performance

An important task in this project is to synthesize and/or fabricate nanostructure materials with a much larger specific surface area, better pore distribution/ structure, higher conductivity, and sounder biocompatibility to significantly increase biocatalysts loading and reduce polarization for high power density. To achieve this task, polymer/nanostructure carbon and polymer/metal oxide composites were investigated.

1.3.2 Exploring new strains of *E. coli* with superior direct electrochemical capacity

Regarding the need to identify a new microorganism to serve as an MFC biocatalyst for rapid direct electrochemistry, allowing for the development of high-power MFCs for practical applications, biologically engineering new bacteria strains may be easier than isolating as-yet unknown strains due to the rapid development of genetic engineering technology. A novel *Escherichia coli* (*E. coli*) strain with superior direct electrochemistry that can be developed using genetic engineering technology can be used to power a mediator-less MFC for achieving higher power density than that using wild *E. coli* cells. As an alternative, long-term electric tension can be applied to genetically evolve the *E. coli* cells to develop new strains that may allow for a rapid direct electron-transfer rate.

1.3.3 Investigation of the direct electrochemistry of *E. coli*

To fully understand the mechanism of the direct electron transfer, it is necessary to design and employ various electrochemical and biological methods guided spectroscopy and atomic force microscopy to study the electrode kinetics while indentifying the redox species responsible for the direct electron transfer. Microarray is a powerful genomic tool very useful for analyzing genetical changes during the electrocatalytic process, and thus providing important molecular biological evidence for better understanding the direct

electron-transfer mechanism.

1.4. Organization

In chapter 1 the study is briefly introduced and an overview of the motivation behind and objective of this PhD research is provided. Chapter 2 reviews recent MFC developments, including configuration optimization, cathode improvement, and membrane and anode development, focusing on recent enhancements in anodic electrocatalysis. In chapter 3 the experimental approaches, materials, and instruments used in this study are described in detail. For chapter 4, the use of a PANI/CNT nano composite in *E. coli* MFCs is evaluated with the investigation of the electrocatalytic behaviour of the composite anode by means of electrochemical impedance spectroscopy (EIS) and discharge experiments. Chapter 5 presents a exploring on a unique nanostructured PANI/mesoporous TiO₂ composite as an anode in *E. coli*-based MFCs. Chapter 6 discusses the direct electrochemistry of *E. coli* cells evolved from long-term discharge in an MFC. In chapter 7 a novel genetically engineered *E. coli* overexpressing glycerol dehydrogenase (GldA) is introduced as an MFC biocatalyst constructed using recombinant plasmid and displaying direct electrochemistry similar to that of evolved *E. coli*. Chapter 8 discusses the results of a time-course transcriptome analysis of *E. coli* with direct electrochemical behaviour in MFCs. In the last chapter, a general conclusion and recommends possible avenues for future research are provided.

Chapter 2 Literature Review

2.1 MFC Overview

2.1.1 MFC principles

A typical MFC consists of two chambers separated by an ion exchange membrane³. In the anodic compartment, the microorganisms located on the biofilm on the electrode surface act as catalysts converting organic fuels into electricity by oxidizing organic substrates, such as glucose, and producing electrons and protons (Eq. 1).

Electrons generated from the substrate's oxidation are transferred to the cathode compartment through an external electric circuit while the protons are transferred to the cathode compartment through the membrane. Electrons and protons consumed in the cathode compartment combine with oxygen to form water (Figure 2.1).

Figure 2.1 Schematic illustration of an MFC

MFCs have operational and functional advantages over the technologies currently used for power generation. They operate efficiently at ambient temperature, do not require energy input for heating, and have high conversion efficiency by the direct conversion of substrate energy into electricity. They also offer the possibility of extracting over 90% of the electrons from organic compounds⁴, a degree much higher than that of enzymatic biofuel cells. Additionally, the diversity of their fuels and regenerating catalysts allow MFCs to have potential for widespread applications. However, due to their low current densities and low power outputs, current MFCs can only power small electronic devices for short periods.

2.1.2 MFC history

The concept of using microbial cells as catalysts in fuel cells was

conceived about 100 years ago. Although the first MFC was constructed by M. C. Potter¹ with *E. coli* and *saccharomyces* as catalysts in 1912, MFCs with enhanced power output have been developed only within the past 20 years. The slow development of MFC research has likely been due to the unclear mechanism of fuel oxidation and electron transfer between microorganisms and the electrode. Until the 1980s, some researchers⁵⁻⁸ found that the addition of electron-transfer mediators could greatly enhance MFC power density. However, the potential toxicity and instability of the artificial redox mediators of these mediator-containing MFCs were major impediments to practical application.

In the late 1990s and early 2000s, the discovery of electrochemically active bacterial strains that can transfer electrons directly to electrodes⁹⁻¹¹ made it possible to construct mediator-less MFCs, which further enhanced the power output of MFCs. Several factors that may affect the overall performance of MFCs, including MFC configuration, the nature of the proton-exchange membrane, and the electrocatalysis of the anode and cathode, are discussed in the following sections.

2.2 Optimization of MFC configuration

Of the many different MFC configurations researchers have developed over the past few years, the most widely used model in lab investigation is the dual-

chamber MFC built in an “H” shape (Figure 2.2 a). In this model of two bottles connected with a tube containing a cation-exchange membrane as a separator¹²⁻¹⁵, the tube structure is used for fixing the membrane, but the tube itself is not required. Furthermore, the two chambers can be directly pressed onto either side of the membrane and clamped together to decrease the electrode distance^{16, 17} (Figure 2.2b) and thus lowering the internal resistance. In some models, a salt bridge, which consists of a U-shaped glass tube filled with agar and salt rather than an ion exchange membrane, connects the two chambers¹⁵. This model is an inexpensive way to join bottles, but the power of a salt-bridge MFC is very low due to its high internal resistance. “H” shape MFCs are acceptable for fundamental research, such as developing new electrode materials and investigating microbial cell behaviour.¹⁸

When using oxygen as an oxidant in the cathode, it is not necessary to place the cathode in a separate chamber. Because it can be placed in direct contact with air, a single-chamber MFC model can be constructed. Liu et al. built a single-chamber MFC with an air cathode¹⁹⁻²¹ for the first time. The simplest single-chamber configuration consists of an anode and a cathode placed on either side of a tube (Figure 2.2c). The anode is sealed against a flat plate, the cathode exposed to air on one side and water on the other, and an ion exchange membrane is used to keep water from leaking through the cathode and for reducing oxygen diffusion into anodic chamber. Much higher power

densities have been achieved with this system in comparison to aqueous-cathodes MFCs.

Generally, the ion exchange membrane is required in both dual-chamber and single-chamber MFCs to separate the anode and the cathode and to balance the charge. The most commonly used membrane is Nafion® Membrane (Dupont Co.) as it possess high proton conductivity and low ohmic resistance. However, due to its high cost, researchers are seeking comparable alternatives with high stability but lower cost. The Ultrex® membrane (Membranes International Incorp. USA), a cost-effective alternative to the Nafion membrane¹⁸, is now widely used in MFCs²²⁻²⁴.

In the development of single-chamber MFCs, researchers found that the presence of the membrane was not essential. It has been reported that removing the membrane increased the maximum power density but greatly decreased the Coulombic efficiency¹⁹. To resolve this problem, Fan et al.²⁵ developed an improved membrane-less configuration by applying a J-cloth (Associated Brands LP.) layer on the water-facing side of the air cathode to fabricate a sandwich cloth-electrode assembly. The cloth-electrode assembly greatly reduced the internal resistance and consequently enhanced the power density as well as the Coulombic efficiency.

Researchers have also developed a form of MFC that could harvest energy from marine sediment²⁶. This MFC has a distinct configuration in that a

graphite-plate anode is buried in marine sediment connecting by an external circuit to a graphite-plate cathode positioned in overlying seawater (Figure 2.2d). The chemical composition is different in the seawater with the sediment due to the microbial decomposition of the organic matter. The seawater is rich in oxidants and the sediment is rich in reductants, resulting in a naturally redox gradient^{26, 27}. In a marine-sediment MFC, cathodic current is attributed to a reduction in the seawater oxygen while the anodic current comes from the oxidation of sediment under catalysis of the microbes on the anode.

Figure 2.2 Different configurations of MFCs. a: “H”-shape dual chamber MFC; b compact dual chamber MFC; c: single chamber MFC; d: marine sediment MFC

2.3 Cathodic electrocatalysis

Cathodic electrocatalysis, including the cathodic electron acceptors and the

catalytic activity of the cathodic materials, greatly affects MFC performance.

The kinetics of the reduction reaction and the standard redox potential of the oxidants determine the performance of the cathode. In an effort to enhance the electrocatalytic capability of the cathode, different oxidants and various kinds of catalysts were investigated.

2.3.1. Oxygen-reduction reaction

In fuel cells, the oxygen-reduction reaction (ORR) at the cathode is the most widely used reaction because oxygen is free to access and its standard redox potential is high (1.229 V vs. NHE for a complete reduction²⁸). Two processes could occur during cathodic oxygen reduction: a complete reduction in a four-electron pathway (Eq. 2) or an incomplete two-electron reduction reaction (Eq. 3) that needs further reduction to H₂O.

The sluggish kinetics of ORR is the main limitation of the power output of low- temperature fuel cells. The application of highly active catalysts such as platinum and the development of strong pH conditions (acid or alkaline) is the most useful strategy for enhancing ORR efficiency. The mechanism of electrocatalysis for ORR is still not completely understood, but the theory proposed by Damjanović²⁹, according to which proton transfer to O₂-producing adsorbed HO₂ or HO₂⁻ occurs prior to cleavage of the O-O bond during the

ORR on Pt-group metals, has been widely accepted.

The high and still increasing cost of Pt also will make an MFC very expensive if Pt is used as the cathode. Extensive research has explored using noble metal-free cathode catalysts for MFCs to lower the cost of the air-cathode and simultaneously improve the ORR kinetics. When Morris et al. smeared PbO_2 paste on Ti sheeting with butanol and Nafion as the binders to fabricate a PbO_2/Ti cathode in a two-chamber MFC³⁰, the PbO_2/Ti cathode delivered more power (1.7-3.9 folds) than PtC/Ti or a commercial Pt-loaded carbon paper cathode. Among the three manganese dioxide materials with three different crystal types (α , β , γ) applied as the MFC cathode³¹, $\beta\text{-MnO}_2$ possessed the highest catalytic activity and delivered relatively high power density in a tube MFC. Besides the metal oxides, several metal porphyrines and metal phthalocyanines examined for ORR activity in the MFC cathode^{32, 33} also exhibited comparable performance with a Pt cathode. Other metals such as cobalt³⁴ that cost less yet exhibit relatively high catalytic activity were also used as alternative catalysts in the MFC cathode. However, as cobalt is not environmentally friendly, its toxicity should be considered, especially in up-scaled MFCs. A great need for exploration of high-performance electrode materials in a neutral solution remains.

2.3.2 Other oxidants

Since the reaction kinetics of ORR in MFCs is poor and oxygen can

diffuse across the proton-exchange membrane, reducing the power by taking electrons from the anode³⁵, oxygen may not be the most desirable electron acceptor in the MFC cathode. Alternative electron acceptors have been used in MFCs for more than 20 years. For example, the Fe(CN)_6^{3+} ion is a good electron acceptor under anaerobic conditions, and has been widely used in dual-chamber MFCs.

The reduction kinetics of Fe(CN)_6^{3+} ion are much more rapid than ORR, obviating the need to use catalysts in ferricyanide cathode. A 50-80% increase in maximum power using a ferricyanide cathode compared to that obtained using an oxygen-dissolved cathode has been reported³⁶. The reason for this increase is that the open-circuit potential of a ferricyanide cathode (332 mV vs. Ag/AgCl) is higher and the mass transfer efficiency greater than those of an oxygen-dissolved cathode (268 mV vs. Ag/AgCl). Similar phenomena were also described by Ringeisen et al.³⁷ and Liu et al.³⁸. However, the ferricyanide cathode is not suitable for up-scaled MFCs because of the greater cost of the container and Fe(CN)_6^{3+} ion regeneration.

To further increase the MFC cell voltage (normally 0.5~0.8V), You et al. applied permanganate as the cathodic electron acceptor³⁹, which has a higher standard redox potential than oxygen. In acidic conditions, permanganate accepts three electrons and is thus reduced to manganese dioxide, as illustrated

in Eq. (5)

The permanganate cathode (pH 3.6) increased the MFC open cell voltage to 1.382V and delivered a 4.5- and 11.3-fold higher power density than that produced by the ferricyanide cathode and the oxygen cathode, respectively

2.3.3 Biocathodes

Microorganisms can also be used as catalysts in the cathode to perform functions similar to those that they perform in the anode. The first research conducted on biocathodes was by Rhoads et al.⁴⁰, who employed a cycle of Mn(IV) reduction on a cathode catalyzed by manganese-oxidizing bacteria (MOB). As the oxygen was required for the biocatalyzed re-oxidation of Mn(II), manganese dioxide actually served as the mediator for the bacteria-catalyzed oxygen reduction.

Similarly, iron compounds can be used as mediators in biocatalyzed oxygen reduction on MFC cathodes. Heijne et al.⁴¹ designed an MFC with a ferric-iron- mediated biocathode catalyzed by *Acidithiobacillus ferrooxidans*. The mechanism of manganese and iron compounds mediated biocathode electrocatalytic process is summarized as Figure 2.3.

Figure 2.3 Metal ions mediated oxygen reduction catalyzed by metal-oxidizing bacteria cathodes

In mediator-less biocathodes, bacteria grab electrons directly from the cathode and catalyze the reduction of oxidants. *Geobacter metallireducens* not only exhibited superior electrocatalytic performance in the anode but also catalyzed the reduction of nitrate and fumarate in the cathode⁴². Despite the pure strains, mixed microorganism communities, such as those found in sewage or waste water, have also been used as biocatalysts in MFC cathodes⁴³. The oxidant often used in the cathode of these mixed-cultures-catalyzed MFCs is nitrate, which has relatively high redox potential, as shown in Eq. (8). Hence, their denitrification function is the major advantage of using these biocathodes⁴⁴.

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2.4 Anodic electrocatalysis

In the MFC anode, organic substrates, which can be sugars and organic acids such as glucose (9) or acetate (10), complex polymers such as starch⁴⁶ and cellulose⁴⁷, or even organic wastes, are oxidized using microorganisms as biocatalysts.

During the development of MFCs, three different types of anodes have been demonstrated. Type I, an early-stage MFC, utilizes the metabolic products of microorganisms to generate electricity. In type II, the anode receives electrons through artificial electron mediators, while in type III, electroactive microorganisms directly transfer electrons from electron donors to the anode. The electrocatalytic mechanisms of these three MFCs are quite different. In type I MFCs, the redox reactions of the metabolic products determinate the energy conversion efficiency and power output. In types II and III, the electrocatalytic performance of the anode is mainly dependent on the electron-transfer process between microorganisms and the anode. Various approaches have been developed to enhance the electrocatalytic performance of the anode.

2.4.1 Development of anodic materials

2.4.1.1 Application of metal-based catalysts

Reducing the activation energy is the key to improving the reaction kinetics of the anode. The use of metal catalysts can lower the activation energy and increase the power output. Early MFCs often utilized electroactive metabolites produced by the bacteria from substrates such as hydrogen. In this case, Pt is widely used as the anode material due to its superior catalytic activity and stability in extreme environments. To increase the active surface area and lower the cost, platinized Pt (Pt black) deposited on the surface of a porous substrate instead of bright platinum sheets or film is often used. A stainless-steel net plated with Pt black has been used as the anode in an MFC with *Enterobacter aerogenes*, a bacterial strain possessing high hydrogen productivity from glucose⁴⁸. The hydrogen evolution rate of this bacterium is reported to be ca. 11 mol (L culture)⁻¹ h⁻¹. However, the current density obtained from this form of MFC is only about 60 $\mu\text{A}/\text{cm}^2$ because only ca. 0.5% hydrogen is utilized by the electrode.

Improving the loading process of Pt can enhance the catalytic efficiency and reduce the Pt loading amount. A 100-nm of Pt layer deposited on carbon paper electrodes by using an e-beam evaporator, which is thinner than that of Pt-black (150 nm) and commercial electrode (250 nm)⁴⁹. While in MFC testing, the e-beam deposited Pt anode produced about two times higher than that of the

Pt-black anode or E-Tek commercial Pt electrode. The catalytic capacity improvement is simply due to the extremely large surface area.

Researchers who used platinum black as the anode catalyst in *E. coli* K-12 catalyzed MFCs have found that the catalyst is rapidly deactivated under fermentative conditions⁵⁰. This is possibly due to the deterioration of the electroactivity of the working electrode by microbial catabolic byproducts. To resolve this problem, researchers have used conducting polymer to protect the platinum black layer⁵¹. The conductive polymer layer not only slows the deactivation of the platinum but also forms a redox active, biocompatible layer that functions as an electron-transfer mediator. MFCs with a polyaniline-modified platinum anode enhanced current output up to 1.5 mA/cm². The conducting polymer-modified platinum anode also can be used in photobiological MFCs^{52, 53}.

Due to its high cost, platinum is not a cost-effective MFC electrocatalyst. Researchers have made significant efforts in searching for replacements for platinum. Rosenbaum et al^{54, 55} utilized tungsten carbide (WC), which also demonstrates good electrocatalytic properties toward the oxidation of formate, as an electrocatalyst for hydrogen oxidation in MFCs. A soil-based bacteria-catalyzed MFC with the WC anode delivered an OCP of 864 mV and a maximum power density of 5.85 W m⁻². However, WC is not stable in high phosphate concentrations (100mM) at neutral pH, and thus can only be used in

weak acidic pH conditions and low concentrations of phosphate ions.⁵⁵

2.4.1.2 Increasing the electrochemically active surface area

Carbon materials are the most broadly used electrode materials, especially in type II and type III MFCs, due to their high conductivity, good biocompatibility, low cost, and easy handling. For non-modified carbon-based anodes, electrocatalytic capability depends on the oxidation of microorganisms on the anode. The discovery of metal-reducing bacteria^{13, 26, 56} has boosted the development of mediator-less MFCs with high efficiency and power output. These bacterial cells can transfer electrons directly to the electrode surface, a direct electrochemical process in which the current is often related to the physical contact between the bacterial cells and electrode surface. Hence, increasing the surface area of anode is an efficient and widely utilized approach, and various strategies have been used to make carbon materials with a large electrochemically active surface area.

Porous carbon materials have been used as the anode in different MFCs, such as dual-chamber-mode MFCs⁵⁷, continuous-flow single-chamber MFCs⁵⁸, and tubular MFCs⁵⁹. The most favourable materials are carbon cloth and carbon paper, both of which are carbon-fibre-based porous materials. Alternatively, reticulated vitreous carbon (RVC) can be used due to its open network structure and large surface area, which allow the reaction to occur and provide the

microorganisms with easy access to the electrode surface. A mini MFC³⁷ (1.2 cm³) using an RVC anode and *shewanella oneidensis* can deliver a maximum power density of 24 mW m⁻² and a current density of 44 mA m⁻². RVC also can be used in upflow microbial fuel cells (UMFC) for continuous power generation⁶⁰. The UMFC was developed to generate electricity while simultaneously treating wastewater at a maximum power density of 170 mW m⁻² and a current density of 516 mA m⁻².

Although these porous carbon materials can enhance the power and current density, the clogging of the material can be a problem, especially in saturated flow systems. To resolve this problem, Logan et al.⁶¹ developed a new type of brush electrode that bundled large-surface-area graphite fibres into a central core (Figure 2.4). When they examined this brush anode in cube and bottle-air-cathode MFCs inoculated with wastewater, they found that the cube MFCs achieved a maximum power density of 2400 mW m⁻² or 73 W m⁻³ based on liquid volume. This brush architecture could be scaled up for use in larger reactors.

Figure 2.4 Graphite fiber brush anode electrode used in a single chamber MFC (Adapted with permission from Ref. 61, Copyright 2007, American Chemical Society).

Anode pretreatment has also been proved to significantly increase the active surface area. For a non-porous graphite electrode, a method of electrochemical oxidation at high potential in a high concentration of sulfuric acid was applied⁶² to increase the surface roughness of graphite to provide more surface area for the colonization of micro-organisms. It has been reported that higher power density could be achieved by using an PPy/RVC anode⁶³, as electrodeposited PPy film increased not only the effective surface area of RVC but also the adhesion of bacteria to the surface through electrostatic attraction.

2.4.1.3 Incorporating artificial mediators

Generally, because the cell wall and plasma membrane of bacteria are non-conductive, the electrons produced in cells need carriers to move them out

to reach the electrode surface. Artificial electron mediators are diffusive, small, and biocompatible molecules that can easily access both cells and electrodes, working as external electron acceptors of the electron-transfer chain and rapidly oxidizing mediators on the electrode surface to enhance the kinetics of anodic reactions (Figure 2.5)^{12,64,65}. Employing mediator-modified electrodes could lower the anode overpotential, thus increasing the current density.

Figure 2.5 Schematic of electron-transfer process from bacteria to anode through mediators
(Reproduced from Ref. 65 by permission of The Royal Society of Chemistry).

An organic mediator-linked anode can be used to enhance MFC performance. For example, when the graphite disc of a marine MFC with a plain graphite-disc anode was modified with anthraquinone-1,6-disulfonic acid (AQDS), the power density increased from $\sim 20 \text{ mW m}^{-2}$ to $\sim 98 \text{ mW m}^{-2}$ ⁶⁵. Similar results were reported in an *E. coli*-catalyzed MFC with an Fe^{3+} -graphite

cathode. The neutral red (NR)-linked woven-graphite anode increased the power density from 0.44 to 1.2 mW m⁻², as the electron transfer from the bacterial cells to the anode was enhanced by neutral red⁶⁶. Metal ions can also serve as more effective electron mediators than organic mediators. In the same report regarding the NR-graphite anode, the authors found that the Mn⁴⁺-modified woven graphite enhanced the power density to 91 mW m⁻². The maximum power density of the marine-sediment MFC with an Mn²⁺- and Ni²⁺-modified graphite-disc anode was higher (~105 mW m⁻²) than that of an AQDS-modified anode (~98 mW m⁻²).

A good redox mediator should possess the following characteristics:

1. The potential of the redox mediator should be positive enough to provide rapid electron transfer from the substrates but not so positive as to affect the potential of the anode.
2. The oxidized and reduced state of the redox mediator should be able to move across the cell membrane.
3. The redox mediator should not interfere with cell metabolism.
4. The redox mediator should be stable and be soluble for long MFC run times⁶⁷⁻⁶⁹.

The mediators applied in MFCs have been summarized in several review papers^{70, 71} and their performance in the anode have been compared. However, there is very little understanding of interface reaction mechanisms such as rate

constants and diffusion processes, or the effects of convection. Recently, researchers examined the electrochemical mechanisms and limitations of anodic currents with rotating disc electrodes in a kinetic study of yeast-catalyzed MFC using methylene blue as a mediator⁷². Their results indicate that both convection and mediator adsorption play important roles in determining the anodic current for microbial reactions.

2.4.1.4 Challenges to anodic material development

To date, the most popular anodic materials in MFCs remain porous carbon-based materials such as carbon cloth, carbon paste, and graphite felt, which are very stable in MFCs, easy to fabricate, and less costly than most metal-based electrodes. However, because the electrocatalytic activity of carbon is very low, carbon-based materials simply serve as current collectors accepting the electrons from the biofilm. In addition, they cannot provide a greater number of active reaction centers because their active surface area is too small. Developing nanostructured anode materials could be a promising way to resolve these problems. Because nanostructured materials provide a larger active surface area and superior electrocatalytic activity, they are widely used in fuel cells to decrease the activation energy and improve the power output. Accordingly, several nanostructured anode materials have been developed applied in MFCs in this study, the results of which will be presented in

following chapters.

2.4.2 Development of biocatalysts

2.4.2.1 Electroactive microorganisms

Microorganisms that do not require an exogenous redox mediator to transfer electrons to an electrode are currently sparking great research interest. In 1999, Kim et al. reported the first bacterial strain with electrochemical activity, the Fe(III)-reducing bacterium *Shewanella putrefaciens* IR-1⁹, with which they developed a mediator-less MFC⁵⁶. Whereas the anaerobically grown cells showed electrochemical activities, the aerobically grown cells did not. To date, this bacterium has been widely used in mediator-less MFCs. Several years later, two novel electrochemically active and Fe(III)-reducing bacterial strains, one phylogenetically related to *Clostridium butyricum*¹⁰ and one phylogenetically related to *Aeromonas hydrophila*⁷³, were isolated from an MFC by the same researchers.

Another widely researched MFC bacterium is *Geobacter sulfurreducens*, which was first introduced into MFCs by D. R. Bond and D. R. Lovley¹¹. Lovley also reported the novel Fe(III)-reducing bacterium *Rhodoferrax ferrireducens*, which had been isolated from anoxic subsurface sediment on Oyster Bay⁷⁴. *Rhodoferrax ferrireducens* can oxidize glucose to CO₂ and quantitatively transfer electrons to graphite electrodes without a redox

mediator⁷⁵. In the following years, many new strains were identified that possess electrochemical activity, such as *Pseudomonas aeruginosa*⁷⁶, *Desulfobulbus propionicus*⁷⁷, *Geopsychrobacter electrodiphilus*⁷⁸, *Shewanella oneidensis* dsp10⁷⁹, *Shewanella oneidensis* mr-1⁸⁰, and *Klebsiella pneumoniae*⁸¹.

2.4.2.2 Mixed communities

In 2002, Tender et al.²⁶ reported the development of a fuel cell consisting of an anode embedded in marine sediment and a cathode embedded in overlying seawater. The marine sediment contained various kinds of microbes, some of which served as MFC biocatalysts. The power generation resulted in specific enrichment of electroactive microorganisms on the anode surface¹³. Later, another kind of mixed culture contained in wastewater was used for electricity generation by Liu et al.^{19, 20} As bacteria present in waste water have been demonstrated to possess electroactivity, they have been widely used as mixed biocatalysts in mediator-less MFCs over the past several years⁸². Besides various waste waters, other organic wastes such as manure sludge waste⁸³, heat-treated soil⁸⁴, and anaerobic sludge have also provided mixed bacteria communities for use in MFCs. Although researchers are far from understanding the mechanism behind the electron transfer in this complicated system, the mixed communities apparently deliver much higher power densities

than do pure strains⁸⁵.

2.4.3 Direct electrochemistry in mediator-less MFCs

As mentioned above, electron transfer between the biofilm and electrode is critical for enhancing MFC power output. Since the mediators employed in MFCs have many drawbacks, mediator-less MFCs are currently of great research interest. Because the anodic direct electrochemical process is the key to improving the performance of mediator-less MFCs, researchers have made great efforts to identify the mechanisms behind this process, which involves two means of electron transfer: direct electron transfer via cell membrane and self-mediated electron transfer via metabolites.

2.4.3.1 Direct electron transfer

It has been proposed that the direct electron transfer occurs via physical contact of the bacterial cell membrane or a membrane organelle with the MFC anode (Figure 2.6). In the first pathway, the microorganisms possess a membrane-bounded electron transport protein relays that transfer electrons from the inside of the bacterial cell to the outside⁷¹ (Figure 2.6a). The Fe(III)-reducing bacterium *Shewanella putrefaciens* is known to localize the majority of its membrane-bound cytochromes on its outer membrane^{73, 86}. The cytochromes on the outer membrane are believed to be involved in the reduction of water-soluble Fe(III). Similar studies have used *Geobacter*

sulfurreducens, another Fe(III)-reducing bacterium⁸⁷⁻⁸⁹. In these studies, the electroactive enzymes present in the outer membrane of the cell were found responsible for the direct electron transfer between the microorganisms and the electrode.

Direct electron transfer has also been observed in yeast cells; *Hansenula anomala* cells have been demonstrated to engage in direct electron transfer via the redox enzymes present in their outer membrane⁹⁰. Although the outer membrane bound proteins provide rapid electron transfer, this pathway requires that bacteria adhere to the electrode, meaning that only the bacteria in the first monolayer on the electrode surface contribute to the current generation. Therefore, the power output is limited by the cell density in the monolayer. Recently, it has been reported that some *Geobacter*⁹¹ and *Shewanella*⁹² strains can evolve electronically conducting pili that may allow the organisms to use an electrode that is not in direct cell contact (Figure 2.6b). The formation of the pili may allow the development of thicker electroactive biofilms and thus higher anodic performance⁹³.

Figure 2.6 Two pathways of direct electron transfer in the MFC anode.

It should be noted that no direct evidence has been found so far to support the speculated direct electron transfer pathways. The current diagrams are proposed according to related research findings through genetic analysis, which indicate what genes might take part in the electron transfer process. Further investigations are required to find out the exact proteins involved in the direct electron transfer and how the direct electron transfer could be happen between those proteins and MFC anodes.

2.4.3.2 Self-mediated electron transfer via metabolites

Microorganisms may produce low-molecular weight, electron-shuttling compounds through metabolic pathways to conduct extracellular electron transfer. For example, the *Shewanella oneidensis* strain MR-1 was found to excrete a quinone-like molecule that is hypothesized to serve this purpose⁹⁴. The involvement of pyocynaine and phenazine-1-carboxamide, which is produced by *Pseudomonas aeruginosa* in the electron transfer to an MFC anode, has been proved¹⁷. A recent study demonstrated that metabolites produced by one kind of bacteria can enable another kind of bacteria to achieve extracellular electron transfer. MFCs operated with only the *Brevibacillus* strain in their anodes demonstrated relatively low electricity generation, but after replacement of the anodic aqueous part with the cell-free anodic supernatants of MFCs operated with *Pseudomonas sp.* CMR12a, their electricity generation improved significantly. *Pseudomonas sp.* CMR12a produces considerable amounts of phenazine-1-carboxamide and biosurfactants believed to facilitate electron transfer from the bacterial cells to the MFC anode⁹⁵.

Several studies have reported that the extracellular electron-transfer capacity of *E. coli* cells can be evolved in MFCs with different anodic constructions. In general, *E. coli* must be used in combination with suitable electron mediators in MFCs or the MFC electricity production will be relatively low. Zhang et al.⁹⁶ employed *E. coli* cells in a mediator-less single-chamber

MFC with a carbon/PTFE composite film anode and a conventional air cathode. For the original bacteria, the initial cell voltage was only 0.4 V under a constant load of 1.98 k Ω , and decreased to 0.1 V within several hours. When the bacteria were treated with repeated discharge cycles, the initial cell voltage increased to 0.6 V until it reached the first discharge plateau at 0.55 V for 2 hours and the second plateau at 0.25 V for 50 hours. A similar phenomenon was observed on the surface of a carbon felt anode; *E. coli* cells growing under anaerobic conditions formed biofilm on carbon fibres, and the anodic current increased with the development of the biofilm⁹⁷. The mechanism behind the electrochemical activation of *E. coli* cells remains unclear, although it is possible that self-excreted mediators respond to the direct electron-transfer process. The details will be discussed along with the results of several experiments in the following chapters.

2.5 Perspectives on MFC development

In spite of the development of many approaches to improving anodic electrocatalysis that effectively enhance MFC power output, there remains room for further improvement. The electrode materials should satisfy the requirements of both electrocatalysis and biocatalysis. The main MFC challenge lies in the efficiency of collecting the electrons produced by the catalysts—the bacterial cells. Specifically, the capacity of the anode oxidizing

the bacteria limits the MFC anodic performance. Inserting precious metal or artificial mediators into the electrode materials is an effective method for decreasing the activation energy and thus improving electrocatalysis. Because the drawbacks discussed above restrict its practical application, new strategies must be developed to enhance MFC electrocatalysis.

To obtain high electrocatalytic activity, the anodic materials must have good biocompatibility with the biofilm so that the bacterial cells can successfully grow on the electrode surface. Furthermore, very large surface areas are needed for supporting the biofilm, and the structure must be able to provide easy access to nutrition. Novel structure architectures, such as brush carbon anodes, are good choices. Because nanostructured material is also a promising solution for this problem, further investigation into interface electron transfer on nanostructured anodes is necessary, and the supporting materials and loading processes may need further improvements.

In addition, using materials in practical or industrial applications should be considered. Some materials are not suitable for large-scale operations because of their inherent lack of durability or structural strength or their high cost. Future designers should consider using conductive coatings on structurally strong supporting materials. System scale-up will also require that the design and application of these materials be adaptable to mass manufacturing approaches.

Moreover, further investigation is needed into the mechanism behind the direct electrochemistry of MFC anodes. Gaining understanding of this mechanism would allow for the development of high-performance anodes and the development of MFC technology for industrial applications. The use of biotechnological tools, such as genomics and proteomics associated with electrochemical analysis, is necessary for such research.

With the development of bio-nanotechnology, more strategies will be developed to enhance MFC power density. High-power MFCs are the most promising alternative power sources because they can utilize various energy sources. With some modifications, MFC technologies will have applications ranging from hydrogen production to the simultaneous treatment of waste and the generation of renewable energy.

Chapter 3 Experimental Approaches

3.1 Materials and equipments

3.1.1 Reagents and chemicals

3.1.1.1 Chemicals

Aniline ($\geq 99.0\%$), 2-hydrox-1,4-naphthoquinone (HNQ, 97%), ammonium persulfate (APS, ACS reagent, $\geq 98.0\%$), tetraethyl orthosilicate ($\text{Si}(\text{OC}_2\text{H}_5)_4$, $\geq 98.0\%$), tetrabutyl titanate ($\text{Ti}(\text{OC}_4\text{H}_9)_4$, $\geq 97.0\%$), polytetrafluoroethylene (PTFE, 60 wt % dispersion in H_2O), lysozyme (from chicken egg white, BioChemika, dialyzed, lyophilized, powder, ~100000 units/mg), dithiothreitol (for molecular biology, $\geq 99.5\%$), beta-mercaptoethanol ($\geq 99.0\%$), were purchased from Sigma-Aldrich. Multi-walled CNTs (95%, 10-20 nm) were purchased from Shenzhen Nanotech Co. Ltd. (Shenzhen, China). All other chemicals were of analytical grade and used as received. Deionized (DI) water (resistance over 18 $\text{M}\Omega$ cm) from a Millipore Q water purification system was used in all experiments.

3.1.1.2 Reagents and kits

AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen biosciences, CA), pET-21b vector (Novagen, Merck, Germany), IPTG (isopropyl β -D-1-thiogalactopyranoside), RNeasy Mini Kit (Qiagen), FairPlay® III Microarray Labeling Kit (Stratagene), Agilent hybridization kit, AxyPrep Bacterial

Genomic DNA Miniprep kit (Axygen biosciences, Union City, CA), and nuclease-free water (Ambion) were used for genetic analysis experiments.

3.1.1.3 Buffers and medium

The Lysogeny broth (LB) medium for engineered *E. coli* growth was prepared as following: Add 10 g NaCl, 10 g Tryptone and 5 g yeast extract into 900 mL DI water and adjust pH to 7.4 with NaOH. Adjust volume to 1L with DI water and sterilize by autoclaving (120°C, 20 min).

Standard glucose medium for *E. coli* culture and MFC operation was prepared as following: Add 10 g glucose, 5 g yeast extract, 10 g NaHCO₃ and 8.5 g NaH₂PO₄ into 1L DI water and sterilize by autoclaving (120°C, 20 min). The pH value of this medium is 7.4.

The buffers used in SDS-PAGE include sample buffer, running buffer and running gel solution.

- (1) 5× Sample Buffer was protein sample preparation, it was mixed with protein as 1:4. The 5× Sample Buffer contains:

10% w/v	SDS
10 mM	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenolblue

- (2) 1x Running Buffer for electrophoresis was prepared as following: 3.01 g

Tris base, 14.4g Glycine and 1g SDS was dissolved in 1L DI water.

- (3) Running Gel Solution (12%) used for resolving gel preparation consists of:

H ₂ O	1.2 ml
1.5 M Tris-HCl, pH 8.8	3 ml
10% (w/v) SDS	40 μ l
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	3.1 ml
10% (w/v) ammonium persulfate (APS)	40 μ l
TEMED	5 μ l

Stacking gel was prepared as following: 1ml Acrylamide/Bis-acrylamide (30%/0.8% w/v), 630 μ l Tris-HCl (1M, pH6.8), 25 μ l 10% SDS and 3.6 ml DI water were mixed together and then add 25 μ l 10% APS and 5 μ l TEMED just before gel.

3.1.2 Other materials

The proton-exchange membrane (Nafion 117, Dupont, Wilmington, DE) was purchased from Sigma-Aldrich and the carbon cloth (plain, non-waterproof) was purchased from E-TEK Inc.

3.1.3 Equipments

Field-emission scanning electron microscopy (FESEM) (JSM-6700F JOEL, Japan) was used to produce the SEM photos.

Atomic force microscopy (AFM; SPM 3100, Veeco Instruments Inc., USA) was used to characterize the morphology of the samples.

Fourier transform infrared (ATR-FT-IR) spectroscopy was performed using the Nicolet Magna IR 560 ESP spectrophotometer (Thermo Nicolet, USA) incorporated with a “Golden Gate” ATR module (Graseby Specac, USA).

X-ray diffraction (XRD) spectra were recorded at room temperature on

a Bruker AXS X-ray diffractometer (USA) using Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$) with a θ -2 θ scan configuration.

UV-VIS was measured on HITACHI U-2800 double-beam system.

The NanoDropTM 1000 Spectrophotometer (Thermo Scientific) and the Agilent 2100 Bioanalyzer (Agilent Technologies) were used to maintain RNA and cDNA sample quality control.

A hybridization oven (Agilent) and a microarray scanner (Agilent G2565BA) were used for the microarray experiment.

A PGSTAT30 Autolab system (Ecochemie, Netherlands) was used for all of the electrochemical measurement.

3.2 Methodology

The common methods that were used in experiments of different chapters are summarized here. The specific experimental approaches only used in one chapter are introduced in the corresponding chapters.

3.2.1 Bacteria culture

E. coli K-12 (ATCC 29181) was inoculated from an agar plate and grown overnight (12 h) at 37 °C in an LB medium. The overnight culture was used as inoculation for further bacteria culture in a standard glucose medium, which was also used as an anodic electrolyte in the MFCs. In this project, *E. coli* cell cultures with different growth phases were used in different

experiments. The growth phases (lag, log, and stationary) were indicated in the growth curve that shown as a plot of absorbance at 600 nm vs. growth time (Figure 3.1). In the log phase, the *E. coli* cells grow rapidly and multiply exponentially.

Figure 3.1 Growth curve of *E. coli* K-12 cells in LB medium at 37°C

In chronoamperometric measurements and anode-limited discharge experiments, *E. coli* cell cultures at mid-log phase were used to explore the current generation and discharge profile during the *E. coli* propagation. The anaerobic *E. coli* culture ($OD_{600} = 0.5$) was transferred from the flask to the electrochemical cell, and nitrogen gas was pumped into the cell during the experiments. To conduct electrochemical measurements such as CV and EIS, cell cultures at an early stationary phase ($OD_{600} = 1.2$) were collected by

centrifugation at 4°C (6000 rpm, 5 min) and resuspended in a fresh standard glucose medium or a 0.1M phosphate buffer with 55 mM glucose after three washes. The concentration of *E. coli* cells was about 10^9 cells mL⁻¹. Before each test, nitrogen gas was pumped into the cell suspension for at least 30 min to remove the oxygen.

3.2.2 Physical characterization technologies

FTIR is specifically used to measure the absorption of various infrared light wavelengths by the material being studied. These infrared absorption bands identify specific molecular components and structures. In this study, FTIR was used to survey the functional groups on the surfaces of nanomaterials and conduct spectral analysis of cell supernatant. FTIR spectra were recorded in the range of 400-4000 cm⁻¹ using a Nicolet Magna IR 560 ESP spectrophotometer. A minimum of 64 scans was averaged using a signal resolution of 0.5 cm⁻¹.

XRD is a versatile, non-destructive technique that reveals detailed information regarding the chemical composition and crystallographic structure of natural and manufactured materials. In this study, XRD was used to characterize the crystallographic structure of nanomaterials. The wide-angle XRD patterns were measured from 5 to 90 degrees (2θ) at a scan rate of 0.5 degree min⁻¹.

The morphological studies were conducted using FESEM, HRTEM and

AFM. The morphologies of nanomaterials were observed by using FESEM and HRTEM. The surface morphologies of *E. coli* cells were observed with AFM (contact mode) and FESEM.

To characterize the specific surface area and pore distribution, nitrogen adsorption-desorption experiments were carried out at 77.3 K using an automated gas sorption system (AUTOSORB-1, Quantachrome Instruments). The surface area was calculated using the Brunauer-Emmett-Teller (BET) equation and pore size distribution was calculated using Barrett–Joyner–Halenda (BJH) methods.

3.2.3 MFC electrode preparation

Nickel foam was used as an electrode substrate to fabricate the powder electrode. The nickel foam was cleaned using acetone, 1M HCl, and DI water successively before use. To produce nanostructured electrodes, the nanomaterials powders were mixed with a PTFE solution to prepare pastes that were coated on the surface of nickel foam (1 cm × 1 cm × 0.1 cm) to produce uniform films that were then pressed to fabricate nanostructured electrodes. The film covered one side of the foam and the other side was insulated with epoxy resin. After drying at 120 °C to remove water, the electrodes were used as MFC anodes.

Plain carbon cloth was used in the MFC cathode in the investigation of *E. coli* direct electrochemical behaviour. The carbon cloth was cut into pieces and

cleaned with acetone, 2M HCl, and DI water sequentially to obtain a hydrophilic surface. After drying at 100 °C, the carbon cloth pieces were connected to copper core conductive wires and applied in MFCs.

3.2.4 Electrochemical measurements

All electrochemical experiments in this research project were conducted using the PGSTAT30 Autolab system. A three-electrode cell consisting of a working electrode, a reference electrode (a Ag/AgCl electrode, sat. KCl or saturated calomel electrode, SCE) and a Pt counter electrode was used to characterize the electrocatalytic performance of the nanostructured materials. For the direct electrochemistry behaviour analysis discussed in chapter 6, a three-electrode system with a plain carbon cloth working electrode, an SCE reference electrode, and Pt film counter electrode was used. The glass bottle together with the carbon cloth and Pt film were autoclaved (120°C, 20 min) before testing and the reference electrode was sterilized with 70% ethanol.

Chronoamperometric, chronopotential, cyclic voltammetry (CV), and electrochemical impedance spectra (EIS) methods were applied to investigate the electrochemical behaviour of the nano-structured anodes. EIS measurements were performed over a frequency range of 0.1 Hz – 100 KHz at open circuit potential with a perturbation DC signal of 10 mV. The simulation of EIS data was conducted with analysis software (Z-view, Solartron).

3.2.5 MFC set up and operation

Dual-chamber MFC devices were used in these experiments (Figure 3.2).

Figure 3.2 A typical dual-chamber MFC device used in this project.

The capacity of the chambers varied in the different experiments. For the PANI/CNT/*E. coli* MFC, the capacity of the anodic chamber was 30 mL. For the PANI/TiO₂/*E. coli* MFC, the capacity of the anodic chamber was 450 mL. The capacity of the MFC used in the *E. coli* direct electrochemistry behaviour analysis was 100 mL, the same as that of the engineered *E. coli* MFC. A Nafion 117 membrane clamped between the two chambers was used as a cation-exchange membrane. The Nafion 117 membrane was pretreated successively in boiling 5% H₂O₂, DI water, and 0.5 M H₂SO₄ DI water for 30 min. The electrode material used in ferricyanide cathode was plain carbon cloth

for all experiments while the anode varied. The catholyte was $K_3[Fe(CN)_6]$ and the anolyte was the cell culture containing glucose. HNQ was used as the electron mediator in the PANI/CNT/*E. coli* MFC and the PANI/TiO₂/*E. coli* MFC. In the three-electrode system analysis (eg. CV, EIS), the reference electrode (SCE) was inserted into the anodic chamber and the anode and cathode served as the working and counter electrodes, respectively.

For the current generation measurement, an external load of 1.96 k Ω was connected into the MFC and the current and potential measurements on the MFC were conducted using a bench-top digital multimeter (ESCORT 3146A) during discharge. The MFC polarization curve and power density were examined using various external loads of 10 ~ 20,000 Ω .

3.2.6 Characterization of cell surface

The *E. coli* cell cultures were harvested by centrifugation at 5000 $\times$ g, 4 °C. After three washes, the cell pellets were resuspended in a 0.05 M phosphate buffer. For the FESEM observation, the cell pellets were resuspended in a 0.05 M phosphate buffer containing 5% glutaraldehyde as a fixative and fixed for 4~6 hours. The fixed cell suspensions were then dropped onto the substrate, a piece of filter membrane with a pore size of 100 nm. After drying in the incubator, the membranes with the cells were gradually dehydrated using 30% ~ 100% ethanol and dried in a vacuum oven at room temperature. Before observation, the membranes were coated with Pt to make them conductive. For

the AFM observation, the cells did not need pretreatment. The cell suspensions were dropped onto mica and rinsed with DI water several times. After air drying, the mica containing the cells could be directly observed.

Chapter 4 PANI/CNT Nanocomposite as Anodic Material in MFCs^a

4.1 Introduction

CNTs have exhibited very promising properties as catalyst supports in fuel cell applications due to their unique electrical and structural properties⁹⁸. For example, CNTs have displayed superior performance compared to carbon blacks as catalyst supports for proton-exchange membrane fuel cells (PEMFCs)^{99, 100} and have served as anodic materials for enzymatic biofuel cells^{101, 102}. However, it has been reported that CNTs have a cellular toxicity that could lead to proliferation inhibition and cell death^{103, 104}. Thus, they should be modified to improve their biocompatibility before used in MFCs. Coating with a biocompatible conducting polymer film is an effective approach to achieve this objective.

Polyaniline (PANI) is a popular conducting polymer due to its simple synthesis process, good electrical conductivity, and environmental stability.¹⁰⁵ It is also used in biosensors and biomedical devices according to its good biocompatibility.^{106, 107} Recently, Schröder *et al.* employed PANI to modify a platinum anode for MFC⁵⁰ and achieved a current density 1 order of magnitude

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higher than the previously reported value. PANI/inorganic composites are also reported to have better conductivity.^{108, 109} Their study suggests that conductive PANI not only provides a protective function layer for catalysts in the MFC, but also directly benefits the anodic electrocatalysis to achieve a high current density^{50, 51}.

Incorporation of CNTs into PANI has been proved an effective approach to enhancing the electronic properties of PANI in neutral buffer. For example, it has been reported¹¹⁰ that PANI fibres containing CNTs displayed significant improvements in both mechanical strength and conductivity. When Liu et al.¹¹¹ constructed PANI/CNT multilayer films by means of a layer-by-layer assembly, the PANI displayed considerable electroactivity in a neutral electrolyte due to the presence of CNTs. Thus, a PANI/CNT composite combining the advantages of PANI with the enhanced conductivity conferred by CNTs could possibly be used in MFCs for performance improvement. In the work reported in this chapter, a PANI/CNT composite is used as an anode in an MFC and its electrocatalytic properties associated with a bacterium biocatalyst are examined for the first time.

4.2 Synthesis of materials

4.2.1 Synthesis of PANI

Aniline monomer was distilled under reduced pressure before polymerization. The PANI was chemically synthesized as follows: aniline (1 mL) was mixed with HCl (0.3 mL) in 50mL DI water in an ice bath. An APS solution (2.3 g in 25mL DI water) was added to the mixture. The polymerization was conducted for 6 h in the ice bath (0–5 °C). A green solid of proton-doped PANI was obtained after several rinses with DI water. The reaction of aniline polymerization is given as following:

4.2.2 Synthesis of PANI/CNT composite

The CNTs were ultrasonically treated using a mixture of 3:1 of H_2SO_4 : HNO_3 at 50 °C for 24 h to produce carboxylic acid groups at the defect sites and thereby improve solubility in HCl solution. The composite of proton-doped PANI/CNT was synthesized *in situ* via chemical (APS) oxidation using different weight ratios of CNT to aniline. The CNTs were dissolved in 1.0 M HCl, subjected to ultrasonic treatment for 3 h, and then transferred to a 250-mL

flask placed in the ice bath. A 1.0 M HCl aniline monomer solution was added to the prepared CNT–HCl suspension. The APS solution was added to the suspension and constantly stirred at 0–5 °C for 6 h until a precipitate was produced. After filtering and several rinses with DI water and methanol, the precipitate was vacuum-dried at 60 °C for 24 h, yielding a powder containing CNT and PANI ready for application to the MFC anode preparation.

4.3 Physical and electrochemical characterizations

4.3.1 FTIR spectroscopy and morphology characterization

The FTIR spectra of plain PANI and the PANI/CNT composite are shown in Figure 4.1. The peak at 835 cm^{-1} can be attributed to the N–H out-of-plane bending absorption and the peaks at 1500 and 1600 cm^{-1} can be attributed to the stretching vibration of the quinoid ring and benzenoid ring, respectively, both of which are characteristic of PANI. For the composites, the presence of the absorption band at 1240 cm^{-1} is very prominent, which is derived from the C–N stretching vibration in proton-doped PANI. The remarkable enhancement of this peak indicates the formation of C–N coordinate-covalent bonds between the polymer chain and the radical cation CNT fragments, which in turn confirms the successful synthesis of the PANI/CNT composite^{112, 113}. The broad band at 1730 cm^{-1} in PANI (C=O vibration) is drastically enhanced for the PANI/CNT composite and becomes the most prominent peak for the 20 wt %

PANI/CNT powder. The band near 3000 cm^{-1} is due to the C–H stretching absorption. This signal is broad and strong in the composite samples and relatively weak in the plain PANI. This phenomenon was reported recently and it was explained that the sp^2 carbons of the carbon nanotubes perturbed the H-bonding environment and then increased the N–H stretch intensity. These results strongly support the formation of PANI/CNT through the chemical oxidation method.

Figure 4.1 FTIR spectra of PANI and composite powders. (a) plain PANI; (b) 1 wt % PANI/CNT composite; (c) 20 wt % PANI/CNT composite.

FESEM micrographs of films made from plain PANI and the PANI/CNT

composite powders are shown in Figure 4.2. The pure PANI film is compact and fibrillar, while the PANI/CNT composite films have a networked-rod nanostructure in which the outer layer is PANI and the inner layer is constructed by CNTs. The rough, amorphous outer PANI layer has an average thickness of about several tens of nanometers. To verify the effect of CNT doping on the structure of the polymer, the specific surface areas of plain PANI and the nanocomposite were measured. The cumulative adsorption surface area (BJH Method) of plain PANI is $34.1 \text{ m}^2 \text{ g}^{-1}$. For the nanocomposite, the value is $50.2 \text{ m}^2 \text{ g}^{-1}$. These values show that the specific surface area of the composite is much larger than that of the plain polymer. Therefore, it is reasonable to expect that a porous PANI/CNT composite with a larger specific surface area may facilitate the accommodation and electron transfer of bacteria, resulting in a higher discharge profile for an MFC built on it.

Figure 4.2 SEM images of PANI and PANI/CNT composite films (a) plain PANI; (b) 20 wt % PANI/CNT composite.

4.3.2 EIS studies

EIS measurements were conducted to investigate the characteristics of charge transfer and ion transport in plain and composite polymers. The Nyquist plots (Figure 4.3a) show well-defined single semicircles over the high-frequency range, followed by short straight lines in the low-frequency region for all samples. The diameter of the semicircle corresponds to the interfacial charge-transfer resistance (R_{ct})¹¹⁴. Since there was no glucose or bacteria in the electrolyte, the R_{ct} counts for the easiness degree of doping/de-doping reaction of PANI. Although the impedance spectra have similar shapes, the R_{ct} declines remarkably with increasing content of CNTs in the composite films. The R_{ct} s of the three electrodes were 1317 (PANI), 827 (PANI/CNT 1 wt %) and 434 Ω (PANI/CNT 20 wt %), respectively. Obviously, the R_{ct} of the PANI/CNT

composite is much lower than that of the pure polyaniline, suggesting that the incorporation of CNTs into the composite facilitates electron transfer at the material/solution interface due to enhanced conductivity. The impedance plane plots in Figure 4.3a show only a very short part of a straight line region, which is an indication of diffusion control for the doping/de-doping process¹¹⁴. It is known that the reactants in the doping/de-doping reaction are anions. The narrow region of the diffusion-controlled process indicates that all the tested anodes have a good micro/nanostructure that provides sufficient access for the reactant to the reaction centers, which has been confirmed by the SEM images. Therefore, slight potential perturbation does not induce a concentration gradient over a wide frequency range. In a conducting polymer/CNT composite, it has been suggested that a charge transfer occurs between the two constituents in either the polymer-functionalized CNTs or in the CNT-doped conducting polymers¹¹⁵. The CNTs illustrate an obvious improvement effect on apparent charge transfer rate.

The Nyquist plots of the PANI/CNT (20 wt %) electrode in phosphate buffer (pH 7.4) in the presence of bacteria with and without 5.5mM glucose were also investigated at a fixed potential of 0.1 V versus the SCE (Figure 4.3a). When the electrode was tested in the phosphate buffer with both bacteria and glucose, the R_{ct} was about 156 Ω , which is significantly smaller than that in the presence of bacteria but without glucose in the electrolyte (400 Ω). As

discussed above, the electrochemical reactions without glucose in the solution are due to the doping/de-doping process of PANI. The significant reduction in R_{ct} indicates that the glucose oxidation in such a PANI/CNT/HNQ/*E. coli* K-12 anode system has an even faster reaction rate than that of the doping/de-doping redox reaction. The result reveals that the composite anode not only improves the electrode conductivity and specific surface area but also provides unique active centers, possibly due to its specific nanostructure (Figure 4.3b), which host the bacteria for more efficient electrocatalysis.

Figure 4.3 (a) Nyquist plots of PANI, PANI/CNT (1 wt %) and PANI/CNT (20 wt %) composite electrodes in 0.1M phosphate buffer (pH 7.4) at open-circuit potential (obtained over a frequency range of 0.1 Hz – 100 KHz with a perturbation DC signal of 10 mV at room temperature). The equivalent circuit shown as inset is used to fit the EIS results. (b) Nyquist plots of PANI/CNT (20 wt %) in phosphate buffer with or without bacteria and glucose.

4.4 MFC performance examination

4.4.1 MFC Anode discharge performance

In order to evaluate the discharge performance of different MFC anodes, an anode-limiting MFC (volume of 30 mL) was designed which had a Pt cathode with a much larger surface area than that of the anodes, making the polarization of the cathode insignificant. The catalytic current from glucose oxidation at a constant potential was measured. As shown in Figure 4.4, the current increases with the growth and proliferation of the bacteria on the electrode surface. The current-time curves of the PANI and composite anodes are significantly different from each other. For the plain PANI anode, the current increased very slowly and the current density was much lower than that of the composites. For the two PANI/CNT composite anodes, when the current of the PANI/CNT (1 wt %) anode reached a plateau, the current of the PANI/CNT (20 wt %) anode was still increasing and the current density was much higher than that of the PANI/CNT (1 wt %). This can be explained by the fact that the nanocomposite electrodes have a greater number of reaction activity sites (a larger specific surface-area) for the bacteria-catalytic oxidation of glucose, and the number of active sites increases with increased doping of the CNTs. This result is in agreement with the impedance analysis and the BET results.

Figure 4.4 Chronoamperometric plots of PANI and PANI/CNT composite electrodes placed in stirred anaerobic culture of *E. coli* K-12 in a standard glucose medium (pH 7.4) at room temperature. The potential applied to electrode is 0.1V (vs. SCE). (a) plain PANI; (b) PANI/CNT composite containing 1 wt % CNT; (c) PANI/CNT composite containing 20 wt % CNT.

The constant-current discharge experiments of the MFCs with three different anodes in 5.5 mM glucose solution were conducted at 50 mA m^{-2} . The anode potential versus time data (Figure 4.5) show different discharge profiles for the three anodes. Within 180 min, the discharge potentials for plain PANI, PANI/CNT (1 wt %) and PANI/CNT (20 wt %) change from -0.01 to -0.2, -0.36, and -0.38V, respectively. For a fuel cell system, the more negative the anode discharge potential, the higher the operational voltage of the fuel cell. The

discharge results show that the composite anode can provide a higher power density due to its lower polarization, which further indicates that the nanostructured composites have a more rapid reaction rate. It is also seen that the composite with the higher CNT content (20 wt %) exhibits better discharge performance than that with the lower CNT content (1 wt %), and the discharge profile of the bacteria anode is fundamentally different from that of conventional anode behavior. Specifically, the potential of bacteria anode increases to a more negative value as the discharge time elapses due to the bacteria growth process, as the discharge process starts immediately after the

Figure 4.5 Constant current (50 mA m^{-2}) discharging curve of PANI and PANI/CNT composite electrodes placed in a stirred anaerobic culture of *E. coli* K-12 in 0.1M phosphate buffer (pH 7.4) with 5.5mM glucose at room temperature. (a) plain PANI; (b) PANI/CNT composite containing 1 wt % CNT; (c) PANI/CNT composite containing 20 wt % CNT.

addition of the glucose solution and the bacteria. The bacteria require time to grow to their maximum level and to distribute into the inner surface of the anode, as shown in Figure 4.5.

4.4.2 Power output of PANI/CNT (20 wt %) MFC

As the PANI/CNT (20 wt %) anode exhibited the best performance, its power output and polarization were examined with the anode-limiting MFC. The results are presented in Figure 4.6. The polarization curve shows that the cell voltage drops to 250 mV at a current density of 145 mA m^{-2} . The power density of the PANI/CNT (20 wt %) MFC was calculated from the results of chronopotential measurements under different current densities. The plot of power density versus current density has a volcanic shape, with the power density increasing with an increase in current density, reaching a maximum value, and then sharply falling with further increase in current density. This is the typical shape of the relationship between output power density and the current density. The maximum power density is 42 mW m^{-2} , which is obtained at a current density of about 100 mA m^{-2} with a cell voltage of 450 mV. It has been reported⁶⁶ that woven graphite electrodes in *E. coli* MFCs could deliver a maximum power density of $0.47\text{--}2.6 \text{ mW m}^{-2}$ with cell voltages of 0.6–3.3V; the power density has reached 91 mW m^{-2} with Mn^{4+} modified woven graphite as the anode but the cell voltage was only about 280 mV. Given that the thickness of the anode used in this experiment was much smaller than 1 mm in

comparison with the 1 cm thickness of the anodes used in the literature⁶⁶, the PANI/CNT anode has superior electrocatalytic activity. Due to the cellular toxicity of CNTs to *E. coli* cells, the composites containing a ratio of CNTs higher than 20 wt % were not used in this investigation.

Figure 4.6 Power output and polarization curve of MFC with 20 wt % PANI/CNT anode measured at room temperature.

Table 4.1 Comparison of the PANI/CNT/*E. Coli*-K12 MFC with a reported dual-chamber *E.coli*-MFC

	Anode		Cathode		Current Density (mA m ⁻²)	Power Density (mW m ⁻²)	OCV (mV)
	Supporting electrode	Electrolyte volume	Electrode	Oxidant			
Ref. 66	woven graphite felt (2.7g, 80 cm ²)	1300 mL	woven graphite felt (2.7g, 80 cm ²)	50 mM ferricyanide in 0.1M PBS (pH7)	0.47	0.3	680
Present work	10 mg PANI/CNT with nickel foam support (1cm×1cm×0.1cm)	30 mL	Carbon cloth (2 cm ²)	50 mM ferricyanide in 0.1M PBS (pH7)	100	42	700

4.5 Conclusions

This study has shown that a nanostructured PANI/CNT nanocomposite can be used as the anode for a MFC. The addition of CNTs to PANI increases the specific surface area of the electrode and enhances the charge transfer capability, thereby significantly increasing the electrochemical activity of the anode reaction in an MFC. A PANI/CNT/*E. coli* K-12/HNQ anode system yields a much higher current density than a PANI/*E. coli* K-12/HNQ system. This demonstrates the superior and specific electrocatalytic effect of the nanocomposite for MFCs in comparison with that of the existing systems. A composite containing 20 wt % CNTs yields the best discharge performance and delivers a power output as high as 42 mWm^{-2} with a cell voltage of 450 mV. The CNT-doped PANI nanocomposite therefore offers good prospects for application in MFCs.

Chapter 5 PANI/TiO₂ Nanoporous Composite as MFC

Anodic Material^b

5.1 Introduction

Over the past few years, some scientists have improved the MFC anodic material by impregnating it with different chemical catalysts^{54, 65, 66}. Optimization of the porous structure of the electrode with a larger specific surface area increases the apparent power density, but the entering bacteria clog the cell pores, resulting in cell death and a significant reduction in the electrochemical reaction surface.³ Conventional carbon-based anodes, such as carbon felt and porous carbon papers, also face this very challenging problem.³

It is still a great challenge so far to develop a new anode material that can further increase MFC power density as the catalytic mechanism of a MFC anode involves not only a bio- but also an electrocatalytic process. An optimal nanostructure with a large specific surface area favorable for both catalytic processes, which could play a critical role in improving the MFC power density; must host the bacteria with high bioactivity while enhancing the electron-transfer rate. Among mesoporous structured inorganic materials, which have a large specific surface area and uniform pore distribution, TiO₂ is biocompatible,

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stable, and environmentally friendly,¹¹⁶ and its electronic, optical, and dielectric properties can be enhanced by surface modifications¹¹⁷⁻¹²⁰ to allow its use as an MFC anode.

The author's previous work¹²¹ described in chapter 4 has demonstrated that a favorable nanostructure of a PANI/CNT composite anode improves the MFC performance, especially the power density. In this work, a new mesoporous TiO₂ electrode material with uniform nanopore distribution and high specific surface area¹²² is used to fabricate a unique nanostructured PANI/TiO₂ composite for the MFC anode. After optimization on both the biocatalytic and electrocatalytic properties of the composite by tailoring the composition ratio, the optimal composite will be employed in an *E. coli* MFC. To the best of the author's knowledge, this is the first time that this new hybrid polymer/inorganic mesoporous composite is fabricated and applied to MFC development.

5.2 Synthesis of materials

5.2.1 Synthesis of mesoporous TiO₂

First, a 35.5-mL solution containing tetraethyl orthosilicate (Si(OC₂H₅)₄), tetrabutyl titanate (Ti(OC₄H₉)₄) and anhydrous ethanol was prepared at a volume ratio of 1:10:60 before 120-mg acid-treated multi-walled carbon nanotubes (MWCNTs) was immersed into the mixture, followed by sonicate for 10 min. Another mixture of 30 mL anhydrous ethanol, 3 mL 28%

$\text{NH}_3 \cdot \text{H}_2\text{O}$, and 30 μL 30% H_2O_2 was slowly added to the MWCNT suspension and stirred at room temperature until it formed a gel. The gel mixture was then transferred into a stainless steel autoclave and heated at 170 $^\circ\text{C}$ for 30 h. After it was cooled to room temperature, the sample was removed, rinsed with ethanol, and dried overnight in a vacuum oven at 80 $^\circ\text{C}$. Finally, the composite was calcined for 2 h at 650 $^\circ\text{C}$ to remove the MWCNT template. According to the XRD pattern, the main peaks can be indexed to the anatase TiO_2 phase (JCPDS, card no: 21-1272), suggesting the anatase TiO_2 and no characteristic peaks of impurities are observed¹²². Possibly, a little of SiO_2 from the raw material might exist but could not affect the crystal structure of the product in terms of the results. Hence, the synthesized material was still denoted as TiO_2 .

5.2.2 Synthesis of PANI/ TiO_2 composite

After the TiO_2 was obtained, different volumes of aniline were injected into 50 mL of an aqueous TiO_2 dispersion with 1 M hydrochloric acid under ultrasonic operation to reduce agglomeration. Ammonium persulfate solution with a mole ratio of 1:1.25 monomer was added drop wise into the mixture with constant stirring at 0-5 $^\circ\text{C}$. After 12 h of reaction, the precipitate was harvested by filtration and rinsed several times with deionized water and methanol. Finally, the precipitate was vacuum-dried at 60 $^\circ\text{C}$ for 24 h, and the PANI/ TiO_2 composites were obtained.

5.3 Characterization of PANI/TiO₂ composites

5.3.1 XRD patterns

The phases of both TiO₂ and the PANI/TiO₂ composite were determined by X-ray diffraction (XRD, Figure 5.1). Curve a displays the characteristic patterns of TiO₂, and the main peaks can be indexed to the anatase TiO₂ phase (JCPDS, card no. 21-1272). Curve f shows that the plain PANI has a certain degree of crystallinity. The broad peak can be ascribed to the scattering from polyaniline chains at interplanar spacing. The patterns of the four composites indicate that PANI deposited on the surface of TiO₂ has no effect on the crystallization characteristics of TiO₂. With increasing PANI percentage in the composite, the intensity of the broad peak of PANI is enhanced, which may suggest that the crystalline behaviour of PANI is not hampered by the restrictive effect of the surface of TiO₂ as reported in the literature.¹¹⁸ However, the intensity of the characteristic peaks of TiO₂ is dampened by the increase in the thickness of the polymer layer.

Figure 5.1 X-ray diffraction of (a) TiO_2 , (b) 10 wt % PANI/ TiO_2 , (c) 20 wt % PANI/ TiO_2 , (d) 30 wt % PANI/ TiO_2 , (e) 50 wt % PANI/ TiO_2 , and (f) PANI.

5.3.2 Morphology

The morphologies (Figure 5.2) of both TiO_2 and the PANI/ TiO_2 composite were examined by FESEM and TEM. Figure 5.2a demonstrates that the TiO_2 agglomerates form a loose nanostructure with a uniform nanopore distribution. The SEM micrograph at high magnification (inset of Figure 5.2a) clearly shows that the TiO_2 cluster consists of flakes that cross-link with each other to form a porous network. The composite structure (Figure 5.2b) is more compact than TiO_2 , and high-magnification graph (inset of Figure 5.2b) reveals that the porous flake cross-linked structure of the mesoporous TiO_2 is retained after PANI modification. The TEM micrographs (Figure 5.2 c, d) also illustrate

Figure 5.2 SEM (a,b) and TEM (c,d) micrographs of TiO₂ (a,c) and PANI/ TiO₂ composite (b,d) with 30 wt % PANI. The insets are the high-magnification graphs.

that the nanoflake structure of the composite is not significantly different from that of the TiO₂.

5.3.3 BET surface area and pore distribution

The results of the investigation of the specific surface area and the average pore diameter are shown in Figure 5.3, with the inset showing the N₂ adsorption– desorption isotherm and the pore distribution of the PANI/TiO₂ nanocomposite with 30 wt % PANI. The composite has a high specific surface area larger than 150 m²/g, which is 300 times larger than that of woven graphite felt (about 0.5 m²/g),^{64, 66} a widely used MFC anodic material. The specific surface area decreased with increasing PANI content in the composite, which

could possibly be attributed to the smaller specific surface area of PANI than TiO₂, the deposition of which could reduce the specific surface area of the composite. The isotherm displays a hysteric loop, which is a characteristic of the adsorption-desorption of a porous material. The inset of Figure 5.3 also shows the narrow and uniform pore-size distribution of the composite, with an average pore diameter in the range of 6-8 nm, which is much smaller than the diameter of the bacteria (about 0.5-2 μ m). Thus, the bacteria could not enter into and clog the pores to cause their death from lack of nutrients,³ while reducing the reaction surface area as well. The results obtained for TiO₂ and other

Figure 5.3 Specific surface area and average pore diameter of PANI/TiO₂ nanocomposites.

The insets are the N₂ adsorption– desorption isotherm (large) and BJH pore size distribution (small) of the composites containing 30 wt % PANI.

composites with different percentages of PANI which are not presented here, are similar to those shown in Figure 5.3.

5.4 Catalytic behaviour of PANI/TiO₂ composite electrodes.

5.4.1 Electrochemical impedance spectra

The complex impedance (Z) vs. frequency, known as a Nyquist plot, was measured with TiO₂ and four composite electrodes in an anaerobic culture of *E. coli* K-12 with 55 mM glucose and 5 mM HNQ. In Figure 5.4 the inset illustrates the high-frequency part of the result. The Nyquist plots of all four composite electrodes represent well-defined frequency-dependent semicircle impedance curves over high frequencies followed by straight lines, but the TiO₂ electrode has no defined semicircle. A Randle equivalent circuit¹¹⁴ is often used to model the complex impedance in an electrochemical cell, in which the charge-transfer resistance (R_{ct}) at the electrode/electrolyte interface is equal to the diameter of the semicircle. A smaller R_{ct} indicates a faster electron-transfer rate. The result in Figure 5.4 indicates that R_{ct} is remarkably reduced after PANI is deposited onto TiO₂ and is further decreased with increasing PANI content in the composite electrode. However, after 20 wt % PANI is deposited on TiO₂, further increasing the PANI content has no significant effect on reducing R_{ct} . The results here reveal that TiO₂ has no electrocatalysis on glucose oxidation,

and its catalytic performance can be significantly improved by PANI deposition on its surface. This can possibly be ascribed to the fact that the addition of PANI forms a nanostructured network (Figure 5.2), which could enhance the electron-transfer rate. At low frequencies, the inclined line with a slope higher than 45° and close to 90° demonstrates a non-ideal capacitive behavior, a characteristic of a porous conducting film.¹²³⁻¹²⁵ For the PANI/TiO₂ electrodes, the slope is lower than that of TiO₂ electrode and closer to 45° , indicating an anion Warburg resistance of the PANI modified electrodes.

Figure 5.4 Nyquist plots (measured over a frequency range of 0.1 Hz – 100 KHz at -310 mV vs. Ag/AgCl with a perturbation DC signal of 10 mV, room temperature) of different electrodes in an anaerobic culture of *E. coli* K-12 containing 55 mM glucose, 0.5 mM HNQ, and 0.1 M phosphate buffer (pH 7.4).

5.4.2 Cyclic voltammograms

The CVs shown in Figure 5.5A, which were measured in 0.1 M phosphate buffer with *E. coli* cells containing 5 mM HNQ, all have a pair of well defined redox waves, obviously due to the redox reaction of HNQ. The charge capacities calculated from the CVs (inset of Figure 5.5A) show that the charge capacity increased with increasing PANI content in the composites up to 30 wt %, at which the maximum value was reached and subsequently decreased with further increases in the PANI content. Because the charge capacity of an electrode for a specific reaction is known to be proportional to the electrode surface, this result indicates that the reaction surface area increases with increasing PANI content until reaching its maximum and then decreases with further increase in the PANI content. The CVs in Figure 5.5B were measured in a 0.1 M phosphate buffer with *E. coli* cells containing 5 mM HNQ and 55 mM glucose, with the current axis having the same scale as that in Figure 5.5A for comparison. Similarly to panel A, panel B also exhibits a pair of well-defined redox waves, but also exhibits significant differences. Panel B's anodic peak current is much larger than its cathodic peak and, for the electrodes with same percentage of PANI, the anodic peak current in panel B is much larger than that in panel A, although their cathodic peak currents are almost identical. It could be clearly observed in Figure 5.5C that the larger anodic current is due to the glucose oxidation. After subtracting the background produced by HNQ, only

the anodic waves can be observed (not shown in Figure 5.5), revealing that the glucose oxidation on the *E. coli*/PANI/TiO₂ electrode is a completely irreversible electrochemical reaction. This is in agreement with our reported works.^{126, 127} The redox potential range of the glucose oxidation, from -0.4 to -0.2 V (Figure 5.5B), is the same as that of HNQ (Figure 5.5A), indicating that HNQ is an electron-transfer mediator between the nonconductive *E. coli* cells and the electrode.^{128, 129} The inset in Figure 5.5B clearly shows that the relationship between the anodic peak current of the glucose oxidation and the PANI percentage in the composites follows the same trend as the relationship between the charge capacity and the PANI percentage (inset in Figure 5.5A). The highest peak current, for glucose oxidation with a 30% PANI composite electrode indicates the best bio- and electrocatalysis of the glucose oxidation.

The peak current of a completely irreversible electrochemical reaction in CV is proportional to the electrode surface (A), bulk reactant concentration (C_0), and reactant diffusion coefficient (D_0).¹¹⁴ In Figure 5.5, C_0 and D_0 can be considered roughly constant. As discussed above, the effective reaction surface of the electrode is proportional to its charge capacity, which is why the relationship between the peak current of glucose oxidation and the PANI percentage in the composites follows the same change trend as the relationship between the charge capacity and the PANI percentage (insets in Figure 5.5 A, B). However, both charge capacity (Figure 5.5 A inset) and peak current

Figure 5.5 Cyclic voltammograms of different PANI/TiO₂ composite electrodes (1 cm² of working electrode area) in an anaerobic suspension of *E. coli* K-12 containing 5 mM HNQ without glucose (A) and with 55 mM glucose (B): (a) 10 wt % PANI/TiO₂; (b) 20 wt % PANI/TiO₂; (c) 30 wt % PANI/TiO₂; and (d) 50 wt % PANI/TiO₂. The inset of panel A displays the charge capacity of the different electrodes. The inset of panel B represents the baseline-subtracted peak current of glucose oxidation. (C): Cyclic voltammograms of 30 wt % PANI/TiO₂ electrode in different solutions. (Scan rate: 5 mV/s, counter electrode: 2cm × 2cm Pt film, room temperature.)

increase with increasing PANI content in the composite. It is known that a porous electrode has a large internal surface area and therefore yields a high electrochemical rate per apparent unit surface area of electrode. However the

internal area cannot, in general, be completely utilized at high current densities due to the internal effect from mass transfer and ohmic polarization in an electrolyte that is simply dependent on the pore structure¹³⁰.

On the basis of the porous electrode theory, it is possible to explain why there is an optimal PANI percentage in the composite for the best bio- and electrocatalytic behaviour. Figure 5.3 illustrates that the average pore size of the electrodes remains constant with increasing PANI content in the composite up to 30% but then decreases with further PANI increases. This may suggest that the pore structure and the utility of the electrodes are mainly determined by the TiO₂ microstructure when the PANI presents up to 30 wt % in the composite. In fact, FESEM and TEM results (Figure 5.2) show that the fundamental nanoflake pore structure of TiO₂ is not changed significantly after modification with PANI, and thus the electrode surface area can increase with increasing PANI deposit. However, when the PANI content is more than 30%, the average pore size decreases, and the internal effect could become the controlling factor in decreasing the electrode utility, which caused by reduced mass transport and higher IR polarization in the electrolyte, which suppresses the electrocatalytic current.

5.4.3 Constant current discharge

In order to explore the discharge profile of different composite anodes, an anode-limiting MFC was designed in which the Pt cathode had much larger

surface area than that of the anode, making the cathode polarization is insignificant. The volume of the MFC anode was 30 mL, the concentration of *E. coli* cells was about 1×10^9 cells mL⁻¹, and the concentrations of HNQ and glucose were as same as those in the experiments described above. The constant current discharge experiments using a MFC with four different anodes were conducted at 0.1 mA/cm². The change of the anode potential (vs. Ag/AgCl) vs. the discharge time is shown in Figure 5.6. For an anodic reaction, the more negative the anode discharge potential, the better the electrocatalysis. Apparently, the composite anode with 30 wt % PANI yields much more negative potential (-0.5 V vs. Ag/AgCl) during the discharge, demonstrating that it has the best electrocatalytic performance among the tested composites, which agrees with the CV results shown in Figure 5.5. As the discussions above, the best electrocatalytic behaviour of the *E. coli*-30 wt % PANI/TiO₂ electrode could be ascribed to its optimal pore structure for good mass transfer and low IR drops in the inner pores. Figure 5.6 also illustrates that the discharge profile of the composite/ bacteria anode is totally different from that of a conventional anode: the bacteria anode initially has a high polarization potential that gradually decreases with increasing discharge time and finally becomes constant at its lowest polarization potential, which is possibly due to the bacteria growth process. In these experiments, the discharge process started immediately after addition of the glucose solution and bacteria, which need

time to grow to their maximum metabolic level and to distribute into the inner surface of the anode for the best performance. In addition, Figure 5.6 also shows a prominent feature of the 30 wt % PANI/TiO₂ composite: after reaching its maximum, its catalytic effect improves significantly more than that of the other composite electrodes relative to their initial electroactivity, possibly indicating that it offers the best nanostructure environment for bacteria growth, which will be discussed more in the following section.

Figure 5.6 Constant current (0.1 mA cm^{-2}) discharging curve of different PANI/TiO₂ composite electrodes placed in a stirred anaerobic culture of *E. coli* K-12 in 0.1M phosphate buffer (pH 7.4) with 55mM glucose at room temperature.

5.5 Bacteria behaviors on PANI/TiO₂ composite surface.

After discharge, the surface morphology of the PANI/TiO₂ composite anode was immediately examined with FESEM (Figure 5.7). It was observed that the *E. coli* cells on the electrode surface produced some hair-like structures, reported as pili, which allow bacteria to attach to other cells and thus play a key role in mediation of the bacterial movement and biofilm formation^{131, 132}. Obviously, the pili could also promote cell adhesion on the composite surface, the host substrate. Figure 5.7a shows that many *E. coli* cells accumulated on the electrode surface and adhere to one another by means of the pili. The graphs with high magnification (Figure 5.7 b, c) clearly illustrate that the extended pili attached the cells on the electrode surface and cross-linked each other to form a network, behaviours not exhibited by free-floating cells in the electrolyte (Figure 5.7d). One possible reason is that the rough surface of the electrode could stimulate the cell to produce the pili and then to firmly attach the cells onto the electrode surface to form a superior biofilm by pili cross-linking¹³³, providing the bacteria with a sound environment for extracellular electron transfer during the electrochemical reaction.^{93, 134} It has been reported that the pili of some metal-reducing bacteria are highly conductive, and thus can directly transfer electrons to electrodes like a cable.^{91, 92} It is possible that the pili of *E. coli* cells on the composite electrode surface could also facilitate electron transfer between the cells and electrode, which could play a key role in

mediatorless *E. coli* MFCs^{96, 135} and is currently under investigation in the author's laboratory. Figure 5.7c shows a superior *E. coli* pili network, indicating that the nanostructured PANI/TiO₂ composite electrode is an excellent host for cell growth.

Figure 5.7 SEM micrographs of *E. coli* cells adhered to a 30 wt % PANI/TiO₂ electrode surface (a-c) and plain carbon cloth (d).

5.6 Performance of the PANI/TiO₂ anode in an MFC.

The performance of the PANI/TiO₂ anode was tested in a dual-chamber

MFC system that was constructed from two glass bottles (450 mL capacity) joined by a glass tube installed with a 1.5-cm diameter Nafion membrane. The anode (1.5 cm × 1.5 cm) was made of 30 wt % PANI/ TiO₂ composite (the best performer as discussed above), the compartment of which contained an anaerobic growing suspension of *E. coli* K-12 cells inoculated in a medium immediately before the test. The cathode was plain carbon cloth (2 cm × 2 cm), and the catholyte was a 50-mM ferricyanide solution with phosphate buffer identical to that in the bacterial medium. Current and potential measurements on the MFC were conducted by using a bench-top digital multimeter during discharge in constant-load mode with an external resistance of 1.95 kΩ. As shown in Figure 5.8, after bacteria enrichment for about 48 h, the power density rose to a plateau and remained at it for about 450 h without the addition of glucose or other nutrition. After 500 h from microbes inoculation, the power density dropped sharply as glucose was depleted in the anodic compartment. The discharge duration is much longer than those of the previously reported *E. coli* MFCs under the same conditions^{16, 64, 135}. This remarkable improvement indicates high energy conversion efficiency, likely resulting from the *E. coli* biofilm formed on the electrode surface, as discussed above.

Figure 5.8 Constant-load (1.95 k Ω) discharge curves of the MFC with 30 wt % PANI/TiO₂ composite anode at room temperature.

To determine the power output, various values of resistances (10–5000 Ω) were applied as external loads. The polarization curve and the power density curve of the MFC (Figure 5.9) show that the open-circuit voltage is 880 mV and the maximum power density is 1495 mW/m², corresponding to a current density of 3650 mA/m² at a cell voltage of 410 mV. As the maximal power density of *E. coli*-catalyzed MFCs¹³⁵ reported to date is 760 mW/m², the PANI/TiO₂ nanocomposite anode in this study significantly improves the power density by almost 2-fold. This new composite anode also has a greater power density than that of the PANI/CNT composite in my previous work.¹²¹ The results demonstrate that a nanoporous PANI/TiO₂ composite with an optimal PANI

content (30 wt %) can be a superior anodic material that yields a high power output.

Figure 5.9 Power output and polarization curve of the MFC with 30 wt % PANI/TiO₂ composite anode.

Table 5.1 Comparison of PANI/TiO₂/*E. coli* MFC with reported best *E. coli*-catalyzed MFC

	Anode		Cathode		Current Density (mA m ⁻²)	Power Density (mW m ⁻²)	OCV (V)
	Supporting electrode	Electrolyte volume	Electrode	Oxidant			
Ref. 135	Graphite particles film of about 150μm (thick) × 2.54 cm ² (area)	Continuous flow at 3.8 mL min ⁻¹ .	Thin layer of Pt/C catalysts	O ₂ (air)	1209	750	0.75
Present work	5 mg PANI/TiO ₂ with nickel foam support (1cm×1cm×0.1cm)	450 mL	Carbon cloth (plain, 2 cm ²)	50 mM ferricyanide in 0.1M PBS (pH7)	3650	1495	0.88

5.7 Conclusions

This chapter presented a unique nanostructured PANI/TiO₂ composite with a large specific surface area, uniform nanopore distribution, and good

biocatalytic performance. As previously described, the catalytic performance of an MFC composite anode can be optimized by adjusting the PANI percentage in the composite, and a composite with 30 wt % PANI yields the best bio- and electrocatalytic performance. In comparison to other anodes, this composite anode delivers 2-fold higher power output (1495 mW/m^2) in an *E. coli* MFC and thus has great potential for use as a high-power MFC anode. This finding may also offer a universal approach for improving other MFCs.

Chapter 6 Direct Electrochemistry of Evolved *E. coli* in MFCs^c

6.1 Introduction

Among various forms of MFCs, the mediatorless MFC, in which the metal-reducing microorganisms *Geobacter*⁹¹, *Rhodospirillum rubrum*⁷⁵ and *Shewanella*⁵⁶ directly transfer electrons to the electrode in the electrocatalytic process, is particularly attractive because of its high energy conversion efficiency and low manufacturing cost. Two models have been proposed to explain the electron-transfer process in the mediatorless MFC. One is direct electron transfer via physical contact between the bacterial outer membrane or membrane appendages and the electrode surface. This model is based on the capacity of some *Geobacter* and *Shewanella* strains that evolve electronically conducting molecular or pili to facilitate distant electron transfer.^{91, 92} Another model involves a secondary metabolites (endogenous redox mediators)-mediated electron-transfer process, such as a model in which pyocyanine and phenazine-1-carboxamide produced by *Pseudomonas aeruginosa* mediate the electron transfer to the MFC anode.¹⁷

Recently, Zhang et al.^{96, 140} reported the development of a mediatorless

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MFC using *E. coli* as a biocatalyst, to which they attributed the direct electrochemistry of the evolved *E. coli* and possible mediator molecules excreted by the *E. coli* cells to a natural selection process. A similar phenomenon was suggested by Wang et al.,⁹⁷ in which an *E. coli*-excreted redox compound functions as a mediator for the electron transfer.

However, the direct electrochemistry of *E. coli* cells has not been systematically examined as a mechanism behind the direct electron transfer process of the cells. To fill this research gap, this study investigated the direct electrochemical behavior of electro-active *E. coli* cells evolved through the electrochemical tension during fuel cell operation. Based on the finding that a hydroquinone type endogenous compound produced by the evolved cells is responsible for the direct electrochemical redox behavior of *E. coli* cells, the author proposed that a membrane related mechanism is responsible for the quinone excretion. This study then proceeded to fabricate a mediatorless MFC to demonstrate its excellent electrocatalytic performance.

6.2 Bacteria culture

6.2.1 Isolation and culture of evolved *E. coli*

Evolved *E. coli* K-12 cells were obtained from a long term operated MFC in the previous experiment¹³⁸ described in chapter 5. After the discharge experiment, the cell suspension was collected from the MFC anode and

centrifuged to remove the medium. The cell pellets were resuspended in fresh glucose medium and inoculated on a standard glucose medium agar plate. After being cultured at 37 °C for 16~18 h, a single clone from the agar plate was inoculated into a liquid medium and cultured at 37 °C for 12 h. Then the overnight bacterial culture was harvested by centrifugation at 6000 rpm at 4 °C. After washing and resuspension in a 0.1 M phosphate buffer, the cell suspension containing 10^9 cells mL⁻¹ was used for the experiment. The CV was measured after cell density reached a plateau.

6.2.2 Culture of original *E. coli*

Original *E. coli* K-12 (ATCC 29181) was grown anaerobically at 37 °C for 12 h in a standard glucose medium. The bacterial culture was harvested by centrifugation at 6000 rpm at 4 °C and then suspended in 0.1 M phosphate buffer. After three washes, the *E. coli* cell suspension (having a concentration of about 10^9 cells mL⁻¹) was used for electrochemical experiments. The CV was measured after cell density reached a plateau.

6.3 Electrochemical behaviour characterization

The electrochemical behavior of *E. coli* cells was investigated by using cyclic voltammetry technology in a three-electrode cell with carbon cloth working electrode. Figure 6.1a shows the CVs of the evolved and original *E. coli* cells. Whereas the CVs of evolved cells display a pair of well-defined

redox waves, the CV of original cells display no redox waves. The ratio of the anodic peak current to the cathodic peak current produced from the evolved cells is about one and the peak potential is independent of the scan rate, indicating a reversible redox reaction. The peak potential separation is around 30 mV, which is equal to the theoretical value for a two-electron reversible reaction. The linear relationship between the peak current and the square root of the scan rate (Figure 6.1b) reveals that the electrochemical redox reaction is a diffusion controlled process indicating that the direct electrochemistry of the evolved *E. coli* cells involves a diffusive, two-electron transfer redox species.

Figure 6.1 Electrochemical behaviour of *E. coli* cells on carbon cloth electrode (0.5 cm²).

(a) Cyclic voltammograms of original *E. coli* cells at 5 mV s⁻¹ (dot) and evolved *E. coli* cells at 5 mV s⁻¹ (solid), 20 mV s⁻¹ (dash), 80 mV s⁻¹ (dash-dot) in 0.1 M phosphate buffer (pH 7.4, room temperature). (b) Function of peak current vs. (scan rate)^{1/2}.

Additionally, the CVs of evolved cells show a strong dependence on the solution pH value (Figure 6.2a, b). Both oxidation and reduction peaks of the evolved cells shift negatively with an increase in pH, indicating that protons are involved in the redox reaction. The slope for the linear regression equation is -

60.7 mV, suggesting that the number of electrons transferred in the redox reaction equaled the number of protons involved.

All the results discussed above reveal that the direct electrochemistry of the cells is enabled by a diffusive redox species, which should be excreted from the evolved cells. The relationship between the growth (at room temperature) of the evolved *E. coli* cells and the corresponding anodic peak current vs. time is shown in Figure 6.2c. During the first 24 hours, the anodic current increased with the increase in cell density. After 24 hours, the cell density remained almost constant (at the stationary phase) but the oxidation current still increased until it reached a plateau at 60 h, which may indicate that the excretion process continues producing the diffusive redox species even after the cells stopped growing.

When the evolved *E. coli* cells were removed from the electrolyte, the supernatant continued exhibiting similar redox behavior (Figure 6.2d), which may be a direct and solid evidence that the direct electrochemistry is caused by certain kinds of endogenous mediators produced from the evolved *E. coli* cells rather than the cell membrane or appendages. The endogenous redox mediator may serve as a reversible terminal electron acceptor that transfer electrons from the bacterial cell to the MFC anode, especially in batch cultures, effectively facilitating the electron transfer.⁷¹

Figure 6.2 (a) Cyclic voltammograms of evolved *E. coli* cells on carbon cloth electrode (0.5 cm^2) in 0.1M phosphate buffers with different pH values at room temperature (scan rate 5 mV s^{-1}). (b) The relationship between E^0 and pH. (c) Variation in cell density (sphere) and oxidation current (diamond) of evolved *E. coli* cells during anaerobic growth at room temperature. (d) Cyclic voltammograms of carbon cloth electrode (1 cm^2) in different cell supernatants obtained at 5 mV s^{-1} , room temperature.

6.4 Mechanism analysis

6.4.1 Spectral analysis of cell supernatant

Bacterial cells can synthesize redox compounds such as cytochromes and quinones, which are the mobile electron carriers in electron transport chains¹³⁹. It is difficult to identify through CV experiments which of them is

responsible for the direct electron transfer of *E. coli* cells. Cytochrome c3, which has low redox potential ($E^0 = -233$ mV), has been isolated from anaerobically grown *Shewanella putrefaciens*¹⁴⁰. Some quinones are also widely used in MFCs as low-redox-potential mediators.

To determine which type of molecules play the key role in the direct electron- transferring process, the UV - Vis and the FTIR spectra of the supernatant isolated from the electrochemical cells, shown in Figure 6.3, were examined. Whereas the supernatant of the original *E. coli* cells displayed no obvious signal over the background in both spectral measurements, the supernatant of the evolved cells displayed strong absorption at 263 nm in the UV - Vis spectrum, indicating the aromatic structure of the compound. When compared to the spectrum of HNQ (50 nM), a widely used exogenous mediator, the supernatant of the evolved cells exhibited almost the same peak as that of HNQ near 263 nm. It displayed no absorption in the visible area, suggesting that the supernatant may not contain cytochromes. The strong band at 1590 cm^{-1} in the FTIR spectrum indicates the vibration of the C=C stretch in the aromatic ring, while the broad strong peak at 3284 cm^{-1} should be assigned to -OH stretching. The characteristic bands (-C=O-) of quinone within the frequency range of $1600\text{-}1800\text{ cm}^{-1}$ are not shown in the spectrum, which suggests that the redox compounds in the supernatant might be hydroquinones rather than quinones. Combining the electrochemical data that pH dependent

electrochemical behavior of the evolved cells is similar to that of quinone – hydroquinone redox couples and results of the UV – Vis and FTIR spectra, it can be concluded that the compounds in the supernatant are hydroquinone derivatives and according to the absorption value at 263 nm, it can be estimated that the concentration of the hydroquinone derivatives is around 150 nM. Further investigation is needed to confirm the exact chemical structures of the redox compounds.

Figure 6.3 UV-Vis absorption and FTIR spectra of different forms of cell supernatant.

6.4.2 Surface morphology of *E. coli* cells

In bacterial cells, quinones and hydroquinones are always in the quinone pool located at the cytoplasm membrane. Because the redox state of the quinone pool depends on aeration, the quinone pool is reduced in the absence of oxygen, allowing the hydroquinones to become the dominant molecules in the quinone pool. This is in accordance with the results described above. Although hydroquinones can diffuse freely in the membrane and periplasmic space, it is difficult for them to move across the cell wall. However, as evolved *E. coli* cells have been observed to excrete endogenous hydroquinone derivatives while the original cells could not, there must be a pathway for the hydroquinones to move through the cell wall. To investigate this mechanism, the surface of *E. coli* cells was examined with an AFM and FESEM. The AFM images of the cell surfaces show that the evolved cells have a much rougher surface morphology than the original cells (Figure 6.4 a, b). Some large clusters appear on the surface of the evolved cells with pores deeper and larger than those of the original cell. The SEM micrographs display the same phenomena with AFM images (Figure 6.4 c, d). From the section curves, it can be observed that pores on the surface of evolved *E. coli* cells are about 5-7 nm in depth, whereas the cell wall of an typical *E. coli* cell is generally 9-10 nm thick, and consists of a 2 - 3 nm peptidoglycan inner wall and a 7-nm outer membrane. It is possible that the outer membrane of the evolved cells has changed in responses to long-term

electrochemical tension in the environment, making their pores increasingly permeable to hydroquinones and thus allowing excreted hydroquinones to reach the electrode surface to transfer electrons through the membrane.

Figure 6.4 AFM topographs and section analysis of original (a) and evolved (b) *E. coli* cells together with the SEM micrographs of original (c) and evolved (d) *E. coli* cells. The insets of (c), (d) are micrographs at high magnification.

6.4.3 Proposed mechanism for direct electrochemistry

In the electron transport chain of bacteria, quinones accept electrons from donors such as NADH to become hydroquinones and then re-oxidized to quinones by electron acceptors such as O₂ (aerobic) or fumarate, NO₂, NO₃ (anaerobic). The cycling of quinone/hydroquinone is essential for electron transport within *E. coli* cells. Because an anaerobic electrochemical cell only containing a phosphate buffer solution, there is no electron acceptor for accumulated hydroquinones except the anode. This may account for the electrochemical redox behavior of the evolved *E. coli* cells in phosphate buffer even without glucose. When glucose was added to the suspension, it increased the oxidative current and enhanced the electron transfer rate. Apparently, this can be attributed to the increased amount of an electron donor-NADH (nicotinamide adenine dinucleotide hydrogen), the product of glucose oxidation in *E. coli* cells. Because the reduction of quinones in the plasma membrane is accompanied by dehydrogenation of NADH¹⁴¹, the NADH concentration is the limiting factor for the quinone redox reaction. This indicates that the electron flow in this system might be: NADH → quinone → hydroquinone → electrode. The electro-oxidized quinones can move into cells and be reduced by the NADH dependent quinone reductase¹⁴² located at the plasma membrane to again become hydroquinones again. The possible quinone-redox cycle and electron-transport path in this membrane-electrode system is illustrated in

Figure 6.5. The hydrophilicity of the derivative is important for the cross-membrane-diffusion and related reductases in the redox reaction need to be identified in further investigations.

Figure 6.5 A hypothetical mechanism for extracellular electron transport of evolved *E. coli* cells.

6.5 Performance of evolved *E. coli* in an MFC

To evaluate the performance of these evolved *E. coli* cells in power generation, a mediatorless MFC with the same configuration as that in the previous experiment was constructed and the mesoporous PANI/TiO₂ nano composite was used as anodic material. This mediatorless MFC delivered a maximum power density of 1300 mW m⁻² (Figure 6.6), corresponding to a current density of 3390 mA m⁻² at a cell potential of 340 mV, which was much

higher than that previously reported for an *E. coli* mediatorless MFC¹³⁵ and comparable with the original *E. coli*/HNQ MFC¹³⁸. Considering that the concentration of HNQ might be much higher than that of the endogenous mediators produced by evolved *E. coli* cells as previously described, these endogenous mediators may be able to efficiently enhance the electron transfer rate between the electrode and *E. coli* cells.

Figure 6.6 Power output and polarization curve of a mediator-less MFC using evolved *E. coli* cells as biocatalyst (anode: PANI/TiO₂/nickel foam, 2.25cm² with 55 mM glucose; cathode: carbon cloth, 4cm² with 50 mM ferricyanide solution with 0.1 M phosphate buffer).

6.6 Conclusions

The *E. coli* cells evolved under electrochemical tension in an MFC possess direct electrochemical behaviour. According to the electrochemical results and spectral analysis data, some hydroquinone derivatives generated by *E. coli* cells may serve as electron mediators. The highly permeable outer membrane shown in AFM and SEM micrographs facilitates the diffusion of mediators. The MFC catalyzed by evolved *E. coli* cells displays excellent performance, demonstrating that the endogenous mediators can efficiently enhance the electron-transfer rate between the electrode and *E. coli* cells.

Chapter 7 GldA Overexpressing-Engineered *E.coli* as a Superior Electrocatalyst for MFCs^d

7.1 Introduction

Since MFCs are catalyzed by bacteria, their performance is greatly dependent on the catalytic activity of bacteria cells⁴. *E. coli* is a readily available and easily grown bacterium and has become a popular biocatalyst used in MFCs^{12, 50}. Due to its non-conductive nature, the *E. coli*-based MFC usually requires mediators to transfer electrons generated from redox reactions inside *E. coli* cells on to an electrode. The application of the artificial mediators in MFCs suffers from potential cell toxicity, high operation cost and thus difficult for scale-up^{11, 56}. In chapter 6 the author has reported an evolved *E. coli* possesses direct electrochemistry behavior through self-generated hydroquinone derivatives as electron transfer mediators¹⁴³. The endogenous redox mediators are of great importance in MFC applications as they are regenerating and avoiding the addition of the artificial redox shuttles. Particularly, these mediators are more efficient than artificial mediators in an electrocatalytic process, thus increasing the current density⁷⁶. However, generating a large number of the bacteria-excreted mediators in order to significantly improve the MFC

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performance is a great challenge¹⁴³.

The direct electrochemistry behavior of evolved *E. coli* strain inspired the author to create a new strain *E. coli* producing more endogenous electron mediators by introducing specific enzyme genes. The gene *gldA* that encodes the glycerol dehydrogenase (GldA) was selected to construct engineered *E. coli* strain. GldA is known as NADH-linked dehydrogenase, which has similar function with the quinone reductase mentioned in chapter 6. While GldA can catalyze various substrates and most of the products are small water-soluble redox molecules like aminoacetone and 1,2-propanediol^{144, 145}, which have properties similar to those used as the electron mediators in MFCs. The GldA-overexpressed engineered *E. coli* strain may be able to serve as MFC biocatalysts.

7.2 Preparation of bacteria cultures

7.2.1 Construction of engineered *E. coli*

The GldA-overexpressed recombinant *E. coli* strain was generated with a standard procedure as shown in Figure 7.1. The genomic DNA of *E. coli* K-12 strain was isolated by using the AxyPrep Bacterial Genomic DNA Miniprep kit. Gene *gldA* (Gene ID: 948440, NCBI) was linked with pET-21b vector and transformed into *E. coli* BL21(DE3), a strain designed as a host for pET expression vectors¹⁴⁶. After selection with a solid LB medium containing 100

$\mu\text{g/ml}$ ampicillin, one positive colony of engineered bacteria was inoculated in a 100-ml LB liquid medium. When the OD_{600} value of the bacterial culture reached 0.5, Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to obtain a final concentration of 0.5 mM to induce overexpression of GldA for about 4 hours. SDS-PAGE was performed to analyze the expression of GldA in BL21 (DE3) and the gel image was captured by a UV Transilluminator (Bio-Rad Laboratories).

Figure 7.1 Scheme of construction of GldA-overexpressed *E. coli* strain.

7.2.2 Bacteria culture

Four different *E. coli* strains including host strain BL21(DE3), GldA-overexpressed recombinant BL21(DE3), BL21(DE3) with vacant plasmid (pET-21b) and evolved *E. coli* were grown at 37°C in flasks containing 100 mL LB

medium for use in MFCs. Before inoculation of GldA-overexpressed *E. coli*, ampicillin (100µg/ml) was added in the LB medium, followed by addition of IPTG at OD₆₀₀ of ~ 0.5 to induce GldA-overexpression. After the cell density reached 1×10^9 cells per ml (OD₆₀₀ =1) the cell cultures were transferred into the MFC anodic chamber, into which nitrogen was purged for one hour to remove the oxygen. Glucose (20 mM) was then added into the chamber before all the MFC tests.

7.3 Overexpression of GldA in *E. coli*

The SDS-PAGE image of the IPTG-induced GldA-overexpressed *E. coli* cells grown from the positive clone showed a dark broad band at ~40 kD, which was different from host *E. coli* cells, indicating the successful overexpression of GldA in the recombinant cells (Figure 7.2).

Figure 7.2 SDS-PAGE analysis of total proteins in host cell (BL21(DE3)) and engineered cell.

7.4 Electrochemical behaviour of engineered *E. coli* in MFCs

The electrochemical behavior of engineered *E. coli* was investigated by using cyclic voltammetry technology in a three-electrode cell with carbon cloth working electrode. As shown in Figure 7.3a, the CVs of the host cells and vacant plasmid contained host cells displayed no redox peak. The CV of the engineered cells displayed a pair of well-defined reversible redox waves at ~ -0.4 V indicating that the overexpression of GldA in host *E. coli* alters the non-conductive host strain into an electroactive one. According to the CVs at different scan rates (Figure 7.3b), a linear relationship exists between the current and the square root of the scan rates, suggesting that a diffusive electroactive reactant is responsible for the redox reaction. The CV of the evolved *E. coli* cells (Figure 7.3a) exhibited a pair of poorly shaped redox peaks and a much lower peak current (0.116 mA cm^{-2}) than that (0.196 mA cm^{-2}) of the GldA-overexpressed *E. coli* cells. The more prominent redox peaks with better similar shape and equal anodic and cathodic peak current of GldA-overexpressed *E. coli* cell indicate their better reversibility while the much larger peak current shows that the engineered cells produce many more redox mediators.

Figure 7.3 (a) Cyclic Voltammograms of MFC anodes using different *E. coli* strains as biocatalysts (supporting material: carbon cloth, 1.7 cm²). Scan rate is 30 mV s⁻¹. (b) Cyclic Voltammograms of GldA-overexpressed *E. coli* catalyzed anode at different scan rates (inset) and plots of peak current density versus square root of scan rates.

EIS was measured at open-circuit potentials to evaluate the charge-transfer resistance (R_{ct}) of different cells on the anode. The Nyquist plots showed a frequency dependent semicircle over high frequencies (Figure 7.4), in which the R_{ct} is equal to the semicircle diameter. A smaller R_{ct} indicates a faster charge-transfer rate. Figure 7.4 clearly shows that both the engineered and evolved *E. coli* cells had a smaller R_{ct} (125 Ω and 127 Ω respectively) than the host cells and vacant-plasmid contained host cells (485 Ω and 510 Ω respectively). These results demonstrate that the redox mediators from both the engineered and evolved cells facilitate the electron transfer between cells and the electrode, with the genetically engineered *E. coli* cells producing many more mediators due to the overexpression of GldA.

Figure 7.4 Nyquist plots of MFC anodes using different *E. coli* strains as biocatalysts (obtained over a frequency range of 0.1 Hz – 100 KHz with a perturbation DC signal of 10 mV at 30 °C).

7.5 Performance of MFC catalyzed by engineered *E. coli*

Two mediatorless MFCs were constructed with carbon cloth electrodes, one of which catalyzed by evolved *E. coli* cells and one GldA-overexpressed *E. coli* cells, to evaluate their direct electrochemical catalytic performance. The output current profile of the MFCs shown in Figure 7.5, measured with a fixed output load resistance (1.96 kΩ), displays that although the current of both MFCs increased for the first 12 hours before reaching a plateau, the GldA-overexpressed *E. coli* catalyzed MFC yielded a much larger output current, indicating its high current efficiency. The amount of time necessary to achieve

the maximum output current was possibly due to the cell proliferation and redox-mediators production. Fuel cell performance can be assessed by cell polarization and power curves.

Figure 7.5 Current generation profiles of evolved-*E.coli* MFC and GldA-overexpressed-*E. coli* MFC.

The polarization and power curves shown in Figure 7.6 were obtained by varying the output load resistance. The polarization curves show that although the open circuit voltage (OCV) of evolved-*E. coli*-MFC (0.73V) is slightly higher than that of GldA-overexpressed-*E. coli*-MFC (0.67V), the former operational voltage drops much faster than the later one as increase of the current density. The lower polarization performance of the GldA-overexpressed *E. coli*-catalyzed anode reaction clearly indicates that the engineered cells have better electrocatalytic performance than evolved cells.

Accordingly, the GldA-overexpressed-*E. coli* MFC yielded much higher power density (640 mW m^{-2} per 10^{11} cells), two folds more than the evolved-*E. coli* MFC (271 mW m^{-2} per 10^{11} cells).

Figure 7.6 Power output (solid) and polarization (hollow) curves of evolved-*E.coli* MFC and GldA-overexpressed-*E. coli* MFC with carbon cloth anodes.

Since carbon cloth electrodes have a smaller specific surface area, the performance of the two MFCs was also tested with a carbon nano cage (CNC)¹⁴⁷ anode as shown in Figure 7.7. This test demonstrated that GldA-overexpressed-*E. coli* MFC yielded a much higher maximum power density (1304 mW m^{-2} per 10^{11} cells) than that of evolved-*E. coli* MFC (680 mW m^{-2} per 10^{11} cells). The mesoporous structure and large specific surface area of CNC provide more reactive centres for the redox reaction of redox mediators.

The maximum power density delivered by the GldA-overexpressed-*E. coli* MFC was also much higher than that of other reported mediator-less *E. coli* MFCs⁹⁶. The superior electrocatalytic performance of the GldA-overexpressed *E. coli* cells can be ascribed to their containing many more cellular redox mediators and having better redox reversibility.

Figure 7.7 Power performance curve of evolved-*E.coli* MFC and GldA-overexpressed-*E. coli* MFC with carbon nano cage anodes.

The cellular redox mediator produced from GldA-overexpressed *E. coli* cells has not been clearly identified. The mechanism to produce the superior mediator from the cell engineered by GldA overexpressing could be understandable. The *gldA* gene is cryptic in wild-type *E. coli*¹⁴⁸ and repressed under anaerobic glucose fermentation¹⁴⁹, while GldA is involved in the

metabolism of threonine, aminopropanol, glycerol and L-1,2-propanediol in a few *E. coli* mutants^{144, 145, 150, 151}, in which the GldA is highly expressed. GldA is also directly associated with pentose phosphate pathway and thus very likely resulting in products containing the superior endogenous redox mediator. The metabolic pathways in which GldA participates and the source of the mediators remains under investigation in the author's lab. The activity of glycerol dehydrogenase and the glucose concentration, which are highly important in explaining the mechanism, are also under investigation.

7.6 Conclusions

In conclusion, due to the large amount of endogenous redox mediators, the GldA overexpressing engineered *E. coli* cells demonstrate superior electrocatalytic performance compared to both original *E. coli* cells and the evolved cells previously developed by this author. Engineered *E. coli*-based MFCs yield lower polarization and higher power density in comparison to other *E. coli*-based MFCs. The study described in this chapter demonstrates that engineering *E. coli* cells by introducing the appropriate oxidoreductase via gene manipulation can significantly improve their direct electron-transfer capability and, as such, is an efficient and economic means of biologically engineering bacteria to improve MFC performance.

Chapter 8 A Time-Course Transcriptome Analysis of *E. coli*

Direct Electrochemistry in MFCs^e

8.1 Introduction

In chapter 6 the author has demonstrated that the *E. coli* evolved under long term electrochemical tension possessed direct electrochemical behavior, implying that *E. coli* can transport electrons to electrodes without the need for an additional artificial redox mediator. This mediator-less MFC catalyzed by the evolved *E. coli* yields much higher power output, reduces operation expense and avoids potential toxic effects of artificial mediators¹³⁶. The author has proposed that the self-generated mediators are likely quinone derivatives and that the facilitation of the mediator shuttle between the cells and the electrode¹⁴³ could be attributed to morphological changes on the membrane surface. Zhang *et al.* have attributed the direct electron transfer of *E. coli* to a natural selection process in a fuel-cell operation environment in which the metabolites serves as electron mediators¹³⁷. Despite these explanations, the detailed mechanisms involved in the direct electrochemistry process of *E. coli* in MFCs remain obscure. It is also very important to investigate the changes in *E. coli* cells'

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electrochemical behavior during the evolution process under electrochemical tension. Consequently, to advance understanding, this study investigated the time-dependant electrochemical behavior of original (wild type) *E. coli* K-12 cells in MFCs and the corresponding genome-wide changes in *E. coli* gene transcription.

Microarray-based transcriptome analysis, which enables simultaneous and global examination of the complete transcriptional response at the genome level, has been widely used to explore the cellular responses of *E. coli* and other strains¹⁵². This study used *E. coli* whole-genome microarrays to investigate the dynamics of the global gene expression profiles at 6, 12 and 24 hours during the MFC discharge process. To the best of the author's knowledge, this is the first study to examine the direct electrochemistry behavior of *E. coli* catalysis in MFCs at the genomic level. The results presented here may thus facilitate the elucidation of the cellular mechanisms underlying the direct electron transfer between *E. coli* cells and MFC electrodes.

8.2 Preparation and measurement

8.2.1 MFC operation and sampling

Dual-chambered MFCs with glucose anode and ferricyanide cathode were used to evaluate the performance of the devices. Both electrodes were plain carbon cloth. The MFCs were operated under an 1.96 k Ω external load at

30 °C. The current was recorded by using a bench-top digital multimeter (ESCORT 3146A). The *E. coli* cells (1 mL) near the anode (noted as MFC-*E. coli*) were collected with an autoclaved pipette tip at three time points (6 h, 12 h, 24 h) for RNA isolation. For the control group, original *E. coli* cells were cultured in same MFC devices but without the electrodes.

8.2.2 Total RNA isolation

E. coli cells were harvested by centrifugation (>8,000×g) at different time points, and then incubated in a TE buffer with 1 mg/ml of lysozyme. RNA was isolated immediately using an RNeasy Mini Kit according to the manufacturer's protocol. Finally, the samples were eluted with 50 µl of nuclease-free water (Ambion). RNA quality, purity, and integrity were determined using both a NanoDropTM 1000 Spectrophotometer (Thermo Scientific) and an RNA 6000 Nano LabChips with an Agilent 2100 Bioanalyzer (Agilent Technologies).

8.2.3 cDNA synthesis and labeling

cDNA was synthesized, purified and labeled from 10 µg of RNA with a FairPlay® III Microarray Labeling Kit (Stratagene) according to the protocol provided by the manufacturer. The fluorescent dye-labeled cDNA was purified using the DNA-binding solution and the microspin cups provided in the kit.

8.2.4 Hybridization and scanning

Microarray hybridizations were conducted on Agilent *E. coli* oligo microarrays using a 300-ng Cy₃-labeled “control” sample and a 300-ng Cy₅-labeled “experimental” sample. After the hybridizations were conducted using the Agilent hybridization kit and a hybridization oven, the microarray slides were washed with Gene Expression Wash Buffer 1 & 2 (Agilent) containing 0.005% Triton X-102. The slides were scanned by the Agilent G2565BA Microarray Scanner.

8.2.5 Data analysis

Data analysis was performed using the Feature Expression Software (Agilent Technologies) v. 10.5 and GeneSpring GX v. 10 (Agilent Technologies). Genes that received “present” and “marginal” calls from 50% or more of the replicates in all conditions were used for the analysis. The gene expression changes with statistical significance were identified by an upper one-tailed *t* test (*p* cutoff value, 0.05). “Fold change” was calculated as the ratio between the signal averages of four control and four treated cultures. Genes with a two-fold or greater induction or repression in at least one time point were used in this analysis.

8.3 Time-dependent electrochemical behavior analysis

8.3.1 MFC current generation profile

The current generation profiles in the operated MFC with and without *E. coli* K-12 cells in Figure 8.1a show that the current of the *E. coli* MFC increased rapidly between 6 and 16 hours before reaching a plateau of 0.034 mA/cm² at 24 hours. Clearly, the current density of the MFC without *E. coli* remained at very low level (0.011 mA/cm²). To investigate whether the current generation profile was related to cell growth phase (or cell density), the growth curve of *E. coli* in the anodic chamber was recorded (Figure 8.1b). The control group *E. coli* cells were cultured under the same conditions with the MFC-*E. coli* cells but without the electrodes. The results show that the cell growth in the experimental MFC reached the stationary phases 3 hours later than that in the control group. Interestingly, even after the cell density reached the stationary phase (after 15 hours of growth), the current continued increasing until achieving a plateau after 24 hours of discharge. The results suggest that the generated current is dependent not only on the cell number but also on the cells catalytic activity. Since the current is measured with a fixed external load, the electrode process is under a kinetic control rather than a diffusion limit. Thus, the MFC-*E. coli* achieves its maximum catalytic activity after 24 hours of discharge, clearly indicating its catalytic performance is related to the discharge time.

Figure 8.1 (a) Current generation profile of the MFC. (b) Growth curve of control *E. coli* and MFC-*E. coli* cells at 30°C.

8.3.2 Time-dependent CV and EIS of *E. coli* anode

This study further examined the electrochemical behaviour of the anode at sequential time points, selecting the points of 6, 12 and 24 hours of discharge because they corresponded to the points at which the output current to start increasing, then rapidly increases and then reaches a plateau, respectively. As shown in Figure 8.2, the CVs at 0 and 6 hours display no redox peaks, but after 12 hours a pair of broad redox peaks occurs at around 0.2V, which indicates the production of direct electron shuttles by MFC-*E. coli*. In contrast, the CV after 24 hours has two different pairs of redox peaks, both of which are lower than 0.2 V (Table 8.1). The two anodic half wave potentials ($E_{a1/2}$) of the CV after 24-hour discharge are -36 and -297 mV, which are much more negative than that of the CV after 12 hours (194mV), clearly indicating their better electrocatalytic kinetics. As all the

redox behaviors display diffusion-controlled electrode kinetics, a very likely reason is that more effective electron mediators are generated by MFC-*E. coli* at a longer discharge time, an explanation in good accordance with the author's previous study¹⁴⁶. The peak separations of both pairs of redox peaks at 24 hours (63 and 49 mV) are much smaller than that at 12 hours (139 mV; Table 8.1), further indicating a faster electron-transfer process that allows for better reversibility. This finding may also indicate that the later-generated electron shuttles possess more rapid redox reactions than the early produced (12-hour) shuttles, thus providing greater electrocatalytic activity.

It is noted that the redox peak potential or even the shape of CVs at 12 hours and 24 hours are different with the evolved *E. coli* anode mentioned in Chapter 6 because the evolution is a very slow process. While the CV recorded after 192 hours (Figure 8.2 inset) shows similar redox behavior with the evolved *E. coli* anode at pH 5.2 (same pH with the anode after 192 hours discharge). This provides a solid evidence for the evolution process of *E. coli* under an electrochemical tension.

Figure 8.2 CVs of MFC anode obtained at different time points. The inset shows CVs of MFC anode at 24-hour and 192-hour within potential range of 0V~-0.6V vs. SCE (scan rate: 30 mV s⁻¹, measured at 30 °C with SCE as reference electrode inserted in cathode compartment.)

Table 8.1 Parameters obtained from CVs

	0 hour	6 hours	12 hours	24 hours	
E _{pa} (mV)	-	-	257	-2	-268
E _{pa} /2 (mV)	-	-	194	-36	-297
E _{pc} (mV)	-	123	118	-65	-317
ΔE _p (mV)	-	-	139	63	49
i _{pa} (μA)	-	-	-90.15	-153.9	-120
i _{pc} (μA)	-	68.6	201	91.91	354

The electrochemical impedance spectra of the anode were also

examined at the three time points. The measured plots of complex impedance Z (imaginary) versus Z (real), known as Nyquist plots (Figure 8.3a), were simulated by the commonly used equivalent circuit (Figure 8.3b) for the charge-transfer resistance (R_{ct}). The results indicate that R_{ct} decreases from 285Ω to 107.5Ω as the discharge time increase (Table 8.2), which agrees with the CV results. The supernatant of the cell has the same electrochemical behaviour, which is in accordance with the author's previous report¹⁴³. In line with this result, *Geobacter sulfurreducens* generates different external redox molecules for optimizing the electrical connection and current production when the electrode is polarized at different potentials¹⁵³. Similarly, the variation of the anodic potential during discharge might induce the excretion of different mediators.

Figure 8.3 (a) Nyquist plots of the MFC anode measured at different time points (obtained over a frequency range of 0.1 Hz – 100 KHz with a perturbation DC signal of 10 mV at 30 °C). (b) Simulated Nyquist plots and equivalent circuits used for calculation.

Table 8.2 Calculated impedance values from equivalent circuit

	0-hour	6-hour	12-hour	24-hour
R_{Ω} (Ω)	3.3	2.1	1.9	1.6
R_{ct} (Ω)	285	207	150.5	107.5

8.4 Time-dependent surface morphology of *E. coli*

In the author's previous study, it was reported that the electroactive *E. coli* cells had a much rougher membrane surface than do original (non-electroactive) cells¹⁴³. To explore the time-dependent effects of the discharge process on the cell envelope, the cell-surface morphology was examined by AFM after 6, 12, 24 hours of discharge, respectively (Figure 8.4 a-f). The results illustrate that the roughness of the cell surface for both

the control and the MFC-*E. coli* cells increases during the discharge time course. Although deep hollows appear after 24 hours for both type of *E. coli* cells, the area of the hollows is larger for the MFC-*E. coli* than the control cells. This phenomenon was also observed by SEM (Figure 8.4 g, h). This result suggests that the cell envelopes of *E. coli* cells in MFCs undergo significant morphological changes compared to those in the control cells. These changes may facilitate the diffusion of redox mediators out of the cells, thus improving electrocatalytic performance.¹⁴³ It is in agreement with the results described in chapter 6.

Figure 8.4 Surface morphology micrographs of *E. coli* cells. AFM images of control *E. coli* cells after 6 hours (a), 12 hours (b) and 24 hours (c) growth. AFM images of MFC-*E. coli* cells after 6 hours (d), 12 hours (e) and 24 hours (f) of discharge. The insets show the section analysis. g, h: SEM micrographs of control *E. coli* (g) and MFC-*E. coli* cells (h) at 24-hour.

8.5 Time-dependent transcriptome analysis

To further investigate the cellular mechanisms underlying the direct electrochemistry of *E. coli* in MFCs, a time-course transcriptome analysis with whole-genome microarrays after 6, 12 and 24 hours of discharge was performed. Four independent microarray experiments with the control *E. coli* and MFC-*E. coli* were conducted. For the control samples, separate sets of *E. coli* grown in MFC devices without electrode at the three time points were used because it has been observed that the transcriptional profiles of *E. coli* cells are various as growing time and culture conditions.

The microarray data indicate statistically significant up- and down-regulation of 107 genes in *E. coli* during direct electrocatalysis in MFC. Figure 8.5 shows the number of differentially regulated genes in each functional class. After 6 hours of discharge, 19 genes involved in the “cellular process”, “energy metabolism” and “transcription” displayed statistically significant changes in the expression level. In the “cellular process” class, six important genes (*flgK*, *flgN*, *fliA*, *fliC*, *fliD*, and *fliS*) for flagella biosynthesis and cell motility^{154, 155} were found to be the most down-regulated genes after 6 hours of discharge. The repression of these genes was resumed after 12 and 24 hours of discharge. After 12 hours of discharge when the output current of the MFC rapidly increased, 13 genes displayed significant induction and 44 genes displayed significant repression. Most of the functional classes were affected, excluding the classes

of “fatty acid and phospholipid metabolism”, “mobile and extrachromosomal element functions” and “cell envelope”. The most affected functional classes were “energy metabolism” and “protein fate”. Of the “energy metabolism” class, 5 genes were significantly induced and 6 genes were significantly repressed, four of which, as described below, also displayed significant induction or repression after 6 and 24 hours of discharge. 12 repressed genes in the class of “protein fate” primarily encode heat shock proteins (HSPs) with chaperone function. After 24 hours of discharge, the number of induced genes increased to 22 while the number of repressed genes decreased to 26. The most regulated functional class after 24 hours of discharge was “amino acid biosynthesis”, of which 11 down-regulated genes are related to biosynthesis or transport of arginine and histidine. Because transcriptional repression is the major control mechanism of the arginine and histidine biosynthetic genes^{156, 157}, the repression of these 11 genes might result from the excess accumulation of arginine and histidine.

Figure 8.5 Functional classification of genes with statistically significant increases and decreases in mRNA level after 6, 12, and 24 hours of discharge.

To identify genes with similar transcription patterns during the time course and identify the relationship between genes and cell electrocatalytic activity, k-means clustering analysis was performed on the 107 genes. As shown in Figure 8.6, which displays the average expression patterns of six clusters, significant changes in gene expression levels occurred. From these six clusters, one can find highly induced or repressed functioning gene units (operons), which potentially provide insights into explaining the mechanism behind the direct electrochemistry of *E. coli* in MFCs. The detailed description and functional classes of these 107 genes were listed in Table 8.3 with their fold changes in expression level at three different time points.

Figure 8.6 Cluster profiles shown as average $\log_2$ ratio with standard deviation

The most intriguing operon is *cydAB* (Cluster 5), which is highly induced at all of the three time points. Cytochrome *bd-I* encoded by *cydAB*, which is one of three terminal oxidases in the respiratory chain of *E. coli*¹⁵⁸, is activated by low-oxygen condition¹⁵⁹ and oxidative stress¹⁶⁰. In this study, the same level of oxygen was provided to the control cells and the MFCs. Since the three oxidative stress protection-related genes, *soxR*, *trxC*, and *ahpF*, are repressed in the MFC-*E. coli* cells, it is possible that the MFC anode served as the final acceptor of the electron transport chain of *E. coli*, providing a “pseudo limited oxygen condition” and inducing the expression of cytochrome *bd-I*. Further investigation is needed to be conducted to verify this speculation.

Besides genes related to energy metabolism, operons encoding transport

and binding proteins were identified among the clusters. The genes related to nutrition uptake, such as galactose ABC (ATP-Binding Cassette) transporters (*mglA* and *mglB*), C₄-dicarboxylate transporter (*dctA*) and ribose ABC transporter (*rbsB*), were significantly upregulated after 24 hours of discharge. Given that these genes are up-regulated in cells grown in nutrient-limited chemostat cultures¹⁶¹, this result may imply that the uptake activity of the *E. coli* cells in MFC is higher than that of the control cells. Intriguingly, part of ABC transporter genes have been also reported to be up-regulated in *Geobacter sulfurreducens*¹⁶² during the growth with an electrode compared to the growth with Fe (III) citrate. Furthermore, an outer membrane porin gene *ompC* (Cluster 6) in this work was found to be highly induced at 24 hours. The outer membrane porin-OmpC allows for ions and other hydrophilic solutes (< 500 Daltons) to cross the outer membrane.¹⁶³ The highly expressed *ompC* might have enhanced the permeability of the cell wall so that the shuttle of small electron mediators across the cell wall was induced to transfer electrons. The time-dependent induction of *ompC* may explain why the CV curve after 24 hours of discharge displays more redox peaks than that after 12 hours (Figure 8.2). The result also reveals that a number of genes with unknown function were significantly induced or repressed, such as *yhbP* (Cluster 4), *ybeD* (Cluster 1), *yagI* (Cluster 2), *ybgE* (Cluster 6), and *ygeY* (Cluster 6). Further investigation is necessary to understand the specific roles of these genes in the

direct electrocatalytic activity of *E. coli* in MFCs.

Table 8.3 *E. coli* K-12 genes that showed statistically significant mRNA level changes at one of the three discharge time points.

b number	Gene Name	Description	Functional class	Fold Change		
				6-hr	12-hr	24-hr
Cluster 1						
b0660	ybeZ	putative ATP-binding protein in pho regulon	Cellular processes	-1.30	-2.01	-1.13
b2669	stpA	DNA-binding protein; H-NS-like protein; chaperone activity; RNA splicing?	DNA metabolism	1.16	-2.30	-1.02
b3635	mutM	formamidopyrimidine DNA glycosylase	DNA metabolism	1.27	-2.98	1.59
b0629	ybeF	putative transcriptional regulator LYSR-type	Energy metabolism	1.04	-2.27	-1.20
b0492	ybbN	putative thioredoxin-like protein	Hypothetical proteins	1.04	-2.59	1.43
b0631	ybeD	orf, hypothetical protein	Hypothetical proteins	1.05	-3.52	1.19
b0659	ybeY	orf, hypothetical protein	Hypothetical proteins	-1.11	-2.03	-1.03
b1321	ycjX	putative EC 2.1 enzymes	Hypothetical proteins	-1.14	-2.49	1.23
b1322	ycjF	orf, hypothetical protein	Hypothetical proteins	1.06	-2.38	1.34
b1521	uxaB	altronate oxidoreductase	Hypothetical proteins	-1.01	-3.72	-2.17
b2726	hypA	pleiotrophic effects on 3 hydrogenase isozymes	Hypothetical proteins	1.24	-2.74	-1.08
b2731	fhlA	formate hydrogen-lyase transcriptional activator for fdhF, hyc and hyp operons	Hypothetical proteins	1.09	-2.01	-1.49
b3400	hslR	orf, hypothetical protein	Hypothetical proteins	-1.11	-3.98	1.35
b3401	hslO	orf, hypothetical protein	Hypothetical proteins	1.05	-3.79	1.41
b4173	hflX	GTP - binding subunit of protease specific for phage lambda cII repressor	Hypothetical proteins	1.22	-2.26	-1.24
b0015	dnaJ	chaperone with DnaK; heat shock protein	Protein fate	-1.11	-3.00	-1.07
b0439	lon	DNA-binding, ATP-dependent protease La; heat shock K-protein	Protein fate	-1.13	-2.63	1.20
b0473	htpG	chaperone Hsp90, heat shock protein C 62.5	Protein fate	-1.30	-3.47	1.39
b0630	lipB	protein of lipoate biosynthesis	Protein fate	-1.01	-2.86	1.17

b2592	<i>clpB</i>	heat shock protein	Protein fate	-1.16	-2.96	1.20
b2614	<i>grpE</i>	phage lambda replication; host DNA synthesis; heat shock protein; protein repair	Protein fate	-1.24	-3.67	1.42
b3931	<i>hslU</i>	heat shock protein hslVU, ATPase subunit, homologous to chaperones	Protein fate	-1.05	-2.23	1.21
b3932	<i>hslV</i>	heat shock protein hslVU, proteasome-related peptidase subunit	Protein fate	-1.02	-2.72	-1.05
b4143	<i>groL</i>	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein	Protein fate	-1.24	-2.24	1.57
b4174	<i>hflK</i>	protease specific for phage lambda cII repressor	Protein fate	1.08	-2.56	-1.14
b4175	<i>hflC</i>	protease specific for phage lambda cII repressor	Protein fate	1.17	-2.11	-1.20
b4142	<i>groS</i>	GroES, 10 Kd chaperone binds to Hsp60 in pres. Mg-ATP, suppressing its ATPase activity	Protein fate	-1.61	-2.49	1.17
b4171	<i>miaA</i>	delta	Protein synthesis	1.11	-2.17	1.11
b4237	<i>nrdG</i>	anaerobic ribonucleotide reductase activating protein	Purines, pyrimidines, nucleosides, and nucleotides	1.11	-2.47	-1.45
b3292	<i>zntR</i>	putative transcriptional regulator	Regulatory functions	-1.02	-2.01	1.19
b3071	<i>yqiI</i>	orf, hypothetical protein	Transcription	1.03	-2.59	-1.70
Cluster 2						
b0273	<i>argF</i>	ornithine carbamoyltransferase 2, chain F	Amino acid biosynthesis	1.39	-1.55	-4.41
b2019	<i>hisG</i>	ATP phosphoribosyltransferase	Amino acid biosynthesis	1.22	-1.53	-2.71
b2020	<i>hisD</i>	L-histidinal:NAD+ oxidoreductase; L-histidinol:NAD+ oxidoreductase	Amino acid biosynthesis	1.14	-1.53	-3.36
b2818	<i>argA</i>	N-acetylglutamate synthase; amino acid acetyltransferase	Amino acid biosynthesis	1.14	-1.15	-2.43
b3172	<i>argG</i>	argininosuccinate synthetase	Amino acid biosynthesis	1.45	-1.13	-2.75
b3359	<i>argD</i>	acetylornithine delta-aminotransferase	Amino acid biosynthesis	1.35	-1.13	-2.06
b3958	<i>argC</i>	N-acetyl-gamma-glutamylphosphate reductase	Amino acid biosynthesis	1.26	-1.34	-3.38
b3959	<i>argB</i>	acetylglutamate kinase	Amino acid biosynthesis	1.30	-1.39	-3.79
b3960	<i>argH</i>	argininosuccinate lyase	Amino acid biosynthesis	1.50	1.03	-2.39
b4254	<i>argI</i>	ornithine carbamoyltransferase 1	Amino acid	1.23	-1.23	-2.96

			biosynthesis; Energy metabolism			
b0272	<i>yagI</i>	putative regulator	Regulatory functions	1.38	-1.18	-5.30
b3405	<i>ompR</i>	response regulator	Regulatory functions	1.01	-1.54	-2.12
b0860	<i>artJ</i>	arginine 3rd transport system periplasmic binding protein	Transport and binding proteins	1.23	-1.35	-3.26
b0862	<i>artQ</i>	arginine 3rd transport system permease protein	Transport and binding proteins	1.08	1.10	-2.08
b2309	<i>hisJ</i>	histidine-binding periplasmic protein of high-affinity histidine transport system	Transport and binding proteins	-1.07	-1.32	-2.13
b3957	<i>argE</i>	acetylornithine deacetylase	Unclassified	1.09	1.01	-2.28
Cluster 3						
b0606	<i>ahpF</i>	alkyl hydroperoxide reductase, F52a subunit; detoxification of hydroperoxides	Cellular processes	-2.06	-1.76	1.13
b2582	<i>trxC</i>	putative thioredoxin-like protein	Energy metabolism	-2.35	-2.97	-1.14
b0802	<i>ybiJ</i>	orf, hypothetical protein	Hypothetical proteins	-1.09	-1.19	2.51
b4104	<i>phnE</i>	membrane channel protein component of Pn transporter	Unclassified	1.59	1.12	2.04
b4367	<i>fhuF</i>	orf, hypothetical protein	Unknown function	-2.13	-1.88	1.04
b3913		orf, hypothetical protein	Unknown function	1.41	1.57	2.23
Cluster 4						
b3767	<i>ilvG</i>	acetolactate synthase II, large subunit, cryptic, interrupted	Amino acid biosynthesis	1.15	-1.50	-2.01
b2727	<i>hypB</i>	guanine-nucleotide binding protein, functions as nickel donor for large subunit of hydrogenase 3	Central intermediary metabolism; Energy metabolism	1.08	-2.07	-2.45
b2724	<i>hycB</i>	probable small subunit of hydrogenase-3, iron-sulfur protein	Energy metabolism	1.15	-2.17	-1.59
b2719	<i>hycG</i>	hydrogenase activity	Energy metabolism	1.15	-1.77	-2.12
b2729	<i>hypD</i>	pleiotrophic effects on 3 hydrogenase isozymes	Energy metabolism	1.09	-2.15	-2.23
b4079	<i>fdhF</i>	selenopolypeptide subunit of formate dehydrogenase H	Energy metabolism	1.09	-2.23	-2.23
b2997	<i>hybO</i>	putative hydrogenase subunit	Energy metabolism	1.04	-2.06	-1.78
b3673	<i>emrD</i>	2-module integral membrane pump; multidrug resistance	Hypothetical proteins	2.02	-1.23	-1.10
b4218	<i>ytfL</i>	putative transport protein	Hypothetical proteins	1.22	-2.10	-1.55

b4212	<i>ytfH</i>	orf, hypothetical protein	Hypothetical proteins	1.14	-2.19	-1.96
b2728	<i>hypC</i>	pleiotrophic effects on 3 hydrogenase isozymes	Hypothetical proteins	1.02	-2.45	-2.41
b3010	<i>yqhC</i>	putative ARAC-type regulatory protein	Regulatory functions	1.12	-1.68	-2.18
b4063	<i>soxR</i>	redox-sensing activator of soxS	Unclassified	1.22	-2.33	-1.65
b0674	<i>asnB</i>	asparagine synthetase B	Unclassified	1.09	-2.08	-1.69
b2730	<i>hypE</i>	plays structural role in maturation of all 3 hydrogenases	Unclassified	-1.08	-1.96	-2.06
b3154	<i>yhbP</i>	orf, hypothetical protein	Unknown function	1.17	-3.20	-2.97
b3596	<i>yibG</i>	orf, hypothetical protein	Unknown function	1.14	-2.24	-1.88

Cluster 5

b0003	<i>thrB</i>	homoserine kinase	Amino acid biosynthesis	1.06	2.40	-1.36
b0002	<i>thrA</i>	aspartokinase I, homoserine dehydrogenase I	Amino acid biosynthesis	1.12	2.32	-1.24
b0775	<i>bioB</i>	biotin synthesis, sulfur insertion?	Biosynthesis of cofactors, prosthetic groups, and carriers	2.37	2.70	1.58
b2867	<i>xdhB</i>	putative dehydrogenase	Energy metabolism	1.04	2.00	1.36
b0734	<i>cydB</i>	cytochrome d terminal oxidase polypeptide subunit II	Energy metabolism	2.47	2.99	2.08
b2870	<i>ygeW</i>	putative carbamoyl transferase	Energy metabolism	-1.71	2.73	1.76
b0733	<i>cydA</i>	cytochrome d terminal oxidase, polypeptide subunit I	Energy metabolism	2.80	2.19	1.47
b2579	<i>yfiD</i>	putative formate acetyltransferase	Hypothetical proteins	3.35	5.09	2.81
b0953	<i>rmf</i>	ribosome modulation factor	Unknown function	1.06	3.83	2.47
b1823	<i>cspC</i>	cold shock protein	Unknown function	2.04	1.29	-1.18

Cluster 6

b2871	<i>ygeX</i>	putative dehydratase	Amino acid biosynthesis; Energy metabolism	-1.20	2.59	1.00
b1925	<i>fliS</i>	flagellar biosynthesis; repressor of class 3a and 3b operons	Cellular processes	-2.25	-1.04	1.30
b1924	<i>fliD</i>	flagellar biosynthesis; filament capping protein; enables filament assembly	Cellular processes	-2.20	-1.05	-1.25
b1887	<i>cheW</i>	positive regulator of CheA protein activity	Cellular processes	-2.39	-1.07	1.18

b1070	<i>flgN</i>	protein of flagellar biosynthesis	Cellular processes	-2.25	-1.08	-1.38
b1082	<i>flgK</i>	flagellar biosynthesis, hook-filament junction protein 1	Cellular processes	-2.33	-1.11	-1.13
b1923	<i>fliC</i>	flagellar biosynthesis; flagellin, filament structural protein	Cellular processes	-2.53	-1.11	-1.04
b1882	<i>cheY</i>	chemotaxis regulator transmits chemoreceptor signals to flagellar motor components	Cellular processes	-2.01	-1.23	-1.05
b1922	<i>fliA</i>	flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons	Cellular processes; Transcription	-3.11	-1.05	-1.04
b1397	<i>paaJ</i>	putative acyltransferase	Central intermediary metabolism	-1.19	1.39	2.25
b1676	<i>pykF</i>	pyruvate kinase I	Energy metabolism	-1.37	1.38	2.30
b0849	<i>grxA</i>	glutaredoxin1 redox coenzyme for glutathione-dependent ribonucleotide reductase	Energy metabolism	-3.09	-1.64	1.05
b1789	<i>yeaL</i>	orf, hypothetical protein	Hypothetical proteins	1.07	1.74	2.07
b2151	<i>galS</i>	mgI repressor, galactose operon inducer	Regulatory functions	-1.12	1.83	2.22
b1818	<i>manY</i>	PTS enzyme IIC, mannose-specific	Transport and binding proteins	-1.14	2.53	2.51
b1819	<i>manZ</i>	PTS enzyme IID, mannose-specific	Transport and binding proteins	-1.20	2.19	2.88
b1817	<i>manX</i>	PTS enzyme IIAB, mannose- specific	Transport and binding proteins	-1.28	2.18	2.23
b3528	<i>dctA</i>	uptake of C4-dicarboxylic acids	Transport and binding proteins	1.04	1.87	2.18
b3751	<i>rbsB</i>	D-ribose periplasmic binding protein	Transport and binding proteins	-1.06	1.83	2.47
b2215	<i>ompC</i>	outer membrane protein 1b	Transport and binding proteins	1.30	1.81	2.62
b3748	<i>rbsD</i>	D-ribose high-affinity transport system; membrane-associated protein	Transport and binding proteins	-1.69	1.47	2.61
b2150	<i>mgIB</i>	galactose-binding transport protein; receptor for galactose taxis	Transport and binding proteins	-1.36	1.43	3.75
b2149	<i>mgIA</i>	ATP-binding component of methyl- galactoside transport and galactose taxis	Unclassified	-1.18	1.71	2.23
b3914		orf, hypothetical protein	Unclassified	1.44	1.69	2.21
b1921	<i>fliZ</i>	orf, hypothetical protein	Unclassified	-2.04	1.10	1.16
b2872	<i>ygeY</i>	putative deacetylase	Unclassified	-1.86	2.83	2.65
b0735	<i>ybgE</i>	orf, hypothetical protein	Unknown function	2.04	2.83	3.05

8.6 Conclusions

In conclusion, the time-dependent direct electrochemistry behavior of *E. coli*-catalyzed MFC anodes indicates an evolution process under electrochemical tension. The corresponding variations in gene expression on a transcriptional level strongly support this speculation. Although further investigation is needed to explain this mechanism, the present work provides the groundwork for identification of the genetic mechanisms specifically responsible for the direct electrochemistry behavior of MFC-*E. coli*. It is hoped that such identification may lead to a means of genetically engineering *E. coli* that provides higher catalytic performance and efficiency.

Chapter 9 General Conclusion and Outlook

9.1 General conclusion

The motivations are raised for the synthesis and fabrication of novel anode materials and new strains of bacteria, as well as direct electron-transfer process, as means of overcoming challenges of currently used MFC electrode materials.

A nanostructured PANI/CNT nanocomposite was fabricated and used as an anode in an MFC. It has been found that the addition of CNTs to PANI increased the specific surface area of the electrode and enhanced the charge transfer capability, resulting in considerable improvement in the electrochemical activity necessary for an anodic reaction in MFC. A PANI/CNT/*E. coli* K-12/HNQ anode system yielded much higher power output than a PANI/*E. coli* K-12/HNQ alone, demonstrating the superior electrocatalytic effect of the nanocomposite on MFCs in comparison to the existing systems. The composite containing 20 wt % CNT exhibited the best performance, yielding a power output of 42 mW m^{-2} with a cell voltage of 450mV. The CNT-doped PANI nanocomposite therefore offers good prospects for application in MFCs.

A unique nanostructured PANI/TiO₂ composite was synthesized with a large specific surface area, uniform nanopore distribution, good conductivity, and good biocatalytic performance. It was found that the catalytic performance of the composite anode in MFCs could be optimized by adjusting the PANI

percentage in the composite. The best power density (1495 mW/m^2) in an *E. coli* MFC was with 30 wt % PANI. This was two times that previously reported, and is promising as an anode for a high-power MFC.

The *E. coli* cells evolved under electrochemical tension in an MFC exhibit direct electron transfer to anode without any artificial mediators. Electrochemical results and spectral analysis data reveal that some hydroquinone derivatives generated by the *E. coli* cells may serve as the electron mediator associated through highly permeable outer membranes to enable the direct electron transfer between the cell and the electrode. The MFC catalyzed by the evolved *E. coli* cells displays excellent performance, demonstrating that the endogenous mediators can efficiently enhance the electron transfer rate between the electrode and *E. coli* cells.

When GldA overexpressing-engineered *E. coli* was constructed as a biocatalyst in MFCs, it exhibited electrocatalytic performance superior to that of both original *E. coli* cells and the electro-evolved cells. The improvement was due to a large number of endogenous redox mediators in engineered *E. coli*. The engineered cell-based MFC displayed lower polarization and high power density in comparison to other *E. coli* cell-based MFCs reported. This study is the first to demonstrate that engineering *E. coli* by introduction of an appropriate oxidoreductase via gene manipulation can significantly improve its direct electron-transfer capability, and thus may be an efficient and economic

approach to biologically engineering bacteria for high performance MFCs.

It has been discovered that *E. coli* with direct electrochemistry in the electrocatalysis of MFCs underwent statistically significant up- and down-regulation of 107 genes. The gene transcription data suggest that the underlying cellular mechanisms include the regulation of outer membrane porin genes and terminal oxidase genes. This work provides the groundwork for identification of the genetic mechanisms specifically responsible for the direct electron transfer of MFC-*E. coli*. Such an identification may assist in the design of genetically engineer *E. coli* that yields better MFC performance and higher MFC efficiency.

In summary, novel nanostructured anodic materials were synthesized and new engineered bacterial strains were developed to enhance the power density of MFCs. Systematic investigation of the mechanisms of the direct electrochemistry of the anodic bacterial cells were interpreted to explain the electrode kinetics. This work offers new approaches for improvement of MFC performance and solid evidence of the direct electrochemistry of the bacteria in MFCs.

9.2 Outlook

With further advances in material science and nanoengineering, nanomaterials with superior chemical and physical properties will be developed and utilized in MFCs for continuous performance improvement. The rapid pace

of the development of functional nanomaterials will also lead to the development of novel MFC anodic materials. To be promising candidates for anodic catalysts in MFCs, biocompatible nanomaterials should allow good biocompatibility for strong cell adhesion, a bacteria-favorable pore structure for cell growth without blocking food (fuel) transport channels, and a large specific surface area for high apparent energy and power density. Different types of pore structures—coarse pores for cell growth and mesopores for a large specific surface area and fuel transport—may need to be synthesized. Electrocatalytic activity can be further improved by tailoring the size distributions and nanostructures. Specific nanostructures such as nanorods, nanotubes, and nanoneedles could provide more opportunities to access the redox center inside the bacterium cell membrane through penetration for direct electron transfer. A shape-controlled synthesis of nanostructures is recommended.

Although the electrocatalytic capability of the engineered strain was enhanced during this work, it still requires further improvement. The results of the microarray analysis performed in this study provide some clues regarding gene and protein choices. Overexpressing the target genes can be expressed inside the cells. The genes can also be expressed on the cell surface to form a membrane bounded by electroactive proteins or cell organelles that facilitate the electron-transfer rate. It is also possible that multiple genes could be transferred into the same cell to construct a new strain expressing different types of

proteins/enzymes simultaneously to increase the efficiency along the electron-transfer path.

Last but not least, the electrochemistry mechanism in the direct electron transfer of bacteria, even that pertaining to MFC anodic kinetics, remains unclear. Further work is needed to fully understand bacterial anodic kinetics and the mechanism of the direct electron-transfer process. It is highly desired to isolate self-generated mediators from a mixture containing thousands of metabolites, as well as to clarify the proteins or enzymes that participate in direct electrochemistry. Development of *in situ* scanning probe microscopy and/or optical spectrometry appropriate for electrochemical investigation, in addition to the use of proteomic approaches, will be very helpful in meeting these challenges.

Abbreviate

AFM: atomic force microscope

CNT: carbon nanotube

CV: cyclic voltammetry

DMFC: direct methanol fuel cell

EIS: electrochemical impedance spectra

FESEM: field emission scanning electronic microscope

FTIR: fourier transform infrared

GldA: glycerol dehydrogenase

HNQ: 2-Hydrox-1,4-naphthoquinone

HRTEM: High-resolution transmission electron microscopy

IPTG: Isopropyl β -D-1-thiogalactopyranoside

MFC: microbial fuel cell

MOB: manganese-oxidizing bacteria

ORR: oxygen reduction reaction

PANI: polyaniline

PTFE: polytetrafluoroethylene

RVC: reticulated vitreous carbon

SCE: saturated calomel electrode,

UMFC: upflow microbial fuel cells

XRD: X-ray diffraction

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