Investigation of charge transfer mechanisms for electroconductivity and bioremediation in bacterial biofilms

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**Investigation of charge transfer
mechanisms for electroconductivity and
bioremediation in bacterial biofilms**

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School of Materials Science and Engineering

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**A thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Doctor of Philosophy**

2014

To my family and the late Mrs Yeo Ai Lee

Abstract

This thesis examines extracellular electron transfer (EET) mechanisms and flavins/metabolite exchange in chemically modified biofilms and natural systems to demonstrate syntrophy for energy and bioremediation applications. Recently, chemical modification of bacteria to exhibit enhancement in microbial fuel cells (MFCs), bioremediation of waste products and electrode-driven biosynthesis of by-products has been demonstrated through insertion and self-assembly of unique organic molecules in cellular membranes. The mechanism for enhancement had been attributed to improved charge transfer through the inserted conjugated molecules. Using the novel transmembrane electron transport molecule (TETM), namely, 4, 4'-bis (4'-(*N*, *N*-bis (6''-(*N*, *N*, *N* trimethylammonium)hexyl)amino)-styryl)stilbene tetraiodide, (DSSN+) to chemically modify *Escherichia coli*, the role of the TETM as a potential charge transfer pathway at the microbe-electrode (biotic-abiotic) interface is studied using MFCs. Significant EET enhancement is observed in this platform and confocal microscopy techniques confirms the incorporation of DSSN+ into the cell membranes of *E. coli* biofilms formed at the electrodes. To uncover the dominant EET mechanism in DSSN+ incorporated *E. coli* systems, the bio-reduction process forming gold nanoparticles is monitored. The prevalent mechanism is revealed to be membrane perturbation with the concomitant release of intracellular redox-active components and not enhanced charge transfer as originally proposed. DSSN+ is also unable to restore charge transfer capabilities in the mutant *Shewanella oneidensis* strain with deleted EET genes, pointing to the absence of charge transfer through the conjugated molecules. In natural systems, EET and flavins/metabolite exchange are investigated by coupling fermentative *E. coli* and electrochemically active *S. oneidensis* in a syntrophic community, which significantly affects bioelectricity generation. Notably, the naturally established syntrophic relationship drove preferential colonization in the planktonic/biofilm modes (on the electrode surface), based on the functions of each strain and the metabolite of exchange. In the syntrophic system, EET mechanisms are especially dominated by *S. oneidensis*. Further exploitation of another simple syntrophy between *Pseudomonas putida* and *S. oneidensis* drove concurrent bioelectricity generation and increased rate of bioremediation. In summary, investigation of novel charge transfer mechanisms and flavins/metabolite exchange for electroconductivity and bioremediation in bacterial biofilms was carried out by studying both chemically modified bacteria and natural interactions between cooperative bacterial species.

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To God Be All Glory

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Chapter 1: Introduction

1.1. Background

Contrary to popular belief, bacterial cells do not preferentially exist as single predominant planktonic entities, but as biofilms. In biofilms, bacterial cells (mono- or multi-species) aggregate and proliferate to form a multi-layered structure through attachment onto a surface. Seminal work on biofilms has refuted the conventional paradigm and established them as complex mono- or multi-species communities which are ubiquitous in natural, industrial or engineered settings. Numerous bacterial populations found within surface-attached biofilms further complicate inter-species interactions as microbial cells interact extensively within biofilms[1, 2]. Social attributes such as competition and cooperation are usually observed in microbial communities in biofilms[3, 4]. The aforementioned mechanisms allow different species to coordinate their behaviour or evolve certain adaptive mechanisms under external stresses, which further dictates the functions and survival of the entire community. Cellular interaction can be realized through endogenously secreted metabolites, which is demonstrated by secretion of antimicrobial compounds to inhibit the growth of neighbouring species[5]. Contrastingly, certain microorganisms rely on these secreted metabolites for survival. The phenomena of charge transfer and metabolite exchange/secretion drive much of the fundamental physical and chemical reactions observed in biofilms. In the context of a biofilm interacting with external components, charge transfer constitutes a major consideration factor as bacteria needs to transfer metabolically liberated electrons to extracellular electron acceptors to initiate physiological growth/function and participate in natural environmental processes.

Charge transfer (i.e. electron equivalents) in biofilms is studied by monitoring conductivity, which is determined by the number of charges transferred through (or within) the extracellular polymeric substance (EPS) matrix, which encases the biofilm. Inherently insulating bacterial cellular membranes and the EPS matrix material confer high charge transfer resistance and impedance in biofilms, which hinders extracellular electron transfer (EET) within and out of the matrix[6]. These constraints have allowed microorganisms to adapt and evolve various mechanisms of charge transfer for continual survival in various environments. This is because metabolically released compounds rich in electrons need to move down an energy transport pathway to supply energy for cellular functions. These unique charge transfer mechanisms (discussed in subsequent sections) have been used to circumvent the low rate of transport kinetics or electron transfer across biotic/biotic interfaces in biofilms and biotic/abiotic interfaces when the biofilm interacts with an extracellular terminal electron acceptor.

The molecular electronics approach, which motivated this thesis, allows chemical modification of the bacteria through introduction of artificially synthesized molecules targeted at energy generation applications. It is further extended to investigate charge transfer mechanisms in bacterial cells which are embedded in biofilms. Highly conjugated molecules, which are designed to function as ‘molecular wires’, are utilized to bridge the insulating junctions at the cellular membrane to form an abiotic-biotic interface as these molecules have been shown to conduct high currents between metallic contacts. Examples of such molecules include polyenes, polyacenes, and poly(phenylenevinylenes). When appropriately designed, such synthetic molecular analogues can be further dissolved in aqueous mediums, such as water. This would make the molecules compatible for use in biofilm system studies as they are usually water-based.

Insertion and self-assembly of the molecules in bacterial cellular membranes can be realized by incorporating long alkyl chains and charged ionic groups at the terminal ends of the molecule. The central concept of a ‘molecular wire’ is for efficient charge transfer across long distances along the delocalized π -backbone of the molecule.

A platform to investigate EET in chemically modified bacterial cells and natural systems is through microbial fuel cells (MFCs). In this platform, released electrons from bacterial metabolism are coupled to the reduction of extracellular terminal electron acceptors through EET. Electrons and protons are released during metabolic oxidative breakdown of organic substrates in the bacteria. Through various EET pathways, these electrons can be transferred out of the cell and onto the electrode, which functions as the terminal electron acceptor, in the anaerobic anode chamber. The electrons then move across an external load towards the aerobic cathode, where oxygen is reduced to water together with protons. However, the limiting factor has perennially been inefficient anodic electron transfer processes occurring at the microbe-electrode interface, which still remains inadequately explored and understood to date[7]. To fully understand how charges can be transferred via ‘molecular wires’ embedded in bacterial cells in MFCs, it is critical to monitor the amount of bioelectricity generated by such devices employing chemically modified microbes.

1.2. Motivation

This study is undertaken to investigate alternative and novel charge transfer mechanisms by modulating the insulating microbial cellular membrane through introduction of

transmembrane electron transfer molecules (TETMs). These are artificially synthesized organic molecules which can self-assemble within lipid membranes and are further suggested to function as an alternate charge transfer conduit across the insulating cellular membrane. Released electrons from oxidative metabolism can therefore be transferred from the internal metabolic cycles of the microorganisms to the extracellular charge collecting electrode (or other terminal electron acceptors) for reductive processes to occur. In the context of MFCs, it is hypothesized that TETMs can act as bridging ‘molecular wires’ to couple charge transfer within the biofilm to the extracellular charge collecting electrode to produce energy. Recent work has demonstrated use of such TETMs for chemically modifying non-electrogenic microbial systems to achieve enhanced EET[8-11] and increased ion conductance[12], which results in improved bioremediation of wastewater in MFCs[9]. In addition, these molecules have been utilized in electrode-driven reduction of fumarate to succinate in *Shewanella oneidensis*[13]. However, the mechanisms of such charge transfer phenomenon in these chemically modified systems are still unclear. By detecting the presence and location of the reductive biotransformation of nanoparticles and through use of identified mutants, the exact charge transfer mechanism afforded to the chemically modified microbial system through TETMs can be uncovered further.

The electroconductivity phenomenon can be further studied by investigating syntrophic interactions in a natural biofilm setting. Biofilms constitute a community with specific bacterial strains colonizing each niche area and interacting in syntrophic manners, which are determined by their individual needs and functions. Other than exposing microorganisms to exogenous chemical components, bacterial species found in natural environments can be subjected to engineered environments to manipulate their interactions. The identified species rarely meet of

their own accord naturally but can be brought into a syntrophic interaction through metabolite exchange. Compositions of various species in the planktonic and biofilm phase can then be further analyzed and correlated to bioelectricity generation. Understanding such syntrophic relations can drive better designs to harness the power of such communities where biofilms and planktonic cells coexist and thrive.

Bioremediation coupled to energy generation is another microbial activity with specific mechanisms which can be investigated in the MFC platform. Although encumbered by low power generation which limits widespread application, biofilms employed in MFCs also allow for bioremediation of waste contaminants concurrently coupled to power generation in a single platform. A recent discovery reports the charging of a commercially available mobile phone by using MFCs with electroconductive biofilms fed with real urine[14]. This breakthrough undergirds the untapped potential in electroconductive biofilms feeding on organic waste substrates to produce energy. Heterogeneous microbial diversity prevalent in biofilms further allows a diverse range of organic substrates to be broken down for power generation. Virtually any substrate that can be broken down by electrogenic microorganisms can be utilized to generate bioelectricity. Moreover, in contrast to extracting electrons from electroconductive biofilms, biofilms can be ‘fed with electrons’ to achieve microbial electrosynthesis[15, 16]. This breakthrough allows the microorganism to convert carbon dioxide and water in the atmosphere to functional extracellular multi-carbon compounds, which can be utilized for useful purposes. Other than these applications, electroconductive biofilms have also been demonstrated to form gold nanoparticles and hydrogen gas in MFCs[17]. These illustrations highlight the ubiquitous nature and usefulness of biofilms in energy generation and bioremediation, which are achieved

through inherent EET mechanisms. Although the kinetics of EET mechanisms in bacterial systems is poor, they can be induced by introducing chemical methods or through exploiting natural cooperative systems.

1.3. Problem statement and approach

Although there has been much research dedicated to characterizing various inherent EET mechanisms in microbial systems, there has been a lack of targeted effort and specialized knowledge in direct chemical modulation of the inherently insulating microbial cellular membrane and syntrophic relations, which directly affects EET. Two approaches can be adopted to enhance and probe EET mechanisms in biofilms which is the singular unifying theme in this thesis, – (1) By employing the molecular electronics approach, this strategy can chemically modify microbial systems, which can confer novel EET mechanisms to open new vistas in bioelectronics applications. (2) The natural syntrophy between cooperative microbial species can be utilized to enhance EET and bioremediation, with specific investigation into community structure and dynamics based on functional expression of identified microbial species. **The aim of this thesis is therefore to understand chemically modified biofilm systems and natural microbial interactions so as to improve charge transfer and metabolite exchange to drive enhanced EET and syntrophic interactions for effective bioelectricity generation and bioremediation.**

1.4. Objectives

In order to address the questions in the previous section, this thesis has been designed according to the following objectives.

- 1) Investigate insertion of novel TETMs in microbial cell membrane and subsequent interaction in MFCs
 - Ascertain insertion of TETMs via confocal microscopy characterization in *Escherichia coli* cell membrane in both planktonic and biofilm configuration (on the electrode surface)
 - Establish enhancement of abiotic-biotic interactions in *E. coli* systems using TETMs and further investigation of its role in EET through a concentration study using MFCs
- 2) Resolve the charge transfer mechanism in TETM – modified bacterial systems
 - Employ presence and location of bio-reduction reactions which form gold nanoparticles as markers to study EET mechanism in chemically modified *E. coli*
 - Electrical characterization of TETMs to investigate conductivity across the delocalized π -backbone. Utilize electrochemical techniques and a modified MFC platform to characterize the intracellular components isolated after cellular interactions with TETMs and ultra-sonication of cells
 - Investigate TETM performance in restoring EET pathways by using electrochemical cells employing specific *S. oneidensis* mutants with deleted genes responsible for such EET mechanisms

- Correlate results and observations from various analyses to probe the change in structure-property relationship afforded by TETMs after interaction with the membrane
- 3) Investigate EET mechanism and flavins/metabolite exchange by defining common microbial species for syntrophic interactions in MFCs
- Develop relationship between identified microbial species (fermentative *E. coli* and electrochemically active *S. oneidensis*) in the dual-species system
 - Investigate the role of the Metal-Reducing (Mtr)-pathway and identification of the main metabolite of exchange in the syntrophy system
 - Extend understanding of the syntrophic system in shaping the community dynamics in the anode media (planktonic phase) and on the electrode surface (biofilm phase)
- 4) Understanding syntrophic interactions for bioelectricity generation and bioremediation
- Develop a single platform for a defined dual-species microbial system (*Pseudomonas putida* for bioremediation and electrochemically active *S. oneidensis*) for concurrent azo dye degradation and bioelectricity generation in MFCs
 - Investigate activity of each species and probe syntrophic interactions and further understand the role of the biofilm in bioelectricity generation by employing a mutant species deficient in biofilm adhesion and formation

1.5. Scope

The goal of this thesis is to investigate the application of novel TETMs in MFCs, understand its role as an EET conduit in enhanced bioelectricity production and the possibility of restoring EET mechanisms in genetically modified strains through bioelectrochemical platforms, electrical characterization and electrochemical techniques, respectively. The most pertinent aspect is to elucidate the EET mechanism that these TETMs confer to the bacterial system. In order to do so, bio-reduction to form gold nanoparticles and the location of these nanoparticles have been employed. Further, specific bacterial species have also been identified to interact in syntrophic function-driven environments with targeted applications in concurrent bioelectricity generation and bioremediation in the planktonic and biofilm phases. Synthesis of TETMs and its chemical characterization will not be elaborated.

1.6. Thesis Organization

This thesis is organized in the following order. Chapter 1 provides a brief overview and motivation for this work, and further outlines the objectives and scope. Chapter 2 surveys relevant literature in biofilms, exoelectrogenic bacteria, EET mechanisms within the biofilm matrix, bioelectrochemical systems in the form of MFCs as a platform for power generation and bioremediation and conjugated oligoelectrolytes designed to function as potential TETMs, etc. Chapter 3 presents the various experimental techniques, process parameters and conditions adopted during various analyses employed in this thesis. In Chapter 4, detailed results and discussion will be presented and are broken down into the subsequent sections:

1. Enhancing electroconductivity in chemically modified *E. coli*
2. Electrical characterization of 4, 4'-bis (4'-(*N*, *N*-bis (6''-(*N*, *N*, *N* trimethylammonium)hexyl)amino)-styryl)stilbene tetraiodide (DSSN+)
3. Using gold nanoparticles as a marker to investigate EET in *E. coli* chemically modified with DSSN+
4. Electrochemical characterization of *S. oneidensis* mutants modified with DSSN+
5. Function-driven structural assembly of a syntrophic microbial community established by planktonic cells interacting with biofilms
6. Simultaneous azo dye bioremediation and bioelectricity generation through a stable syntrophic microbial consortium of *P. putia* and *S. oneidensis*

Chapter 5 provides the overall conclusion and further summarizes the key findings and significant contributions of this thesis. Future recommendations will also be suggested for exploration.

Chapter 2: Literature Review

This chapter explores relevant topics and concepts which are essential for further development of this thesis. The sections include electrochemically active biofilms, bioelectrochemical systems, various forms of EET mechanisms and transmembrane electron transfer analogues, etc.

2.1. Biofilms

The ubiquitous biofilm can be found in both natural and engineered environmental settings. Biofilms – a structurally complex composition of microbial diversity encased in an endogenous matrix adhering to surfaces or interfaces – portrays a unique form of life that was previously uncharacterized[18]. In this configuration, genetically heterogeneous species in the community adapt and interact to ensure continued existence against external threats in a dynamic and hostile environment[3]. Surfaces are critical components for biofilm formation and most surfaces thrive with microbial life, which eventually forms diverse ecosystems constituting biofilms, when there is adequate moisture and nutrients. For example, the dental plaque coating the tooth surface comprises a plethora of microorganisms in a biofilm which exhibits strong adhesion and antibiotics tolerance during tooth decay[19-21]. The slippery crust covering the surfaces of river stones and bacterial coatings on infected wounds or organs are further examples of biofilms forming in favourable environments (Figure 2.1).

Figure 2.1: Schematic depicting biofilm (green) formation after infiltrating the human body. Image credited to P. Dirckx, Montana State University, Center for Biofilm Engineering.

Various forms of microscopy characterization further depicted the biofilm to be encased and supported in a fibrous, highly hydrated, exopolysaccharide matrix (or EPS), where the chemical constituents are unique to each species (Figure 2.2).

The discovery, confirmation and characterization of biofilms in the past decades have created a sharp deviation from conventional microbiology concepts, where bacterial life form

and physiology have traditionally been depicted as liquid cultures of dispersed planktonic cells. Now, it is widely recognized that most microbial species exist in the form of biofilms. This fundamental revelation is significant as cells in the biofilm mode are shown to be at least 1000 times more resistant to antibacterial agents than the same cells existing in the planktonic mode[22, 23]. This is partly because these biocides are unable to fully penetrate the entire thickness of the biofilm to generate stimulus to ensure complete eradication of all cells[24]. Inter-species cooperation also allows evasion of such antibacterial agents[25]. This constitutes a fundamental adjustment in traditional microbiology, as some form of cooperation or syntrophy, resulting in added ecology advantage, must be considered in the biofilm mode of life. Each immobile cell in the biofilm thrives in a tailored and unique micro-environment which further forms the complex microbial macro-environment. In this stage, simple homeostasis occurs along with a system for circulating essential nutrients necessary for growth. Metabolic synergy is also present and each immobile cell in the biofilm form responds specifically to its unique environment, which forms fundamental differences when observed from the same species in its planktonic form[3, 26].

2.1.1. Biofilm growth, structure and dispersal

Planktonic bacterial cells can attach to surfaces with adequate moisture and nutrients for subsequent growth and aggregation to form biofilms. In order to select the surface with the best conditions for proliferation, a planktonic bacterial cell must be able to ‘sense’ its surroundings (Figure 2.2, Stage 1). This is realized through sharp gradients or differing concentrations of either protons or signaling molecules, which can be picked up by the bacterial cell[27-29]. Once

the surface has been ascertained to be favourable for growth, the bacterial cell will adhere to the surface by up-regulating specific adhesion genes once it is on the surface (Figure 2.2, Stage 2). These cells will then produce EPS material through endogenous secretion to envelop the community of cells (Figure 2.2, Stage 3). Nutrient exchange is promoted thorough the biofilm architecture, which causes the biofilm to grow physically thicker and increase in complexity. After the initial stages of biofilm formation have been established, the cells can mature through redistribution of attached cells by surface motility, binary division of attached cells or recruitment of cells from the bulk fluid to the developing biofilm. In this environment, cell-cell communication can occur through a variety of mechanisms, such as mediators or signaling molecules.

The inherent presence of micro-environments in various locations in the biofilm produces a community with cells of different phenotypes from an initial single bacterial cell of a single genetic complement. This arises partly due to differences in nutrient access and concentrations or stressed induced mutations, such as oxidative stresses, which causes the bacterial cells to grow differently. However, this phenomenon is favourable as genetic diversity can be achieved within the biofilm. This can provide insurance against unstable environmental conditions as the community can adapt better to sudden environmental changes[30].

Figure 2.2: Stages of biofilm development. Stage 1: Initial attachment; Stage 2: Irreversible attachment; Stage 3: Maturation 1; Stage 4: Maturation 2; Stage 5: Dispersal. Adapted from [31].

Biofilms can adopt various structures or architectures which are heavily reliant on the condition of survival and are further influenced by the environment and species involved. This causes the biofilm to take up certain shapes, such as mushroom-like or aerial projections. These shapes have been studied through the use of confocal laser scanning microscopy (CLSM) techniques. Analyses have revealed that micro-colonies constitute the basic structural unit of biofilms. These are discontinuous matrix-encased communities of bacterial cells of one or many species. The composition of the micro-colony is dependent on the specific species involved. Composition of cells may range from 10 – 25% and the EPS matrix may range from 75 – 90% and comprises polysaccharides, proteins and nucleic acids[6, 32]. As biofilms develop, CLSM imaging further revealed a basic structure where cells grow in matrix-encased micro-colonies

with a network of open and inter-dispersed water channels (Figure 2.2, Stage 3)[6, 31]. Bacterial cells change their internal physiological processes according to conditions in different micro-niches. This form of self-regulation, coupled with cell-cell signaling, helps to ensure optimum biofilm growth. For example, ensuring open and dynamic water channels to facilitate full circulation of essential nutrients to all parts of the biofilm requires the cells to prevent cellular growth and EPS secretion away from the channels. Upon full maturation, individual micro-colonies or cells may detach from the biofilm surface, which allows the planktonic cells to search for and colonize new habitats[6, 31]. The detachment process leaves voids which may become part of the water channels (Figure 2.2, Stages 4, 5) and completes the biofilm developmental life cycle[6, 31].

2.1.2. Exoelectrogenic bacteria

Electrochemically active biofilms, which are capable of EET through various means of charge transfer, are critical in natural environments as they are important in the carbon cycle and metal oxidation/reduction processes amongst other functions. They can also contribute towards bioelectricity production in bioelectrochemical systems.

The biofilm is a natural microcosm of microbial diversity. Bacteria with various forms of exoelectrogenic mechanisms can exist within biofilms and have been shown to facilitate electron flow via EET. Exoelectrogens are defined as bacteria which possess the ability to transfer electrons to an external chemical or material that is not the immediate electron acceptor. This is different from other anaerobes which can only transfer electrons to soluble compounds that

diffuse across the cell membrane and into the cell interior to function as acceptors. Exoelectrogens are further distinguished by their capabilities to directly or indirectly (mediated) transport electrons out of their cells to an extracellular or insoluble terminal electron acceptor. This capability allows the use of such bacteria in MFCs[33].

Common exoelectrogenic and dissimilatory iron reducing bacteria, such as *Geobacter sulfurreducens*[34] and *Shewanella* spp.[35, 36] are inherently exoelectrogenic and electrochemically active due to their ability to reduce solid terminal electron acceptors[37, 38], allowing these bacteria to be commonly used in MFCs. With such species, exoelectrogenic character through EET is demonstrated via a few widely recognized mechanisms, such as membrane associated cytochromes[39, 40], secretion of soluble redox mediators[41, 42] and physical appendages such as conductive pili[43, 44].

For bacteria to ultimately produce bioelectricity in MFCs, electrons need to be transported externally via two mechanisms (Figure 2.3), namely, direct electron transport and indirect (mediated) electron transport.

Figure 2.3: Direct and indirect (mediated) forms of EET in exoelectrogenic bacteria. Adapted from [7].

2.2. Extracellular electron transfer (EET)

Bacteria are known to produce electrons and protons through oxidative metabolism of nutrients which pass through the water channels in the biofilm structure. As the electrons cascade down the energy transport pathway in the metabolic cycle, useful energy is harnessed for growth and essential cellular processes. However, these electrons need to be transported out of the cell

eventually. This is realized through EET, where bacteria couple their metabolic pathways to external terminal electron acceptors under anaerobic conditions, such as soluble or solid electron acceptors in soil sediments or in the electrode housed in the anode chambers of MFCs.

2.2.1. Direct electron transport

Conducting surface appendages on the cellular membranes, such as nanowires produced by *Geobacter* and *Shewanella* spp., and c-type cytochromes (shown as red dots in Figure 2.3) produced by *Geobacter*, provide means by which electrons can be transferred through intimate and direct contact with the extracellular terminal electron acceptor.

Nanowires are depicted as extracellular appendages emanating from the cell membrane of *Shewanella* spp. (Figure 2.4A). These appendages were shown to be highly conductive using scanning tunneling microscopy (STM) techniques (Figure 2.4B). This was demonstrated by loading the sample onto a highly ordered pyrolytic graphite surface, where a conductive tip was moved across the sample surface under constant-current imaging conditions. The resultant current-voltage graph illustrates the part of the scan that was conductive relative to the surface. Although this mechanism would require the bacteria to be directly situated on the electrode, the bacteria are not limited to short range interactions. This is because nanowires produced by *G. sulfurreducens* can conduct electrons in anode biofilms that are more than 50 μm thick. This direct mode of EET allows for higher kinetic rates of electron transfer, as compared to diffusion of mediators. In general, these nanowires consist of polymeric protein assemblies from the type IVa family of pilin proteins and mainly comprise of one subunit protein, PilA[45]. This protein is

more than 85% α -helical and consists of a conserved N-terminal helical region, followed by a divergent globular head domain. The organization of the aromatic amino acid structure in the α -helical region is critical for electrical conduction. Two mechanisms exist to account for the charge transfer mechanism afforded by the nanowires[46]. One model argues for superexchange conductivity between redox active sites, such as the heme groups in cytochromes, that are found on the nanowires fibers[47]. For example, in *S. oneidensis*, it is suggested that the CymA-MtrA-MtrC complex functions as a multistep conduit that conducts respired electrons originating in the cytoplasm from the inner membrane through the periplasm and outer membrane to the cell outer surface[48-51]. The electrons are seen to be ‘hopping’ between heme groups in cytochromes closely aligned with the pilus as the support structure.

Another different model suggests metallic-like conduction along the nanowires due to stacking of aromatic residues which align the π -orbitals in the quaternary structure of the pilus, hence allowing electrons to be conducted along these π - π stackings[52]. It is further suggested that the attached multiheme cytochromes mediate direct electron transfer to the final electron acceptor[53]. This is different from the superexchange model as it visualizes electrons as delocalized entities across the entire nanowire as compared to hopping or tunneling between discrete redox centers[54].

Figure 2.4: (A) Scanning electron microscopy (SEM) image of a wild-type *S. oneidensis* strain showing nanowire formation. (B) Scanning tunneling microscopy (STM) image of extracellular appendages showing high conductivity. Adapted from [55].

Other than conducting nanowires, other modes of direct EET are realized through outer-membrane cytochromes, which are made of conducting proteins. These are defined as protrusions on the membrane surface but are not classified as nanowires.

2.2.2. Indirect electron transport

As the biofilm grows thicker, more electrochemically active cells are positioned further away from the terminal electron acceptor, hence EET through direct physical contact becomes inefficient due to distance limitations. The need for an alternative mode of EET arises. Endogenous chemical mediators can be naturally secreted by certain bacteria to overcome these physical constraints to facilitate EET. For example, pyocyanin which is self-regulated and

produced by *P. aeruginosa* can function as an electron shuttle to promote EET[56]. Pyocyanin is a typical phenazine compound and exhibits characteristic blue-green colour (Figure 2.5).

Figure 2.5: Observation of pyocyanin production in *P. aeruginosa* strains. Strains were grown in LB medium for 18 h, and aliquots of respective cultures were recorded to document the production of the blue-green pigment, pyocyanin. Over-expressing sPqsE significantly improves pyocyanin production, as seen from the intense blue-green colour, with respect to the wild-type PAO1. Adapted from [57].

Shewanella spp. has also been shown to reduce insoluble terminal electron acceptors, in the form of metal oxides, to achieve bioremediation[39]. This is achieved without physical contact between surfaces. It was further demonstrated that flavins secreted by the species function as redox mediators or electron shuttles, which aids in EET[58].

Externally introduced mediators can also be added to facilitate EET across the microbe-electrode interface. Exogenous mediators, such as neutral red[59, 60], methylene blue[61], anthraquinone-2-6, disulfonate (AQDS)[62], etc are typical redox mediators which are capable of entering the cell interior, picking up the liberated electrons from metabolism, and are then reduced itself. These reduced compounds will then exit the cell interior and diffuse to the anode where the electrons are passed on to the conducting surface. The mediators are subsequently oxidized again and free to re-enter the cells to pick up more electrons and the cycle continues.

However, these mediators are usually toxic, expensive and require constant renewal, hence limiting their use in practical applications.

2.3. Bioelectrochemical systems – Microbial Fuel Cells (MFCs)

Bioelectrochemical systems (BESs) employ microorganisms to convert chemical energy (stored in the organic substrate) to electrical energy. If BESs produce electrical energy, they are called MFCs. In another case, if BESs use electrical energy to drive certain electrochemical reactions (bioelectrosynthesis), they are called microbial electrolysis cells (MECs). In the context of this thesis, MFCs are employed as the platform for investigation of EET mechanisms in TETM – modified bacterial systems and syntrophic communities for bioelectricity generation and bioremediation.

MFC is a device which is able to exploit bacteria as biocatalysts to generate energy. Exploitation of bacteria offers several advantages, as they can utilize a wide range of substrates[63]. The ubiquitous and robust nature of bacteria also allows ease of access and cultivation respectively. These microorganisms release electrons and protons by metabolizing the organic source in the anaerobic anode chamber through catalyzed oxidation. The electrons are transferred from the internal metabolic cycles of the microbes by a variety of EET pathways to the external anode and subsequently move across an imposed load towards the cathode. Protons travel across the proton exchange membrane (PEM) to the aerobic cathode chamber. At the cathode, electrons and protons combine with oxygen to produce water to complete the charge balance, according to the following reaction:

In this manner, electrons that are liberated during oxidative microbial metabolism can be transferred across the inherently insulating lipid membrane and harnessed to produce a useful electromotive force as they move through an external circuit across an electrical load. The flow of charges and the general working principle of MFCs are illustrated (Figure 2.6). In this particular scenario, acetate is used as the carbon source and *G. sulfurreducens* is used as the bacteria.

Figure 2.6: Schematic representation of a MFC using *G. sulfurreducens*. Adapted from [64].

In addition to generating power through microbial breakdown of substrates, MFC is also recognized as a technique for removing various contaminants[65, 66]. This complementary platform of concurrent power generation and bioremediation puts MFCs in a unique position as an alternative and compelling avenue for green energy production. MFCs can be constructed in

various configurations and architectures with a diverse array of materials, which are all aimed at improving device performance. MFCs can also be operated under many varying conditions, such as differences in temperature, pH, electron donors/acceptors, etc[67]. The type of bacteria used in the anode chamber can also affect the amount of energy generated by the MFC.

However, the relatively low magnitude of bioelectricity generated and low efficiency of MFCs have hindered the widespread implementation of this technology. Current research efforts aimed at improving MFC performance have focused mainly on electrode engineering – for example, fabrication of networked nano-structured electrodes to increase surface area for biofilm attachment, optimization of device architectures, or natural selection and electrochemical evolution of electrochemically active microbes/biofilms[68]. However, these efforts have either failed or demonstrated varying degrees of success. Other common methods to improve MFC performance include the incorporation of soluble redox mediators, such as methylene blue[61] or neutral red[59, 60] to aid in electron shuttling. However, these mediators are expensive and need constant replenishment, which is impractical for long term applications. Alternative options for improving MFC performance are therefore of great interest and importance to the advancement of bioelectronic technology. Given that charge transfer across the insulating cellular membrane (from the interior of the microbe to an external charge-collecting electrode) is a significant barrier limiting MFC performance, investigations into the modulation of structural properties at the microbe-electrode interface should reveal means to better optimize charge extraction from microorganisms, which can maximize electrical output[69, 70].

Inherently exoelectrogenic bacteria, such as *S. oneidensis* and *G. sulfurreducens* have been extensively used in MFCs as they possess various forms of EET mechanisms, such as conductive pili[53], membrane bound cytochromes[71, 72], or secretion of soluble redox mediators[42, 58]. Novel routes to generate energy from common, non-pathogenic microorganisms that are not inherently exoelectrogenic are also interesting. Recent studies on integrating conjugated oligoelectrolytes with such microorganisms to drive bioelectronic interactions have also received much recent attention due to enhanced electrical output in MFCs[8-10], applications in wastewater bioremediation[9] and biosynthesis of reduced metabolites[13]. This synthetic system relies on the ability of the molecules to spontaneously intercalate within the microbial cell membrane due to their amphiphilic nature.

Some typical factors affecting MFC performance loss include ohmic voltage losses due to hindered electron transfer through the electrodes and ion transfer through the electrolyte[73, 74]. These are directly influenced by the components in the MFCs. Other losses include electrode over-potentials arising from activation polarization, concentration polarization (or mass transport) and bacterial metabolism. Activation polarization is due to the amount of invested energy that is required to initiate redox reactions or to transfer an electron to the terminal electron acceptor. Concentration polarization is attributed to slow or insufficient mass transfer of reactants and products which are caused by difficulties in maintaining the different concentration gradients. These factors result in a limitation in EET rates, which directly affects the performance of the MFCs.

2.3.1. Summary of methodologies to enhance performance of MFCs

Although the dual functionality of MFC technology may seem attractive, commercialization has met with hindrances due to bottlenecks in power output. Other than inherently slow cell metabolism, another limitation has been identified to be the insulating abiotic-biotic interface at the cellular membrane, which needs to be bridged effectively to circumvent inferior EET[75].

Park and co-workers have demonstrated enhanced electrical output by employing neutral red as an exogenous redox mediator[59, 60]. By genetically engineering the wild-type *P. aeruginosa* strain, Wang *et al* and Yong *et al* have also demonstrated enhanced electrical output in MFCs by employing *P. aeruginosa* mutant strains with over-expressed quorum sensing systems which up-regulates endogenous synthesis of phenazines[41, 57]. The higher concentration of phenazines, which functions as an electron mediator, contributed to the enhanced electrical output in MFCs. Recently, cisplatin has also been shown to elongate *S. oneidensis*, which improves microbe-electrode interactions in MFCs as larger bacterial cells could produce more flavins and robust nanowires for enhanced EET[76].

Other common forms of material engineering methods include electrode modification or surface functionalization to increase available area for biofilm attachment and growth or promotion of bacteria adhesion by enhancing hydrophilicity[77]. For example, polyaniline nanowires[78], carbon nanotubes[79, 80] and graphene[81, 82] have been demonstrated to improve electrical output by increasing the surface area for biofilm colonization. The

requirements of the anode material are: high conductivity, non-corrosive, high specific area, high porosity, non-fouling, long term stability, inexpensive, facile synthesis and scaling up.

The oxygen reduction reaction at the cathode is also a typical limiting factor as electrons, protons and oxygen must interface in a tri-phase reaction (solid electrode, air and oxygen). Oxygen is recognized as the ideal terminal electron acceptor due to its high oxidation potential, low cost and formation of water as the waste product. However, oxygen has very low diffusivity in water and reduction is very slow on carbon electrode surfaces. Platinum has conventionally been used as the catalyst but this area is still in need of further exploration, as platinum is an expensive material choice[79, 83]. Recently, efforts to circumvent these limitations have been explored through the use of a biofilm at the cathode electrode, i.e. biocathode[84-86] or the use of different materials for the biocathode[87].

Various MFC architectures have also been adopted to boost the energy output of MFCs[88-90]. For example, single-chamber air-cathode MFCs[91], cube and bed reactors of varying sizes have all been employed to varying degrees of success. Recently, twelve ceramic cylindrical MFCs were arranged in various series and parallel configurations. These were then used to power a mobile phone using urine[14]. Novel miniature MFCs in microarrays have also been employed to simultaneously assess the electrical performances of potential environmental exoelectrogens. This highly compact assay has been validated through identification of a *Shewanella* isolate that produces twice as much energy as the reference strain in conventional and microarray configurations[90, 92].

2.3.2. MFCs as a platform for bioremediation

Bioremediation taps on the capability of microorganisms to eliminate, detoxify or immobilize pollutants through their secreted by-products or other means, which negates the need for addition of toxic chemicals. This method is in contrast to conventional physicochemical remediation methods, such as advanced oxidation techniques, which are expensive and environmentally unfriendly. Bioremediation may occur through various *in situ* approaches, such as natural attenuation, bioaugmentation, air venting, and interactions between electron acceptors/donors.

Azo dyes are constituents frequently utilized in textile industries and contain the aromatic functionality with one or more N=N groups. Seventy percent (by weight) of all the dye used by society are composed of these compounds and 15% are eventually discharged into water bodies[93, 94]. The environmental release of dye-containing wastewater causes major pollution and public health concerns as these compounds and their by-products are poisonous and mutagenic[95, 96]. Discharge into water bodies can also interfere with natural light penetration and oxygen diffusion, which will have adverse impacts on aquatic life forms. These compounds are characterized by high stability and cannot be attenuated in natural or conventional engineered treatment systems. Various effective physicochemical methodologies have been implemented to degrade these dyes, however inherent high costs and tedious procedures hinder the widespread application of these techniques. Further, secondary waste contaminants generated after dye degradation have yet to be adequately neutralized, hence remaining an environmental threat.

Biological reactions have emerged as cheap, environmentally-benign and effective alternatives for dye degradation, as specific azo dye degrading microorganisms can break down azo dyes by reductive cleavage of the molecular bonds[97]. Clear understanding of various microbial interactions within a process setting can facilitate optimal manipulation of selected microbial communities for azo dye biodegradation. The bioremediation process would prove more attractive when realized with simultaneous bioelectricity generation, which can be harnessed for use. This would realize a dual-purpose system which employs microbial degradation of waste products to drive useful energy production. Recent reports have demonstrated that MFCs can be used to couple bioremediation of azo dyes with bioelectricity generation[98, 99]. Other than bioremediation of azo dyes, MFCs is also widely recognized as a platform for wastewater bioremediation[65]. In one of the objectives defined in this thesis, MFCs were used as a platform to develop a defined dual-species microbial system for concurrent bioelectricity generation and bioremediation of Congo red.

2.4. EET investigation through MFCs and electrochemistry techniques

MFCs are ideal platforms for identifying exoelectrogens and their intrinsic modes of EET mechanisms. Hou *et al* have utilized micro-fabrication techniques to demonstrate high throughput identification and characterization of exoelectrogens using a MFC microarray[90]. In MFCs, the non-corrosive anode electrode also provides a surface that can be modified through various methodologies for optimum biofilm growth and maturation for facile investigation. Further, in this configuration, biofilm formation and morphology on the electrodes can be characterized by a variety of microscopy techniques to understand how EET occurs.

However, there are limitations to the MFC platform. Although experiments with two-chamber MFCs inoculated with yeast[8], *E. coli*[10, 11] or wastewater consortia[9] showed increase in current output when DSSN+ was added, singular current output is insufficient in explaining the effect of DSSN+ on EET mechanisms. This is because the anodic and cathodic potentials cannot be set precisely in MFCs, therefore the effect of electrode potential on EET increase cannot be eliminated. Hence, electrochemistry techniques such as chronoamperometry, voltammetry or impedance measurements in a controlled three-electrode electrochemical system are needed to reveal the mechanisms through which DSSN+ improves EET in viable microorganisms. This is achieved by monitoring generated current over a time period (chronoamperometry), signature peaks in current in cyclic voltammograms and elucidating values of charge transfer resistances through impedance spectroscopy measurements.

2.5. Transmembrane electron transfer molecules (TETMs)

Conjugated polyelectrolytes consist of a pre-determined number and choice of repeating unit from a polymer with ionizable constituents. This class of material has been recently employed as critical components in a variety of novel technological platforms, such as organic electronics in the areas of bio-imaging, solar cells and thin film transistors, etc. The structure of these molecular analogues typically contains an electronically delocalized π -conjugated aromatic backbone which can be further functionalized with various pendant groups or ionic functionalities to impart solubility to the molecules in aqueous media or solvents. The resultant product is a class of macromolecules which incorporates the properties of organic semiconductors with the physicochemical behaviour of polyelectrolytes (Figure 2.7).

Investigations into single molecule conductance have revealed insights into factors influencing charge transfer and has further aided in design and development of these ‘molecular wires’, which may have potential roles as charge transporting components in new bioelectronic applications[100-102].

Figure 2.7: Pictorial representation of a conjugated oligoelectrolyte with delocalized π -backbone in the middle and terminated by ionic functionalities at the 4 ends (circles). Red lines depict pendant side groups, such as alkane chains.

Charge transfer can occur across a range of potential systems[102, 103]. The highly conjugated oligo(phenylenevinylene) system presents itself as an attractive option for efficient charge transfer as it is made up of co-planar phenyl groups linked by vinylene bonds, resulting in reduced rotation[102]. The conformational rigidity confers conjugation of the π -backbone and allows enhanced charge transfer. These conjugated oligoelectrolytes, designed to function as TETMs, have great potential for improving bioelectronic interaction between microorganisms and electrodes as they have been suggested to act as alternative charge transfer conduits. These are essentially ‘wire-shaped’ molecules that self-align and facilitate efficient charge transfer across the cellular membrane (Figure 2.8). The π -backbone (hydrophobic region) of the molecule is the charge transporting site, whereas the pendant ionic end groups and long alkyl chains (hydrophilic region) imparts and enhance solubility characteristics. The resultant amphiphilic

nature of the molecules further affords the ability to insert into, and align within, the lipid membrane.

Figure 2.8: Illustration of a single molecule attached to two electrodes as a basic component in molecular electronics. Adapted from [104].

In the application context of MFCs, these ‘wires’ should intercalate and align in the microbial cellular membrane, forming a conducting channel from the microbe to the extracellular charge collecting electrode (Figure 2.9). Pendant charged ionic functionalities and long alkyl chains aid in molecule solubility in polar media, such as water, which is the main solvent for media in MFCs. These molecules have strong propensity for insertion and self-assembly into lipid membranes of living microorganisms due to carefully designed hydrophobic aromatic core and well defined hydrophilic side chains. A schematic depicting a TETM used in this thesis, 4, 4’-bis (4’-(*N*, *N*-bis (6’’-(*N*, *N*, *N*-trimethylammonium) hexyl) amino)-styryl) stilbene tetraiodide, (DSSN⁺), intercalating in the bilayer lipid membrane is shown (Figure 2.9)

Figure 2.9: Schematic representation of DSSN+ spanning a bilayer membrane (Drawing not to scale).

MFCs inoculated with yeast and DSSN+ have demonstrated enhanced electrical output[8]. Notably, MFCs incorporated with nanomolar concentrations of DSSN+ were able to generate approximately 7× more bioelectricity than those without DSSN+, and MFCs with micromolar concentrations of conventional diffusion based redox mediator, i.e. methylene blue. Various electrochemical, optical and microscopy techniques were also performed to prove that these ‘molecular wires’ self-assemble in the lipid membranes[8]. Intercalation of DSSN+ into a microbial membrane has been further demonstrated with *E. coli* and has been applied in MFCs demonstrating improved charge collection[10, 11]. It has been proposed that the intercalation of

DSSN+ allows an intimate bioelectronic interaction between the microorganism and external electrodes, with DSSN+ acting as TETMs and not as conventional redox shuttles. Enhanced EET has also been attributed in the electrode driven reduction of fumarate to succinate in DSSN+ incorporated *S. oneidensis*[13]. In summary, by employing a series of conjugated oligoelectrolytes to chemically modify *E. coli* in conventional and/or high throughput MFCs[10, 11], *S. oneidensis*[11] or the microbial consortia in wastewater[9], an enhancement of the electrical output as well as biosynthesis of reduced metabolites and waste contaminant bioremediation have been demonstrated respectively. Recently, the differences in length of repeat units of the conjugated oligoelectrolytes have been shown to affect the rate of insertion into mammalian membrane patches and its relative stability[12]. The study further proposes that these molecules can facilitate facile transmembrane movement of ions or substrates across mammalian cells[12]. It can therefore be expected that the same system of chemically modified microorganisms using DSSN+ could be used to drive other reduction reactions such as formation of metal nanoparticles[105].

However, the exact mechanisms responsible for these charge extraction/injection reactions, afforded by incorporation of these novel TETMs, have not been pin-pointed. To this end, this thesis sought to elucidate a dominant mode of EET, which is prevalent in such chemically modified microbial systems by studying insertion and fluorescence of these molecules in *E. coli* employed in MFCs. Formation of gold nanoparticles will be further monitored to deepen understanding on charge transfer mechanisms. Electrical characterization will be performed on DSSN+ to ascertain its charge transfer characteristics. The ability of

DSSN+ in restoring deleted EET pathways in specific *S. oneidensis* mutants will also be ascertained.

Chapter 3: Materials and Methods

This chapter details materials and experimental methods used in this thesis. All materials and chemicals were used as received, unless otherwise stated.

3.1. Synthesis and preparation of DSSN+ for use in MFCs

DSSN+ (Figure 3.1) was produced through a series of organic synthesis processes and further characterized[8]. Coupling was performed between a bis(methylene)phosphonate and the amino-functionalized benzaldehyde via a *trans*-selective Horner-Wadsworth-Emmons reaction to produce DSSN. In order to prepare DSSN+, quaternization was performed using trimethylamine. 1 mM stock solution of DSSN+ was prepared in ultrapure water and filter-sterilized prior to use. If required, only the appropriate volume is added to the anode chamber or reaction tubes to achieve the desired concentration.

Figure 3.1: Chemical structure of DSSN+.

3.2. MFC Construction and Data Acquisition

The bioelectrochemical system employed in this thesis is MFCs. Dual chambered, U-tube devices were constructed as reported previously[11, 57, 106-109] (Figure 3.1). This particular architecture has been adopted from other configurations due to its ease of assembly. Further, the whole device can be autoclaved as a unit after assembly to ensure full sterility. This is in contrast to other configurations which requires assembly from scratch after autoclaving the individual components. These additional steps may compromise sterility during the assembly process. Other MFC architecture and systems have been used in trials, however sterility is usually compromised and there are extensive leakage issues.

The anode and cathode chambers were made of two 90° 28/15 ball-to-plain-end and socket-to-plain-end glass adapters (17 mm O. D. × 1.8 mm wall thickness) (VWR, Singapore) separated from each other by a circular piece of Nafion® N117 (Ion Power, USA) proton exchange membrane (PEM). These membranes must meet several desired properties such as high proton conductivity, low electronic conductivity, impermeability to fuel gas or liquid, good mechanical toughness in both the dry and hydrated states, and high oxidative and hydrolytic stability in the actual MFC environment. Commercial PEMs, such as Nafion® N117 are widely used in MFCs and have many advantages such as high proton conductivities at moderate operating temperatures and a wide range of relative humidity as well as good physical and chemical stabilities.

The glass tubes were sealed against the PEM with high-vacuum silicone grease and held in place with a 28/15 stainless steel pinch clamp (#28) (VWR, Singapore). Carbon felt electrodes (#43199, 3.18 mm thickness) (VWR, Singapore) were cut to 2 cm × 5 cm dimensions (width × length) and connected at one end with titanium wire (#460400, 0.25 mm diameter) (Sigma Aldrich, Singapore) with nylon screws and nuts (Small Parts, Inc., USA). The electrodes were then seated inside the glass tubes (Figure 3.1).

Prior to MFC operation, the devices were filled with ultrapure water and autoclaved to sterilize the devices and internal components as a whole unit. After sterilization and decanting off the ultrapure water, the anode and cathode chambers were each filled with the respective sterile medium required for specific experiments. The required volume of live cell culture solution was then inoculated into the anode chamber. The final total volume of solution in each of the anode and cathode chambers was maintained at 20 mL. The anode chamber was sealed with a serrated silicone septum (Z512877, 18 mm O. D.) (Sigma Aldrich, Singapore) through which the titanium wire was threaded, while the cathode chamber was loosely capped with an inverted glass scintillation vial. The cathode electrodes were only partly submerged (~70%) in the catholyte to allow for an ‘air-wicking’ aerobic configuration. Typical connection resistances were less than 1 Ω . The electrodes were then connected to resistors of known resistances and voltage measurements were recorded at a rate of 1 point per 5 min using an eDAQ e-corder® data acquisition system (ED 1621 – 16 channel recorder) equipped with Chart™ software (Bronjo Medi, Singapore). Data collection started immediately after inoculation of the devices. The MFCs were operated inside an incubator at temperatures required for specific experiments, which are dependent on the inoculated bacterial strain. All experiments were conducted in

triplicates and data was further averaged. Voltage readings are collected as raw data and further converted to current density for presentation according to the equation and following explanation below. The MFC is connected across a 1 k Ω resistor.

$$I = \frac{V}{R}$$

where I is the current in amperes (A), V is the potential difference in volts (V) and R is the resistance in ohms (Ω). Current density is obtained by dividing the equation above by the projected geometrical surface area of the electrode (in cm²). The number of Coulombs of transferred electrons by individual MFCs across the operational time was acquired by integrating the area under the graph of the current (A) versus time (s) plots of the MFCs, according to the following equation.

$$C_e = \int_0^t I dt$$

where C_e is the number of Coulombs of electrons (C), t is the operational end time of the MFC in seconds (s), I is the current in amperes (A).

Figure 3.2: (A) Photograph of assembled MFC. (B) Schematic of MFC depicting *S. oneidensis* as the microorganism.

3.3. Assembly of electrochemical cell setup, instrumentation and data acquisition

1 cm × 1 cm carbon felt (#43199, 3.18 mm thickness) (VWR, Singapore) were used as the working electrode (WE). These were soaked overnight in 1 M HCl to remove residual metals and other contaminants before storage in deionized water. The WE was connected to titanium wire (0.25 mm diameter) (Sigma Aldrich, Singapore) via a nylon screw and nut (Small Parts, USA). The other end was soldered to a copper wire after housing the assembly in a glass capillary. Robust interconnects were confirmed by probing with a multi-meter to ensure low resistance (less than 1 Ω) in the assembly.

The counter electrode consisted of a titanium wire wound into 20 coils of ~ 3 mm diameter. The Ag/AgCl reference electrode (RE-1B, Alpha Analytical, Singapore) were connected to the electrochemical cells via a conducting salt bridge (2 g lysogeny broth agar in 1 M KCl) assembled from a glass capillary, heat shrink tube (50-60 mm length, Bioanalytical Systems, USA) and vycor frit (4 mm diameter, 4 mm length, Bioanalytical Systems, USA). The electrode capillaries were inserted through ports in a custom made Teflon lid sealed with an O-ring gasket (Shenton Engineering, Singapore). The lid was designed to fit the 20 mL conical electrochemical cell (MF-1084, Bioanalytical Systems, USA), which had been thoroughly washed previously. After assembly of the various components and addition of a magnetic stirring bar, the electrochemical cell was autoclaved for sterility. The salt bridge and reference electrode were then subsequently added, prior to inoculation.

Electrochemical cells were operated under a constant flow of sterile (passed through sterile 0.2 μm syringe filter) and humidified N_2 gas to ensure full anaerobic conditions in the headspace and solution in the electrochemical cell. The solution in the electrochemical cells was constantly stirred with magnetic stirring bars during chronoamperometry (CA) measurements. Stirring was only stopped temporarily during cyclic voltammetry (CV) measurements to minimize noise during analysis. The electrochemical cells were maintained at 30°C in a circulating water bath for optimum biofilm growth.

Data acquisition was achieved through a 5-channel potentiostat (VSP, Bio-Logic, France) connected to the electrochemical cells. The software from the same manufacturer (EC-Lab) was used for data acquisition. The parameters for each technique were:

CA: Potential poised at the working electrode = 0 (or 200) mV versus Ag/AgCl

CV: Potential swept from -800 mV to 200 mV versus Ag/AgCl; scan rate = 1 mV/s

3.4. Toxicology analysis

Minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) of DSSN+ were determined for *E. coli* using a modified protocol[110]. Both results indicated that DSSN+ showed toxicities against *E. coli* with MIC at 64 μ M and MBC at 128 μ M. It should be noted that the concentrations used in the MFC and gold nanoparticle formation studies were much less than the determined MIC and MBC. However membrane perturbation may still be expected at low concentrations, rather than complete cell lysis.

Cells were cultured overnight in LB. Cells used for the MIC assays were diluted in phosphate buffered saline (PBS) to a cell density of $\sim 1.0 \times 10^6$ cells/mL. 50 μ L of LB was first added to a 96 well plate (Nunc, flat bottom clear). 50 μ L of 512 μ M DSSN+ was added to column 1 and serially diluted in a 96 well plate (Nunc, flat bottom clear). 50 μ L of the diluted bacterial suspension was then added to columns 1–11. The final concentration of DSSN+ ranges from 1 μ M to 128 μ M while the final density of bacterial suspension in each well was $\sim 5 \times 10^5$ cells/mL. A growth control well with no DSSN+ was also included to assess suitability of the broth for growth. In addition, a sterile control well that was not inoculated was also included to ensure medium sterility. Growth within each well was monitored spectrophotometrically by measuring absorbance at 600 nm using a microplate reader (Tecan M200 Pro, Austria) for 24 h at 40 minute intervals. The MIC end-point used was IC₅₀, which is the lowest DSSN+

concentration that reduced growth of a bacterial species to less than 50% of the appropriate control (100%)[111, 112].

MBCs were determined by streaking samples from each well onto LB agar and observing growth after 24 h of incubation. MBC is defined as the minimum concentration at which there is no growth on the plates after 24 h.

3.5. Confocal imaging of stained biofilms

Confocal laser scanning microscopy (CLSM) techniques were employed to acquire confocal fluorescence images of *E. coli* biofilm stained with DSSN+. Carbon felt electrodes were removed from operating MFCs and fibres colonized by *E. coli* stained with DSSN+ were obtained from these electrodes for imaging purposes. A Carl Zeiss CLSM model LSM 780 with 34 channels (32-GaAsP array detector + 2 × PMTs) and transmitted light-PMT (T-PMT), equipped with an Axio Observer inverted microscope, laser diode 405 nm, laser argon multiline (458, 488 and 514 nm), diode- pumped solid-state (DPSS) laser (561 nm), helium/neon laser (633 nm) and different objective lenses (20×/0.4 N.A, 40×/0.6 N.A, 40×/1.3 N.A Oil, 63×/1.4 N.A. Oil and 100×/1.4 N.A. Oil), was used to acquire images. Image processing was performed with the software package, Zen 2011 (Carl Zeiss). Specifically, an excitation wavelength of 488 nm and Plan-Apochromat 100×/1.4 NA oil immersion objective lens were used to acquire images.

The same technique was employed to acquire images of the carbon felt electrodes, which were colonized by green fluorescent protein (GFP)-tagged *S. oneidensis* and *E. coli*. The electrodes were stained with 4', 6-diamidino-2-phenylindole (DAPI) to facilitate independent analysis of the mixed species colony. GFP and DAPI image channels were illuminated with 488 nm and 359 nm laser excitation respectively. To determine the syntrophic association on the electrode, GFP and DAPI images were acquired simultaneously on two detection channels with the DAPI channel accounting for both *E. coli* and *S. oneidensis* and GFP channel only for wild-type *S. oneidensis* MR-1. Image stacks were acquired using 20×/0.4 N.A objective lens. The images were then re-constructed using Zen 2011. IMARIS (Bitplane AG, Zurich, CH) software was used to stimulate the three-dimensional images and compute essential parameters such as bio-volume. Simultaneous imaging on two separate channels enabled the processing of the exact compositions of *S. oneidensis* and *E. coli* on the electrode.

3.6. Bacterial strains and growth conditions used in the syntrophic microbial community

In Section 4.6, wild-type *E. coli* and *S. oneidensis* (tagged with GFP) bacterial strains were cultured aerobically overnight in sterile LB medium at 37°C and 30°C respectively. *S. oneidensis* mutant strains lacking CymA outer-membrane cytochrome expression ($\Delta cymA$), MtrA outer-membrane cytochrome expression ($\Delta mtrA$) and MtrC-OmcA outer-membrane cytochrome expression ($\Delta mtrComcA$) were also cultured in the same manner. Other *S. oneidensis* MR-1 strains lacking hydrogenase enzymes include $\Delta hydA$, $\Delta hyaB$ and $\Delta hydA/hyaB$. These mutants were employed to verify whether hydrogen is a major metabolite of exchange in the syntrophic relationship between *E. coli* and *S. oneidensis*. Prior to aerobic culturing, the stock

cultures were stored in LB medium with 50% glycerol at -80°C. The list of bacterial strains used in this study is listed in Table 1.

Table 3.1: Bacterial strains used in Section 4.5

Strains	Locus tag	Description	Reference
<i>Escherichia coli</i> K-12	-	Wild-type fermentative strain	[113]
<i>Shewanella oneidensis</i> MR-1	-	Manganese reducing strain isolated from Lake Oneida, NY	[114]
$\Delta cymA$	SO4591	CymA gene deletion derivative of <i>S. oneidensis</i> MR-1	[115]
$\Delta mtrA$	SO1777	MtrA gene deletion derivative of <i>S. oneidensis</i> MR-1	[115]
$\Delta mtrC/omcA$	SO1778 SO1779	MtrC-OmcA gene deletion derivative of <i>S. oneidensis</i> MR-1	[115]
$\Delta hydA$	SO3920	HydA gene deletion derivative of <i>S. oneidensis</i> MR-1. Also referred to as Fe-only hydrogenase mutant	[115]
$\Delta hyaB$	SO2098	HyaB gene deletion derivative of <i>S. oneidensis</i> MR-1. Also referred to as NiFe hydrogenase mutant	[115]
$\Delta hydA/hyaB$	SO3920 SO2098	HydA-HyaB gene deletion derivative of <i>S. oneidensis</i> MR-1	[115]

Chapter 4: Results and Discussion

This chapter investigates how enhanced electroconductivity was demonstrated through the synthetic molecular electronics approach in MFCs. Formation of gold nanoparticles through bio-reduction was used as a marker to probe the EET mechanism in chemically modified *E. coli*. The formation of gold nanoparticles was then further confirmed through control experiments. A *S. oneidensis* mutant strain lacking specific genes (MtrC/OmcA) for EET was also employed to uncover the charge transfer mechanism in DSSN+ incorporated microbial systems. Electroconductivity was further demonstrated through an alternative route via rational choice of specific microbial species. Syntrophic interactions between the species with a specific electron donor (glucose) affect electroconductivity and structural compositions/dynamics. By extending the application of a co-culture system, concurrent bioelectricity generation and bioremediation were subsequently demonstrated.

Common tools employed include MFCs, electrochemical techniques, current-voltage characterization, absorption spectroscopy measurement and various microscopy techniques (confocal laser scanning microscopy and field emission scanning electron microscopy). The consistently low current densities from all MFCs were attributed to high internal resistances within the bioelectrochemical devices of this particular architecture, which impede charge movement. Various methodologies can be adopted to enhance electrical output, as discussed in Chapter 2. However, the focus of this work is to study (1) the specific EET mechanism brought about by DSSN+ in microbial systems, and (2) the enhanced electroconductivity and/or concurrent bioremediation through a syntrophic microbial community.

4.1. Enhancing electroconductivity in chemically modified E. coli with DSSN+

Water-soluble conjugated oligoelectrolytes have been demonstrated to intercalate spontaneously in amphiphilic lipid bilayers and were further suggested to function as alternative charge transfer conduits in microbial systems whose membranes constitute lipid bilayers. As discussed in Chapter 2, intercalation and self-assembly in lipid membranes are aided by pendant ionic groups, long alkyl side chains and the amphiphilic nature of the molecule, arising from a hydrophobic core and hydrophilic side chains. Charge transfer is speculated to occur across the central delocalized π -conjugated aromatic backbone. Although the mechanism has not been fully explored, it is hypothesized that the TETMs have modulated the cell membrane which causes a transmembrane electron transfer phenomenon, driving the increase in electroconductivity in these chemically modified bacterial systems. The TETMs employed in the subsequent studies to investigate the charge transfer mechanism is DSSN+.

4.1.1. Confirming DSSN+ incorporation in E. coli with confocal and electron microscopy images

From literature, the absorption wavelength of DSSN+ is 412 nm[8]. Given this information and using the equation:

$$E = h \times f$$

where E is the energy in eV, h is Planck's constant (6.63×10^{-34} m² kg / s) and f is the frequency, which is also equivalent to c/λ (c is the speed of light (3×10^8 m / s) and λ is the wavelength in nm), the band gap of DSSN+ is 3.01 eV. In order to investigate the ability of DSSN+ to

intercalate in a real microbial cell membrane, confocal microscopy images of planktonic *E. coli* cells stained with DSSN+ were taken at excitation wavelength of 488 nm. *E. coli* K-12 (ATCC #10798) was purchased from American Type Culture Collection (Manassas, VA). The stock culture was stored in lysogeny broth (LB) medium with 25% glycerol at -80°C. LB medium was prepared from trypticase peptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L) dissolved in ultrapure water and sterilized by autoclaving. Although it is a complex media, LB was chosen due to its relative ease to culture most bacterial strains successfully. When needed, *E. coli* was grown aerobically in LB medium at 37°C overnight. Images of the *E. coli* biofilm were collected using a FEI XL40 Sirion FEG Digital Electron Scanning Microscope at acceleration voltage of 5 kV. At the end of the MFC runs, the carbon felt anodes were removed from the MFCs and soaked for 24 h in a 2% formaldehyde solution prepared in 10 mM phosphate buffer for cell fixation. The electrodes were then dehydrated by soaking in a 70% ethanol solution and subsequently dried under ambient conditions for 72 h in a sterile environment prior to image acquisition.

The confocal and corresponding bright-field microscopy images show DSSN+ readily incorporating in the membranes of planktonic *E. coli* bacterial cells and exhibiting fluorescence under excitation (Figures 4.1A and B). This strongly suggests that the introduced DSSN+ has spontaneously intercalated in the cell membranes of *E. coli* without external influences to the microbial system. No other fluorophores have been introduced to the system, hence the resultant fluorescence has been attributed to DSSN+. In addition, only *E. coli* has been introduced to the system. SEM images at various magnifications of 2000× and 10000× show *E. coli* colonizing the surfaces of carbon felt electrodes to form a biofilm (Figures 4.1C and D).

Figure 4.1: (A) Confocal microscopy image of planktonic *E. coli* bacterial cells showing DSSN+ fluorescence. (B) Corresponding brightfield microscopy image. (C, D) Scanning electron microscopy (SEM) images showing *E. coli* biofilm on fibers of carbon felt. Adapted from [11].

In addition, bright-field, corresponding confocal and overlaid microscopy images of DSSN+ intercalating in cell membranes of *E. coli* colonized on fibers of carbon felt electrodes are collected (Figures 4.2A, B and C). These collective images strongly suggest that DSSN+ are indeed present in *E. coli* cell membranes and could potentially facilitate transmembrane charge transfer out of the bacterial cell and onto the conductive carbon felt, which functions as the terminal electron acceptor. It should be noted that no external influences were introduced to the system. Independent fluorescence was observed solely from DSSN+ interaction within *E. coli*.

Figure 4.2: (A) Brightfield image of *E. coli* biofilm colonized on a strand of carbon felt electrode surface. (B) Corresponding confocal fluorescence image. (C) Overlaid confocal fluorescence and brightfield image. Adapted from [11].

4.1.2. Employing DSSN+ to enhance bioelectronic performance in *E. coli* MFCs

After confirming the ability of DSSN+ in intercalating in the microbial membrane of *E. coli*, a study was conducted to investigate how DSSN+ affect microbial systems in terms of EET. MFCs were chosen as the platform to investigate the role and impact of DSSN+ in modulating interaction at the microbe-electrode (biotic-abiotic) interface. At this point, it is still unclear if enhanced EET was brought about by (1) improved charge transfer across the conducting backbone of DSSN+, or (2) through structural alteration of the inherently insulating microbial cellular membrane, resulting in release of electroactive redox components via the perturbed membrane.

The degree of interaction was determined by the extent of EET from the internal metabolic cycles of *E. coli* to the extracellular carbon felt electrode. Current densities generated by the MFCs over ~ 120 h were recorded (Figure 4.3). *E. coli* MFCs with 5 µM DSSN+ consistently produced higher current densities (average current density of ~ 2.25 µA/cm²) than

MFCs without DSSN+ incorporated (average current density of $\sim 1.25 \mu\text{A}/\text{cm}^2$). These observations suggest that DSSN+ could have induced transmembrane charge transfer by either mechanism mentioned above.

Periodical spikes during MFC operation may be attributed to daily dislodging of gas bubbles (hydrogen and carbon dioxide), which tend to accumulate in the anode chambers after prolonged operation as the microbes respire. Movement of the MFCs to dislodge these bubbles to reflect true performance of the system could have formed spikes during daily maintenance of the devices. Although there may be some form of electrical cross-talk, proper cable shielding and adequate spacing between cables used in the data acquisition system have been ensured to eliminate this phenomenon.

Figure 4.3: Current density versus time plots of *E. coli* MFCs without DSSN+ and 5 μM DSSN+.

The average numbers of Coulombs of charge collected were determined for each system over ~ 120 h (Table 4.1). These values were calculated by integrating the area under the curve of the current (A) versus time (number of s) graph over ~ 120 h to obtain number of Coulombs of harvested electrons.

DSSN+ incorporated *E. coli* MFCs were able to harvest $\sim 62\%$ more electrons (Table 4.1), as compared to systems without DSSN+. Although the mechanism is still unclear, this analysis suggests that DSSN+ could have functioned as alternative charge transfer conduits to enhance bioelectronic performance. Further studies were undertaken in subsequent sections to

investigate the proposed charge transfer mechanisms. MFCs were operated in triplicates and high reproducibility was achieved for each set of experimental parameter (Figure 4.3).

Table 4.1: Average amount of electrons collected from *E. coli* MFCs without/with DSSN+

Average number of Coulombs of electrons collected (up till Day 5) (C)	
<i>E. coli</i> without DSSN+	1.01±0.0839
<i>E. coli</i> with 5 µM DSSN+	1.64±0.0140

4.1.3. Concentration effect of DSSN+ on number of charges harvested from *E. coli* MFCs

After DSSN+ has been established to be associated with improved bioelectronic performance, a concentration effect study of DSSN+ on bioelectricity output was carried out to further probe its effect in modulating the microbe-electrode interface. *E. coli* MFCs were constructed as described previously and average current densities are recorded with varying DSSN+ concentrations (Figure 4.4). The average numbers of Coulombs of electrons harvested versus DSSN+ concentration were determined (Table 4.2), with the corresponding graph (Figure 4.5). It is observed that the average current density increases with higher DSSN+ concentration. This suggests that with increased DSSN+ concentration in the system, more charges can be hypothetically siphoned from the microbial systems. However, a saturation point was reached at a concentration of 10 µM. This possibly implies that the DSSN+ molecules have fully saturated the cell membranes at this particular concentration. It is interesting to note that the average number of Coulombs of harvested electrons at 25 µM DSSN+ is slightly lower than that at 10 µM. This is a possible indication that at high DSSN+ concentrations, charge transfer across the cell membranes could be hindered or slight cell toxicity could be induced. However, the

correlation between numbers of Coulombs of harvested electrons with varying DSSN⁺ concentration corroborates the central role of DSSN⁺ in modulating transmembrane electron transfer.

Although incorporation of DSSN⁺ at higher concentrations may induce cell toxicity as shown above, cell toxicity and cell lysis are not expected at the concentrations employed in this study. This is because they are well below the minimum inhibitory and minimum biocidal concentrations of 64 μ M and 128 μ M which have been determined for DSSN⁺ with *E. coli*, respectively[11]. Therefore, the enhanced bioelectronic activity observed is not attributed to redox active molecules released from *fully* lysed cells. However, the possibility of leaked redox active molecules from membrane perturbation (*without full cell lysis*) caused by DSSN⁺ intercalation in *E. coli* cannot be ruled out.

Figure 4.4: Average current density versus time plots of *E. coli* MFCs with varying DSSN+ concentration.

Table 4.2: Average amount of electrons collected from *E. coli* MFCs with varying DSSN+ concentration

Average number of Coulombs of electrons collected (up till Day 5) (C)	
<i>E. coli</i> without DSSN+	0.96002 $\pm$ 0.0322
<i>E. coli</i> with 1 μM DSSN+	1.31016 $\pm$ 0.308
<i>E. coli</i> with 5 μM DSSN+	1.62885 $\pm$ 0.21657
<i>E. coli</i> with 10 μM DSSN+	1.91381 $\pm$ 0.0078
<i>E. coli</i> with 25 μM DSSN+	1.78041 $\pm$ 0.14115

Figure 4.5: Graph of average number of Coulombs of electrons collected versus DSSN+ concentration.

A schematic of the discussion earlier is summarized for this section (Figure 4.6). In brief, incorporation of the novel conjugated oligoelectrolyte, DSSN+, was demonstrated in the context of a bioelectrochemical device, i.e. MFCs, to improve bioelectronic performance by modifying the microbe-electrode interface. Spontaneous intercalation was confirmed via confocal microscopy and electrical performance was investigated by using MFCs. This is the first time that DSSN+ has been incorporated in *E. coli* MFCs to investigate the electrical performance in a modified microbial system. Concentration effects were found to affect device performance in terms of average number of Coulombs of electrons collected and a trend is followed, up to a certain degree. However, until this point, only the MFC platform has been used to elucidate the charge transfer mechanism afforded by DSSN+. The mechanism by which charge transfer is

enhanced is still unclear and will be investigated in subsequent sections using further experiments, specifically, 3-electrode electrochemical cells.

Figure 4.6: (A) Schematic depicting spontaneous intercalation of DSSN+ into the bacterial cell membrane. (B) Incorporation of membrane-modified microorganisms in a MFC resulting in enhanced power generation. Adapted from [11].

4.2. Investigation of electrical properties of DSSN+

To further evaluate if DSSN+ is able to function as an alternate charge transfer conduit through the mechanism of transferring charge across its backbone, its electrical conductivity was probed by a 2 terminal electrode measurement (Figure 4.7). Based on the hypothesis that DSSN+ can facilitate charge transfer across its conjugated backbone, which is inherently conducting, the electrical conductivity of the molecules should be higher.

Molecular monolayer junctions overlaid with nanowires of an oligo(phenylene vinylene) (OPV) backbone have been electrically characterized previously[116]. This system has been

compared against backbones of dithiolated dodecane and oligo(phenylene ethylene) (OPE) molecules. Conductance from π -conjugated oligomers (μA region) was several orders of magnitudes higher than saturated alkanes (nA region) as revealed from current-voltage measurements of these individual nanowires. OPV has the highest conductance of all 3 systems, which can be attributed to higher coplanarity, which results in improved π -conjugation. Shorter bond-length alteration which causes a smaller HOMO-LUMO (highest occupied molecular orbital - lowest unoccupied molecular orbital) gap also improves electrical conductivity. Other electrochemical characterization have also ascertained the superior conductivity of the OPV backbone with respect to the OPE backbone[102, 103].

Experiments were thus designed to deposit and electrically probe a thin layer of DSSN+ across conducting gold electrodes to test its conductivity. DSSN+ was first dissolved in Ultrapure water and drop-casted onto a 200 nm silicon nitride surface. In this manner, DSSN+ molecules can be dispersed uniformly across the channel length. The test platform was in the form of a bottom gate – bottom contact thin film transistor configuration (Figure 4.7). A 200 nm silicon nitride dielectric surface was deposited on top of the heavily doped silicon bottom gate through plasma enhanced chemical vapour deposition method. Chromium was employed as the adhesion layer for the interdigitated gold electrode (channel width: 1000 μm ; channel length: 30-100 μm). This layer was fabricated through a series of lithography and electron beam deposition procedures.

The substrates went through a standard cleaning process: acetone sonication (5 min), isopropanol sonication (5 min), deionized water sonication (5 min) and ultraviolet air plasma (2

min). 100 μL of 1 mM DSSN+ (dissolved in water) was then drop-casted onto the cleaned substrate and allowed to dry overnight in the dark in ambient conditions to achieve a thin DSSN+ film through complete solvent evaporation.

Figure 4.7: Schematic depicting electrical characterization of DSSN+.

Electrical characterization of the DSSN+ thin film was performed through 2 terminal electrode measurements by a Keithley 4200 Semiconductor Characterization System (SCS) connected to a Desert Cryogenic Probe Station. DSSN+ exhibited extremely low currents (~ 200 pA) when swept through a systematic and independent series of voltages (Figure 4.8A). The pA amplitude is comparable to noise in the characterization system and is even lower than the nA region measured from saturated alkane system mentioned above. Figure 4.8B shows the current-voltage characteristics of the DSSN+ thin film measured in the dark and under illumination. Sweep 1 (black trace) was performed in the dark for an initial comparison (similar to previous

measurements illustrated in Figure 4.8A). Sweeps 2 and 3 (red and blue traces) were subsequently performed in full and continuous illumination under a xenon/halogen lamp. Photons emitted from intense light would normally generate photo-excited charges in organic molecules which would result in generation of increased photo-currents. Comparing the red and blue traces against the black trace, it is evident that no significant photo-currents were generated with continuous illumination.

Figure 4.8: Current-voltage (I-V) curves of DSSN+ in a 2 electrode system. (A) I-V characteristics under systematic voltage sweeps. (B) I-V characteristics under dark and illumination conditions.

Based on low currents (pA region) measured (Figure 4.8A) and the lack of photo-currents (Figure 4.8B) in DSSN+ after intense and continuous illumination, it is likely that the molecules do not exhibit any conducting or even semi-conducting behaviour. Functioning organic molecules will usually exhibit photo-excitation when a strong light source is introduced. The electrons will be excited by the photons and hop across the band gap from the HOMO to the LUMO and tunnel to the metal contact. This phenomenon will then be reflected in a spike in current production over exposure time to the light source. To argue for the low voltage ranges that were scanned in these experiments, an assumed linear extrapolation in the I-V graphs will still yield extremely low currents (nA region), similar to that of saturated alkanes, which is insulating in nature. In summary, based on the observations, it is suggested that DSSN+ is non-conducting, suggesting that enhanced EET was not due to the improved charge transfer across the conducting backbone of DSSN+. The analyses in this section add to the overarching argument of this thesis and do not serve as an immediate conclusion.

4.3. Using gold nanoparticles as a marker to investigate EET in E. coli chemically modified with DSSN+

In this section, the specific EET mechanism in chemically modified *E. coli* was investigated with gold nanoparticles as the marker[105]. *E. coli* K-12 (ATCC #10798) was purchased from American Type Culture Collection (Manassas, VA) and treated as described in Section 4.1.1. 100 μ L of filter-sterilized DSSN+ stock solution (1 mM prepared in ultrapure water) was added to the culture solution ($OD_{600} \sim 1.0$) to achieve a $\sim 5 \mu$ M DSSN+ concentration. The mixture was further incubated at 37°C (shaking at 200 rpm) for 5 h to ensure

full intercalation of DSSN+ in the microbial membrane, and the resultant microbial system was subsequently employed in forming gold nanoparticles. All reaction tubes, bungs and ultrapure water were autoclaved for sterility after thorough cleansing. For the formation of gold nanoparticles, 5 mM of chloroauric acid solution (HAuCl_4 , Sigma Aldrich) was prepared in ultrapure water. The solution was then filter-sterilized. 50 μL of DSSN+ stock solution was added to 2 mL of *E. coli* culture and 2 mL of as-prepared chloroauric acid solution as control experiments. Control experiments with unmodified *E. coli* under the same reaction conditions were also prepared. Experimental procedures for water-based formation of gold nanoparticles were modified from reported literature[117].

Characterization of the bio-synthesized gold nanoparticles was performed through optical absorption spectroscopy, field-emission scanning electron microscopy (FESEM) and energy-dispersive x-ray (EDX) spectroscopy techniques with a JEOL, JSM-7600 F field emission scanning electron microscopy (FESEM) with an Oxford attachment at an operating voltage of 5 kV. MFCs were further employed to confirm the specific EET mechanism driving bio-reduction of AuCl_4^- ions to form gold nanoparticles. By comparing *E. coli* cells with DSSN+ incorporated into the membrane and the isolated supernatant after DSSN+ interaction with *E. coli*, the mechanism for EET, leading to reductive biotransformation of AuCl_4^- ions based on leaked cytosolic components was suggested. Building on these observations, an alternative explanation for the enhancement of bioelectronic performance, which has been observed in Section 4.1, is subsequently suggested to be due to leaked electro-active cytosolic components.

Location of the formed nanoparticles will reveal where the reduction reaction has occurred. Extracellular nanoparticle synthesis is therefore possible when transmembrane electron transfer has taken place. If intracellular nanoparticles are revealed, it would suggest that bio-reduction has occurred within the confines of the cells as the ions had diffused into the cell interior. Therefore, it can be expected that DSSN+ incorporated microorganisms can form extracellular nanoparticles, if the hypothesis of imparting or enhancing transmembrane charge transfer characteristics is valid.

E. coli was chosen as the model microorganism for experimental continuity. Through systematic control experiments (Figure 4.9), the mechanism behind extracellular reduction of chloroaurate ions to form gold nanoparticles by chemically modified *E. coli* was resolved. Based on the results, a model for reductive biotransformation of aqueous AuCl_4^- ions to gold nanoparticles via leakage of redox molecules from the perturbation of microbial membrane by DSSN+ insertion is suggested. This was further extended to explain enhanced bioelectronic performance in MFCs using DSSN+ modified microorganisms.

4.3.1. Bio-reduction by DSSN+ modified *E. coli* to form gold nanoparticles

Incorporation of DSSN+ into the membrane of *E. coli* was achieved through an extended incubation period of 5 h and the resultant microbial system was subsequently introduced to the reaction tubes containing HAuCl_4 . After ~ 15 min, purple precipitates could be observed in the reaction tube containing *E. coli*, DSSN+ and HAuCl_4 (Figure 4.9, Tube 2). Tube 1 (control) did not contain any DSSN+ and did not reveal any purple precipitates.

Figure 4.9: Photograph depicting reaction tubes at 0 h. (1) *E. coli* + HAuCl₄; (2) *E. coli* + HAuCl₄ + DSSN+; (3) HAuCl₄; (4) Supernatant + HAuCl₄; (5) *E. coli*/DSSN+ pellet + HAuCl₄; (6) HAuCl₄ + DSSN+. Supernatant in Tube 4 was collected by centrifugation after DSSN+ interaction with *E. coli*. Tubes 1, 3, 5 and 6 did not show any purple precipitates even after 6 days of incubation, indicating absence of gold nanoparticles (Appendix, Figure A1).

After 4 h of incubation, distinct purple precipitates were observed in the aqueous media of the reaction tube containing *E. coli*, DSSN+ and HAuCl₄ (inset of Figure 4.10A). The inset compares the reaction tube containing HAuCl₄ mixed with *E. coli* (1) and DSSN+ incorporated *E. coli* (2) after 4 h. No colour change was observed in the control reaction tubes without DSSN+, indicating the absence of such a reaction. Optical absorption spectroscopy analysis performed on

the nanoparticles at the wavelength range of 450 nm to 800 nm, using a Shimadzu UV 3600 UV-visible absorption spectrometer, showed an optical absorption spectrum with a distinct absorption peak at ~ 550 nm (Figure 4.10A). This peak is a feature of gold nanoparticles as they exhibit absorption in the visible region of the electromagnetic spectrum (~ 520 nm) due to the excitation of surface plasmons[118]. This observation was supported by FESEM characterization, which indicated extracellular formation of discrete nanoparticles around *E. coli* in the aqueous media in the size regime of ~ 50 nm (Figure 4.10B). After incubation for 6 days (Appendix, Figure A1), the gold nanoparticles ripened and grew to ~ 150 nm (Figure 4.10C). This characteristic is expected since there are no ligands attached and passivating the growth surface of the nanoparticles[119, 120]. Spot-profile EDX analysis was performed on the nanoparticles at 20 kV to reconfirm them as gold nanoparticles (Figure 4.10D). Formation of gold nanoparticles was used to indicate EET occurring in the DSSN+ incorporated microbial system. A series of control experiments was performed to identify the source of the gold nanoparticles (Figure 4.9). As indicated above, only reaction tubes containing DSSN+ incorporated *E. coli* yielded gold nanoparticles when mixed with HAuCl₄. Reaction tubes containing mixtures of DSSN+ and HAuCl₄ (Tube 6) and HAuCl₄ alone in ultrapure water (Tube 3) did not produce any reduction reaction. This clearly illustrates the basic need for *E. coli* to interact with DSSN+ to facilitate reduction of AuCl₄⁻ ions.

Figure 4.10: (A) Optical absorption spectrum of nanoparticles formed in external aqueous media by bio-reduction of HAuCl_4 via interaction of DSSN+ with *E. coli*. The inset photographs depict reaction tubes with *E. coli* (1) and DSSN+ incorporated *E. coli* (2) after 4 h. Field emission scanning electron microscopy images of gold nanoparticles formed via interaction of DSSN+ with *E. coli* at 20 000 $\times$ magnification after 4 h (B) and 6 days (C) of incubation. (D) EDX spectrum of gold nanoparticles.

4.3.2. Elucidation of EET mechanism through gold nanoparticle markers

Two possible pathways are proposed for the extracellular formation of gold nanoparticles by DSSN⁺ incorporated *E. coli* (Figure 4.11). One such pathway is through EET via charge transfer across the delocalized backbone of incorporated DSSN⁺ (Mechanism 1). The second possible pathway is due to release of intracellular cytosolic components, such as redox molecules, from the cell interior, after DSSN⁺ incorporation. The intracellular components could then drive the HAuCl₄ reduction reaction (Mechanism 2). Various redox molecules that are present within the cell interior have been well documented[121, 122]. Hence, during DSSN⁺ incorporation, redox molecules could be released through membrane perturbation and quantified using liquid chromatography mass spectroscopy (LCMS), which will be discussed in later sections.

Figure 4.11: Proposed mechanistic model of EET by chemical modification of *E. coli* with DSSN+ for biotransformation of $HAuCl_4$ to gold nanoparticles. Chemical structure of DSSN+ is shown in the hatched box.

To further study the dominant EET mechanism, the reaction tube that incubated DSSN+ intercalation into *E. coli* was centrifuged to separate the DSSN+ incorporated *E. coli* bacterial cells from the intracellular components released into the supernatant. The centrifuged *E. coli*/DSSN+ pellet (after washing 3×) was further re-suspended in deionized water and vortexed to form a homogenous mixture. This was done to isolate solely DSSN+ incorporated *E. coli* cells

(without other residues). The addition of HAuCl_4 to these isolated *E. coli*/DSSN+ cells did not yield any nanoparticles (Figure 4.12A, 1). This further suggests that the enhancement of bioelectronic performance (through hypothesized charge transfer across the delocalized backbone of incorporated DSSN+) is not the primary mechanism for gold nanoparticle synthesis, as no nanoparticle formation was shown on the cell periphery by FESEM characterization (Figure 4.12B). Consequently, the supernatant which did not contain any DSSN+ incorporated *E. coli* cells yielded dark purple precipitates when HAuCl_4 was added to it (Figure 4.12A, 2; Appendix, Figure A1). The FESEM image (Figure 4.12C) and spot-profile EDX analysis (Figure 4.12D) of the dark purple precipitates confirmed the formation of gold nanoparticles. This indicates that the reductive mechanism was predominantly driven by intracellular cytosolic components released into the supernatant (Mechanism 2).

Figure 4.12: (A) Reaction tubes containing re-suspended *E. coli*/DSSN+ pellet (1) and supernatant (2) with HAuCl_4 precursor showing absence (1) and formation (2) of nanoparticles. (B) Field emission scanning electron microscopy image of re-suspended *E. coli*/DSSN+ pellet showing absence of nanoparticle formation. (C) Field emission scanning electron microscopy image of agglomerated nanoparticles collected from reaction tube containing supernatant and HAuCl_4 precursor. (D) EDX spectrum of gold nanoparticles.

If EET had indeed been facilitated via charge transfer across the backbone of DSSN+ as previously suggested, the HAuCl_4 precursor would have been reduced to gold nanoparticles by DSSN+ incorporated *E. coli* cells, resulting in distinct purple precipitates. However, no

precipitates were observed. It has been previously demonstrated that metallic biotransformation can be driven by secreted redox molecules, such as reductases[123, 124], flavins[122], or quinones[125]. These compounds are capable of reducing a variety of metals or metal oxides. By incorporating DSSN⁺ into *E. coli* cells, interaction between these components could have triggered the release of these redox compounds, which reduced the HAuCl₄ precursor to gold nanoparticles.

Due to its amphiphilic nature, DSSN⁺ can spontaneously intercalate in the *E. coli* cell membrane, as shown through confocal microscopy characterization in Section 4.1. However, the mechanical rigidity and limited molecular flexibility of its backbone could potentially perturb the cell membrane and compromise the structural integrity which causes cell membrane collapse or pinching[126]. This is caused by the pendant phenyl rings along the backbone moiety which limits molecular flexibility due to steric repulsion. Further, although the molecular length of DSSN⁺ (~ 3.8 - 4 nm) may seem to coincide with the thickness of the *E. coli* cell membrane (~ 4 nm)[127], the length of the conducting core (~ 1.8 - 2.1 nm) is in fact much shorter as the molecule is also made up of long alkyl chains at the terminal ends of the molecule to aid in solubility (Figure 4.13). These insulating alkyl chains are unable to contribute to charge transfer. The length mismatch between the conducting core of DSSN⁺ molecules and the *E. coli* membrane thickness impedes extracellular charge transfer and hinders electron injection. Based on these observations, it is therefore suggested that the re-suspended *E. coli*/DSSN⁺ pellet did not drive the HAuCl₄ reduction reaction. The reaction tube was monitored for 10 days and showed no signs of nanoparticle reduction. Mechanism 2 is therefore proposed to be the primary mechanism driving the bio-reduction of AuCl₄⁻ ions.

Figure 4.13: Schematic depicting lengths of DSSN+ and its conducting core in a microbial membrane.

Ultra-sonication was further employed to disrupt the *E. coli* membrane in an effort to mimic the hypothesized effect of DSSN+ intercalation, causing perturbation in microbial membranes. No DSSN+ was introduced in these subsequent experiments. Prior to ultra-sonication with a Sonics & MaterialM Vibracell VCX750 Probe Ultrasonicator, the culture was centrifuged (8000 rpm for 5 min) and washed 3× with ultrapure water to remove any residual components on the cell periphery. The resulting pellet was re-suspended in 20 mL of ultrapure water and ultra-sonication was carried out for 20 min at 30% amplitude at 20 kHz. The total process was 40 min with 5 s of pulsing, followed by 5 s of rest. In addition to this precaution to prevent overheating the system, which may cause the intracellular components to denature, ice was used to cool the system during ultra-sonication. Centrifugation at 12 000 rpm for 10 min was then performed to isolate the supernatant containing the released intracellular components. H_{Au}Cl₄ was then introduced to the supernatant and resulting pellet separately. No change was

observed in the control reaction tube (Figure 4.14A) containing supernatant isolated from unsonicated *E. coli*, however precipitates (Figure 4.14B) were observed after ~ 24 h in the system containing the supernatant collected after ultra-sonication. The FESEM image (Figure 4.14E) and spot-profile EDX analysis (Figure 4.14F) of the precipitates confirmed the formation of gold nanoparticles. Further, the reaction tubes containing the centrifuged pellets and HAuCl₄ (Figure 4.14C and D) did not produce any precipitates overnight. These observations support the above hypothesis, which states that it is the released intracellular cytosolic components containing redox molecules primarily driving the bio-reduction of AuCl₄⁻ ions. This set of experiments did not involve any DSSN+. Instead, ultra-sonication was employed as a means to substitute the effect of DSSN+.

involved the same architecture as that of a MFC, but *without* the presence of microorganisms (Figure 4.15A). The supernatant isolated from the DSSN+ incubation process was introduced to the anode chambers of the MFCs and electrical performances of the devices were monitored over time (Figure 4.15B). The device with 20 mL of undiluted supernatant generated a maximum current density of $\sim 0.04 \mu\text{A}/\text{cm}^2$, which stabilized to a steady state of $\sim 0.02 \mu\text{A}/\text{cm}^2$ (red trace, Figure 4.15B). The MFC containing supernatant from ultra-sonicated *E. coli* also generated a maximum current density of $\sim 0.14 \mu\text{A}/\text{cm}^2$, which stabilized to a steady state of $\sim 0.10 \mu\text{A}/\text{cm}^2$ (orange trace, Figure 4.15B). However, the device with re-suspended DSSN+ incorporated *E. coli* generated negligible current density (black trace, Figure 4.15B).

These bioelectrochemical data suggest that the intracellular cytosolic components are electron rich and are able to transfer charges to the terminal electron accepting electrodes. On the other hand, DSSN+ incorporated *E. coli*, which has been thoroughly washed to remove peripheral residues, did not transfer charges to the electrodes, resulting in negligible current density (black trace, Figure 4.15B). It should be noted that DSSN+ is still intercalated in the cellular membrane at this juncture. The minimum inhibitory and minimum biocidal concentrations of DSSN+ with *E. coli* have previously been determined to be $64 \mu\text{M}$ and $128 \mu\text{M}$ respectively[11]. Although the working concentrations employed were between $5 \mu\text{M}$ - $25 \mu\text{M}$, a certain degree of membrane perturbation can still be observed. However, due to the low concentration of DSSN+ introduced here, perturbation is restricted to a minimum, hence the amount of released cytosolic redox molecules is also low, which directly affects the current densities recorded in the MFCs. The uniformly low energy output from all MFCs was attributed to high internal resistance of the bioelectrochemical devices. DSSN+ incorporated MFCs with

the adopted architecture typically show low electrical output in the range of few $\mu\text{A}/\text{cm}^2$ (1-3 $\mu\text{A}/\text{cm}^2$)[8, 9, 11, 13]. However, the focus of this study was to investigate the effect of the supernatant in bioelectrochemical devices. Variations have only been made to the anode chamber of the MFCs and it is evident that electrical signals could be detected from the isolated redox-active supernatants introduced to the anode of the MFCs (Figure 4.15B, red and orange traces). It is clear that the electrical signals originate from the interaction of these redox-active components with the electrodes in the anode chamber.

Figure 4.15: (A) Schematic of MFC setup *without microorganisms*. (B) Current density versus time traces of MFCs with various supernatant concentrations and re-suspended *E. coli*/DSSN+ cells.

These findings strongly suggest that the bio-reduction of $AuCl_4^-$ ions was a result of cytosolic redox molecules being released via membrane perturbation. It is critical to note that DSSN+ incorporated *E. coli* did not contribute to any electrical signals in the MFCs or in the biotransformation of $AuCl_4^-$ ions after thorough washing. Therefore, improvements in

performances of MFCs incorporating such conjugated oligoelectrolytes should be carefully evaluated and corroborated in the future with parallel experiments, where transmembrane electron transfer is confirmed by using the chemically modified microbial system to drive other reduction reactions.

Cyclic voltammetry (CV) was further employed to probe the presence of electroactive redox components in the leaked cytosolic components after ultra-sonication and DSSN+ incorporation (Figure 4.16). CV was performed using a three-electrode setup with a CHI-660D Electrochemical Workstation (CH Instrument). The working electrode was platinum, the reference electrode was Ag/AgCl and glassy carbon was used as the counter electrode. The scan rate was 10 mV/s and untreated *E. coli* was used as a control experiment. CV traces revealed redox processes at ~ -250 mV (versus Ag/AgCl reference electrode). This suggests the presence of electroactive components, such as quinones, which can be found abundantly in the cytosol of *E. coli*[121, 125, 128, 129]. Similar redox potentials revealed from CV traces of the supernatant obtained after separate ultra-sonication and DSSN+ incorporation of *E. coli* suggest that both systems have released similar cytosolic components due to microbial membrane disruption. These redox potentials are different from those reported for DSSN+[8] ($\sim +550$ mV, $\sim +600$ mV and $\sim +800$ mV vs Ag/AgCl), indicating that DSSN+ could not have contributed to the revealed redox potentials. LCMS was also performed to identify the electroactive redox components. The presence of menaquinones (Molecular weight: 444.65 g/mol) was revealed in the leaked cytosolic components (Appendix, Figure A2). The peak intensity was less significant in the supernatant acquired after DSSN+ incubation (Appendix, Figure A2B) as compared to the supernatant acquired after ultra-sonication (Appendix, Figure A2A). This could be attributed to

more menaquinones released via intense ultra-sonication, as compared to lesser menaquinones released via 5 μ M DSSN+ intercalation. The aggressive action of ultra-sonication disrupted the bacterial membrane as compared to lesser degree of cell membrane disruption by DSSN+, which is in low concentration. Other peaks in the spectroscopy data have been attributed to signatures of other components, which have not been identified. However, by establishing the presence of menaquinone amongst other components, it can be suggested that both mechanisms (DSSN+ incorporation and ultra-sonication) released redox components which are found in the intracellular component of the bacterial cell, suggesting a strong correlation effect between both mechanisms. The same volume and optical density of cells were used for each procedure.

Figure 4.16: Individual cyclic voltammetry traces of supernatants collected from untreated *E. coli*, after ultra-sonication and DSSN+ incorporation, suggesting presence of electroactive components, such as quinones. Supernatants were mixed with 5 $\times$ phosphate buffered saline (PBS), pH 7. Scan rate was fixed at 10 mV/s.

In summary, the dominant EET mechanism was revealed through reductive biotransformation of AuCl_4^- ions using DSSN+ modified *E. coli*. These findings suggest a shift from previous studies which suggest the facilitation of bioelectronic interactions via improved charge transfer across the TETMs. Based on the findings, it is concluded that previously reported enhanced electrical output in MFCs, or formation of reduced metabolites, are dominant effects of microbial membrane perturbation caused by DSSN+, rather than charge transfer across the backbone. Structural integrity of the cell membrane was compromised, which caused leakage of intracellular cytosolic redox molecules through the microbial membrane. Careful and rational design of functional molecules is thus required to realize desired bioelectronic communication via enhanced charge transfer between microorganisms and terminal electron acceptors.

4.4. Electrochemical characterization of S. oneidensis mutants modified with DSSN+

To further understand if DSSN+ can chemically restore the metal reducing (Mtr) pathway in *S. oneidensis*, the mutant strain lacking MtrC/OmcA expression was employed. This particular strain was used to evaluate the hypothesis of DSSN+ replacing the deleted EET pathway by bridging the gap as an alternative or replacement charge transfer conduit. In brief, MtrC and OmcA are outer-membrane cytochromes, which are known to facilitate electron transfer to external extracellular electron acceptors through direct conductive contact across the bacterial cellular envelope[15, 130, 131]. MtrC is a decaheme c-type cytochrome localized in the outer cellular membrane of *S. oneidensis* and is proposed to be involved in anaerobic respiration by mediating electron transfer to extracellular mineral oxides that can serve as terminal electron acceptors[50]. The ability of both outer-membrane cytochromes to bind to and transfer electrons

to mineral oxides suggests that they function as terminal reductases when these oxides are used as respiratory substrates. However differences in their redox behaviour and binding properties indicate that they have different roles in the electron transfer reactions. A study has suggested that MtrC but not OmcA is required for biofilm development and growth when mineral oxides serve as the terminal electron acceptor[132]. Although an OmcA deficiency does not hinder cellular replication and growth, OmcA is required for efficient electron transfer and cellular attachment to the mineral oxide once maximum surface cell density is achieved. It is thus suggested that OmcA is important for overcoming barriers to electron transfer and cellular attachment caused by reductive dissolution of the mineral oxide surface from cellular respiration associated with achievement of high surface cell densities[132].

Potentiostat-controlled electrochemical cells were employed as the test platform as the half-cell potential poised at the anode can be controlled independently and decoupled from biofilm activity, cathode potential and chosen external resistance for a simplified platform to study EET mechanisms.

Chronoamperometry (CA) traces of wild-type and mutant strains were recorded (Figure 4.17). Current generation was observed for both poised potentials, indicating an expected microbial oxidative breakdown of sodium lactate and subsequent electron transfer to the working electrode. With a higher potential poised at 200 mV, both strains (Figures 4.17A, C) were capable of generating currents $\sim 7\times$ more than biofilms poised at 0 mV (Figures 4.17B, D), as the applied higher potential provides a more favourable electrochemical potential for electron transfer. The wild-type strain (Figures 4.17A, B) was able to produce $\sim 10\text{-}15\times$ more current than

the mutant strain (Figures 4.17C, D) as the MtrC-OmcA Mtr pathway is present for EET to occur. After a medium change was performed to remove secreted redox mediators and planktonic cells, 5 μ M DSSN⁺ was added to the electrochemical cells. The medium change was required to remove all secreted flavins in the solution as these compounds have been shown to mediate charge transfer. There should be no external interference contributing to charge transfer as only the influence of DSSN⁺ to charge transfer in these strains is to be studied. Planktonic cells were also removed as these cells are able to transfer electrons via direct contact mechanisms through the MtrC and/or OmcA pathway. The objective was to solely investigate the role of incorporated DSSN⁺ in facilitating charge transfer across the microbial membrane. After medium change, the current decreased significantly, indicating that planktonic cells and secreted riboflavin were primary factors contributing to current production. The anodic (oxidation) current increased significantly upon addition of DSSN⁺ in all circumstances, however it is unsustainable and rapidly declined subsequently. This suggests that the exogenously added DSSN⁺ was unable to sustain prolonged EET as all currents (red traces) decreased to that of the controls without DSSN⁺ (black traces) towards the end of the experiment. It is interesting to note that in wild-type strains without DSSN⁺ introduced, currents continued to increase after medium change. This indicates viability and robustness of the bacterial cells as they could continue to multiply and contribute to EET through oxidative breakdown of lactate, which could not be observed in DSSN⁺ incorporated cells. This suggests that the introduction of DSSN⁺ has compromised cell viability.

Figure 4.17: Chronoamperometry of *S. oneidensis* and MtrC-OmcA mutant grown at two different potentials. (A) Wild-type *S. oneidensis* poised at 200 mV. (B) Wild-type *S. oneidensis* poised at 0 mV. (C) MtrC-OmcA mutant poised at 200 mV. (D) MtrC-OmcA mutant poised at 0 mV. Poising at a higher potential after DSSN+ addition resulted in higher current production which rapidly declined in both wild-type and mutant systems. EET was not restored in mutant biofilm at both potentials even after DSSN+ addition.

Further, another compelling factor as revealed from the CA traces generated by the mutant strain at both potentials, DSSN+ did not restore the absent Mtr pathway to current levels exhibited by the wild-type strain (Figure 4.17C, D versus A, B). Collectively, these data suggest a deviation from previous claims which hypothesize that DSSN+ is able to bridge the absent EET mechanism.

CV analyses of the mutant strain were performed with variation in DSSN+ introduction (Figure 4.18). Non-catalytic voltammetry traces did not show significant swellings or reversible redox peaks, which indicates absence of charge transfer in both control experiments with/without DSSN+ and at both potentials. Further, the values of generated current were low. This was to be expected as the MtrC-OmcA gene has been deleted, hence the EET mechanism controlled by this particular protein was not functioning[130]. It should be noted that no difference in CV traces were observed after DSSN+ addition as no peaks were observed. If the deleted EET mechanism (MtrC-OmcA gene) was restored by DSSN+, CV analyses would register signature redox peaks at -201 mV or -163 mV vs. Ag/AgCl for OmcA and MtrC respectively[131, 133]. This clearly indicates the incapability of DSSN+ in restoring the absent EET mechanism from the periplasmic membrane to the extracellular environment.

Figure 4.18: Cyclic voltammety traces of MtrC-OmcA mutant. (A) MtrC-OmcA mutant poised at 200 mV. (B) MtrC-OmcA mutant poised at 200 mV with DSSN+ addition. (C) MtrC-OmcA mutant poised at 0 mV. (D) MtrC-OmcA mutant poised at 0 mV with DSSN+ addition.

Non-invasive electrochemical impedance spectroscopy (EIS) was used to characterize the microbe-electrode interface in the biofilm at increasing potentials from -500 to 200 mV, in the frequency range 200 kHz-50 mHz (Figure 4.19). The charge transfer resistances (R_{ct}) after inoculum were $6827 \pm 2 \Omega$ and $3775 \pm 1 \Omega$ for the wild-type *S. oneidensis* poised at 0 and 200 mV, respectively (Figure 4.19A, B). The R_{ct} for the MtrC-OmcA mutant was approximately 100× higher (Figure 4.19C, D), which is consistent with the current output results for both strains. This was demonstrated in the slight increase in impedance and is attributed to the absence of EET

mechanism, which caused the mutant biofilm to exhibit insulating properties. After ~ 20 h, the R_{ct} of the wild-type *S. oneidensis* poised at 0 and 200 mV decreased to $1816 \pm 1 \Omega$ and $1429 \pm 1 \Omega$, respectively, as planktonic cells and extracellular riboflavin accumulate. Following spent medium removal, R_{ct} increased again to 6654 ± 2 and $5940 \pm 2 \Omega$. Following addition of 5 μ M DSSN+, the R_{ct} increased 7% and decreased 12% for *S. oneidensis* poised at 0 and 200 mV, respectively. While this effect from DSSN+ is not negligible, it is not comparable with the decrease of R_{ct} due to extracellular flavins and planktonic cell accumulation. Interestingly, the addition of 5 μ M DSSN+ to the MtrC-OmcA mutant showed a similar trend, as it increased the R_{ct} of the culture poised at 0 mV and slightly decreased the R_{ct} of the culture poised at 200 mV. Therefore, DSSN+ has small positive effect on EET only when the culture is poised at 200 mV. It should be noted that addition of DSSN+ did not significantly alter the charge transfer resistance in all circumstances, suggesting that the molecule is not functioning as an alternative and effective transmembrane electron transport conduit, as previously suggested. Therefore, the temporarily increased current after DSSN+ addition in CA traces should be attributed to change in flavin concentration, rather than improvement in EET at the biofilm-electrode interface through DSSN+ intercalation[134].

Figure 4.19: Electrochemical impedance spectroscopy (Nyquist plots) of wild-type *S. oneidensis* and MtrC-OmcA mutant. (A) Wild-type *S. oneidensis* poised at 200 mV (B) Wild-type *S. oneidensis* poised at 0 mV (C) MtrC-OmcA mutant poised at 200 mV (D) MtrC-OmcA mutant poised at 0 mV.

To further confirm the role of DSSN+ in electrochemically based *S. oneidensis* systems at various electrode potentials, short term CA steps were employed after ~ 48 h of operation in electrochemical cells with and without DSSN+ addition. A typical short-term CA experiment lasts ~ 2 h, therefore current output is not significantly affected by cell growth. In both wild-type (Figure 4.20A) and mutant (Figure 4.20B) systems, no significant difference in current generation was observed after DSSN+ addition. This indicates that DSSN+ did not facilitate charge transfer at any poised potential. In particular, it did not restore the EET mechanism in the mutant MtrC-OmcA strain, as the low current level was not significantly increased to that of the wild-type strain. Observed current output is attributed to secretion of riboflavin from *S.*

oneidensis, which functions as a mediator. This observation corroborates previously discussed data. Slightly higher currents at higher voltages may be attributed to the release of intracellular components after DSSN+ incorporation, which are subsequently detected at such potentials. This set of short term experiments also validates long term CA results discussed previously. In both short and long term analyses, DSSN+ did not significantly alter the current generation in wild-type and mutant systems.

Figure 4.20: Short term CA steps of wild-type *S. oneidensis* and MtrC-OmcA mutant. (A) Wild-type *S. oneidensis* (B) MtrC-OmcA mutant. Representative results are reported.

In summary, electrochemical characterization of wild-type and mutant *S. oneidensis* strains revealed unsustainable bioelectronic performance. Improvement after chemical

modulation was only temporal with DSSN+ incorporation. Moreover, DSSN+ did not restore the absent EET mechanism in the mutant strain, resulting from the deleted MtrC-OmcA gene.

4.5. Effects of polarization and DSSN+ on *E. coli* cell viability

To further study the effect DSSN+ has on perturbing the cellular membrane, which may affect bacterial cell viability, polarization experiments were performed with *E. coli* modified with DSSN+. Electrochemical cells (preparation details listed in Chapter 3) were employed with *E. coli* (OD₆₀₀ ~ 0.1) which are poised at ± 200 mV, with and without DSSN+. Control experiments (Conditions 5 and 6) were performed in sterile beakers and without the presence of electrodes and external bias. Poising of external biases makes the electrodes thermodynamically favorable for biofilm formation and electrons to flow from the biofilm (formed at the electrodes) to the terminal electrode through various EET mechanisms.

CA traces of *E. coli* over 12 h (Figure 4.21A) showed increased current generation with 10 μ M DSSN+, which validates previous electrical data, indicating enhanced charge transfer processes with DSSN+. Observed current spikes could be attributed to instrumental variations. The low current generated is attributed to the limited EET capabilities and low concentration of *E. coli*. CFU counts of each parameter were performed with the drop-plate method (Figure 4.21B), where serial dilutions of the reaction mixture were expelled in 10 μ L droplets on agar plates with divided rows and columns to represent different dilutions. *E. coli* cells with DSSN+ and poising (at ± 200 mV) exhibited ~ 1000 $\times$ less CFU counts after 12 h of operation (Columns 2 and 4 in Figure 4.21B). However it should be noted that absence of external bias and electrodes,

which discourage biofilm formation, did not produce any significant decrease in CFU counts, regardless of the presence of DSSN+. This suggests that DSSN+ action could be more prevalent in a biofilm mode. However, the observation in the significant decrease in CFU counts, in both cases of ± 200 mV poisoning, illustrates the decreased viability of the *E. coli* cells after introduction to DSSN+. Although the employed DSSN+ concentration ($10\ \mu\text{M}$) is unable to cause ***complete*** lysis of the cells, it is sufficient to induce increased susceptibility through ***partial*** lysis via membrane perturbation.

Figure 4.21: (A) Chronoamperometry of *E. coli* grown at ± 200 mV with and without DSSN+. Representative results from electrochemical cells are reported. (B) Semi-log plot of CFU counts before and after 12 h of polarization with/without poisoning and with/without DSSN+. Data represent the average of triplicates.

4.6. Investigation of a function-driven syntrophic microbial community established by planktonic cells interacting with biofilms

Demonstration of electroconductivity and possible syntrophy were investigated through an alternative route via rational choice of specific microbial species, such as fermenting bacteria and anaerobically respiring bacteria. Syntrophic interactions coupled with a specific electron donor affects electroconductivity and population dynamics in the planktonic and biofilm phases.

Syntrophy between microorganisms involves closely coupled cell-cell associated interactions in microbial communities which play critical roles in biogeochemical cycles, waste decomposition and biofuel production. Diverse interactions have been observed to drive syntrophic systems. These include a resource-service system, which is a common syntrophic model, where one species produces a chemical compound which is consumed by another species for mutual benefits. The collective output through syntrophic interactions is typically greater than that of each of the monoculture systems. For example, a defined binary culture of the cellulolytic fermenter *Clostridium cellulolyticum* and the electrochemically active *G. sulfurreducens* respire on an extracellular electrode to convert a specific cellulosic biomass to useful energy[135]. However, there is an extensive focus on syntrophic microorganisms in planktonic configurations, which do not represent many of the natural environments that microorganisms inhabit. Hence, syntrophic interactions between fermenting and anaerobically respiring microorganisms residing in biofilms, commonly found in natural and engineered settings, needs to be studied. Such interactions are critical in a mixed species biofilm

environment as their collective interactions can ensure the viability and survival of the biofilm community.

E. coli was chosen as a representative fermentative microorganism as it is easy to culture with simple nutritional requirements. On the other hand, *S. oneidensis*, a well-studied metal reducing bacterium capable of reducing a wide range of metal ions, was used as a model anaerobically respiring bacteria. The *S. oneidensis* genome encodes a large number of membrane-bound multiheme c-type cytochromes that form an electrical conduit, allowing the microorganism to use various extracellular terminal electron acceptors, such as solid state electrodes as electron sinks. In addition, *S. oneidensis* produces soluble electron mediators such as riboflavin that can facilitate electron transfer between outer-membrane cytochromes and solid electron acceptors.

By using *E. coli* and *S. oneidensis*, the establishment of biofilm-mediated syntrophic interactions between fermentative bacteria and dissimilatory metal-reducing bacteria in MFCs is investigated. The assembly of the syntrophic structure is driven by specific microbial functions of the interacting microorganisms.

The selective media in the MFCs was specifically formulated to facilitate optimal growth of *E. coli* and *S. oneidensis*. The media was a 1:1 (v/v) mixture of M9 and M1 salt solution, containing 20 mM glucose as the sole carbon source. 2 mL of 1 M magnesium sulphate and 100 µL of 1 M calcium chloride were added last to the selective media. The carbon felt electrode in the anode chamber served as the terminal electron acceptor.

M9 salts consisted of sodium monohydrogen phosphate heptahydrate (64 g/L), monopotassium phosphate (15 g/L), sodium chloride (2.5 g/L) and ammonium chloride (5 g/L). All components were dissolved in ultrapure water. M1 salts consisted HEPES (7.2 g/L), sodium hydroxide (0.3 g/L), ammonium chloride (1.5 g/L), potassium chloride (0.1 g/L), monosodium phosphate (0.52 g/L), and further supplemented with trace amounts (1% v/v) of mineral stock solution, vitamin stock solution and amino acids stock solution. Both salt solutions were autoclaved for sterility.

4.6.1. Investigation of syntrophic interactions between co-cultures in MFCs

To test the syntrophic co-culture relationship between *E. coli* and *S. oneidensis*, MFCs were employed as a platform to determine the electrical output with both species inoculated only into the anode chamber. Both anode and cathode chambers contained the same selective media mentioned earlier. MFCs were operated for each experimental parameter and the temporal profiles of the average current densities by each of the combination of wild-type or mutant strains of *S. oneidensis* were recorded (Figure 4.22). The co-culture MFCs produced a significantly higher average maximum current density ($\sim 2.0 \mu\text{A}/\text{cm}^2$), whereas monoculture MFCs produced negligible current densities (inset of Figure 4.22). These findings suggest that there may be syntrophic interactions between *E. coli* and *S. oneidensis*. Further, the data revealed a rapid increase ($\sim 0.17 \mu\text{A}/\text{cm}^2/\text{h}$) in the average current density generated by the co-culture system, suggesting an immediate establishment of the syntrophy. Current density of the co-culture system saturated at a maximum of $\sim 2.0 \mu\text{A}/\text{cm}^2$ before decreasing after ~ 72 h due to the consumption of limited organic substrates in the modified media. This is because the single fed

batch MFC system was employed in this thesis. This is in contrast to a continuous fed system, where the energy source can be renewed through a steady exchange of spent and fresh medium.

Figure 4.22: Electrical output of MFCs inoculated with mono- and co-cultures of *E. coli* and *S. oneidensis* microbial strains. The inset depicts maximum current density generated by each parameter (average $\pm$ standard deviation). Data represent the average of triplicates. Biological replicates, showing consistent highest current generation from co-culture system, are included in Appendix, Figure A3.

4.6.2. Investigation of the role of metal-reducing (*Mtr*)-pathway in syntrophy of the co-culture system

The dissimilatory metal reducing bacterium, *S. oneidensis*, can reduce various forms of external terminal electron acceptors, such as extracellular metal(loid)s or electrodes, through

direct and/or mediated electron transfer mechanisms[72]. Among which, the Mtr-pathway consisting membrane-bound c-type cytochromes, including CymA, MtrA, MtrC and OmcA, is one of the best characterized electron transport chains[49, 136-138]. CymA is bound in the cytoplasmic membrane and is essential in electron transfer from the quinone/quinol pool to the periplasmic space where MtrA will continue the electron transfer. MtrA, a periplasmic decaheme cytochrome *c* lipoprotein, can associate tightly with the trans-outer-membrane protein MtrB and it is hypothesized that electrons can be transported across the outer-membrane directly to MtrC[15]. Both MtrC and OmcA are outer-membrane cytochromes, which facilitate electron transfer to external electron acceptors through direct contact. To investigate the role of the Mtr-pathway in the syntrophic interactions in the MFC systems, *S. oneidensis* mutant strains lacking various CymA, MtrA, or MtrC/OmcA genes were employed to investigate their interactions with *E. coli*. MFCs employing these mutants in the syntrophic system exhibited a significant attenuation of average maximum current densities as compared to the wild-type syntrophic system (<0.5 vs. $\sim 2.0 \mu\text{A}/\text{cm}^2$) (Figure 4.23). This suggests a direct involvement of the Mtr-pathway in the syntrophic interactions between *E. coli* and *S. oneidensis*.

Figure 4.23: Electrical output of MFCs inoculated with co-cultures of *E. coli*, *S. oneidensis* and mutant Mtr microbial strains. The inset depicts maximum current density generated by each parameter (average $\pm$ standard deviation). Data represent the average of triplicates.

4.6.3. Identification of the metabolite of exchange in the syntrophic co-culture system

Hydrogen and formate are common metabolites of exchange in syntrophic systems and are produced from glucose fermentation by *E. coli*[139, 140]. To investigate the role of hydrogen transfer in the MFC-based syntrophic system, *S. oneidensis* mutant strains lacking hydrogenases were used to interact with *E. coli*. MFCs containing co-cultures of *E. coli* and *S. oneidensis* hydrogenase mutants generated current densities of $\sim 3.0 \mu\text{A}/\text{cm}^2$, which is comparable to the wild-type co-cultures (Figure 4.22, 4.24A and B), suggesting that hydrogen is not a key

metabolite of exchange in this syntrophic system. It is interesting to note that MFCs containing *E. coli* and hydrogenase mutants produced higher current densities and lasted longer. Based on additional experiments, the specific growth rate of wild-type and hydrogenase mutants are similar. However, towards the end of the stationary phase (~ 24 h), the hydrogenase mutants exhibited a higher biomass than the wild-type (OD₆₀₀ – wild-type: 0.327±0.0050; $\Delta hydA$: 0.369±0.0100; $\Delta hyaB$: 0.505±0.0148; $\Delta hydA/hyaB$: 0.438±0.0018).

Figure 4.24: Electrical output of MFCs inoculated with co-cultures of *E. coli*, *S. oneidensis* and mutant hydrogenases microbial strains. (A) Current density generated as a function of time. (B) Maximum current density (average $\pm$ standard deviation). Data represent the average of triplicates.

To further elucidate the metabolite of exchange, the main fermented products (namely, lactate, formate and acetate) were first identified and monitored over the operation time of the MFCs using high performance liquid chromatography (HPLC), which has been equipped with a HPX-87 H (Bio-Rad) ion exchange column (300 mm $\times$ 7.8 mm) and UV detector with 8 mM

H₂SO₄ as the mobile phase at a flow rate of 0.6 mL/min. Formate, acetate and lactate were identified as the three main metabolites (Figure 4.25). The concentration of lactate was low (< ~ 1.0 mM) throughout the MFC operation. Acetate concentration remained relatively constant (~ 10.0 mM) as it cannot be utilized by *S. oneidensis*. In contrast, the concentration of formate increased rapidly to ~ 45.0 mM over ~ 48 h and decreased thereafter to ~ 0 mM within ~ 100 h. This observation agrees well with the electrical output of co-culture MFCs which started to decrease after ~ 48 h (Figure 4.22). Taken together, these results suggest that formate is the main metabolite of exchange in this MFC-based syntrophic system.

Figure 4.25: Plot of metabolite concentration in MFC anode chamber containing the syntrophic system.

4.6.4. Characterization of the community structure across anode media and electrode

Community dynamics and interactions between specific bacterial species are fundamental in determining how syntrophic systems function as an entity in various ecological settings. To elucidate the structure-function relationship of the syntrophic system, the community structure in terms of CFU and biovolume of samples from the anode media and the electrodes were quantified.

A small representative section of the electrode, periodically retrieved from the anode chamber of the co-culture MFCs, was rinsed with sterile selective M9/M1 salt solution (1:1 v/v) into 2 mL of M9/M1 solution to generate slurry of bacterial cells and carbon felt. This reflects bacterial cells in the biofilm state. Four serial dilutions of the slurry mixture were prepared for the drop-plate method. Each of the dilution was expelled at a respective volume of 10 μ L onto LB agar plates with surfaces divided into rows and columns with each dilution occupying one row. The dispensed drops of slurry were allowed to dry on the plates before turning it over for incubation at 30°C for ~ 16-20 h. The typical dilutions consisting of colonies within the range of 5-50 were only considered for each 10 μ L drop. The GFP-tagged *S. oneidensis* bacterial cells were counted in the dark by using a fluorescent torch and the non-fluorescent *E. coli* cells were counted in light. The viable bacterial cells were expressed as colony forming units per mL (CFU/mL).

Seven serial dilutions of the anode media were prepared for the anodic media suspension which is rich in bacteria in the planktonic state. The drop-plate method was performed with each

row on LB agar plates catering to each dilution. The typical dilutions consisting of colonies within the range of 5-50 were only considered for each 10 μ L drop. The GFP-tagged *S. oneidensis* bacterial cells were counted in the dark by using a fluorescent torch and the non-fluorescent *E. coli* cells were counted in light. The viable bacterial cells were expressed as colony forming units per mL (CFU/mL).

Although both *E. coli* and *S. oneidensis* are classically shown to be good biofilm forming organisms[122, 141, 142], *E. coli* was found to represent the majority of the cells (~ 98%) (Figure 4.26A) in the planktonic fraction of the MFC-based syntrophic community, while *S. oneidensis* was dominant in the biofilm state on the electrode (biovolume fraction ~ 60%) (Figure 4.26B). It is noteworthy to mention that the electrode serves as the sole electron acceptor for *S. oneidensis* respiration while the sole carbon source (glucose) for *E. coli* fermentation is in the aqueous media. This observation implies that the distribution of each species in the syntrophic community could be driven by metabolic functions towards an optimum communal metabolism (Figure 4.26A, B). It has been reported that the combination of fermentation and anaerobic respiration across different species is more thermodynamically favorable than a sole fermentative process by a single bacterial species, based on the energy yield per electron transferred in the respective reactions[143]. Hence, the establishment of a dual species biofilm community of *E. coli* and *S. oneidensis* on the electrode may have an advantage over monospecies biofilms formed by *E. coli*. Over the course of the MFCs operation, *S. oneidensis* was preferentially localized on the anode electrode (Figure 4.26B), suggesting that it has developed an ecological niche at the locale of the electrode, probably due to its capability to utilize formate (from glucose fermentation by *E. coli*) for respiration at the anode electrode.

To understand how communal metabolism can be harnessed by the syntrophic system studied here, a material balance was performed on the glucose fermentation by *E. coli* planktonic cells. Based on the dry *E. coli* weight (derived from the CFU count in Figure 4.26a) and biomass yield of *E. coli* in glucose fermentation[144], it was revealed that ~ 60% of glucose is consumed by planktonic *E. coli* cells within 24 h, leading to the release of fermentation products. The number of electrons transferred from *S. oneidensis* to the electrode is equivalent to the number of electrons released by the oxidation of formate and lactate by *S. oneidensis*. Based on the generated concentrations of formate and lactate (Figure 4.25), it was observed that the number of electrons derived from *S. oneidensis* varied from 4.36×10^{17} (24 h), 6.05×10^{17} (48 h) and 2.41×10^{17} (72 h), which accounted for ~ 3-4% of the total electrons derived from the co-culture system. This suggests that the majority of electrons (~ 96-97%) are derived from *E. coli*, which utilizes flavins as electron shuttles. This points towards flavins-mediated electron transfer as the main extracellular electron transfer mechanism in the co-culture system. These results provide a novel perspective for syntrophic interactions. Although *E. coli* is considered an inferior electrogenic bacterium, the presence of *S. oneidensis* improves the electrogenic properties of *E. coli* through the release of flavins. This in turn releases simple metabolites for *S. oneidensis* to undergo anaerobic respiration. Hence, the syntrophic community which has been enriched with species of diverse metabolic functions is able to drive itself towards an optimum communal metabolism.

Figure 4.26: Community dynamics in the MFC-based syntrophic system. (A) CFU counts and relative proportion (%) of *E. coli* and *S. oneidensis* strains in anode media. (B) CFU counts and relative proportion (%) of *E. coli* and *S. oneidensis* strains on the anode electrode.

4.6.5. Proposed syntrophic model between *E. coli* and *S. oneidensis*

Based on the results obtained from this study, a syntrophic model between fermentative *E. coli* and a dissimilatory metal reducing bacterium *S. oneidensis* is proposed (Figure 4.27). *S. oneidensis* is unable to metabolize glucose in the anode media, while *E. coli* ferments glucose to produce metabolites, such as formate, which is selectively utilized by *S. oneidensis* as an electron donor in MFCs, where the anode electrode serves as the terminal electron acceptor (Figure 4.27, Pathway 1). The Mtr-pathway in *S. oneidensis* plays a critical role in the syntrophic interactions. *S. oneidensis* also produces soluble electron mediators (flavins) that can facilitate *E. coli* EET to the electrode. The electron mediators can also enhance the metabolic activity of *E. coli* in the syntrophic community (Figure 4.27, Pathway 2). To further elucidate whether flavins is critical in generating high current density in the syntrophic system, the concentration of extracellular flavins were quantified in monoculture and syntrophic systems. The highest flavins concentrations were observed in syntrophic systems after ~ 24 h, reaching up to $\sim 25 \pm 2.44$ nM, whereas monoculture systems produced significantly lesser flavins (*E. coli*: 8.0 ± 0.6 nM and *S. oneidensis*: 8.2 ± 0.3 nM) after ~ 24 h. The presence of low concentrations of extracellular flavins in *E. coli* monocultures may in part be attributed to a certain degree of cell lysis, leading to release of flavins. The enhanced current generation observed in the syntrophic system may thus be attributed to increased extracellular flavins secreted by *S. oneidensis*, which is consistent with previous reports[58, 136, 145]. It has also been observed that the addition of flavins boost the current production in *E. coli* monoculture systems[146]. Flavins were quantified using fluorescence measurements. 200 μ L of the cell free supernatant was transferred to a clear 96 well

plate and read at 440 nm excitation and 525 nm emission[147]. The background fluorescence was corrected by using the selective minimal media as the blank.

Figure 4.27: Model of the syntrophic relationship between *E. coli* and *S. oneidensis*. Glucose is fermented by *E. coli* to yield metabolites such as formate, which is taken up by *S. oneidensis* as electron donor. Flavin mediator molecules are secreted to facilitate electron movement to the external charge collecting electrode (1) to derive energy in MFCs. Flavins can also be taken up by *E. coli* to facilitate its respiration on electrodes as *E. coli* does not secrete flavins (2). Inset depicts the role of the Mtr pathway in *S. oneidensis* for syntrophic interactions with *E. coli*.

To further establish formate as the metabolite of exchange in the syntrophic model, a ‘starvation study’ was performed using the MFCs containing both wild-type strains (Figure 4.28). The average current density of the system was allowed to reach the peak value of $\sim 3 \mu\text{A}/\text{cm}^2$ before natural decline due to intentional starvation of available electron donors in the closed

system. A closed system refers to the single fed batch MFC system which was employed in this study. This differs from a continuous fed system, where the energy source can be renewed through a steady replacement of spent medium with fresh medium. Only sodium formate was subsequently introduced to the system and the current density was restored to its approximate original value. The subsequent increases of current density are slightly higher at $\sim 4 \mu\text{A}/\text{cm}^2$ after the injections of sodium formate as the anode chamber has become more anaerobic over time due to complete oxygen scavenging by the bacteria and the bacterial populations have matured. More released electrons could then be passed to the terminal electrode to produce energy.

Figure 4.28: Average current density of MFCs using the co-culture system, illustrating deliberate starvation and effect of subsequent formate introduction.

In summary, a syntrophic system between fermentative *E. coli* and the dissimilatory metal reducing microorganism, *S. oneidensis*, was established in MFCs with interacting planktonic culture in the anode media and biofilms on the electrodes. The Mtr-pathway and transfer of flavins play a critical role in the syntrophic interactions. Formate transfer, and not hydrogen, is the key mechanism in this syntrophic system. Further, the structural assembly of this syntrophic community is driven by metabolic functions towards an optimum communal metabolism. This study reports a function-driven structural assembly of syntrophic communities consisting of planktonic cells interacting with biofilms. A comprehensive understanding of such syntrophic interactions will inform better process designs for harnessing the power of such communities in various applications where both biofilms and planktonic cells coexist in natural and engineered settings.

*4.7. Investigation of simultaneous azo dye bioremediation and bioelectricity generation through a stable syntrophic microbial consortium of *P. putida* and *S. oneidensis**

In this section, a simple dual-species microbial consortium was defined, based on a model bioremediation microorganism (*P. putida*) and electrochemically active *S. oneidensis*, to examine concurrent bioremediation and generation of bioelectricity. Both species were cultivated in MFCs with the azo dye (Congo red) added to the MFCs with both planktonic *P. putida* and *S. oneidensis* biofilm to investigate the inter-species interactions within the co-culture system. *P. putida* is an established azo dye degrading microorganism (ADM) that can break down azo dyes by reductive cleavage of the molecular bonds[97]. Power generation, Congo red bioremediation

and anode biofilm formation were observed to study inter-species interactions to achieve simultaneous power generation and bioremediation.

Congo red was purchased from Sigma-Aldrich, Singapore (#C6277). 5 mM stock solution was prepared with ultrapure water and filter sterilized subsequently. As reported in literature, 2 mL of Congo red solution (5 mM stock solution) was introduced only to the anode chamber containing M1 minimal media with 20 mM sodium lactate (as the carbon source) to achieve a final Congo red concentration of 0.5 mM[148-151].

To examine the inter-species interactions of planktonic and biofilm cultures, co-cultures were employed in MFCs and bioelectricity production was recorded. Each experimental parameter was operated in sets of triplicates and the average current densities versus time profiles were recorded after inoculation of both species into the anode chamber (Figure 4.29). MFCs inoculated with only *P. putida* produced negligible energy output. Monoculture *S. oneidensis* MFCs produced an average current density of $\sim 0.75 \mu\text{A}/\text{cm}^2$ over 72 h, whereas the co-culture MFCs produced significant bioelectricity output ($\sim 1\text{-}2 \mu\text{A}/\text{cm}^2$).

S. oneidensis biofilms formed on the anode surface is critical in power generation in MFCs. Studies have reported that the increase in anodic current is related to the amount of biofilm formed on the electrode surface[152]. However, *S. oneidensis* biofilm formation might be affected by the presence of *P. putida* as it is known to form biofilms extensively with the help of its large secreted adhesion protein, LapA[153, 154]. The co-culture MFCs employing *P. putida* OUS82 $\Delta lapA$ and wild-type *S. oneidensis* were thus examined. This combination

produced a maximum average current density of $\sim 2 \mu\text{A}/\text{cm}^2$, which is approximately twice that of wild-type *P. putida* OUS82 and *S. oneidensis* co-culture systems (Figure 4.29). The higher bioelectricity output from this particular system suggests inter-species interactions occurring in the co-culture MFCs. The ability of *P. putida* OUS82 $\Delta lapA$ mutant to form biofilms on the electrode surface has been suppressed and this might favour *S. oneidensis* in forming an electroconductive biofilm over a larger area.

Figure 4.29: Current density versus time traces of MFCs with various combinations of *S. oneidensis* and *P. putida* mutant strains. Data represent the average of triplicates. Biological replicates showing consistent highest current generation from *P. putida* OUS82 $\Delta lapA$ + Wild-type *S. oneidensis* included in Appendix, Figure A4. Adapted from [109].

As control experiments, MFCs were further used to determine if Congo red is an extra electron donor for the dual-species system, and if *P. putida* utilizes sodium lactate as an

alternative electron donor (Appendix, Figure A5A). No appreciable bioelectricity was generated by monocultures of *S. oneidensis* and *P. putida* with Congo red (Appendix, Figure A5A). This is a clear indication that both species do not employ Congo red as an alternative electron donor. *P. putida* also produces negligible bioelectricity with sodium lactate, however *S. oneidensis* produces significant bioelectricity with sodium lactate, which is in agreement with previous data (Figure 4.29). Growth curves of *P. putida* OUS82 and *P. putida* OUS82 $\Delta lapA$ showed no difference in growth rates. Both strains were grown in M1 salt solution (incubated at 30°C, shaking at 200 rpm). OD₆₀₀ was measured hourly by a microplate reader over 7 h (Appendix, Figure A5B).

4.7.1. Congo red bioremediation in the dual-species MFC system

Quantification of Congo red degradation through bioremediation action of *P. putida* was analyzed through absorption spectroscopy measurements over 72 h (Figure 4.30A). Samples were collected from the anode chambers at different time intervals. These aliquots were centrifuged at 10 000 rpm for 10 min to remove suspended biomass from the liquid media. Decolourization of Congo red was monitored through optical absorbance spectroscopy at $\lambda = 496$ nm using a Shimadzu UV-visible spectrophotometer (UVmini-1240). Actual concentration was acquired against a standard calibration curve (absorbance vs. concentration). The initial concentration of Congo red in the anode chamber was 0.5 mM which produced an intense red colour (Figure 4.32A). After 72 h, Congo red concentration had decreased significantly in all co-culture systems employing wild-type *P. putida* OUS82/wild-type *S. oneidensis* and *P. putida* OUS82 $\Delta lapA$ /wild-type *S. oneidensis*. The solution in the anode chambers turned milky white,

further indicating decrease in Congo red concentration. However, the concentration of Congo red in MFCs containing monocultures did not decrease. This indicates a possible syntrophy in the co-culture systems which could have facilitated Congo red bioremediation. This hypothesis is supported through MFCs which were operated (Figure 4.30B). Syntrophic mechanisms could be inferred from the orange and purple traces.

S. oneidensis and *P. putida* OUS82 were inoculated into separate aerobic containers with M1 salt solution (with 20 mM sodium lactate) and 0.5 mM Congo red. Another set of MFCs containing individual *S. oneidensis* and *P. putida* OUS82 monocultures were operated concurrently for ~ 48 h. The supernatant from the separate aerobic containers were then collected after centrifugation and inoculated into the MFCs. Current densities (orange and purple traces) increased to values similar to a wild-type co-culture system (red trace). This observation suggests a syntrophic system where by-products of Congo red degradation (by *P. putida* OUS82) could be utilized by *S. oneidensis* to produce bioelectricity and vice versa. It is noteworthy to mention that wild-type *S. oneidensis* did not show decrease in Congo red concentration over 72 h, although studies have suggested secretion of flavins can facilitate azo dye bioremediation[42, 155]. However, demonstration of concurrent Congo red bioremediation with bioelectricity generation validates the choices of these specific bacterial strains in the co-culture system. Contrary to systems identified to date, which have mainly utilized diverse and complex microbial communities in the form of sludge, this study was based on the electroconductive microorganism, *S. oneidensis*, and azo dye degrading *P. putida*. The platform for further investigation into the mechanisms or possible syntrophy has been laid through identification of the microbial species responsible for dye degradation and power generation.

Figure 4.30: (A) Congo red degradation profile in MFCs with various combinations of *S. oneidensis* and *P. putida* mutant strains. (B) MFCs with various supernatants. Data represent the average of triplicates. Adapted from [109].

4.7.2. Biofilm characterization

Representative epifluorescence images were collected (using Zeiss Observer.Z1 inverted epifluorescence microscope at 20× magnification) from random strands of carbon felt electrodes in respective MFCs to identify the role of the biofilm in these systems (Figure 4.31). Biofilm formation could be observed in all systems as seen from the brightfield images. To differentiate between each species, *S. oneidensis* was tagged with red fluorescent protein (RFP), whereas *P. putida* was tagged with green fluorescent protein (GFP). From the collected images, GFP-tagged *P. putida* was absent in the *P. putida* OUS82/wild-type *S. oneidensis* system (Figure 4.31A, GFP). This is attributed to instantaneous biofilm dispersal due to decrease in surface adhesion when the biofilm is removed from the native environment which provides nutrients[156]. This is because electrode strands were removed from the M1 media housed in the anode chamber and transported to a separate facility for epifluorescence imaging. However, RFP-tagged *S. oneidensis* biofilm was evident (Figure 4.31A, RFP and Merged). In the *P. putida* OUS82 $\Delta lapA$ /wild-type *S. oneidensis* system, a thick RFP-tagged *S. oneidensis* biofilm could be observed (Figure 4.31B, RFP), whereas no GFP-tagged *P. putida* was present (Figure 4.31B, GFP). This analysis supports the hypothesis that more *S. oneidensis* biofilm could form (Figure 4.31B, RFP and Merged) to produce more bioelectricity (Figure 4.29). This is due to more available space on the electrode surface as *P. putida* OUS82 $\Delta lapA$ is unable to form a stable biofilm. The collected epifluorescence images agree well with the electrical data (Figure 4.29).

Figure 4.31: Representative epifluorescence images of RFP-tagged *S. oneidensis* and GFP-tagged *P. putida* mutant strains on electrodes from respective MFCs. (A) *P. putida* OUS82/wild-type *S. oneidensis*. (B) *P. putida* OUS82 Δ lapA/wild-type *S. oneidensis*. Adapted from [109].

4.7.3. Mechanistic insights into functional dual-species system

Based on the results and observations, the following mechanisms in the functional dual-species system were proposed (Figure 4.32). The actual setup of the MFC is also illustrated (Figure 4.32A). Congo red bioremediation is caused by *P. putida* (ADM) activity, whereas bioelectricity is generated through microbial oxidative breakdown of lactate by electrochemically active *S. oneidensis* (EAM) (Figure 4.32B). The metabolites of Congo red degradation after *P. putida* activity are potential electron donors for *S. oneidensis*. *P. putida* biofilm on the anode surface might occupy the physical space where electron transfer by *S. oneidensis* can occur. Thus, the *P. putida* mutant strain which is deficient in biofilm forming capability has better interaction with *S. oneidensis* in establishing a stable syntrophic microbial consortium for simultaneous azo dye removal and bioelectricity generation.

Figure 4.32: Proposed mechanisms of concurrent Congo red bioremediation and bioelectricity production by the dual-species system. (A) Photograph of MFC used in this study. (B) Schematic illustration of inter-species interaction in the MFC depicting Congo red bioremediation (by *P. putida*) and bioelectricity generation (by *S. oneidensis*). Adapted from [109].

In summary, a defined dual-species system was identified and demonstrated concurrent Congo red bioremediation with bioelectricity production by *P. putida* and *S. oneidensis* respectively in a single bioelectrochemical platform. By employing a mutant species, the biofilm and its spatial coverage are shown to be critical in this particular application through

epifluorescent images. Competition for space and the ability to form biofilms affect the amount of bioelectricity. This investigation is a deviation from conventional studies[99, 148, 149, 151]. Although oxygen flux/permeability may be critical to this system, this study was not undertaken as the main focus was to investigate a simplified microbial system for concurrent bioremediation and bioelectricity generation. Additional insights on the importance of biofilm formation and possible synergy were also gleaned from this study. Divergence from complex communities to bioremediate azo dyes and generate bioelectricity clearly illustrates that a complete understanding of inter-species interactions in identified planktonic and biofilm cultures will allow for manipulation of syntrophic microbial consortium for applications in engineered systems.

Chapter 5: Conclusion and Recommendations

5.1. Conclusion

In conclusion, facile incorporation of novel TETMs (DSSN+) into the cellular membranes of *E. coli* has been demonstrated for the first time. This characteristic is attributed to the nature of the artificially designed molecular system. The chemically modified *E. coli* has been further applied in MFCs to realize improved device performance through enhanced EET by modulation of the abiotic/biotic interface. Varying DSSN+ concentrations were revealed to affect electrical performance until a certain degree. An alternative EET mechanism was probed by the presence and location of chemically transformed gold nanoparticles after reductive bio-reduction of HAuCl₄ by DSSN+ incorporated *E. coli*. SEM and EDX characterization confirmed the formation of gold nanoparticles. The EET mechanism was subsequently uncovered to be predominantly driven by the release of cytosolic components through DSSN+ interaction with the membrane. Cyclic voltammetry data of supernatants isolated from both DSSN+ incorporated and ultra-sonicated (to mimic DSSN+ perturbation on membranes) *E. coli* systems confirmed similarly released cytosolic components, which also contributed to electrical signals in a MFC configuration. LCMS analysis revealed the presence of electroactive quinones. These findings suggest a shift from previous studies which hypothesize facilitation of bioelectronic interactions via improved charge transfer across the electrically conducting backbone of DSSN+.

Electrical characterization of DSSN+ in a 2 terminal electrode configuration showed that the molecule was not conducting, as indicated by extremely low currents. Photo-excitation of

DSSN+ did not produce any noticeable photo-currents. In addition, electrochemical characterization of wild-type and DSSN+ modified *S. oneidensis* strains also showed unsustainable bioelectronic performance. The *S. oneidensis* mutant strain with absent EET mechanisms was also employed. However, DSSN+ did not restore the deleted electron transfer mechanism. Further electrical polarization of *E. coli* with DSSN+ revealed significant attenuation in cell viability based on CFU counts, strongly suggesting DSSN+ has weakened the cells through perturbation mechanisms. Based on these findings, previously reported phenomena related to DSSN+ modified systems, such as enhanced charge transfer to carbon electrodes in MFCs, resulting in enhanced electrical output, and bioremediation of waste contaminants analyzed through decrease in chemical oxygen demand (COD), or electrode-driven formation of reduced metabolites, are concluded to be predominantly driven by microbial membrane perturbation caused by DSSN+, in addition to possible enhanced charge transfer. Structural integrity of the membrane was compromised by DSSN+, causing a change in the structural-property relationship, which allowed leakage of intracellular cytosolic components. In addition, although DSSN+ contains a conducting backbone, the long alkyl chains which aid in solubility are insulating and cannot facilitate charge transfer even when DSSN+ is fully spanned across the membrane space. In other words, the conducting core is shorter than the membrane width.

Congruent to defined objectives to study metabolite exchange and enhanced EET in natural microbial systems, defined bacterial species have been allowed to interact in a MFC setting. The syntrophic relationship between wild-type fermentative *E. coli* and electrochemically active *S. oneidensis* has been shown to be primarily driven by the exchange of formate, which is produced as a metabolite after *E. coli* metabolism with a defined electron

donor (glucose). The dominant driver for syntrophic EET between both species has also been identified to be flavins. These are electron shuttles which are secreted by *S. oneidensis*, and subsequently affect *E. coli* EET mechanisms. This points towards flavins-mediated electron transfer as the main extracellular electron transfer mechanism in the co-culture system. In addition, EET has been shown to be affected by the Mtr pathway in *S. oneidensis*. The roles of outer-membrane cytochromes and hydrogenases in this particular syntrophic system have also been studied through the use of various mutants. Community dynamics between both strains have been characterized and the results show a natural driving force in the syntrophic system, which influences the ability of individual strains in populating niche areas (independently in the planktonic and biofilm phases) in the community. HPLC analysis and a starvation study have been employed to determine the role of formate as the main metabolite of exchange. These collective findings could significantly influence and chart out future paradigms in interactions between bacterial strains in unique ecological settings where planktonic cultures interact with biofilms.

Further studies into syntrophic co-cultures for specific applications established a simple platform using a defined dual-species system (*P. putida* and *S. oneidensis*) for bioelectricity production and concurrent Congo red bioremediation. The role and spatial coverage of the biofilm was further revealed to be critical for bioelectricity generation.

To this end, two approaches have been employed to demonstrate enhanced EET and metabolite exchange in microbial systems. Based on the merit of magnitude of bioelectricity generation, both strategies achieved similar results. However, it should be noted that the

chemically modified microbial system (through DSSN+ incorporation) does not reflect true EET as the dominant charge transfer mechanism has been strongly suggested to be caused by release of intracellular electroactive components via membrane perturbation after DSSN+ incorporation. A high concentration of such artificial molecules is likely to induce toxicity in individual microbial systems, which will have dire consequences on EET. Consequently, EET and metabolite exchange demonstrated by the cooperative co-culture system of *E. coli* and *S. oneidensis* is a natural syntrophic interaction between both species in the absence of exogenous mediators. Further, this syntrophic system proves a strong community dynamics relationship between the planktonic (*E. coli*) and biofilm (*S. oneidensis*) communities based on functional needs. In natural or engineered microbial systems, where biofilms interact extensively with planktonic cells in a syntrophic manner, such knowledge gleaned from this study can aid in manipulating specific strains for enhanced EET, and even bioremediation.

5.2. Recommendations

5.2.1. Chemically modified systems

The chemical approach to modify bacterial species for enhanced EET can still be adopted with more stringent molecular design and deeper understanding of charge transfer between bacterial cells and external electron acceptors. One recommendation would be to adopt a less rigid chemical moiety as the conducting conjugated backbone so as to minimize membrane perturbation. By removing the insulating alkyl end-chains, a direct electrical conduit can be further established between the released electrons within the bacterial cell to the external

terminal electron acceptor. However, this would result in loss in solubility and the ability to self-assemble in the cell membrane. Hence, a compromise between these parameters has to be made. As a further test to validate these molecules, they should also be monitored through the formation of metal halide reduction, as detailed in this thesis.

5.2.2. Natural syntrophic systems

Understanding various forms of EET mechanisms in natural systems is critical for understanding how various microbial species can potentially interact and develop in a biofilm. *P. aeruginosa* is a common opportunistic pathogen and is known to have type IV pili on the surface of its cellular membrane. This facilitates twitching motility which allows the bacteria to move in various environments to colonize suitable areas[157-160]. The biofilm of this species is developed with these cell appendages. Knowledge of how EET mechanisms affect biofilm growth can aid in controlling the spread of this pathogen, which is the primary cause of persistent lung infections, such as cystic fibrosis.

Other than motility, the role of type IV pili can be investigated for its role in EET. Preliminary current density versus time data using MFCs inoculated with various *Pseudomonas* mutant strains reveal that type IV pili can indeed facilitate EET, in addition to its role in motility (Figure 5.1). $\Delta pilA$ MFCs produced the least average current density at $\sim 0.01 \mu\text{A}/\text{cm}^2$ (Figure 5.1A, blue trace) as the type IV pili gene has been deleted. The presence of minimum current density for this case is attributed to the role of charge transfer from pyocyanin, which is known to be an endogenously secreted charge transfer molecule[56]. MFCs inoculated with the $\Delta pilA$ +

pDA2 strain produced the highest average current density at $\sim 0.14 \mu\text{A}/\text{cm}^2$ (Figure 5.1A, red trace) and highest power density at $\sim 13 \mu\text{W}/\text{cm}^2$ (Figure 5.1B, red trace). This strain had the gene for pili production replaced, hence the possible restoration of EET via the charge transfer along the pili. These preliminary data suggests that type IV pili can be responsible for both motility and EET. However, more work needs to be conducted to validate this system. For example, conducting AFM has to be performed on the various strains so as to prove the presence and conductivity of the type IV pili.

Figure 5.1: (A) Average current density versus time graphs of various *Pseudomonas* strains. (B) Power curves of various *Pseudomonas* strains

EET at the cellular interface has inherent rate limiting factors, resulting in inferior charge transfer kinetics. This has created bottlenecks in the widespread limitation of MFC technology. As much as the technique is appealing in concurrent bioelectricity generation and waste contaminant bioremediation, MFCs have not been commonly implemented. In order to circumvent these problems, various modifications to minimize energy losses can be made on the electrode surface[161-163], device configuration[89], architecture[164, 165] and on the material aspect[74, 166]. Much effort has been centered on engineering the electrode surface to make it

more compatible and favourable for biofilm attachment and growth. Increasing the surface area and enhancing hydrophilic characteristics will allow the biofilm to be more robust, which will in turn enhance EET rates.

5.3. Novelty and scientific contributions

- Characterized the interaction of novel TETM (DSSN+) in microbial cellular membrane in *E. coli* bacterial system in the planktonic and biofilm mode. First demonstration of DSSN+ in MFCs to establish enhanced EET.
- Resolved charge transfer mechanism in chemically modified *E. coli* system. Established novel EET mechanism in DSSN+ modified microbial system through studying presence and location of gold nanoparticles (confirmed by SEM and EDX characterization) formed by reductive chemical transformation of HAuCl_4 . Utilized a series of electrical and electrochemical characterization techniques to further confirm molecular conductivity and investigate the effect of DSSN+ modulating charge transfer resistance at the abiotic-biotic interface in chemically modified mutant *S. oneidensis* systems, respectively.
- Established a working syntrophic relationship between *E. coli* (fermentative bacteria) and *S. oneidensis* (electrochemically active bacteria). Based on the natural driving forces in the co-culture system, a functional community comprising species occupying each niche area in different ratios in the planktonic and biofilm mode was characterized. Formate was determined to be the main metabolite of exchange and the Mtr pathway in *S. oneidensis* was revealed to be critical in driving EET in this syntrophic relationship. Flavins were identified to be the main driver for EET in the co-culture system.

- Concurrent bioelectricity generation and azo dye bioremediation were demonstrated in a simple dual-species system of *P. putida* (azo dye degrading microorganism) and *S. oneidensis* (electrochemically active bacteria) with enhanced degradation of the azo dye attributed to syntrophic interactions. The role and spatial coverage of the biofilm affecting the system were further established.

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1. **Wang, V.B.**, Du, J., Chen, X., Thomas, A.W., Kirchhofer, N.D., Garner, L.E., Maw, M.T., Poh, W.H., Hinks, J., Wuertz, S., Kjelleberg, S., Zhang, Q., Loo, J.S.C., Bazan, G.C., 2013. Improving charge collection in *Escherichia coli*-carbon electrode devices with conjugated oligoelectrolytes. *Physical Chemistry Chemical Physics*, 15(16), 5867-5872. (*Chapter 4.1*)
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2. "Water-soluble oligo-electrolytes increase extracellular electron transfer in *Shewanella oneidensis* MR-1 biofilms", Poster in 2nd Water Research Conference, Singapore, 2013
3. "Transmembrane Electron Transport Molecules (TETMs) - applications for biofilm reactors", Poster in 9th International Conference on Biofilm Reactors, Paris, France, 2013
4. "The potential for bioelectrochemical detection of fecal contamination indicator organisms in environmental water", Poster and Oral Presentation in The International Conference On Advances in Environment and Bio-Technology – EBT, Hong Kong, 2014

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Appendix

Figure A1: Photograph depicting reaction tubes after 6 days and comparison between various reaction tubes

Figure A2: LCMS analyses of leaked cytosolic components collected from (A) Ultra-sonication of *E. coli* and (B) DSSN+ incorporated *E. coli*.

Figure A3: Biological replicates of electrical output of MFCs inoculated with mono- and co-cultures of *E. coli* and *S. oneidensis* microbial strains. Data represent the average of triplicates.

Figure A4: Biological replicates of current density versus time traces of MFCs with various combinations of *S. oneidensis* and *P. putida* mutant strains. Data represent the average of triplicates.

Figure A5: (A) Average current density versus time traces of MFCs with *S. oneidensis* and *P. putida* in sodium lactate or Congo red. (B) Growth curves of wild-type bacterial strains in various media. Data represent the average of triplicates.