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**NANYANG
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SINGAPORE

**ORDERED MICROSTRUCTURED METAL ARRAY
ELECTRODES FOR BIOELECTROCHEMICAL SYSTEMS**

SOLANGE ELIZABETH ASTORGA

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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ELECTRODES FOR BIOELECTROCHEMICAL
SYSTEMS**

SOLANGE ELIZABETH ASTORGA

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Solange Elizabeth Astorga

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I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

05 Aug 2019

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Date

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A/Prof. Huang Yizhong

Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals where I am the first author.

Chapter 4 is published as S. E. Astorga; L. X. Hu; E. Marsili; Y.Z. Huang. Ordered micropillar array gold electrode increases electrochemical signature of early biofilm attachment. *Materials and Design journal*, 185, 108256. (2020).

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The contributions of the co-authors are as follows:

- I, Prof. Marsili, and A/Prof. Huang provided the initial project direction and conceptualized the study.
- I and Dr. Hu fabricated the microstructured gold film electrodes.
- I performed all the laboratory work at the School of Materials Science and Engineering and Singapore Center for Environmental Life Science and Engineering (SCELSE). I also analyzed the data
- I and Prof. Marsili wrote the manuscript drafts.
- All the authors reviewed the manuscript.

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- I and Prof. Marsili analyzed the data.
- I and Prof. Marsili wrote the manuscript drafts.
- All the authors reviewed the manuscript.

23 Feb 2020

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Date

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Solange Elizabeth Astorga

Abstract

Innovative applications like energy production from wastewater via microbial fuel cells, production of high-added value products, biosensors for bacteria detection in food, environment, and clinical samples, as well as understanding microbially influenced corrosion can be achieved through bioelectrochemical systems (BES). BES are powered by the extracellular electron transfer (EET) from bacteria into electrodes. Bacteria accumulate at interfaces to form biofilms. When they grow on electrodes, biofilms are termed electrochemically active biofilms (EAB). While biofilm formation and electrochemical activity are thought to be affected by the material and the microstructured of the interface, this interaction is not well-understood for early bacterial biofilms.

This dissertation aims to investigate early bacterial biofilms at electrode surfaces, through the fabrication and testing of ordered array microstructured electrodes with different topography and materials. The goals of this research were to understand (i) the EET from bacteria to the electrode, (ii) the role of the metal surface in the transfer mechanism, and (iii) the effect of electrode microstructure on early biofilm formation.

The first study involved three different gold microstructured electrodes fabricated with photolithography. The charge production showed that micropillar structures enhanced the charge transfer up to 22% higher than without any microstructure. The characterization of the electrode surface using electrochemical impedance spectroscopy (EIS) showed a higher conductivity and lower impedance for the microstructured electrodes after early biofilm growth at 24 h. Microscope analysis of the electrodes showed that *Escherichia coli* biofilm formation ensued at the base of the pillar microstructures. Confocal laser scanning microscopy images revealed that 41% of the cells on the electrode were alive, composing an early biofilm of 400 nm thickness. Further, the combination of the microstructures, electrochemical analysis, and imaging shed light on the EET at the biofilm/gold electrode interface.

The second study employed nickel microstructured electrodes analog to the gold electrodes used in the first study, to assess the effect of a different material on early biofilm formation. Microstructured electrodes showed a minimal enhancement on the

charge transferred (3%) for the chronocoulometry test, although the surface area was increased by 3 and 6% for small and large pillars, respectively. The cell/electrode interface was monitored through EIS at 0, 8, and 24 after cell inoculum at three different temperatures (23, 30, and 37 °C). The results showed a drop of the impedance during the first hours of the experimental runs, followed by a constant increase of the impedance with time. EIS analysis was carried on a range of electric potentials, temperatures and stirring conditions, to gain further information on the electrochemical signature at the cell/electrode interface. The results showed that non-stirring conditions favored reproducible results, rather than stirring conditions. Experiments at 37 °C displayed a redox active species at 0 V vs. Ag/AgCl, which is formed after 24 h.

The third study tested topographic arrangement of the features on the electrode. Microstructures of 10 µm height spaced by approximately 10 µm produced more current than bigger 100 µm gaps. However, the higher surface area available when using 10 µm vs 100 µm gaps (91% vs 3%) did not correspond with the increase in current measured (38% vs. 15%). This implies that the available surface is under-utilized during early bacterial biofilm detection, which suggests that the use of shorter pillars may be more efficient. Thus, efforts should be put into optimizing gap distance rather than the surface area of the pillars. Additionally, chronocoulometry revealed an increase in current with glucose concentration, while EIS analysis showed that capacitance increased when glucose concentration decreased. These results highlighted the sensibility of *E. coli* to the substrate concentrations in bioelectrochemical studies.

Overall, this dissertation advances the understanding of the bacteria-surface interaction in early biofilm formation, for a weak electrogenic model organism, using microstructured array electrodes fabricated on Au and Ni. The findings in this dissertation should have implications for bacterial biofilm sensing developments, as well as for preventing microbially influenced corrosion in drinking water systems and biomedical devices.

Lay Summary

Nature is the main inspiration for innovation. Looking into natural environments, researchers have found that bacteria are able to produce enough current to power small devices. Technologies such as energy recovery from wastewater, biosensors for bacteria detection in food, environment, and clinical samples can be derived from them. The working principle is based on the bacterial respiration metabolism known as extracellular electron transfer (EET). The electric charge can be deposited on metallic surfaces through oxidation and reduction (redox) processes. In a closed system, the electric charge can be measured through an electrochemical circuit, using electrodes and a conductive electrolyte which contains bacteria. Such schemes receive the name of bioelectrochemical systems (BES). The bacteria that accumulate and grow on the electrodes receive the name of electrochemically active biofilms (EAB). Researchers aim to understand the reasons behind poor BES performance in comparison with theoretical calculations. Performance is assessed by current, charge, potential, or impedance measurements depending on the applications. Although the electrode is a key component of BES, the effects of an early biofilm formation on its surface and the biofilm's capacity to transfer electrons are poorly understood, requiring more research.

This dissertation aims to explore early bacterial biofilm formation on microstructured electrodes that facilitate cell attachment and increase the electrochemical signature. *Escherichia coli* was used as a model organism that can be found almost in any ecosystem. The microstructure modification consisted on micro sized pillars fabrication on top of the surface of electrodes made of different metallic materials. The pillars varied in diameter and height, and were spaced at varying distances. The goals of this research were to understand, for early biofilm formation and electrode performance, (i) the effects on the EET mechanisms, (ii) the role that electrode materials play, and (iii) the effect of microstructured arrangements and surface modifications.

The first study involved the fabrication of gold micropillar arrangements on top of the electrode. The charge performance of the electrode in the BES showed that the use of short micropillar structures enhanced the charge transfer up to 22% higher than without any microstructure. Similarly, impedance performance showed higher conductivity for the microstructured electrodes. Later, the biofilm was observed attached on the

modified electrodes using two different microscopy techniques (confocal laser scanning microscopy and scanning electron microscopy). Bacterial cells accumulation was observed to occur particularly at the base of the pillar microstructures. Also, it was seen that 41% of the cells on the electrode were alive and the biofilm thickness was 400 nm. These findings contributed to the understanding of EET mechanisms occurring during early stages of *E. coli* biofilm attachment on gold electrodes.

The second study employed an arrangement of micropillars on nickel electrodes analog to the one used on gold, in order to assess the effect of a different material under the same prior conditions. Ni microstructured electrodes showed a minimal enhancement on the charge performance (3%), although the surface area was increased in 3 and 6%. To examine impedance, its performance was tested at short intervals of time. The results showed that the impedance dropped after the first hours of the early biofilm formation, followed by a constant increase over time. Also, impedance performance was measured under a range of temperature and stirring conditions. The performance showed an improvement under non-stirring conditions, while impedance at 37 °C suggested the presence of an unidentified chemical compound at 300 mV in the EET process.

The third study tested the effect of the micropillars of 10 µm height with a different arrangement in terms of spacing. Gap distances among microstructures of approximately 10 µm produced more current than bigger 100 µm gaps. However, the higher surface area available when using 10 µm vs. 100 µm gaps (91% vs. 3%) did not correspond to the increase in current measured (38% vs. 15%). This may be due to diffusional limitations under stirring conditions, which led to sub-optimal biofilm formation. Thus, future efforts should be put into optimizing gap distance rather than surface area. Further measurements showed that current increased with glucose concentration, while capacitance -a calculated component of the impedance- was inversely proportional to the glucose concentration. These results highlighted the sensibility of *E. coli* to the substrate concentrations in bioelectrochemical studies.

Overall, this dissertation advances the understanding of the bacteria-surface interactions in early biofilm formation, using microstructured array electrodes fabricated with Au and Ni. The findings in this dissertation should have implications for biosensing developments on electrochemical detection of early biofilm formation.

*To my Mom and her Mom (my Grandma), Lizzy Echazú and Julia Marta Álvarez,
to my Grandmas Albina del Carmen Campos and Rosa “Yaya” Silva,
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Abbreviations

2-HNQ	2-hydroxy-1,4-naphthoquinone
BEI	Backscatter Electron Images
BES	Bioelectrochemical systems
CA	Chronoamperometry
CC	Chronocoulometry
CLSM	Confocal Laser Scanning Microscopy
CV	Cyclic Voltammetry
EAB	Electroactive biofilm
EET	Extracellular Electron Trasfer
EIS	Electrochemical Impedance Spectroscopy
MFC	Microbial Fuel Cell
SEM	Scanning Electron Microscopy

Nomenclature

EET: Extracellular electron transfer (EET) is the process by which some microorganisms exchange electrons with an extracellular electron donor/acceptor, including naturally occurring metal compounds and artificial electrodes, across the cell membrane (Lovley, 2008; Kato, 2015)

BES: Bioelectrochemical systems (BES) are systems that use EET for sensing, biosynthesis or energy conversion. Certain BES combine waste and external electrical energy for the synthesis of chemical products (e.g., hydrogen, volatile fatty acids) formed at the cathode by an electrochemical reduction process (microbial electrolysis cells (MECs). BES can be used as environmental sensors to detect organic chemicals and contaminants that affect the EET process (Pant et al. 2012, and Kannan et al. 2019)

EIS: Potentiostatic Electrochemical Impedance spectroscopy (EIS) rely on the analysis and modelling of the AC current (e.g, the phase shift and amplitude change) that is generated across the liquid/electrode interface when an AC potential with defined amplitude is applied to the interface over a broad range of applied frequencies. The interface, comprising the attached cells or biofilm, is described through the equivalent circuit methodology, which allow a semi-quantitative description of the interface and biofilm properties (Christie L.C.Ellis, 2018)

Chapter 1

Introduction

This chapter gives an overview of the dissertation. Bioelectrochemical systems are powered by the extracellular electron transfer from bacteria into electrodes. Bacteria accumulate at interfaces to form biofilms, which when they grow on electrodes receive the name of electroactive biofilms (EAB). Early stages of EAB elicit a bioelectrochemical response which is thought to be affected by the interaction with the electrode. This framework leads to define the main hypotheses addressed. The objectives and scope are defined based on the hypotheses. Then, overviews are given for the seven chapters of the dissertation. Finally, the findings and outcomes based on the dissertation's content are summarized.

1.1 Problem/Hypothesis statement

In an anodic process, many microorganisms, particularly bacteria can transfer extracellularly electrons to a metallic surfaces^[1-3]. The extracellular electron transfer (EET) process has been investigated in bioelectrochemical systems (BES)^[4-7]. BES are hybrid systems of increasing interest for innovative applications, including: energy production from wastewater via microbial fuel cells^[8]; production of high-added value products^[9]; biosensors for bacteria detection in food, environmental, and clinical samples^[10]; as well as understanding microbially influenced corrosion processes^[11]. Although they have high potential, bioelectrochemical processes are limited by the slow EET rate between microorganisms and solid electrode donors/acceptors, which increase the operational cost and pose a challenge towards industrial application^[12].

Investigation of the EET process in potentiostat-controlled BES allows to control the EET rate and characterize precisely the EET-related biological phenomena using electrochemical methods^[13-15]. BES are powered by interactions between viable cells (e.g., bacteria) and electrically conductive materials (e.g., metals)^[16-20]. Anodic EET originates from bacterial respiration processes in which microorganisms oxidize organic carbon sources and, in absence of soluble electron acceptors (e.g., oxygen or nitrates), reduce extracellular electron acceptors at a suitable redox potential^[21, 22]. EET in microorganisms is generally thought to occur through direct or indirect mechanisms. The electron transfer strategies include (not mutually exclusive): (i) direct electron transfer through a either transmembrane cytochrome complex^[23, 24] or hopping via pili-like conductive appendages termed nanowires^[2, 25]; (ii) indirect electron transfer through mediated electron transfer via exogenous or microbially produced redox mediators^[1, 26]. Beyond increasing the understanding of this process, improving BES efficiency requires a thorough understanding of the mechanisms of electron transfer into inert electrodes in BES, yet deconvoluting these mechanisms with accuracy remains a challenge.

Bacteria tend to accumulate at interfaces to form polymicrobial aggregates deemed biofilms^[27]. Biofilms that grow on electrodes are also known as electrochemically active biofilms (EAB)^[28]. EAB is a biofilm that interacts with the electrode either by migrating electrons to or from the electrode in order to achieve EET within the biofilm.

Studies in EAB are challenged by the complexity of bacteria metabolisms in the biofilm and its heterogeneity. The electron transfer rate is known to change as the biofilm forms^[29]. Frequently, studies on EET use grown EAB at the electrode due to the stability and high current that a grown biofilm provides^[15, 30]. However, very little is known for early stages of EAB formation and the establishment of the early interfacial layer. Particularly, the use of microstructured electrodes for EET on thick biofilms with full surface coverage hinder the effect of the microstructures on EET in EAB ^[31, 32], making the study of early biofilm attachment an attractive alternative. Additionally, the electrode surface is thought to influence the electrochemical performance during early biofilm attachment^[33], yet little research has been carried to understand its effect.

Therefore, the first hypothesis of this dissertation is that:

- ❖ For early biofilm formation in aqueous media, the coulombic charge of EET will increase at electrodes modified with microstructures, as the latter favor their formation. The microstructure dimensions can be achieved with microelectronics methods, such as lithography, to obtain an ordered array.

Bacteria can grow on electrodes via potentiostat-controlled electrochemical cells leveraging on the EET process. In this regard, the higher the current output at the electrode, the more efficient the applications (e.g., microbial fuel cells and biosensors) would be ^[7, 13]. In practice, the current output can be generated when a mixed microbial community is used in a wastewater system, which can degrade not only acetate and glucose, but also complex organic wastewater mixture ^[34-37]. However, producing reproducible mixed communities to study the biofilm and electrode surface interactions is difficult. Alternatively, *E. coli* is commonly found in almost every environment (including wastewater) which displays electrogenic activity using Hydroxy-1,4-naphthoquinone (HNQ) as an external redox mediator ^[38], and can also be grown consistently thus being suitable to study the effect of microstructural modification of the electrode on the overall EET.

The electrode material affects electron transfer processes at the interface, where surface charge transfer resistance and biocompatibility determine the current output and the biofilm attachment, respectively. Biocompatible electrodes are inert and do not have a

toxic effect on the biological systems. Most bioelectrochemical studies have been carried out on inert electrodes of carbon materials, such as graphite. Carbon electrodes have been widely used in energy-producing BES for their low-cost and excellent biocompatibility. However, except for glassy carbon, most carbon based electrodes are not suitable to detect early biofilm formation as they have high capacitive background current (in comparison to metals), which hinders the EET process in the early stage of bacterial colonization [28]. On the other hand, gold (Au) thin film electrodes are biocompatible and have low capacitance and low electrical resistance [39, 40], thus allowing sensitive EET measure [41]. Moreover, Au thin film deposition allows the systematic fabrication of electrodes with specifically designed features [31, 42-44]. Therefore, Au electrodes represent a suitable option for biofilm and electrode-surface interaction studies. Yet, despite their benefits in practice, the trend in BES is moving away from using precious metals as electrodes due to their high cost [45]. In this regard, nickel (Ni) offers a lower-cost option compared to gold, with a broad range of industrial applications, but at the expense of a lower biocompatibility and higher capacitance and electrical resistance in comparison. Ni foam was employed as a platform for biofilm growth in BES, with promising current output [46]. More research is needed to better understand the trade-offs in early bacterial biofilm formation and electron transfer when using different metal materials as electrodes in BES. To date, the electrochemical characterization of early biofilm formation on Au and Ni microstructured electrodes has not been reported.

Thus, the second hypothesis for this work is that:

- ❖ A thin layer of Au and Ni deposited on the electrode surface will improve the charge transfer resistance at the interface due to their metallic properties allowing the characterization of early biofilm formation and EET.

It is known that electrode surface characteristics have influenced the bacteria early biofilm formation [33]. Recent research shows that both nano- and micro- features determine the current output of anodic biofilms [42, 43, 47] and that the roughness of the electrode surface contributes to the electrochemical signature and EET rate during early biofilm attachment [48]. Additionally, electrode surface engineering enables the fabrication of micropillars of comparable size with bacteria, providing a favorable

scenario to study biofilm-surface interactions. However, microstructures with high aspect ratio increase diffusional limitation for the transport of nutrients and redox mediators from the bulk solution to the biofilm. Thus, the attachment of bacterial cells and the extracellular electron transfer do not correlate linearly with the type of microstructure. Since most studies have focused on improving the current output in BES, little attention has been given to the bacteria-surface interaction in the early stage of biofilm formation. This aspect is particularly relevant to biomedical applications, in which early detection of biofilms on devices such as catheters and prosthetic implants allows rapid and life-saving intervention. Previous research on the topography of the electrode surface and its influence on the bioelectrochemical process included studying the adherence of the cell to the surface, the formation and structure of the biofilm, and the electrical connectivity^[49]. However, most studies do not explain the interaction between early biofilms and electrodes' surfaces with systematic topographic arrangement, nor how the functioning and efficiency of these systems is determined.

Therefore, the third hypothesis of this dissertation is that:

- ❖ Modification of electrode surfaces, in the form of topographical arrangements of a pattern, affects the bacterial early biofilm attachment as well as the extracellular electron transfer detected by the electrochemical setup.

1.2 Objective and Scope

The main objective of this dissertation is to investigate the bioelectrochemical characterization of early bacterial biofilms on electrode surfaces, through the fabrication and testing of ordered array microstructured electrodes of different materials and topography. Based on the hypotheses above, the detailed objective and scope are listed below:

First, ordered array gold electrodes with different topography arrangement will be fabricated using photolithography. The electrodes will be tested under electrochemical treatments for characterization. The liquid solution will contain a weak electrogenic bacteria (*E. coli*), which would grow as a biofilm on the electrode surfaces. The first 24 h of the biofilm formation will be used for the characterization. Imaging via scanning

electron microscopy and confocal laser scanning microscopy will be used for biofilm characterization as well. The electrochemical results (cyclic voltammetry, chronocoulometry, electrochemical impedance spectroscopy) will be used to assess the extracellular electron transfer performance, while images will be employed to match such electrochemical measurements to the different stages of biofilm growth.

Second, electrodes with different ordered array microstructures will be fabricated using nickel metal thin film deposition. The bacterial biofilm growth and their extracellular electron transfer will be studied under the effect of nickel thin film with electrochemical impedance spectroscopy. Additionally, the effect of temperature will be evaluated. The changes of the electrochemical interface due to early biofilm formation on the electrode's surface will be measured through time and closely monitored with electrochemical impedance spectroscopy (EIS) treatment. The EIS characterization will include a broad range of potentials in the search for changes on the charge transfer efficiency besides the biofilm deposition.

Third, nickel electrodes with different topography arrangement and aspect ratio microstructures will be fabricated. Their biocatalytic capabilities will be measured by both the extracellular electron transfer and early bacterial biofilm growth onto their surfaces, which will be tested using a bioelectrochemical system. The early biofilm will be characterized under a potential-controlled environment. Its growth will be monitored by EIS at short and long intervals, which will help to understand the changes through time on the electrode's surface. In addition, the effect of the carbon source will be tested by employing different concentrations of glucose.

1.3 Dissertation Overview

The present dissertation addresses the characterization of early biofilm attachment of weak electrogenic bacteria (*E. coli*) on Au and Ni ordered array electrodes of customized fabrication. It also assesses the effect of the topographic arrangement of the electrode's microstructures on both extracellular electron transfer and biofilm growth. Further, it includes the analysis of early biofilm formation and charge transfer efficiency under different conditions of carbon energy source, temperature and electric-potential.

Chapter 1 has given an overview on this dissertation which begins with the introduction of the hypotheses, followed by the description of the objectives and scope based on the hypotheses. Here, the dissertation overview for each chapter is included. Finally, the main findings and outcomes of this dissertation are summarized.

Chapter 2 displays a literature review on the recent progress in microstructured-surface electrodes for early biofilm detection. Relevant studies in biofilms are presented first, followed by electrochemical detection and bioelectrochemical advances. Subsequently, works on microstructure and materials used are presented. Finally, the research questions of the present dissertation are discussed based on the literature review.

Chapter 3 discusses the rationale behind the selection of methods for the electrode fabrication, while the techniques for characterization and measurement are summarized. First, the photolithography principle of operation is described, followed by nanoimprint lithography. Then, the bioelectrochemical principles are explained, as well as the electrochemical techniques used for characterization. Finally, measurement and complementary techniques are included.

Chapter 4 elaborates on the microstructured gold electrode fabrication to support the growth of *E. coli*. The electrodes are maintained at oxidative potential, the electrochemical output is measured, and the EET rate during early biofilm formation is analyzed. The charge output of the modified electrodes is enhanced up to 22% higher than the control electrode, enabling the electrochemical detection of early *E. coli* biofilms. The electrode microstructures promote biofilm attachment as shown by Field Emission Scanning Electron Microscope (FESEM) and Confocal laser scanning microscopy (CLSM) imaging. Following biofilm formation, the resistance to charge transfer at the biofilm-electrode interface decreases and the capacitance increases as shown by EIS analysis.

Chapter 5 explores the fabrication of nickel micropillar-array electrodes to detect early attachment of *E. coli* biofilm through mediated EET in potentiostat-controlled electrochemical cells. The biofilm-surface interaction is studied via EIS across a broad range of electric potentials and three different temperatures (23, 30 and 37 °C) over a

24 hour period. The micropillared electrodes show a higher charge transfer in comparison with the flat ones. The analysis of the EIS signature suggests an effect of temperature on mediated EET transfer in biofilms.

Chapter 6 elaborates on the production of nickel electrodes with a micropillar array of 10 μm height to study the early surface attachment of *E. coli* biofilms, with a monitored growth through mediated EET in potentiostat-controlled electrochemical cells. Also, the biofilm-surface interaction is evaluated using EIS at short and long intervals, as well as under different concentrations of glucose. The micropillared electrodes showed that interfacial capacitance was inversely proportional to the glucose concentration. Further, Ni micropillared electrodes allow earlier biofilm detection in comparison to flat ones.

Chapter 7 presents the main conclusions of this dissertation. It further elaborates on the opportunities and strategies for future research.

1.4 Findings and outcomes/originality

This dissertation presents the following findings:

1. Gold and nickel electrodes are both suitable for the characterization of early bacterial biofilm attachment using potentiostat-controlled anodic BES. Ordered array size-controlled micropillars enhance the charge transfer at the electrode surface in microstructured electrodes in comparison with flat electrodes for both materials. This effect is stronger for gold electrodes, due to its higher electrical conductivity
2. The type of microstructure used matters, as a smaller surface increase was found to have a greater impact on the electrode charge transfer, in comparison with high aspect ratio microstructures. The electron transfer and early biofilm growth are dependent on the topographic distribution of microstructures on the electrode. Surface area increase is not as important as the gap distance between microstructures, which seems to be key. Distances of approximately 10 μm among micro-features are preferable for biofilm growth. Further, imaging supported that

the special arrangement of the microstructures is determinant for *E. coli* early biofilm formation.

3. Short intervals monitoring uncovers the dynamics of the EET process, in which the impedance drops in the first hours followed by a continuous increase. Experiments at a temperature of 37 °C show potential features of an unidentified component which may correspond to endogenous mediators or other redox-active species. In addition, EET analyzed with EIS at long and short intervals indicates that EET presents an inverse correlation of the capacitance with the carbon source concentration. Further, non-stirring conditions can reduce the signal to noise ratio in the electrochemical settings, posing a good operational recommendation for future studies.

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Chapter 2

Literature Review

This chapter includes a literature review on the recent progress in microstructured-surface electrodes for early biofilm detection. Relevant studies in biofilms are presented first, followed by electrochemical detection and bioelectrochemical advances. Subsequently, works on microstructure and materials used are presented. Finally, the research questions of the present dissertation are discussed based on the literature review.

2.1 Overview:

Energy recovery from wastewater, green production of high-added value chemicals, and biosensors for food, environmental, and clinical purposes are applications enabled by bacterial biocatalysis and its ability to transfer electrons to and from a solid surface. In the environment and in real-world applications, bacteria occur as self-assembled microstructured communities termed biofilms. The aim of this chapter is to evaluate recent advances on biofilm characterization in bioelectrochemical systems with emphasis on the importance of the electrode, improvements achieved, and future research paths.

2.1.1 Early biofilms

Biofilms are self-assembled microstructured bacterial communities that grow on surfaces or interfaces. In biofilms, bacterial cells are enclosed in a self-produced matrix of extracellular polymeric substance (EPS)^[1]. The EPS functions as adhesion for the biofilm and as three-dimensional structure. Also, EPS is involved into the molecular interaction among the bacteria within the biofilm which is significantly different from the microorganisms dispersed in the planktonic phase. The biofilm life mode has been demonstrated through functions of the EPS such as: adhesion, aggregation of bacterial cells, and cohesion of biofilms among others^[1], demonstrating the cell to cell communication. The EPS controls the diffusion of nutrients and energy source elements dissolved in water phase. It also keeps components of the lysed cells such as DNA for expected gene transfer^[2]. The EPS also serves as a protection system against the environment such as oxidation, antibiotics, and other predatory cells. Each biofilm develops a particular EPS whose characteristics depend on the microorganism consortia, the environmental shear forces, temperature, and nutrients available.

A biofilm presents five stages through its formation^[3, 4] (Figure 2.1). First, the planktonic cells attach to the surface (1) for the first time with a reversible attachment. Motility and virulence factors are downregulated. Then, the attachment is irreversible (2-3) and bacterial division starts. The first microcolonies are formed which start to secrete EPS containing proteins, polysaccharides, and DNA. Then, the biofilm grows (4) to a mature state with a mushroom-like structure. At the final stage, some cells

detach from the biofilm and disperse back to the planktonic state, (5) and the cycle re-starts. In this dissertation, the term *early biofilm* is adopted referring to stages 1 to 3.

Figure 2.1: Five stages of biofilm formation (from Vasudevan, 2014^[4]).

Biofilms were first described by H. Heukelekian and A. Heller through a publication in the *Journal of Bacteriology* in 1940^[5]. Biofilms research bloomed in the 90's when the development of new technologies allowed scientists to study biofilms further and understand their mechanisms. Currently, the increased awareness of the impact of biofilms on human health and industrial damage-costs have propelled a rapid expansion of this field of study.

Biofilms are of interest for disciplines like health, environment, and energy. They are present in all sorts of environments going from soils, oceans, and the atmosphere, to human skin, colon, and dental plaque among others^[6]. Biofilms were first explored in the healthcare field due to problems caused by pathogenic bacteria. In such cases, the extracellular polymeric substance (EPS) protects the bacterial colonies and increase their resistance to traditional solutions. Thus, biofilms can cause lung infections^[7] and even colonize indwelling medical devices such as pacemakers^[8]. For environmental sciences, biofilms are used for bioremediation purposes. For example, applications in wastewater treatment includes the use of biofilms to degrade contaminants to alleviate the environment^[9, 10]. Biofilms can also be used for green energy generation through a microbial fuel cell (MFC) where the microbes in the biofilm degrade the organic matter

to produce clean energy^[11]. Also, the oil and gas industry is looking into biofilms as well due to the degradation of materials suffered via biofilms attachment to pipelines, ships, and other metallic surfaces causing microbial influenced corrosion (MIC)^[12].

2.1.2 Biosensors

Biosensors are technologies dedicated to identifying a biological target, thus can be used to identify and characterize biofilms. Biosensors were developed as an innovative subject in research to detect food toxins, environmental contaminants, and potential sources of diseases rapidly and accurately. A biosensor consists of three main parts: a *biorecognition* which is a biological component which will specifically recognize the analyte wanted; a *transducer* which will detect the attachment of the analyte into the biorecognition and transduce it into an electrical signal; and the *display* which shows the electrical signal. These three key components are illustrated in Figure 2.2. The blood glucose monitor is one of the most widely known and commercially available biosensors, which allows diabetic users to have a rapid and accurate control of the level of glucose on their blood ^[13].

Figure 2.2: Biosensor components. (a) Biorecognition: interacts with the analyte to be sense. (b) Signal transduction: translate the interaction between biorecognition and analyte into a quantifiable signal. (c) Display: shows the specific signal generated by the transducer (from Conroy P.J., et al, 2009 used with permission from Elsevier^[13]).

A variety of transduction platforms have been explored in biosensors including electrochemical, optical, piezoelectric, and magnetic platforms. Electrochemical platforms are the oldest and most commonly used due to their high specificity, low-detection limits, and low cost, therefore being suitable for industrial applications. The electrochemical electrode sensing system has evolved and developed over three generations (Figure 2.3). *First generation biosensors* are defined by direct detection of the target or oxygen consumption, *second generation biosensors* involve a single mediator (M) between the prokaryotic microbe target and the electrode (transducer), while *third generation biosensors* involve eukaryotic microbes and a double mediator. Yet some challenges remain to be solved including achieving high performance, ultra-sensitivity, high selectivity, and a lower cost^[14, 15]. The research in electrochemical biosensors serves as a base for research translation into bioelectrochemical systems so as to allow a closer study of bacteria and biofilms interaction.

Figure 2.3: Electrochemical Biosensors generations: (a) 1st generation, (b) 2nd generation, and (c) 3rd generation (from Nakamura H., 2010 used with permission from Royal Society of Chemistry^[16]).

2.1.3 Electrochemical biosensors

An electrochemical sensing system fundamentally involves a closed electrical circuit which enables the current to flow during measurements as shown in Figure 2.4. The electrochemical system is formed by three electrodes: (i) the working electrode (WE) where the chemical reaction in study and its potential is measured, (ii) the reference electrode (RE) which is an electrode of known potential that serves as a reference to measure the potential in WE without passing current, and (iii) the counter or auxiliary electrode (CE) which allows the current to pass thorough and close the electrical circuit.

The electrochemical circuit is completed with the electrolyte or media. The electrochemical cell can be configured to extract an electrical signal such as potential, current or impedance/capacitance.

Figure 2.4: a) Three-electrodes electrochemical set up: working electrode (WE), reference electrode (RE), and counter electrode (CE). b) Equivalent electronic circuit of the electrochemical cell. *I* is the current measured and *V* is the potential applied (adapted from Bard and Faulkner, 1980 with permission from Wiley^[17]).

2.1.3.1 Potentiometric sensors

The working principle of a potentiometric sensor relies on the potential equilibrium ΔE of the electrochemical components in solution at the surface of the electrode where charge is accumulated. The potential difference between the working electrode (WE) and the reference electrode (RE) change accordingly to the Nernst equation^[17] (Equation 2.1).

$$\Delta E = \Delta E^\circ - \frac{RT}{nF} \ln \frac{[Red]}{[Ox]} ; [\text{unit: V}] \quad (2.1)$$

Where ΔE° is the standard cell potential, *R* is the universal gas constant, *T* is temperature, *n* is number of electrons transferred in the cell reaction, *F* is the Faraday constant, $\frac{[Red]}{[Ox]}$ is the ratio of reduced to oxidized molecules. The Nernst equation shows

a logarithm scale as a function of the ion activity. In a biosensor, the biological component is immobilized on the surface of the sensor which measures the potential difference proportional to the analyte concentration [18].

A common potentiometric sensor is the field effect transistor (FET) which is the result of an important contribution from the semiconductor field. The FET working principle involves the electron running from the electrode ‘source’ to the ‘drain’, passing by an electrode ‘gate’ which is controlled by the potential charge. In the case of a chemical FET sensor, the gate electrode is changed by an ion-conducting liquid solution and a reference electrode which replaces the electron conduction [19]. Afterwards, FET evolved to ISFET or ion-sensitive field-effect transistor. A membrane was incorporated that is chemically sensitivity (ion-selective) to an specific analyte in the gate [20, 21]. Then, MOSFET (metal oxide silicon field effect transistor) introduced the novelty of a covalent surface due to the SiO_2 at the gate that facilitates the biological recognition attachment [21]. Therefore, the conductivity at the electrode surface increased when the biological recognition was bound to the gate surface proportionally to the gate’s material and the concentration of the analyte [22, 23].

Figure 2.5: Schematic diagram of a chemical ISFET used for pH sensing (left), and a microscopic image of the ZnO - based ISFET (right). Electrons run from ‘source’ to ‘drain’ passing by the ‘channel’ ZnO:In (indium (In) doped ZnO) electrode. The electrons run based on the potential at the ‘gate’ (Ta_2O_5). I_D : drain current, V_{DS} : drain-source potential, V_G : ground potential. SU-8 is epoxy resin to protect the electrodes (from Yano, *et al.*, 2014 with permission from Wiley [24]).

The literature shows that ISFET with zinc oxide (ZnO) can be used for human immunoglobulin (IgG), glucose, and pH detection reaching higher sensitivity and stability for pH measurements^[24] (Figure 2.5). IgG biosensors sensitive to concentration changes were developed using Indium oxide (In_2O_3) nanoparticles based on label detection^[25]. FET devices have been studied also for bacteria detection. Due to the bacteria size the device signal changes dramatically when the cells attach to the surface. However, achieving a reliable attachment of bacterial cells in the sensing area remains a challenge. CNT (carbon-nanotube) and graphene materials have been explored for this application functionalizing the surface with linker molecules and electrostatic interaction of functional groups such as carboxyl, carbonyl hydroxyl, and epoxide groups^[26-28]. Even though these sensors showed sensitivity and selectivity sensing performance, the bacteria detection reported still used indirect recognition methods like aptamers or antibodies functionalized on the electrode surface, which slow down sensing capabilities.

2.1.3.2 Amperometric sensors

Amperometric electrochemical sensors measure the current output when a constant potential is applied to the WE. The applied potential serves as the driving force for the electron transfer process between the liquid phase and the electrode. Under well-defined conditions, the current output is proportional to the concentration of the target analyte when it is reduced or oxidized during the amperometric measurement. The direct current measured made the amperometric sensors suitable for miniaturization. An important limitation is the concentration gradient at the electrode surface which may affect the current output. Hence, constant stirring is recommended. Another limitation is the dependency on the applied potential, which may lead to other species to redox and thus lead to inaccurate results^[29].

Figure 2.6: Schematic diagram of a wearable amperometric sensor. I_{IN} : current generated at WE, V_{OUT} : voltage converted from I_{IN} and a resistor R , V_M : switching voltage from the inverter (from Shiwaku, *et al.*, 2018 with license under the Creative commons Attribution 4.0 International License [30]).

Biological information can be detected using a wearable amperometric sensor (Figure 2.6) where a flexible circuit system based on organic thin-film transistors detects lactate with sensitivity and rapidness[30]. Among the amperometric sensors is found a platform with graphene supported Pd/Magnetite ($\text{Pd-Fe}_3\text{O}_4\text{-GS}$). The platform allows the amperometric detection of IgG corresponding to ultralow levels of tumor markers so to diagnostic different cancer at earlier stage[31]. Also, a sandwich assay amperometric sensor was developed including an aptamer–protein–antibody complex modifying a gold nanoparticle (NP) modified electrode[32]. The system achieved higher sensitivity and selectivity of viral proteins detection (e.g. virus subtype (H5N1)). A free label biosensor for α -fetoprotein (AFP) was developed using a glassy carbon electrode modified with graphene/ SnO_2 /Au nanocomposite and immobilized α -fetoprotein antibody (anti-AFP)[33]. For bacteria detection, enzymatic biorecognition is used however the whole bacteria sensing is not widely used [34]. Amperometric bacteria detection was reported using an immunomagnetic microfluidic system that uses gold chemically modified electrode which avoids electrode fouling [35]. Other biosensor uses bacteriophages which infect specific bacteria strain (*E. coli* K12) thus enzyme is release and measured by the electrochemical system using screen printed carbon electrodes [36].

2.1.3.3 Impedimetric sensors

Impedimetric sensors measure the impedance or capacitance change on the electrode surface. The electrochemical impedance is measured by applying an alternating current (AC) potential to the electrochemical cell and measuring the current through the cell. Using impedance for a biosensor was first reported for the antibody-antigen complex (Ab/Ag) detection. The measure is based on the increase of the dielectric layer thickness which induce changes in capacitance proportional to the size and the concentration of antibodies^[37]. Impedance spectroscopy show great selectivity of two different carbohydrates (galactose and sialic acid) lectin biosensors^[38] For extremely low concentrations of histamine in buffer solution was used a very low-cost platform sensor using reduced ultra-thin graphene oxide (GO)^[39]. Graphene is a material with great potential for achieving high sensitivity and selectivity in biosensors fabrication. Thermally reduced graphene oxide has shown higher performance than chemically modified graphene materials ^[40].

Summarizing, potentiometric, amperometric, and impedimetric electrochemical sensors provide a broad range of applications. The materials used vary according to the applications, however the biocompatible material commonly used include gold, carbon (CNT), and graphene. Majority of the sensors, the electrode is functionalized for specific components for bacteria detection rather than whole bacteria detection. Whole bacteria and biofilms detection and their interaction with the electrode surface have not been explored deeply in the literature.

2.1.4 Bioelectrochemistry

The field of bioelectrochemistry is believed to be born after Luigi Galvani's demonstration on the frog's leg movement. However, its relevance increased after the 2000's when the advanced developments of biosensors urged the field on the classification based on faradaic currents^[41]. Faradaic biosensors allowed the physicochemical description of kinetics and energetic reactions of the solutions under study. Then, the electron transfer was monitor for single cells, tissue slides, and brains. It was known at that time that small electrons carriers such as cytochromes proteins have reversible electrochemistry depending on the electrode used. The catalytic

properties of the bacteria for current generation was the ground for the further development of microbial fuel cell (MFC) and bioelectrochemical field^[42-45]. Then, the scientific joined effort aimed to maximized the electron harvesting from the bacteria consortia using MFC ^[16, 46-50].

The bacteria able to transfer electrons outside of the cell are known as exoelectrogenic bacteria. And the process of moving electrons to and from external electron acceptors or donors is known as extracellular electron transfer (EET) and corresponds to the bacteria respiration process. The bacteria oxidize the organic matter ^[51] gaining electrons which will be accepted on the electrode depending on the redox potential applied. Also, the opposite happens as well when bacteria reduce nitrate, perchlorate or metals as reported ^[52-55]. The Figure 2.7 shows a schematic of possible biological induced reactions involving electron transfer from the bacteria to the electrode at the anode and cathode. As the electrons are unable to enter the cell directly the extracellular electron transfer plays an important role. The EET transfer mechanism can be direct or indirect: (i) direct electron transfer through either transmembrane cytochrome complex ^[56, 57] or hopping via pili-like conductive appendages termed nanowires^[58, 59]; (ii) indirectly through mediated electron transfer via exogenous or microbially produced redox mediators^[60, 61]. Also, EET are transferred under a controlled-potential of oxidation and reduction according to Nernst equation, although some energy is lost due to electrolyte resistance^[62].

Figure 2.7: Schematic representation of possible biological induced reactions involving the electron transfer from the bacteria to the electrode in both cases as the anode and cathode. The

electrodes can reach the electrode directly or indirectly through electrons shuttle (from Rabaey, *et al.*, 2007 with permission from Springer Nature ^[46]).

2.1.4.1 Extracellular electron transfer mechanisms

The electron transfer generally accepted mechanism are direct transfer mechanisms and indirect pathways^[46] (Figure 2.8). (i) Direct: direct transfer^[63] or electron hopping via nanowires or pili-like appendages^[58, 59], (ii) Indirect through mediators or shuttles via exogenous or microbially produce redox mediator^[61]. In the direct case, or immobilized structure, the bacteria have direct contact with the electron acceptor^[63] which are transferred through special outer-membrane cytochrome (OmcS)^[64-66], pili (nanowires)^[67], flagella^[67] or/and biofilm formation^[68]. On the other hand, indirect case, electrons can be transferred to the electrode using redox mediators or shuttles such as phenazines for *P. aeruginosa*^[61, 69], AQDS (anthraquinone-2,6-disulfonic acid) for *S. putrefaciens*, or HNQ (naphthoquinone) for *E.coli*^[70, 71]. There is a large number of electron shuttle which can be used including sulfur species and hydrogen^[72-75], although the diffusion of the mediator can be critical when a biofilm is created^[67].

Figure 2.8: Electron transfer mechanism. (1) Diffusion of the substrate to and from the carbon source in a microbial cells, (2) metabolic redox reactions, (3) electron transfer from the cell to extracellular redox compound, (4) mediated electron transport through the biofilm matrix, (5) direct electron transfer to the electrode surface direct contact or through pili-like appendage (from Champigneux, *et al.*, 2018 with permission from Elsevier ^[76]).

Inside the cell, the microbial respiration involves subsequent energetic chemical reactions. A glucose molecule can be converted by microorganisms into acetate, and later produce energy following the equation 2.2:

The electrons harvested from the microorganism for energy production does not interfere with the bacteria metabolism. The bacteria with EET are also found in the literature as electricigens, electrochemically active microbes, exoelectrogenic bacteria, and anode-respiring or anodophilic species.

In this work, *E. coli* was used as a model organism as a ubiquitous organism that can be found almost at any ecosystem. *E. coli* is likely to be the most studied organism and is classified as gram-negative. Morphologically, *E. coli* has a rod shape of about 0.25 – 1 μm in diameter and 2 μm long (Figure 2.9). *E. coli* has a fast growth rate. These characteristics provide *E. coli* with the capability of producing theoretically 16,000 kW per m^3 [11] of energy however, it haven't been reached in reality. The limitations are found in the extracellular electron transfer pathways and on the electrode surface used as well. There is evidence that suggest that architecture modification of the electrode surface may enhance the electron exchange by changing the bacteria packing disposition thus enhancing the signal transmission[11, 49] but few systematic and controlled studies of such relationships have been conducted to date.

Figure 2.9: Computer generated image of *E. coli*. Visual example of flagella, and shorter fimbriae (credits: Alissa Eckert and Jennifer Oosthuizen/CDC).

The *E. coli* bioelectrochemistry can be monitor using addition of a biocompatible redox mediator: 2-hydroxy-1,4-naphthoquinone (2-HNQ)^[70], therefore used for this research. The mediator mechanism involved (1) diffusion into the cell oxidized, (2) active respond to the electron source, (3) diffusion out of the cell reduced, (4) deposit the electron at the electrodes surface. The mediator requires also to be chemical stability for long hours, soluble in the medium, biocompatible to the biocatalyst, not being part of the bacteria metabolism, and have a potential redox suitable for the reduction^[77]. 2-HNQ present chemical stability and moderate electron yield^[77, 78].

2.1.5 Bioelectroactive biofilms

Electroactive biofilms (EAB) correspond to the biofilm grown on an electrode so that the electrons can be harvested. EAB use all the terms described previously and is used specifically to refer to the biofilm interaction with the inert solid surface of the electrode (bioelectrochemical system). In the literature, EAB studies are mostly founds in microbial fuel cells (MFC). MFC have shown the potential to power electronic devices from the bacterial biocatalysis however, the power remains low in comparison with the theoretical calculated^[79, 80]. MFC has contributed to maximize the electron transfer from biofilms to solid surfaces. However, numerous challenges are still unsolved. There are two main contribution factors which re being studies in parallel: (1) microbial consortia, and (2) the electrode surfaces. This dissertation would focus on the electrode surface and electron transfer mechanisms between bacteria and solid electrodes which are not well described in the literature^[81].

2.1.6 Electrode topography

Electrodes in bioelectrochemical systems allows the electron collection for application such as biosensors, MFC for remote equipment, or even the study of biocorrosion processes. In search for higher electron harvesting, studies have covered the effect of materials, coatings, and surface functionalization, however, there is not much evidence focused on the effect of the electrode surface topography and the early bacteria attachment interaction on it.

Looking into the electrode surface, porous material electrode present high signal response due to the increased active area that presents. However, usually the pores are blocked when functionalizing the surface limiting the signal response of the electrode. Also, when the electrodes are used for whole bacteria interaction, the size of the bacteria may block pores. Therefore, size-controlled microstructures would increase the surface area and enhance the signal-to-noise ratio. Previously, Bruggemann and Nick have reported significant improvement of signal response when fabricated a gold microstructure arrays to study the biomedical application such as neuronal interface [82, 83]. Other structures biocompatible with cells were reported such as vertically align carbon nanotubes (CNT) [49, 84], silicon nanowires [85-87], metallic nanorods [88]. These microstructured electrodes were developed for increasing sensibility in biological applications. Furthermore, researchers have shown an increase of the energy harvested on MFC from bacteria when changing the micro and nano-structure of the electrode configuration [49]. Also, microstructured electrodes were successfully used for energy harvesting in MFC applications using *Geobacter* as model organism [47]. Although, it has not been reported how energy production and bacteria biomass are related.

The fabrication methodologies for size-controlled microstructured surface modification are photolithography, nanoimprinting and electron beam lithography. Photolithography allows patterning in relatively large surface for electrochemical applications with features of submicron level which allows the modification of the structure/topography to any structure size and it is widely used [49, 89]. Nanoimprint lithography can produce features on the nanoscale however the resolution is low [90]. Electron beam lithography is used to create high resolution patterning but usually is very slow and costly since the features are made individually, it is normally used for mask fabrication for traditional lithography [91].

2.1.6.1 Electrode materials

Despite the topographical shape and rigidity of the microstructure, their biological interaction are highly dependent on the surface material as well as on the bacterial characteristic such as height, width and shape of the bacteria [92]. In addition, the stability and reproducibility of the electrodes material would be reflected on the electrochemical quality results.

Electrochemical sensors studies present conductive and biocompatible options for direct attachment of the bacteria. Although, most of the works presents functionalized surfaces with antibodies and/or aptamers for indirect measurement, they are used to evaluate the material used for the electrode substrate. First, ITO was used as a transparent electrode for MFC application with *Geobacter* as a bacteria catalyst^[48]. The transparent electrode would be useful for imaging of the electrodes and bacterial growth observation. Gold and Carbon materials are the classic biocompatible electrodes. Gold is traditionally an excellent material for electron transfer, whereas carbon present high background currents. Other materials found in the literature were ZnO, SiO₂, I₂O₃, and Au NP which were explored for FETs configuration biosensors. Graphene, such as ultra-thin graphene oxide or thermally reduced graphene oxide presents immense potential for biosensors fabrication, although sized controlled microstructured materials fabrication might be challenging.

The material selected should consider the extended used for biosensor and the capability for miniaturization since smaller devices tend to produce rapid and accurate results. Some examples are the point-of-care (PoC) devices and lab-on-a-chip (LoC) which are able to detect a threat agent without requiring specific trained personnel. Although the biosensors field is highly investigated, still the trends for future research will focus on PoC devices improving cost, portability, rapidness, accuracy, sensitivity, and reusability^[93, 94], as well as miniaturization which would require a smaller sample size and less processing time^[95]. Miniaturization of devices open up remote application of broad expectrum which would bring worldwide benefits of this scientific knowledge for both health or environmental application. While a considerable amount of research is found for medical devices, still environmental application of biofilms detection sensors has huge potential to develop new strategies. Finally, merging the field of engineering sciences, nanotechnology and biotechnology with transduction will drive more applications.

2.2 Research gap identified from literature review

This literature review has covered the topic of bacterial biofilm able to transfer electrons to metallic surfaces which have been of great interest for bioenergy recovery from wastewater, production of high-added value products, and biosensors for bacteria

detection in food, environmental, and clinical samples, also to understand microbial influenced corrosion processes. The electron transfer mechanism is based on the bacteria respiration which may use a mediator to transfer the cellular components to the electrodes for its electrochemical detection. Although theoretical calculations are positive on the electron harvest capabilities and various efforts have been conducted to increase the EET, the transfer rate remains low. However, within the literature on bioelectrochemical system, there is not a clear understanding of the interaction between the electrode surface and the bacteria early attachment.

Much of what is known about the relationship between the biofilm interaction with the surface derives from the microbial fuel cells (MFC) literature which uses a mature biofilm. Early stages of biofilms involve an irreversible attachment achieved after the cells grow, sense the surface and reversibly attach to it. During the growth period, the biofilm produces a stochastic amount of current which is not preferred for MFC applications. However, the number of researchers studying the early biofilm electroactivity is small even though early biofilm attachment may hold the key to understand the electron transfer mechanism at the interface. Therefore, this dissertation investigates the electrochemical activity for early biofilm attachment.

Bioelectrochemical and microbial fuel cells (MFC) present two very distinguished configurations although both use a three-electrode system approach. Independently of the configuration chosen, both are highly dependent on the working electrode characteristic which impacts directly on the quality and interpretation of the results. The electrode material used directly affects the amperometry measurements. Biocompatibility is a very important requirement to meet. Most of the bioelectrochemical research presents carbon-based electrodes while it is a low-cost option, their high background current is not sensible enough to detect low concentrations of redox species. In addition, the material should allow microstructural changes on the topography of the electrode. Therefore, this dissertation explores different types of materials to characterize the bioelectrochemical activity.

Besides the study of electrodes' materials, it is also important to identify the role that the electrode surface's topography plays in the electron transfer and early biofilm growth. Also, addressing the understanding of the biofilm – surface interaction may

enhance the efficiency of bioelectrochemical systems. Most studies in biomedical field studied biofilms and surfaces seeking to avoid the bacteria attachment and claim that the increase of roughness enhance the biofilm attachment. In bioelectrochemical system, roughness modification increased indeed the current harvested supported by systematically roughness-controlled studies^[76]. However, these studies failed to explain their impact and characterization of early biofilm attachment. Therefore, this dissertation aims to provide the first evidence of electrode topographically modified surface for *E. coli* early biofilm attachment.

2.3 Novelty of research

This dissertation addresses the previously described research questions to improve the understanding of the interaction between the electrode surface and the early biofilm. Also, it explores the electrodes characteristics for bioelectrochemical systems and the early biofilm electron transfer mechanisms. My contributions to the research are described as followed:

Electrodes were successfully fabricated with a controlled-size and shaped pattern array. The three-dimensional microstructures were fabricated with photo and nanolithography. The pattern modification shows that the gap distance among features affect the biofilm formation. Small increments in surface area determine a detectable change in the electrochemical impedance of the biofilm/electrode interface across different potential and at different growth temperatures. The impedance change was observed within few hours from inoculation, confirming this method is adequate to investigate early biofilm formation, particularly on conductive thin film metal surfaces.

The biocompatible metal microstructures enable extracellular electron transfer from the bacterial early biofilm to the electrode. The electrochemical signature of the biofilm depends also on the electrode material. This dissertation studied Au and Ni microstructured. Gold is a biocompatible material with high conductivity, which is ideal for laboratory characterization of EET in biofilm and suitable for lithography modification. However, its high cost prevents real-world applications in biomedical devices. Ni is a low-cost biocompatible material that can modified through lithography and is a good candidate to replace gold in applications. Nickel microstructured

electrodes fabrication were the first of its type to be used for bioelectrochemical characterization of biofilms. The Nickel electrodes showed a good sensibility when analyzed through electrochemical impedance spectroscopy, which highlight possible applications in early biofilm sensing.

In this dissertation, an early biofilm was defined after the irreversible attachment and beginning of the growth process. *E. coli* was used as a model organism, as it is ubiquitous organism that can be easily grown on electrodes. The microstructured array electrodes presented a larger surface area and promote biofilm formation. The early biofilm attachment was observed with field emission scanning electron microscopy (FESEM) and confocal laser scanning microscopy (CLSM) that confirmed the biomass increment. The biofilm growth coincides with the electron transfer increase measured by the electrochemical characterization. These results demonstrate that microstructured electrodes allow earlier biofilm detection than flat electrodes, which is relevant for biofilm sensing and investigation of microbially influenced corrosion in drinking water systems and biomedical devices.

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Chapter 3

Experimental Methodology

This chapter discusses the rationale behind the selection of methods for the electrode fabrication and summarizes the techniques for characterization and measurement of the early biofilm/electrode interface. First, the photolithography principle of operation is described, followed by the nanoimprint lithography. Then, the bioelectrochemical principles are explained, as well as the electrochemical techniques used for characterization. Finally, measurement and complementary techniques are included.

3.1 Rationale for selection of Methods

The study of the bacteria – surface interaction is the goal of the research outlined in this dissertation, particularly the understanding of the extracellular electron transfer (EET) between bacteria and the microstructured electrode surface, in presence of early biofilms. *E. coli* was used as a model organism as a ubiquitous organism that can be found in almost any ecosystem. Although *E. coli* is generally very well understood, its electrogenic capabilities are being dismissed based on their lower electroactivity. Indeed, *E. coli* is a weak electrogenic microorganism able to use hydroxy-1,4-naphthoquinone (2-HNQ) as a redox mediator.

In the first part of this research, a gold (Au) micropillar array electrode was selected for its biocompatibility and low electrical resistance. The goal was to enhance the EET from the bacteria to the electrode by increasing the surface area with microstructure sizes resembling the size of the bacteria (0.25-1 μm in diameter and 2 μm long). Microstructures of 1 μm height are comparable in size with the maximum diameter of an *E. coli* individual organism. Also, microstructured electrodes with 1 μm micropillars have higher roughness and surface area, which reduce the shear forces on the surface, thus facilitating bacterial attachment and early biofilm development. Two micropillar diameters of 4 and 8 μm were adopted, these sizes being comparable with that of small micro-aggregates and micro-colonies which are among the first structures spearing in biofilm formation (Figure 3.1). Photolithography was used for the fabrication, allowing the simultaneous production of multiple electrodes and therefore ensuring reproducible quality of the microstructured surfaces. Photolithography also allows the fabrication of high-quality size-controlled micro features over a homogeneous surface free of defects. The fabrication of multiple identical electrodes was important in order to maintain identical conditions for the biological replicates as well. Photolithography uses a photoresist to shape the structure which hardens after UV exposure. Once the structure was achieved, the surface was covered by a thin film of Au using a sputtering system. The system was tested in a batch bioelectrochemical reactor inoculated with *E. coli*. Several electrochemical and microscopy techniques, including field emission scanning electron microscopy (FESEM) and confocal laser scanning microscopy (CLSM) were used for the characterization.

Figure 3.1: Schematic top view of the ordered structure array electrodes with pillars of (a) 8 μm and (b) 4 μm in diameter. (c) 3D schematic tilted view of the electrodes. Pillars are 1 μm high.

In the second part of this research, Nickel (Ni) micropillar array electrodes with micropillars of 1 μm height and 4 and 8 μm diameter were fabricated. These electrodes were fabricated with photolithography and the design of the electrodes was similar to that of the Au electrodes used in the first part of the research (Figure 3.1). These electrodes were used to understand how the early biofilm grows and its EET changes on a Ni substrate. While less conductive than Au (1.43×10^7 vs. 4.1×10^7 S/m, respectively), Ni is much more conductive than the graphite electrodes (2.5×10^5 S/m) commonly used in electrochemical biofilm experiments, thus EET can be more precisely characterized through electrochemical methods using Ni^[1]. This material also allows the deposition of a thin film on top of the structure using an e-beam evaporator, which ensures a free-defect size-controlled pattern and the reproducibility of the electrodes across biological replicates within experiments. Further, Ni was selected as a low-cost material alternative to Au, with potential applications in the biomedical and sensor industry. *E. coli* was grown on Ni thin-film electrodes using 2-hydroxy-1,4-naphthoquinone (2-HNQ) as a redox mediator, similar to what was recently reported for graphite electrodes^[2, 3], and the EET was measured with electrochemical techniques, particularly Electrochemical Impedance Spectroscopy (EIS). The surface morphology was studied using FESEM.

In the third and last part of this research, micropillar array electrodes with 10 μm high features were fabricated. The distance between pillars was set to 10 μm and 100 μm in

two different electrode production batches, respectively. This electrode design was used to determine the effect of the microstructure height and topography on EET and early biofilm formation. The electrodes were fabricated using nano-imprint lithography^[4, 5], which allows a better control over the height increment of the pattern. The *E. coli* culture was inoculated with 2-HNQ as an extracellular mediator as in the first and second set of experiments. The bacterial growth was monitored using electrochemical techniques. The glucose concentration effect on early biofilm formation and EET was also recorded.

3.2 Fabrication

3.2.1 Photolithography

Photolithography is a method that creates micro scale features or patterns on a large scale on wafer surfaces. The process includes several steps (Figure 3.2). Firstly, the bare wafer is cleaned with sulfuric acid solution or “piranha” (sulfuric acid + peroxide) in order to remove any dirt on the surface which may cause fabrication defects later down in the process. Then, the wafer is coated with hexamethyldisilazane (HMDS) to boost the photoresist adhesion. Following, the photoresist is spin coated forming a uniform layer on the silicon wafer. Afterwards, the wafer is exposed to UV light through a mask previously fabricated by a third-party using a laser beam. This mask serves as a filter and demarks the pattern desired. The UV light hardens or softens the photoresist if the photoresist is positive or negative, respectively^[6]. Then, the wafer is removed from the UV and baked to fix the pattern. Later, the pattern is developed with a developer solution (EDM Performance Materials Corp, USA), and the unwanted photoresist is removed. Finally, the pattern is ready for coating and a thin film of the desired material is deposited. Depending on the final design, the photoresist can be removed with acetone, so that the desired metal pattern is left on the surface of the wafer.

The quality of the photolithography process depends on the UV source, mask, photoresist (PR), and defects after fabrication^[7]. The spatial resolution of the pattern depends on the UV source and mask. The mask is made using laser beam on Cu coated over quartz. The usual UV source is a mercury vapor lamp with 400 nm wavelength for

features around 2 μm . Common defects such as step coverage and shadowing occur on the metal deposition step. The etching process can be isotropic or anisotropic depending on the type of etchant used: dry or wet. Dry etchants include plasma or high reactive ions, which result in anisotropic etching, while wet etchants result in isotropic etching.

Figure 3.2: Step by step schematic of photolithography fabrication. Au ordered structure array electrodes using a photolithography process and sputtering for thin film Au deposition.

3.2.2 Nano-imprint Lithography

Nanoimprint lithography (NIL) is a technique used for surface patterned modifications that is high-throughput and low cost^[4, 5]. NIL is a high-resolution pattern alternative allowing the bulk fabrication of features up to 25 nm in size. The patterns are formed by mechanical deformations of a polymer material after the nanoimprint mold is stamped. NIL is also known as soft lithography and allows to achieve better resolution than photolithography. Usually the mold used is a silicon wafer whose pattern has previously been achieved by e-beam lithography. The mold is usually fabricated by a third party. The working principles for NIL are very simple and the steps are explained as follows (Figure 3.3). First, the hard mold is held and pressed against the polycarbonate film at a specific controlled temperature and pressure (160 °C, 40 bar, 300 s). After a few seconds, the temperature is reduced so the polymer cures below glass transition temperature and the stamp is removed. The microstructures created by the mold in the polymer are then ready for any functionalization or further modification.

The quality of the nanoimprint depends on the lifetime of the mold^[5]. After certain time, wearing can be observed and the quality of the stamp would be reduced. Also, fabrication defects and roughness variation may appear depending on the material, temperature and pressure used, as well as the mold stamp.

Figure 3.3: Schematic of a Nanoimprint process for Ni ordered array electrodes fabrication.

3.2.3 Magnetron sputtering

Magnetron sputtering is a technique with high rate vacuum coating used for depositing targets such as metals, alloys and compounds as a thin layer over another material's surface (substrate). The working principle method^[8] starts when the chamber is vacuumed and argon gas is blown in. Then, the magnets behind the targets create a magnetic field through the target which will generate plasma. Plasma is formed when the argon gas (or another inert gas) is ionized due to the high voltage generated. The argon ions are accelerated towards the target so when they collide they cause metal atoms to get ejected and to fly towards the desired surface where they condensate and deposit as a thin film. This way the substrate becomes uniformly coated with the applied target material. Figure 3.4 shows a schematic of the working principle of sputtering.

Figure 3.4: Schematic of the working principle of the magnetron sputtering system (from Pessoa, et al. 2015 with permission from Elsevier^[9]).

3.2.4 Electron beam evaporator

Electron beam evaporator (EBV) is a type of physical vapor deposition tool^[10] (Figure 3.5). It is used to cover a sample with various metals. Commonly used metals in the EBV include Al, Pt, Au, Cr, Ni, and Cu. Once the sample has been loaded, the chamber is closed. The chamber is then vacuumed down to a very low pressure. The EBV then releases electricity through a filament. Several magnets are used to direct the electron beam from the filament to a crucible containing the target metal. The metal then heats up and begins to evaporate onto the shutter. This process can take several minutes. A sensor within the chamber is used to determine the current rate of evaporation. Once the desired rate is reached, the shutter is opened, and the sample is exposed to the evaporating metal. The desired rate of evaporation and the amount of metal to be evaporated can be set under the deposition controller. Once the desired amount of metal has been evaporated, the shutter is closed. The pressure inside the chamber is then increased to atmospheric pressure and the chamber is opened.

Figure 3.5: Electron beam deposition schematic (from Jeol website^[11]).

3.3 Characterization

3.3.1 Bacteria growth media

The bioelectrochemical system used in this dissertation is driven by the metabolism of a weak electrogenic bacteria, *E. coli* K12. First, bacteria are cultivated in Lennox Luria broth at lower sodium chloride medium, which contains casein peptone (enzymatic digestion product of casein), yeast extract, and sodium chloride. After 20 h of incubation in a shaker incubator at 200 rpm at 37°C, the biomass is separated by centrifugation at 6000 rpm for 5 min at room temperature, forming a bacteria pellet. This pellet is re-dissolved in 11 mL of MOPS 1x (3-(N-morpholino)propanesulfonic acid) buffer and the OD₆₀₀ (optical density) of the bacteria in solution is measured.

Into the electrochemical cell, a calculated volume of the suspension with bacteria is added to the final OD₆₀₀ = 0.7, as per the Equation 3.1. The electrochemical cell volume is completed with MOPS and 50μM of HNQ is added as redox mediator.

$$Volume_{bacteria\ suspension} = \frac{Volume_{final} \times OD_{final}}{OD_{bacteria\ suspension}} \quad (3.1)$$

3.3.2 Bacteria concentration measurements

The optical density (OD) is a quick method to measure the bacteria or cells concentration in a liquid solution^[12]. In the OD method, the light absorbance of a sample at a given wavelength is measured. Therefore, OD₆₀₀ refers to the optical density at the wavelength of 600 nm. OD presents some limitations for concentrations higher than 0.5 measured by most of the spectrometers. If the concentration is higher than 0.5, it is recommended to dilute the solution. OD measures have equivalent on cell units for widely used bacteria or cells. For example, it is accepted that for *E. coli* an OD₆₀₀ of 1 is equivalent to 1 x10⁹ cells/mL culture (Equation 3.2).

$$OD_{600} = 1 \approx 1 \times 10^9 \frac{\text{cell}}{\text{mL}} \text{ culture} \quad (3.2)$$

Colony-forming units (CFU), formerly known as standard plate counts, is another method used to estimate the viable bacteria cells concentration in a liquid media. Results are given as CFU/mL (colony-forming units per milliliter) for liquids. For this work, we have followed the single plate-serial dilution spotting (SP-SDS)^[13] which is an adaptation from the standards methods for the Examination of Water and Wastewater #9215^[14]. The steps of this technique include: (i) Sample preparation by marking each plate (disposable petri dish) with sample number, date, and any other necessary information before examination. The plate is marked with vertical lines indicating the corresponding dilution to be examined in each column as shown in Figure 3.6. Each column would include the replicates corresponding to each dilution. (ii) Media preparation by using a plate count agar (tryptone yeast agar) at pH = 7 after autoclaving at 121 °C for 15 min. (iii) Incubation for 24 h (for *E. coli*) to observe and quantify the colonies formed at 37 °C. (iv) Counting of all colonies on selected plates promptly after incubation. Manual counting is acceptable when performed against a darkfield background where the bacteria colonies are bright and readily distinguished from other structures in the agar. For manual counting an auxiliary marker can be used to distinguish the colonies. In preparing plates, sample volumes that yield from 30 to 300 colonies per plate are pipetted. The aim is to have at least one dilution giving colony counts between these limits. However, since SP-SDS aims for the use of one single plate and 10 µL, the sample volume in the last dilution should yield from 3 to 30

colonies per droplets. Droplets of 10 μL are placed in the agar plate on the corresponding dilution column previously indicated. Replicates of the sample would be placed on the same corresponding column as shown in the Figure 3.6. Consider only droplets having 3 to 30 colonies in the corresponding dilution column in determining the plate count. (v) Computing bacterial count per milliliter following Equation 3.3.

$$CFU/ml = \frac{\text{colonies counted}}{\text{actual volume of sample in drop (ml)} \times \text{dilution factor}} \quad (3.3)$$

An individual colony is identified as an isolated dot. The droplets with 3 to 30 colonies represent the bacteria concentration calculated with the previous equation. If the colonies are higher than 30, then further dilution is required.

Figure 3.6: Agar plate CFU counting using a single plate-serial dilution spotting (SP-SDS).

3.4 Electrochemistry

Electrochemistry studies the chemical species in a solution or on the electrode surface based on losing or gaining electrons, namely oxidation or reduction^[15]. In general, a controlled potential or current is applied to the working electrode, and the corresponding current or potential, respectively, is measured. If the media into the electrochemical cell contains living cells and/or cells are attached to the working electrode, the system is denominated bioelectrochemical system (BES)^[16]. In this work, a controlled potential was applied to the working electrode. A three-electrodes set-up was used: working electrode (WE), reference electrode (RE), and counter electrode (CE) (Figure 3.7a). The microstructured working electrode described earlier was held

by a PTFE jacketed electrode holder (IDA Company, China) (Figure 3.7b) with a platinum (Pt) current collector (Figure 3.7c). The reference electrode was made of silver-silver chloride (Ag/AgCl) (IDA Company, China). The counter electrode was a Pt wire (Sigma-Aldrich, Singapore). The three (3) electrodes were connected to a multichannel VSP potentiostat (Bio-Logic, France) at the Singapore Center for Environmental Life Sciences and Engineering (SCELSE). The electrodes were immersed in a media solution which contained *E. coli* bacteria inoculum, MOPS 1X, and 50 μ M 2-HNQ as redox mediator (previously described in section 3.3.1). The electrochemical cells were sealed with parafilm, thus becoming anaerobic as growing microorganisms consume all oxygen. The electrochemical cells were operated at 23°C and stirred with a magnetic stirrer when performing the electrochemical characterization, unless stated otherwise. All the following electrochemical techniques were tested under the same conditions. All electrodes were analyzed using the same sequence of electrochemical methods.

Figure 3.7: (a) Three-electrode system. (b) PTFE jacketed electrode chip as holder electrode with a platinum (Pt) plate. (c) Customized micro-structured electrode grasped in the holder electrode.

3.4.1 Cyclic Voltammetry (CV)

Cyclic Voltammetry (CV) is the most common technique to characterize an electrochemical system^[15]. The potential E of the working electrode is set at a starting potential and increased linearly with a defined scan rate to an upper potential, and then the potential is decreased again to a lower potential. This cyclic process is repeated

several times, if needed. The potentiostat records the value of the current (i) and plot its value against the potential (E) (Figure 3.8). When the potential applied is scanned from low to high potential, anodic processes are shown in the curve as distinctive peaks due to the oxidation processes. Under diffusion-controlled conditions, for each redox-active species, the CV curve returns the cathodic and anodic peak current (I_{pc} and I_{pa} , respectively), cathodic and anodic peak potential (E_{pc} and E_{pa} , respectively), and the potential difference between E_{pc} and E_{pa} (ΔE). This information is used to understand the electroactive species in the solution, as well as the reversible or irreversible nature of the electrochemical reactions that occur in the system.

Figure 3.8: Schematic of a typical CV curve. Adapted from Bard and Faulkner with permission from Wiley^[15].

The electrochemical reaction is usually represented by equation 3.4:

Initially, there is a capacitive current which drives the oxidation of the species O. Once the potential is applied, near the anodic peak potential (E_{pa}) the species O starts oxidizing at the electrode, and a Faradic current is generated. The oxidation becomes stronger when the potential approximates to E_{pa} , increasing the current continuously on the electrode. Under diffusion-controlled conditions, the concentration of O on the electrode surface is considerably smaller than the rest of the bulk solution due to the oxidation, which results in a current peak as the potential increases. In the reverse scan, the opposite process takes place (oxidation of R) and a reduction peak appears.

The parameters used in Cyclic Voltammetry for this research were the following. The potential swipe scan varied from -0.4 V to 0.4 V vs. Ag/AgCl reference electrode. The scan step was 10 mV/s. Current was recorded on 10 potential steps in order to reduce the data file size and smooth the trace. The cycle was repeated three (3) times. Potential range was set from -2.5 to 2.5 V. The current range was 10 μ A.

3.4.2 Chronoamperometry (CA)

Chronoamperometry (CA) is an electrochemical technique used to determine the diffusion coefficients, kinetics, and mechanisms of the species in solution^[15]. A potential step is applied to the working electrode in order to cause an electrochemical reaction. The resulting current is recorded against time. When the system is unstirred, the current is limited by diffusion of the electrochemical species to the electrode surface. However, in this system stirring is applied to reduce diffusional limitations. In ideal systems, with a limited number of redox-active species, the current can be described with Cottrell's equation (Equation 3.5).

$$i = \frac{nFAD^{1/2}C^b}{(\pi t)^{1/2}} \quad (3.5)$$

Where: n = number of electrons, F = faraday's constant, D = diffusion coefficient, C^b = bulk concentration, A = area, t = time.

However, the system investigated in this work was far from ideal, as the electrode reactions were complex and the number of redox-active species was not well-defined. For that reason, CA results deviated significantly from those predicted by Cottrell's equation. Therefore, the analysis of CA results was carried out in a qualitative fashion.

Both chronoamperometry and chronocoulometry used the same parameters, as described in section 3.4.3.

3.4.3 Chronocoulometry (CC)

Chronocoulometry (CC) is derived from the chronoamperometric technique^[15]. In a well-defined electrochemical system, CC allows measuring accurately the kinetic rate constant for electrochemical reactions and detect absorption of species on the electrode. The CC plot shows the charge resulting from the overall oxidation or reduction reactions as a function of time (Equation 3.6). In the first few seconds/minutes of the electrochemical reactions, the charge/discharge of the electrical double layer is observed. At longer time, the faradic current becomes dominant. The CC curve can be calculated from the integration of the CA, after adding the correction for the double layer charge and adsorption charge (interfacial interactions):

$$Q = \int i dt \quad (3.6)$$

The parameters used for Chronoamperometry and Chronocoulometry techniques were the following. The constant initial potential was 0.4 V vs. Ag/AgCl reference electrode applied for 24 h. Current was recorded every 30 s. Potential range was set from -2.5 to 2.5 V. Current range was 10 μ A.

3.4.4 Electrochemical Impedance Spectroscopy (EIS)

Electrochemical Impedance Spectroscopy (EIS) probes the electrochemical system through a small amplitude alternating signal and measures the change in frequency, phase and amplitude of the output to gain information on the electrical interface. Here, potentiostatic EIS was used, which applied a small potential and measured the resulting current. The EIS results are commonly interpreted through the use of equivalent circuits, in which the electrochemical interface is represented as a combination of concentrated electrical elements (*e.g.*, resistance and capacitors) that approximate well the complex transfer function between the applied input (potential) and the measured output (current), across a broad range of frequencies^[15]. The equivalent circuit elements can be then interpreted in terms of interfacial phenomena. The EIS potential applied has a sinusoidal form:

$$E_t = E_0 \sin \omega t \quad (3.7)$$

E_t : potential at time t , E_0 : voltage amplitude, ω : angular frequency, t : time

Such potential yields a current:

$$I_t = I_0 \sin(\omega t + \varphi) \quad (3.8)$$

I_t : current at time t , t : time, ω : angular frequency, φ : phase shift

Following Ohm's law, the circuit impedance is expressed in terms of magnitude and phase shift:

$$Z = \frac{E_t}{I_t} = Z_0 \frac{\sin(\omega t)}{\sin(\omega t + \varphi)} \quad (3.9)$$

Z : impedance, Z_0 : Impedance magnitude, ω : angular frequency, t : time, φ : phase shift

Using Euler's relationships:

$$\exp(j\varphi) = \cos \varphi + j \sin \varphi \quad (3.10)$$

The impedance can be expressed as:

$$E_t = E_0 \exp(j\omega t) \quad (3.11)$$

$$I_t = I_0 \exp(j\omega t - \varphi) \quad (3.12)$$

$$Z(\omega) = \frac{E_t}{I_t} = Z_0 \exp(j\varphi) = Z_0 (\cos \varphi + j \sin \varphi) \quad (3.13)$$

Therefore, the impedance is represented as a complex number.

EIS is used for example to demonstrate the existence of an absorbed reaction intermediate^[17], identify corrosion phenomena^[18], measure viscosity gradients^[19], and detect microorganisms in aqueous solutions^[20].

The parameters used in Electrochemical Impedance Spectroscopy were the following. The constant initial potential was set relatively to the cell equilibrium potential E (V): 0.0000 vs. E_{oc} . Cell current stabilization was set to 3 min. Potential was recorded at the working electrode for 10 seconds before the AC simulation started. Impedance scan was from frequency 100 kHz to 30 mHz with 10 points per decade in logarithmic spacing. Sinus amplitude was set at 10 mV waiting for 0.10 periods before each frequency. Repeated two (2) measurements and averaged the values for each frequency. Drift correction was selected. Potential range was set from -2.5 to 2.5 V. Current range was set in automatic mode.

3.5 Scanning electron microscope

The Scanning Electron Microscope (SEM) is a microscope that scans the surface of the specimen probe with an electron beam generated by an electron gun and forms an electronic image of the specimen^[21]. The system works with a pair of magnetic lenses, the condenser lens which controls the intensity of the electron beam, and the objective lens which reduces the scattered electrons. Both lenses improve the size of the spot, thus enhancing the resolution. At every point of the scanning, secondary electrons and backscattered electrons are generated and collected with different detectors to determine the imaging signal. Secondary electrons are low energy electrons that are ejected from near the surfaces layer thus topographical information of the specimen is collected for imaging. Backscattered electrons arise from deeper interaction volume and represent information about composition, topography and chemistry of the specimen. The Figure 3.9 shows a schematic of the interaction volume.

Figure 3.9: Schematic of the interaction volume of an electron beam from SEM (license under the Creative commons Attribution 4.0 International License^[22]).

SEM was used to characterize the electrode's surface morphology. The equipment pieces used were FESEM 6340 and FESEM 7600 (JEOL, USA) at the Facility for Analysis Characterization Testing & Simulation (FACTS) within the Materials Science

and Engineering School in the Nanyang Technological University. FESEM 6340 counts with a resolution of 3.0 nm, an accelerating voltage of 0.5 to 30 kV, a magnification of 50x to 200,000x, and an electron source of cold cathode FEG. FESEM 7600 counts with a resolution of 0.7 nm, an accelerating voltage of 0.01 to 30 kV, a magnification of 10x to 1,000,000x, and an electron source of Thermal FEG. The parameters used for each SEM micrography displayed in this dissertation are stated together with the corresponding image. Most of the images were taken at 5 kV with different magnifications.

Prior to imaging, the samples were removed from the electrochemical cell and rinsed with phosphate buffered saline (PBS) in order to remove the planktonic cells. Then, the cells followed several dehydration steps in ethanol solutions of 25, 50, 75, and 100 % concentration. Finally, the samples were coated with Pt to increase the conductivity.

3.6 Confocal laser microscopy scanning (CLSM)

CLSM uses a laser to scan the object of interest (Figure 3.10). It allows a very precise view of a layer within the object therefore a larger resolution than traditional light microscopy is achieved. Using CLSM, 3D images of cells can be produced. In addition, specific proteins or structures within cells can be tagged by using special dyes or antibodies.

CLSM working principle is based on the laser which hits the specimen and the reflection light passes by an objective lens. Then, it is reflected in the dichroic mirror that deviates the light to the detector. Before the detector there is a pinhole to filter out the light that is not confocal to the specimen of interest.

Figure 3.10: CLSM working principle schematics. Adapted from Anderson with permission from Elsevier^[23].

Prior to imaging, the samples were removed from the electrochemical cell and rinsed with phosphate buffered saline (PBS) in order to remove planktonic cells. Then the electrode's surface was stained with a mix of fluorescent dyes LIVE/DEAD™ BacLight™ Bacterial Viability Kit to image viable and dead cells attached to the electrode. The dyes (SYTO® 9 and propidium iodide) were mixed in equal parts and placed on the electrode for 15 min protected from light. The electrode was then rinsed with PBS to eliminate the excess of dyes and then imaged. CLSM images were taken using the reflection mode under static and z-stack mode. The biomass was quantified using SYTO® 9 and propidium iodide used to differentiate live and dead cells.

3.7 Statistical Analysis

Statistics is the science that collects, analyzes, organizes, and interprets data in order to infer conclusions about a whole population from a sample. Descriptive statistics are

usually used to describe the properties of the experimental data. A widely used statistical method is the central limit theorem which describes average, standard deviation, and standard error of the samples under analysis. Also, central tendency of the population uses the arithmetic average, which in turn describes the central tendency of the sample. Both methods were used in this dissertation.

In addition, to minimize human error most of the electrodes were fabricated on the same batch unless stated otherwise. Also, the electrodes were randomized before being used during the bioelectrochemistry experiments. Each experiment was inoculated with biologically independent overnight cultures of *E. coli*. Each experimental characterization included a number of independent replicates, usually $N = 4$. Average and standard deviation of the results were calculated.

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Chapter 4

Ordered micropillar array gold electrode increases electrochemical signature of early biofilm attachment

This chapter elaborates on the microstructured gold electrode fabrication to support the growth of E. coli. The electrodes are maintained at oxidative potential, the electrochemical output is measured, and the EET rate during early biofilm formation is analyzed. The charge output of the modified electrodes is enhanced up to 22% higher than the control electrode, enabling the electrochemical detection of early E. coli biofilms. The electrode microstructures promote biofilm attachment as shown by FESEM and CLSM imaging. Following biofilm formation, the resistance to charge transfer at the biofilm-electrode interface decreases and the capacitance increases as shown by EIS analysis.

4.1 Introduction

Bioelectrochemical systems (BES) are relevant to green synthesis and biosensors for food, environmental and clinical applications [1]. BES are powered by interactions between viable bacterial cells and electrically conductive materials [2, 3]. Numerous bacterial species are capable of Extracellular Electron Transfer (EET) to electrodes maintained at a defined electrochemical potential [4, 5]. EET originates from bacterial respiration processes in which microorganisms oxidize organic carbon sources and, in absence of soluble electron acceptors (e.g., oxygen or nitrates), use electrode as terminal electron acceptor [6, 7]. EET in microorganisms is generally thought to occur through one or more of three main mechanisms: (i) direct electron transfer through transmembrane cytochrome complex [8, 9], (ii) electron hopping via pili-like conductive appendages termed nanowires [10, 11]; or (iii) mediated electron transfer via exogenous or microbially produced redox mediators [12, 13]. These three EET mechanisms are not completely distinct from one another, thus deconvoluting them with accuracy remains a challenge [14].

Bacteria accumulate at interfaces to form polymicrobial aggregates deemed biofilms [15]. Biofilms that grow on electrodes and show EET via one of the three mechanism described above are usually termed as electrochemically active biofilms (EAB) [2, 5, 16]. The EET rate increase as the biofilm grows [17], but early biofilm attachment stage is sufficient to elicit a bioelectrochemical response [18]. Carbon electrodes have been widely used in energy-producing BES for their low-cost and biocompatibility. However, they are not suitable to detect early biofilm formation, as they have high (capacitive) background current, which hinder the EET process in the early stage of bacterial colonization [19]. On the other hand, gold (Au) and Au thin film electrodes are biocompatible and have low capacitance and low electrical resistance [20, 21], thus allowing sensitive EET measure [22]. Although the trend is moving away from precious metal [3], Au thin film deposition allow the systematic fabrication of electrodes with specifically designed features [23-26]. Therefore, Au electrodes represent a suitable option for biofilm and electrode-surface interaction studies. Recent research shows that both nano- and micro- features determine the current output of biofilms [23, 24, 27] and the roughness of the electrode surface contribute to the electrochemical signature and EET rate during early biofilm attachment [18]. Also, the surface engineering enables the

fabrication of micropillars of comparable size with bacteria, which create a favorable substrate to study the biofilm-surface interactions. Since most of the studies have focus on increasing the current output, little attention has been given the bacteria-surface interaction in the early stage of biofilm formation. This is particularly relevant to biomedical applications, in which early detection of biofilms on devices such as catheters and prosthetic implants allows rapid and life-saving intervention.

Here, we report the use of an ordered micropillared array gold electrode to study the electrochemical signature of biofilm-surface interaction at early biofilm attachment. We measured the electrochemical charge, impedance, and analyzed the EET rate during early biofilm formation. The micropillared electrodes showed faster biofilm formation, as confirmed by field emission scanning electron microscope (FESEM) and confocal laser scanning microscope (CLSM) images. *Escherichia coli* biofilms were used because of their common occurrence and easy handling. *E. coli* showed low but detectable electrochemical activity in presence of the exogenous redox mediators 2-HNQ [28].

4.2 Experimental Methods

4.2.1 Fabrication of Au ordered structure array electrodes

A schematic diagram of the ordered array microstructures on the electrode surface is shown in Supplementary Figure S4.1. The electrodes are covered with a thin layer of Au, which is patterned into ordered arrays of cylindrical pillars with 8 μm (a) and 4 μm (b) diameter and 1 μm height (Figure S4.1a and S4.1b, respectively). In both cases, the distance between the centers of the pillars was 20 μm . The geometric surface of the electrode modified with 8 μm and 4 μm cylinder was 1.03 and 1.06 cm^2 , with the surface of the flat electrode = 1 cm^2 . Figure S4.1c is a 3D sketch of the pillar array viewed at inclined angle. Au was selected as it is a highly conductive and biocompatible electrode material [29].

Lithography was used for the fabrication (Figure S4.2). The surface of 4-inch silicon (Si) wafer was firstly cleaned in sulfuric acid (H_2SO_4) at the temperature of 120 $^\circ\text{C}$ for 30 min. A 20 nm thick layer of titanium (Ti), followed by 200 nm thick layer of Au was

subsequently deposited on the Si wafer surface by the magnetron sputtering system SP3 (Coaxial Power System Ltd, UK) at 3 bars for 10 min. The Si wafer was then covered with hexamethyldisilazane (HMDS) coating for 2 min using DELTA 150 VPO prime oven (Yields Engineering Systems, Inc, USA) to strengthen the adhesion of the photoresist onto the surface. After the wafer cooled down, a $\sim 1\ \mu\text{m}$ layer of photoresist (PR 7217 positive) was spin-coated on the Au surface at 5000 rpm for 30 s. Then, the photoresist was exposed to 365 nm wavelength ultraviolet (UV) light through the glass photomask for 4 s, which was followed by developing the pattern in AZ 300MIF developer solution (EMD Performance Materials Corp., USA) for 1 min. Finally, the patterned wafer was coated with a 50 nm Au layer by magnetron sputter for 5 min.

4.2.2 Field emission scanning electron scanning (FESEM) of the bare electrodes

The surface morphology of the electrodes was characterized using FESEM 6340 (JEOL, USA) at an accelerating voltage of 30 kV and 5 kV. The FESEM images of Au ordered array electrodes with cylindrical pillars of $8\ \mu\text{m}$ are shown in Figure 4.1(a) (top view, magnification 3500x, 30 kV) and Figure 4.1(b) shows the thickness of the cylindrical feature of $1\ \mu\text{m}$ height approximately (magnification 10000x, 5 kV, tilted at 52°). The electrodes with cylindrical pillars of $4\ \mu\text{m}$ diameter are shown in Figure 4.1(c) (top view, magnification 3500x, 30kV), and Figure 4.1(d) shows the thickness of the cylindrical feature of $1\ \mu\text{m}$ height approximately (magnification 25000x, 5 kV, tilted at 52°).

Figure 4.1: FESEM images of the bare gold cylindrical pillars electrode with 8 μm and 4 μm diameter pillars. (a, c) Top view, 3500x magnification at 30 kV; (b, d) Cross-section showing the height of the pillar on the electrode, tilted 52° at 5 kV at 10000x and 25000x of magnification, respectively.

4.2.3 Bioelectrochemical experiments

E. coli K12 was inoculated in Luria Bertani (LB) broth at low sodium chloride for 20 h in a shaker incubator at 200 rpm at 37°C . Following centrifugation at 6000 rpm for 5 min at room temperature, the bacterial pellets were re-dissolved in 10 mL of MOPS 1x (3-(N-morpholino) propanesulfonic acid) buffer at $\text{pH} = 7.4$, to a final optical density at 600 nm (OD_{600}) of 0.7. The bacterial suspension was then added into the autoclave-sterilized electrochemical cell and 50 μM of 2-Hydroxy-1,4-naphthoquinone (2-HNQ) was added as redox mediator.

The electrochemical cells comprised of an 11 mL glass vial and a custom Teflon lid sealed with Parafilm layer (Bemis Company, Inc., USA). Each cell was fitted with a microstructured Au working electrode (WE) hold by a PTFE jacketed electrode holder, an Ag/AgCl reference electrode (RE) (IDA Company, China), and a Pt wire counter electrode (CE). The three electrodes were connected to a VSP multichannel potentiostat (Bio-Logic, France). The electrochemical cells were operated at 23.0 ± 0.5 °C under constant stirring with a magnetic stirrer. Each condition was tested with at least two independent biological replicates ($n=2$). The WEs are the microstructured electrodes described in Section 4.2.1: 8 μm (a) and 4 μm (b) cylindrical pillar arrays and flat electrode (c) without any microstructure. The WEs were characterized at the beginning (following inoculation) and the end of the experiment through cyclic voltammetry (CV) (Figure S4.3) and electrochemical impedance spectroscopy (EIS), which was performed at open circuit potential (OCP) without stirring. Charge output was measured at 400 mV vs. Ag/AgCl through Chronocoulometry (CC) under stirring conditions. EIS results were fitted to the equivalent circuit model through the EC-Lab software v11.26 (Bio-Logic, France).

4.2.4 Imaging of *E. coli* attachment onto the electrode

At the end of the electrochemical characterization, all the electrodes were imaged through CLSM or FESEM to determine the effect of electrode microstructure on biofilm formation. The electrodes were removed from the electrochemical cells and rinsed with phosphate buffered saline (PBS) to remove the planktonic cells. For FESEM imaging, the biointerface was dehydrated with ethanol at increasing concentrations (25%, 50% 75%, and 100%). Then, the electrode was placed in glutaraldehyde 3% at 5 °C for 24 h for fixation on the surface and then dried at room temperature. Finally, the electrode was coated with platinum (Pt) for FESEM observation.

After the removal of the planktonic cells with PBS, the electrodes were stained with a mixture of fluorescent dyes LIVE/DEAD™ BacLight™ Bacterial Viability Kit to image separately viable and dead cells attached on the surface of the electrode. The dyes (SYTO® 9 and propidium iodide) were mixed in equal part and deposited on the electrodes for 15 min. The electrode was then rinse with PBS to eliminate the excess of dye and then imaged with a 40x magnification CLSM (Zeiss 5780 microscope (Zeiss, Germany)). The reflection technique was used to visualize the metal surface [30].

4.3 Results and Discussion

4.3.1 Effect of microstructure electrodes on the EET process

The potentiostat-controlled electrochemical cells were inoculated with overnight grown *E. coli* culture to a final $OD_{600} = 0.7$, which corresponds to fully grown *E. coli* culture in MOPS [31]. Following inoculum, the bacterial cell concentration in the planktonic phase remained approximately constant during the experiment. The *E. coli* inoculum do not contain soluble mediators [32-34]. Therefore, the difference in the charge output across the Au electrode microstructures originated mainly from the bacterial attachment and initial biofilm formation during the 24 h of the batch experiments. This experimental setup consents deconvoluting the effect of bacterial growth in the planktonic phase from the biofilm growth. In previously reported experiments, in fact, the change on current output with time was the combined effect of growth in planktonic phase and early biofilm formation [35-37].

Three types of electrodes: cylindrical pillars ordered arrays of 8 μm , and 4 μm in diameter, and smooth flat electrode (without microstructure) were tested in potentiostat-controlled electrochemical cells. The working electrodes were maintained at $E = 0.4 \text{ V}$ vs. Ag/AgCl, which is higher than the limiting potential for 2-HNQ but not too high to damage the bacterial membranes and the EET chain [38, 39]. The charge output over 24 h was calculated from the current output via numerical integration using EC-Lab software. Previous experiments show that 24 h at 37 °C gives enough time to observe *E. coli* biofilm formation in flow cells [40]. Under batch conditions, longer experiments could not be performed, as the nutrients are rapidly consumed, leading to a strong decrease in the current output. Longer experiments (36 h) in continuous flow cells at 30 °C have been previously reported, showing complete biofilm formation under these conditions [41], with 70-100 μm thick colonies. However, thick biofilms (multilayers biofilm) with full surface coverage hinder the effect of electrode microstructure on EET [23, 42], thus short experiments at lower temperature are preferable to study the effect of microstructure on EET in biofilms.

After 24 h, the charge output for the 8 μm and 4 μm cylindrical pillars microstructured electrodes, and flat electrodes produced $0.299 \pm 0.018 \text{ C}$, $0.273 \pm 0.028 \text{ C}$ and

0.244±0.021 C per cm² respectively (n=4). The microstructured electrodes showed an improvement of 22% and 11% higher than that of the flat electrodes (Figure 4.2). Considering the electrodes specific surface area of 0.30 (flat electrode), 0.31 (4 μm pillars), and 0.32 cm² (8 μm pillars), the specific surface areas increase in 3% and 6% for 4 and 8 μm pillars microstructure electrodes, respectively. Relating charge output and specific surface area leads to find that the increase of 3% on specific surface area corresponds to 11% more charge for 4 μm cylindrical pillars microstructured electrodes. And an increase of 6% cause 22% in charge for the 8 μm cylindrical pillars microstructured electrodes. Therefore, the effectiveness per specific surface area is 3.66 times higher. This result shows that the microstructures have a synergic effect where the charge output increases likely due to the higher cell attachment and early biofilm formation, and not only to the redox cycling of 2-HNQ mediated by the suspended cells in the planktonic phase.

Also, the effect of a small increment of the specific surface in area on the charge output in *E. coli* has not been reported. Previous works reported about specific surface increase from 50 to 600 % for both low and high aspect ratio. Low aspect ratio microstructured electrodes showed similar results as this work for application with *S. oneidensis* [24] and *G. sulfurreducens* [25], therefore the low aspect ratio microstructures show better efficiency than the high aspect ratio. Although increasing the surface area to improve the charge output is a common procedure, the correlation of charge output may not be proportional to the increase of the specific surface area. For example, high aspect ratio microstructures with 500 μm high offer an increment of 5.73 fold of specific area which turn into only 2.36 fold increase of current for *G. sulfurreducens* [23]. This result is relevant to determine the optimal surface area for the detection of initial biofilm formation in biomedical devices.

Figure 4.2: Charge-time response for microstructured electrodes when media contains *E. coli* at OD600 = 0.7, and 50 µM of 2-HNQ as redox mediator in MOPs buffer. Microstructure of 8 µm diameter (red line), 4 µm diameter (blue line), and flat electrode (black line). Control experiment with flat electrode without bacteria (pink line) is included. All the experiments used a potential step of 0.4 V vs Ag/AgCl over 24 h. Each line represents the average of four independent biological replicates (N = 4)

Each CC curve (Figure 4.2) represents the average of four independent biological replicates (N=4). The charge output variability results in partial overlapping of the 8 µm and 4 µm pillars microstructured electrodes. The charge output variability can be explained by the colonization patterns (isolated cells to patches), and the stochastic nature of initial biofilm attachment^[23, 43], due to the reversible attachment/detachment phenomena. The charge output is comparable with that of other weak electroactive microorganisms such as *Enterococcus faecalis*^[44] in presence of 0.5 mM concentration of Fe(II)/Fe(III) as redox mediator. Sterile control experiment with flat Au electrodes produce less than 10% of the experiment inoculated with viable cells, confirming that the current output is caused by the reduction of 2-HNQ by metabolically active viable cells and its re-oxidation at the electrode maintained at oxidizing potential (Figure 4.2).

The charge output with time follows similar trend for microstructured and flat electrodes. After inoculation, the charge increases gradually in the first 5 h, which is

followed by a rapid increase. Interestingly, this increase did not follow a logarithmic trend (Figure S4.4), indicating that the charge production was not related to bacterial duplication in planktonic phase but rather to bacterial biofilm formation [35]. After 15 h, the charge output rate decrease which can be caused by nutrient limitations, pH decline at the electrode interface, and accumulation of EPS as the biofilm grows. It is possible that pH changes locally in the early biofilm thus causing lower EET rate, then plateau, and eventually decrease of the current output, however there is no clear evidence. Previous reports have suggested that the charge output plateau is due to limitation in EET through thick biofilms [23, 45]. The plateau in charge production has been previously observed for growing bacterial culture, and it is not yet clear whether this is due to the exhaustion of nutrients or the excessive accumulation of biofilm which decrease biofilm conductivity and charge output rate. Charge output plateau might also be caused by coverage of surface microstructure, however the CLSM analysis (Figure 4.6) shows only partial coverage which would lead to the nutrient limitation explanation. However, cells might have not been firmly attached to the electrode surface and could have been easily washed off during the staining prior to the CLSM observation. Also, it is possible that cells leave a non-conductive layer on the electrode which decrease overall current output; however, this hypothesis is out of the scope of this research. The charge plateau effect was observed for flat electrodes as well, further research is needed to understand the charge plateau. The CV analysis (Figure 4.3) shows a poorly reversible oxidation peak at 0.05 V vs. Ag/AgCl after biofilm formation (24 h). The height of the peak is higher for microstructured than flat electrodes. However, no significant difference between 4 and 8 μm was observed.

4.3.2 Early biofilm attachment on electrode surface impedance

The Nyquist plot for the smooth and microstructured electrodes (8 μm and 4 μm diameter cylindrical pillars) immediately after inoculation (time= 0 h) and at 24 h is shown in Figure 4.3. The EIS show features at high frequency (HF) and low frequency (LF). While the HF feature does not change with biofilm formation, the impedance at LF decrease significantly with time as early biofilm attaches onto the electrode. It has been previously shown that *E. coli* does not produce a current output without a redox mediator [1, 28]. Thus, the EIS feature at LF is due to the following processes: 1. Molecular diffusion of 2-HNQ from bulk solution to the biofilm; 2. Reduction of 2-

HNQ in the biofilm; 3. Re-oxidation of 2-HNQ at the electrode polarized at high potential. The EIS feature at HF is likely due to the impedance of the electrode-charge collector connection, which is much smaller than that at LF.

Figure 4.3: Nyquist plot of EIS response and fitting to the equivalent circuit model, for (a) $4\ \mu m$ pillars, (b) $8\ \mu m$ pillars, and (c) control flat electrode, before (0 h) and after biofilm formation (24 h). Insets show the high frequency portion of the EIS scan. Each experiment was repeated with two independent biological replicates ($N=2$).

Prior to equivalent circuit modeling of the EIS result, the distribution function of relaxation times (DFRT) was obtained using the software ISGP [46, 47] to determine the number of time constants in the system and their variation with biofilm growth. The raw EIS data were trimmed to remove the noise caused by the electrode connection (HF) and the diffusional limitations (LF), thus isolating the time constants of the system. The gamma function, corresponding to the total impedance of the RC circuit associated to each time constant, and the characteristic time for the RC circuit decrease with time for all electrodes (Figure 4.4), indicating a more efficient EET process as biofilm forms on the electrode. This is due to the faster redox cycling of 2-HNQ in presence of biofilms, as per the EET process previously discussed. The relaxation time

decrease from 10 s to 5 s approximately after biofilm formation. However, no significant difference in the relaxation time was observed between electrodes of different microstructure. Similarly, the decrease of gamma function after biofilm growth does not show statistical difference across the microstructure. The DFRT results confirm that the EIS data should be modeled with two times constant model, in which only the time constant at LF depends on biofilm formation.

Figure 4.4: (a) Relaxation time (τ) and (b) gamma main-peak area value from electrochemical impedance spectroscopy (EIS) before ($t = 0$ h) and after biofilm formation ($t = 24$ h), using ordered structure array gold electrodes of 4 μm and 8 μm pillars, and flat electrodes; (c-e) Gamma as function of relaxation time ($\text{Log } \tau$) for the 4 μm and 8 μm microstructure and flat electrodes. The reduction and left-displacement of the peak areas after biofilm formation ($t = 24$ h, dashed lines) correspond to a decrease of the impedance and to acceleration of EET at the biofilm-electrode interface.

The two time constants shown in the equivalent circuit of the system was determined by fitting the EIS data described by Eq. (4.1, 4.2) ^[48] (Figure S4.5):

$$Z(\omega) = R_1 + \frac{1}{(i\omega)^{\alpha_2} Q_2 + \frac{1}{R_2}} + \frac{1}{(i\omega)^{\alpha_3} Q_3 + \frac{1}{R_3}} = R_1 + \frac{R_2}{1 + (i\omega)^{\alpha_2} \tau_2} + \frac{R_3}{1 + (i\omega)^{\alpha_3} \tau_3} \quad (4.1)$$

$$\tau_1 = R_1 Q_1, \tau_2 = R_2 Q_2 \quad (4.2)$$

where R_1 represents the ohmic resistance of the bulk solution in the cell. Q_3 is the constant phase element (CPE) that describes the pseudo-capacitance of the solution – biofilm interface and R_3 represent the resistance to charge transfer at the same interface. Q_2 and R_2 represented the biofilm – electrode interface in series with the solution-biofilm interface. Finally, α_2 and α_3 represent the nonideality of pseudo-capacitance Q_2 and Q_3 , respectively. Q_2 and R_2 are shown in the Figure S4.6.

The EIS results confirm that the microstructured electrodes improve the EET rate and the biofilm attachment in comparison with the control flat electrode. The 8 μm pillar microstructure shows the lowest impedance, which corresponds to the lowest R_2 and highest Q_2 values (Table S4.1). These results are consistent with previous observation of EET in *G. sulfurreducens* biofilms. ^[14]. R_3 and Q_3 did not show a well-defined trend, as they do not depend on biofilm formation.

4.3.3 Electrode morphology and biofilm-surface interaction imaging: FESEM and CLSM

The distribution of the attached cell onto the electrode support the results of the electrochemical characterization of the biofilm-electrode interface. The electrodes were imaged before inoculation (0 h) and after early biofilm attachment (24 h). FESEM of the bare electrode confirms the fabrication morphology in concordance with the designed characteristics. Figure 4.1 shows the pillars array with smooth Au thin film deposited on the microstructures. The microstructures are homogeneously distributed with centers equally distant of 20 μm . The distance measured from the edge of the structure to the next one was 16 μm and 12 μm for the 4 μm and 8 μm , respectively, which is consistent with the lithography design. The 1 μm height of the pillar structure

is smaller than previously reported microstructure, e.g., 500 μm height pillar [23], 8 μm height pillar [49], and 20 μm height pillar [27]. Higher pillars increase the geometrical surface area; however, they also increase diffusional limitations of the nutrient toward the biofilm. The optimal size of microstructure can vary depending on the microorganisms used and the characteristics of the biofilm.

After 24 h, the early biofilm formed by *E. coli* was observed through FESEM and CLSM. The electrode was suspended vertically in the growth medium, to minimize bacterial deposition due to gravity and rinsed with PBS to remove planktonic cells and poorly attached cells from the electrode surface. The FESEM images showed different pattern of biofilm formation on the two microstructured electrodes, with higher surface coverage for the 8 μm pillars (Figure 4.5a and 4.5b). The bacteria accumulate especially at the foot of the cylindrical pillars. The 4 μm pillars microstructured electrodes barely showed any biofilm accumulation. (Figure 4.5c). However, bacteria were visible on the tilted image as rod-shaped object (Figure 4.5d). FESEM images do not allow accurate identification of biofilm matrix, therefore the results were characterized with CLSM under live/death cell assays. A cross-section performed with focused ion beam system (FIB) showed the biological layer covering the electrode had a thickness of approximately 500 nm which is consistent with the thickness of *E. coli* monolayer.

Figure 4.5: FESEM images of the ordered structure array gold electrodes with bacteria attached onto their surface after 24 h of growth at 0.4 V vs. Ag/AgCl. (a) 8 μm pillars electrode top view, magnification 999x, 5 kV; (b) 8 μm pillars electrode top view, magnification 6000x, 5 kV; (c) 4 μm pillars electrode top view, magnification 1000x, 5 kV; (d) 4 μm pillars electrode tilted 52°, magnification 7500x, 5 kV.

The FESEM results were confirmed by CLSM images. In fact, the distance between features appear to affect biofilm formation, with higher biofilm growth for 12 μm distance than 16 μm, as shown in Figure 4.6. As in FESEM images, most bacteria were observed as small aggregates at the foot of pillars. Previous results show preference of bacteria for micropatterned surface at initial attachment [\[50\]](#) and for the grain boundaries of stainless steel [\[51\]](#). The viable and dead biomass on the 8 μm pillar electrodes were $6.3 \pm 2.1 \mu\text{m}^3$ and $8.9 \pm 3.6 \mu\text{m}^3$, respectively, resulting in a biofilm thickness of 0.40 μm (Figure 4.6a). In the case of the 4 μm pillar electrodes, the CLSM showed a live cells biomass of $4.3 \pm 0.7 \mu\text{m}^3$ and death cells biomass of $3.4 \pm 0.8 \mu\text{m}^3$ leading to a biofilm thickness of 0.34 μm (Figure 4.6b) which is lower than the 8 μm electrode and

consistent with the lower electroactivity observed. Overall, the FESEM and CLSM results support the electrochemical data, showing that the distance between pillars determine both EET rate and biofilm formation. Interestingly, the significant difference in biofilm formation among different microstructure does not correlate with the small difference in surface area, indicating that the spatial arrangement of microstructures determines early biofilm formation, rather than the overall surface area of the electrodes.

Figure 4.6: CLSM images of the ordered structure array gold electrodes of (a) 8 μm and (b) 4 μm pillars, with bacteria attached onto their surface after 24 h of potential-controlled (0.4 V) electrochemical batch. SYTO® 9 and propidium iodide staining used to differentiate live and dead cells. Right-side panels display the individual channels for live cells, dead cells, and electrode surface.

Conclusion

Electron transfer rate between viable *E. coli* biofilms and gold thin films was measured on flat and microstructured ordered array electrodes poised at oxidative potential. The effect of bacterial growth on EET rate was deconvoluted from the biofilm formation, showing that EET depends on the initial biofilm attachment, which in turn depends on the electrode microstructure. Bacterial attachment and initial biofilm formation were visually confirmed via FESEM and CLSM imaging, while the decrease of interfacial impedance following biofilm formation was experimentally measured through EIS, and then fitted to a two times constant model. Microstructured electrodes enhanced the charge output and help understanding the details of the bacteria-surface interactions in early biofilm formation. Moving forward towards bacterial biosensors with high sensitivity and stability (e.g., water quality monitoring), future research efforts should focus on different surface modifications at a microstructure scale as well as modified electrodes using different materials.

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Chapter 5

Electrochemical signature of *E. coli* Ni micropillar array electrode for early biofilm characterization

*This chapter explores the fabrication of nickel micropillar-array electrodes to detect early attachment of *E. coli* biofilm through mediated EET in potentiostat-controlled electrochemical cells. The biofilm-surface interaction is studied via EIS across a broad range of electric potentials and three different temperatures (23, 30 and 37 °C) over a 24-h period. The micropillared electrodes show a higher charge transfer in comparison with the flat ones. The analysis of the EIS signature suggests an effect of temperature on mediated EET transfer in biofilms.*

5.1 Introduction

Biofilms are microstructured bacterial communities living at interfaces, often encased in a self-produced extracellular matrix, composed of proteins, carbohydrates and extracellular DNA^[1]. The biofilm matrix is responsible for the mechanical stability and adhesion to surfaces. Extracellular matrix allows nutrient capture and immobilization of small metabolites, thus reducing the diffusional limitations in biofilms. Transition from planktonic to attached cells is a key step in the biofilm formation process and accurate monitoring of this transition is needed when assessing contamination of biomedical devices and microbially influenced corrosion^[2].

Biofilm grown on redox-active surfaces and electrodes maintained at defined potential are termed electroactive biofilms (EAB), as they produce a current output, which is correlated to their catabolic activity. Under local anoxic or anaerobic conditions, part of the electron flow can be diverted to the electrode surface via extracellular electron transfer (EET) process, which occur through three mechanisms: i) direct contact biofilm/electrode through transmembrane cytochrome complexes ^[3, 4], ii) conductive pili-like appendages (i.e., nanowires) that connect the cell with the electrode surface ^[5, 6], and iii) mediated electron transfer via microbially produced or exogenous redox-active agents through a redox gradient^[7-9]. Recent studies have shown that the mechanism iii) can be elicited in most single-species and microbial consortia, under favorable experimental conditions (e.g., local anoxic conditions, oxidative electrode potential and exogenous redox mediators)^[10]. While several details of EET are still unclear, the electrochemical principles of this phenomenon have been generally accepted

Emergence of EET in bacterial cultures correlates with early biofilm formation, as bacteria transition from planktonic into sessile state (attached biomass). At this point, the electron-carrying mediators are either adsorbed in the biofilms or at the interface, in few micrometers (μm) thickness. Thus, their local concentration increases, and diffusional limitations of the mediator become less relevant, which leads to increased EET rate.

Detection of early EAB is relevant to drinking water systems and biomedical applications. While it is well known that biofilms are more virulent than planktonic cells, there are evidences of convergence between electroactivity and virulence, which open novel possibility in biofilm control and biomedical applications^[11]. Surface structure determine initial biofilm attachment ^[12-14], in which biofilm formation is enhanced by nano- and micro- roughness modifications. However, large microstructures increase diffusional limitations for the transport of nutrient and redox mediator from bulk solution in the biofilms, thus the cell attachment and EET rate does not correlate linearly with the microstructure ^[15]. Controlled roughness on the electrode can be achieved using microelectromechanical techniques like lithography for a systematic study of biocompatible electrodes with size-controlled micro-features dimensions ^[16, 17]. Biofilm formation and activity is affected, among others, by the specific surface area and microstructure of the substrate^[14]. However, this correlation is not well-understood, particularly for early biofilms.

The electrode material affects biofilm electroactivity, as surface resistance and biocompatibility determine the current output and the biofilm attachment, respectively. Most bioelectrochemical studies have been carried out on inert electrodes of carbon materials, such as graphite. While graphite is an excellent biocompatible material, its conductivity is much lower than metals relevant to early biofilm attachment. Gold present higher conductivity and lower resistivity, however it is an expensive material and cheaper options like graphene are preferred^[18]. Nickel (Ni) is a low-cost option with a broad range of industrial applications. Previously, Ni foam was used as substrate for biofilm growth in BES, with good current output^[19]. Biofilm can slowly solubilize Ni from orthodontic equipment^[20] and Ni ions will promote the biofilm formation at subtoxic concentrations^[21]. Interestingly, *Desulfovibrio desulfuricans* biofilm formation is faster on Ni than on stainless steel, likely because the partial solubilization of Ni increase the availability of this key micronutrient^[22]. To our best knowledge, the electrochemical characterization of early biofilm formation on Ni microstructured electrodes has not been reported.

Here we report a fabricated Ni electrode micropillar array to detect early formation of *Escherichia coli* biofilm through mediated EET in potentiostat-controlled electrochemical cells. The biofilm-surface interaction is monitored using EIS across a

broad range of potentials at 23, 30 and 37 °C over 24 h. The micropillared electrodes showed a higher EIS signature than the flat ones, thus enhancing detection of early biofilm formation. These results are relevant for biofilm sensing and investigation of microbially influenced corrosion.

5.2 Experimental Methods

5.2.1 Fabrication of Ni ordered micropillar structure array electrodes

Two patterned micropillar features electrode were fabricated: cylindrical micropillar Ni structures of 4 μm (Ni4) and 8 μm (Ni8) diameter, and flat electrode for comparison. The distance between the pillars was 20 μm from center to center. The pillar height was of 1 μm approx. The total surface calculated for 1 cm^2 electrode was 1.03 cm^2 for Ni4, 1.06 cm^2 for Ni8, and 1 cm^2 for flat Ni-coated. Supplementary Figure S5.1 show a schematic of the electrode surface top view.

The patterned micropillar Ni electrode was fabricated using photolithography. The fabrication started with a 4-inch silicon (Si) wafer which was first rinsed in 90% sulfuric acid (H_2SO_4) solution at 120 °C for 25 min to ensure a clean surface. Then, 50 nm thick nickel (Ni) layer was deposited on the Si wafer surface by e-beam evaporator (Figure S5.2, step 1) (EDWARDS FL 400, Germany). The wafer was then coated with Hexamethyldisilazane (HMDS) in a Delta 150VPO prime oven to enhance the photoresist adhesion to the wafer (Yield Engineering System, Inc, USA). Thereafter, 1.5 μm thick AZ 7217 photoresist was spin-coated on the Ni-deposited wafer (Figure S5.2, step 2) (AZ electronics materials, Singapore). The wafer was then transferred to the photolithography stage of the mask-aligner for the 365 nm wavelength ultraviolet (UV) light exposure, for 5 s (Figure S5.2, step 3). The UV light through the mask selectively harden parts that will not be removed at the developing step. The exposed wafer was then developed in AZ 300MIF developer solution (AZ electronics materials, Singapore) to form the expected pattern (Figure S5.2, step 4). After the development step, the micropillar array pattern were observed on the wafer. Finally, the micropillar patterned wafer was coated with another 50 nm thick Ni layer using the previously mentioned e-beam to increase electrical conductivity (Figure S5.2, step 5). After the fabrication, the wafer was cut in 1 cm by 1 cm pieces and store at 23 °C until use.

5.2.2 Bioelectrochemical experiments

E. coli K12 (DSMZ GmbH) was grown in Luria Bertani (LB) broth at low sodium chloride for 15 h at 37 °C in a shaker incubator at 200 rpm. Following centrifugation at 6000 rpm for 5 min at 23 °C, the pellet formed was washed with PBS three times to eliminate residual nutrients and impurities. The cells were then resuspended in fresh LB at $OD_{600} = 0.4$ to ensure the homogeneity of the culture [23, 24] and incubated again for 2.5 h at 37 °C. After that, the biomass was centrifuged and washed as described earlier. The biomass pellet was dissolved in 10 mL of 3-(N-morpholino)propanesulfonic acid (MOPS 1x) and the optical density was adjusted to $OD_{600} = 0.7$. The bacterial suspension was placed into the electrochemical cells and 50 μ M of 2-hydroxy-1,4naphthoquinone (2-HNQ) was added as redox mediator[25].

The three-electrodes electrochemical cell comprises of Ag/AgCl as reference electrode (RE) (AIDA company, China), Platinum (Pt) wire as counter electrode (CE), and the customized Ni micropillar array as working electrode (WE), which was held by a PTFE jacketed electrode holder. The electrochemical cell was a 11mL glass vial with a Teflon lid, and sealed with Parafilm film (Bemis company, Inc, USA). The vials were connected to a VSP multichannel potentiostat (Bio-Logic, France). For the stirred experiments, a magnetic stirrer was used at 23°C. The non-stirring experiments used metallic beads bath to control the temperature (Elite Dry Bath, EL-02, from Major Science, USA). At least two independent biological replicates (n=2) were tested for each experimental condition unless otherwise stated.

The electrochemical tests include CV, CA, and EIS. CV was carried out without stirring immediately after inoculation ($t = 0$ h) and after 24 h. CA was measured continuously for 24 h under stirring conditions at 0.4 V vs. Ag/AgCl, and EIS was measured at 0.4 V vs. Ag/AgCl under stirring conditions at time: 0, 2, 4, 6, 21, 23, 25 h at room temperature (23°C). In another set of experiments, EIS was measured at 23, 30, and 37 °C under non-stirring conditions in the potential range of -0.2 to 0.4 V vs. Ag/AgCl at 0 h, 8 h, and 24 h. The same EIS was also performed at 23 °C at 0 h, 8 h, and 24 h under stirring conditions. See Table S1 for a summary of the experimental conditions adopted.

5.3 Result and Discussion

5.3.1 Fabrication of Ni ordered micropillar structure array electrodes

The successful fabrication of the Ni ordered micropillar structure array electrodes as a platform for biofilm formation and electron transfer was demonstrated by FESEM images (Figure 5.1). The size and distance of the micropillars were designed in accordance with the *E. coli* dimension, which present a rod shape of 2 μm long with 0.5 μm diameter in an average cell. The pillar diameters were 4 and 2 folds (e.g. 8 and 4 μm) larger, thus ensuring a higher contact area for the bacteria and protect them from the shear forces, which, in turn, favor biofilm formation^[26]. Previous work developed in Chapter 4 shows that bacteria was established at the foot of the pillar at microstructured electrode, therefore the height of the pillars was designed based on 2 folds (e.g. 1 μm) of the average height of bacteria to increase both surface area and biofilm attachment.

Figure 5.1: FESEM images of the Ni micropillar array electrode, lower secondary electron (LEI) mode. Top view, magnification 1800x, 2 kV (a) Ni4: 4 μm diameter pillar. (a) Ni8: 8 μm diameter pillar.

5.3.2 Deconvolution of biofilm attachment and cell growth

The Ni micropillared electrodes influenced the EET and the electrochemical characterization shows the effect of the micropillars on early biofilm attachment. The current output CA measured over 24 h at 0.4 V vs. Ag/AgCl under stirring conditions

shows similar trends for the early biofilm attachment on micropillared Ni8 and Ni4, and flat electrodes (Figure S5.3a). The results were averaged across four independent biological replicates ($n=4$). The current output remained approximately steady after 14 hours and was $2.15 \pm 0.47 \mu\text{A cm}^{-2}$ for Ni8, $1.25 \pm 0.37 \mu\text{A cm}^{-2}$ for the Ni4, and $2.08 \pm 0.39 \mu\text{A cm}^{-2}$ for the flat electrodes. Therefore, the current alone did not correlate with the surface area of the electrodes. The accumulative charge output (i. e., the integral of current output over time) shows a higher value for the flat electrode (Figure S3b), which is due to the slow capacitive discharge of the flat electrode with respect to the micropillared electrodes. Control experiments without bacteria show very low current output, confirming the electrochemical signature is due microbial respiration and not abiotic reduction of 2-HNQ.

The current output measured under stirring conditions was poorly repeatable, as indicated by the large standard deviation value. This is a common observation in biofilm electrochemistry experiments and microbial fuel cells^[27]. However, there is very little literature on CA of initial biofilm formation. In fact, most experiments reported measures over longer times, in which current and charge output are higher and the effect of experimental conditions or surface area of the electrodes become more evident. We concluded that CA under stirring conditions is not a suitable method for characterization of early biofilm formation. Instead, static conditions may be more informative. In all the experiments, we inoculated bacteria at OD close to their maximum value. Interestingly, the optical density at 600 nm wavelength (OD_{600}) measured at 24 h decreased by $\sim 20\%$ in comparison with the initial OD_{600} (Figure S5.3b) for all the electrodes, without significant differences. This can be due to either the nutrient exhaustion or the pH drops, which in turn depends on the accumulation of volatile fatty acids produced during the fermentation. In most batch bioelectrochemical experiments previously reported^[28, 29], the initial OD was much lower, and bacteria grow together with the current output following a Monod-like trend. These experiments show the combined effect of bacterial growth and biofilm formation on the current output. Conversely, experiments with large initial cell concentration like those reported here allow the deconvolution of the effect of growth from that of biofilm formation and highlights the effect of microstructure on the current output. The methodology was recently validated for a bioelectrochemical sensor setup^[25]

The potential scan characterization cycle voltammetry (CV) was recorded at inoculation time ($t=0$) and 24 h later (Figure S5.5) in the potential range -400 mV to 400 mV, to avoid damaging the microbial membrane^[30]. At inoculation time ($t=0$) the remaining oxygen, dissolved in the medium, produced a cathodic current at low potential $E < -200$ mV^[31]. In absence of biofilms and in presence of oxygen (O_2), the redox peak of 2-HNQ was not observed, which is expected since the 2-HNQ reduction is very slow without bacteria. After 24 h, the O_2 was consumed by the bacteria and the CV was recorded under anoxic conditions. Two oxidation peaks were observed at 0 V and 0.2 V vs Ag/AgCl which may correspond to succinate oxidation (0.032 V) and ubiquinol oxidation (0.045 V), and cytochrome oxidation at 0.25 V^[32]. The 2-HNQ redox reaction at -0.45 V^[33] was observed only after 24 h. There was no significant difference between Ni4, Ni8 and flat electrodes.

5.3.3 Effect of the electrode's material: Nickel vs Gold

The electrodes were fabricated with an ordered array pattern analog to the one used previously on gold electrodes (Chapter 4), in order to assess the effect of a different material under the same prior conditions. Using chronoamperometry methods, the microstructured nickel electrodes showed minimal enhancement on the charge transferred (3%) in comparison with the flat nickel electrodes, although the surface area was increased in 3 and 6% with the addition of the microstructures (Table 5.1). The electrode material was expected to present a strong effect on the results due to their intrinsic metal characteristics. The lower electroactive response of the biofilm on an electrode made of nickel is a direct consequence of its higher resistivity ($7 \times 10^{-8} \Omega \text{ m}$) in comparison with gold's resistivity ($2.2 \times 10^{-8} \Omega \text{ m}$). Also, the intrinsic characteristics of each metal's conductivity was reflected on these results where nickel presents $1.4 \times 10^7 \text{ S m}^{-1}$, versus $4.5 \times 10^7 \text{ S m}^{-1}$ for gold.

Gold electrodes present excellent biocompatible and electrical properties which have promoted research on electroactive biofilms^[15, 34, 35]. However, gold is a precious and expensive material, reason why industrial applications would seek the use of lower-cost materials. Nickel is a low-cost option in comparison with gold, with potential practical applications in the industry. Previously, Ni was used for MFC developments with satisfactory current output^[19]. Also, nickel have found applications in orthodontic

equipment^[20] which suggests its biocompatibility. Therefore, Ni is a potential candidate material to develop BES electrodes to study electroactive biofilms.

Table 5.1: Comparison of electrode materials, nickel vs gold

Material	Electrode type	Area [cm ²]	Area increased [%]	Charge Max [C]	Charge performance [%]	Current Max [μA]	Current performance [%]
Gold (Au)	Flat	0.30	0%	0.245	100%	4.979	100%
	4um	0.31	3%	0.273	111%	4.979	112%
	8um	0.32	6%	0.299	122%	8.148	164%
Nickel (Ni)	Flat	0.30	0%	0.107	100%	1.04	100%
	4um	0.31	3%	0.057	53%	0.624	60%
	8um	0.32	6%	0.093	87%	1.074	103%

In addition, from practical point of view, industrial applications are also concerned about the corrosion caused by microbial biofilms in stainless steel. In this regard, the study of early biofilm formation in nickel has the additional potential of expanding our knowledge on microbial induced corrosion in nickel alloys-stainless steels. Hence, to further investigate the effects that might be missed by only focusing on charge transfer when using nickel electrodes, the changes on the electrode's surface were also assessed using electrochemical impedance spectroscopy (EIS) at short intervals of time.

5.3.4 Monitoring biofilm growth onto the electrode surface

The transition from planktonic cell to biofilm can be monitored using electrochemical impedance spectroscopy at fixed electrode potential (PEIS), as the impedance depends on the structure and composition of the biofilm/electrode interface^[36]. The PEIS characterization returns the charge transfer resistance (R_{ct}) and the capacitance Q of the biofilms, thus complementing CV and CA techniques. The EIS at 23 °C under stirring conditions at different times (Figure 5.2) shows the increase of impedance with time in both electrodes. However, the impedance of Ni8 is lower than Ni4, indicating a higher EET rate in the biofilm grown on Ni8. FESEM of Ni4 and Ni8 show good biofilm formation in both cases (Figure S5.6)

Figure 5.2: Nysquit plot of biofilms grown on (A) Ni4 and (B) Ni8 thin film electrodes at different times. Ni8 shows a lower and constant impedance after the initial capacitive discharge.

Prior to modeling with equivalent circuit, the EIS experimental data were fitted with ISGP [37, 38] to determine the distribution function of relaxation times (DFRT) and the corresponding number of time constants. Following this analysis, a two time constant equivalent circuit (Figure 5.3) was used to fit the EIS data using the Eq (5.1, 5.2)^[39]

$$Z(\omega) = R_1 + \frac{1}{(i\omega)^{\alpha_2} Q_2 + \frac{1}{R_2}} + \frac{1}{(i\omega)^{\alpha_3} Q_3 + \frac{1}{R_3}} = R_1 + \frac{R_2}{1 + (i\omega)^{\alpha_2} \tau_2} + \frac{R_3}{1 + (i\omega)^{\alpha_3} \tau_3} \quad (5.1)$$

$$\tau_1 = R_1 Q_1, \tau_2 = R_2 Q_2 \quad (5.2)$$

In which R_1 is the ohmic resistance of the bulk solution Q_2 and α_2 , represent the constant phase element (CPE) that describes the pseudo-capacitance of the solution – biofilm interface, and R_2 is the resistance to charge transfer at the same interface. Similarly, Q_3 and α_3 represent the CPE that describes the pseudo-capacitance of the biofilm-electrode interface, and R_3 is the resistance to charge transfer R_{ct} at the same interface. These results are consistent with previous studies, which report lower impedance when the active surface is increased^[40]. To our best knowledge, this is the first EIS study of *E. coli* biofilms on Ni thin film. However, uneven stirring in electrochemical cells causes

low repeatability of the results and do not allow meaningful comparison between electrodes of different microstructures. Therefore, experiments under static conditions were carried out, which have been shown to be informative in a recent work on weak electroactive biofilms^[11].

Figure 5.3: Equivalent electrical circuit used to fit impedance of *E. coli* biofilm on Ni thin film electrodes, together with physical interpretation of the circuit elements for a complementary understanding (adapted from^[41]).

5.3.5 Effect of stirring and temperature on biofilm impedance

EET in EAB is obviously potential-dependent, thus it makes sense to include potentials in the parameters of impedance spectroscopy, in addition to time, temperature and stirring. EIS at different potential shed light on both biofilm-electrode interactions (e.g., mediated EET) and abiotic effect (e.g., passivation and dissolution of Ni thin film at high potential). Impedance spectroscopy allows also to monitor the behavior of biofilm in terms of capacitance, which has been recently exploited for rapid biomass monitoring^[42].

EIS at different potentials has been used to study the kinetic reactions in analytical electrochemistry for the non-stationary interfaces with faradaic process and in corrosion process^[43, 44]. It was also used to show EET between *Shewanella oneidensis* biofilms and semiconductor iron oxide substrate^[45]. The experiment was set for EIS characterization in the potential range between -200 to 400 mV vs. Ag/AgCl with 50mV

steps. The experimental data was fitted using the equivalent circuit model described in Section 5.3.3. Among the seven parameters of the model, charge transfer resistance in the biofilms R_{ct} showed the greatest change with time, temperature, and stirring, as expected, and was plotted against the potential for all the experimental conditions tested. The measurement was done at three time points: inoculation ($t = 0$ h), 8 h, and 24 h.

Bacteria growth rate under stirring and non-stirring condition at 23 °C was measured for Ni8, Ni4, and flat electrode. The stirring was maintained at all time except during the actual EIS measurement to avoid interference. The data at all potentials were binned and plotted to determine the effect of stirring on the R_{ct} of the biofilms. The greatest effect of stirring was observed at 8 h (Figure 5.4), when the R_{ct} of the stirred biofilms was lower than that of the unstirred experiments. This is due to the formation of a thinner and denser biofilm under stirring conditions ^[46]. However, there was no significant difference between the three microstructures, likely because of the low repeatability of the results under stirring conditions. The same graph at 0 and 24 h did not show any difference between stirred and unstirred experiments (Figure S5.7). These results suggest that EIS measures of mediated EET should be carried out at the time of maximum biofilm activity to obtain the best result, rather than under nutrient depleted conditions ($t = 24$ h). Stirring ensures a homogeneous media and changes both anaerobicity and nutrient delivery at the biointerface, which generate a favorable environment for the bacteria to grow^[47]. Although non-stirring condition reduce the signal to noise ratio in our electrochemical setting, it increases the nutrients gradient in the system, thus slowing down the bacterial growth and increasing R_{ct} at the biofilm/electrode interface. Overall, the effect of stirring on EIS is larger than that of the microstructure, which suggest using non-stirring condition for accurate impedance measure.

Figure 5.4: R_{ct} comparison between stirring and non-stirring conditions at 23 °C at 8 h. Data at all potential from -200 to 400mV in 50mV steps were binned. Results show that R_{ct} under stirring conditions was lower than non-stirring conditions, due to change in biofilm assembly/structure.

It is well-known that incubation temperature changes the growth rate of bacteria in batch culture, as described by the complete Monod equation. It is less understood how temperature affects the final optical density of a microbial culture. Early research shows that this dependency is very small, except for low and high T, where bacteria do not grow at all [48]. The changes in the electrochemical signature of the biofilms at different temperature are due to either higher biofilm formation rate or higher metabolic rate, which in turn correspond to faster turnover of 2-HNQ between its reduced and oxidized state. It is expected the effect of electrode microstructure will be higher as the growth temperature reaches its optimal value, which is approximately 37 °C. Further experiments at higher temperature and with biofilm mutants will help separating the effect of temperature on biofilm formation from that due to metabolic activity.

The SPEIS was measured at three time points 0 h (inoculation time), 8 h, and 24 h. Results show that increasing the temperature from 23 to 37 °C results in a much higher R_{ct} at the biofilm-electrode interface for both microstructured and flat electrodes. The R_{ct} calculated from SPEIS shows two features at 0 and 350mV vs Ag/AgCl, respectively. Figure 5.5 shows the R_{ct} for the Ni8 at 37 °C. The results for Ni4 show a similar pattern. Interestingly, the nonideal capacitance of the biofilm/electrode interface does not show any well-define trend with the growth temperature or time. The effect of microstructure on R_{ct} is maximum at 37 °C and at potential approximately 0 V vs. Ag/AgCl, which correspond to the highest biofilm contribution (Figure 5.5). At 24 h,

the R_{ct} at 0 V vs Ag/AgCl is smallest for Ni8, indicating biofilm formation and 2-HNQ turnover. The feature of R_{ct} at high electrode potential may correspond to the electrochemical activity of other component of the system rather than 2-HNQ. In facts, previous research has shown the production of endogenous mediators in *E. coli* grown on electrode at oxidizing potential such as hydroquinone derivatives^[49] and other unidentified polar compounds^[50].

The binned data shows that R_{ct} is lower for Ni8 than Ni4 in most cases across the whole potential range (FigureS5.8). This effect is explained mainly by the biofilm growth on the electrode since the platonic OD₆₀₀ measured for Ni4 and Ni8 is approximately the same.

Figure 5.5: R_{ct} from EIS at different potential at 37 °C of temperature under non-stirring conditions for Ni8. The EIS spectrum show two features at 0 and 350 mV, respectively. The earlier become more evident as the growth temperature increases. Each measure is the average of at least two independent biological replicates (n=2).

5.4 Conclusion

Successful fabrication of Ni micropillars electrode lead to a better understanding of the EET in *E. coli* early biofilms using electrochemical characterization. Small increments in surface determine a detectable change in the electrochemical impedance of the biofilm/electrode interface, particularly the charge transfer resistance, across different potential and at several growth temperatures. The impedance change was observed few hours from inoculation, confirming this method is adequate to investigate early biofilm formation, particularly on conductive thin film metals surfaces.

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Chapter 6

Effect of micropillar ordered array electrode arrangement for early biofilm

*This chapter elaborates on the production of nickel electrodes with a micropillar array of 10 μm height to study the early surface attachment of *E. coli* biofilms, with a monitored growth through mediated EET in potentiostat-controlled electrochemical cells. Also, the biofilm-surface interaction is evaluated using EIS at short and long intervals, as well as under different concentrations of glucose. The micropillared electrodes showed that interfacial capacitance was inversely proportional to the glucose concentration. Further, Ni micropillared electrodes allow earlier biofilm detection in comparison to flat ones.*

6.1 Introduction

Bioelectrochemical systems (BES) search answers for questions where the biocatalysis from bacteria is combined with biotechnology and electrochemistry for the study of biointerfaces on electrodes surfaces. The biocatalysis allows the harvesting of electrons which often is used to explain process on biosensors and biocorrosion^[1, 2]. Biocatalytic processes are the result of bacteria respiration able to extracellular electron transfer (EET) from and to a solid electrode surface at a specific redox potential in a potentiostat-controlled environment potential^[3-6]. Although BES are highly used to describe microbial biocatalytic processes, understanding the bacteria-surface interface interaction is a hybrid complex that remains as a challenge^[7].

The bacteria agglomeration receives the name of biofilms which attaches to a surface, grow, form a monolayer of cells, and produced polysaccharides (matrix) which protect them from the environment^[8]. When the biofilm grow in the electrode surface of a BES, receives the name of electroactive biofilm attachment (EAB)^[1, 3, 6]. BES use the capability of electron exchange of EAB with solid materials in order to measure the electron or coulombic flow^[3]. The EET from the bacteria at the interface commonly accepted, is described by three mechanisms: i) direct contact when electrons are conducted through the cell membrane to the electrode^[9, 10], ii) pili-like appendence which uses nanowire to connect the cell with the electrode surface^[11, 12], and iii) mediators microbially produced that transfer the electron to the electrode surface through a redox gradient^[13, 14]. Yet, fully understanding the surface that enhanced the ohmic flow remains a challenge^[15-17].

The electron diffusion through the EAB changes as the biofilm grows depending on the electrode surface. Also, the initial bacteria attachment for biofilm formation is enhanced by nano and micro roughness modification onto the electrode which changes the biofilm development hence the electron transfer as well ^[18-21]. A controlled roughness on the electrode can be achieve using microelectromechanicals techniques like nanoimprint photolithography for a systematic study of biocompatible electrodes with size controlled micro-features dimensions ^[22]. The fabrication of platforms sensible to electrical stimuli open opportunities for the development of technological application. Therefore, understanding the biofilm-surface interaction converts the BES

into a potential accurate electrochemical detection system for bacteria and biofilm devices, yet more research is needed.

Here we report a fabricated Ni electrode micropillar array of 10 μm height to study the early attachment of *E. coli* biofilm and to monitor growth on the electrode through mediated EET in potentiostat-controlled electrochemical cells. Also, the biofilm-surface interaction is monitored using Electrochemical Impedance Spectroscopy (EIS) at short and long intervals, as well as under different glucose concentration. The micropillared electrodes showed an enhanced interfacial capacitance depending on the glucose concentration, thus higher early biofilm attachment. Further, Ni micropillared electrodes allow earlier biofilm detection rather than Ni flat electrodes and conventional graphite electrodes, which is relevant for biofilm sensing and investigation of microbially influenced corrosion. This work presents a novel approach on bioelectrochemical characterization of weak electroactive biofilms on Ni substrate.

6.2 Experimental Methods

6.2.1 Fabrication of Ni ordered structure array electrodes

Nickel electrodes with three different types of surface topographies were fabricated: cylindrical pillars 10 μm in diameter and 10 μm in height, spaced 10 μm (Ni10) and 100 μm (Ni100) apart in a hexagonal layout (Figure S6.1), and an electrode without any feature denominated flat electrode which was used as control.

The micro-pillars were fabricated using a thermal nanoimprint lithography process (Figure S6.2). Customized nickel molds (Eulitha, Switzerland), each measuring 25 mm by 75 mm and having 10 mm x 35 mm patterned area of uniform hexagonal array of holes 10 μm in diameter and depth, were pre-coated with an anti-stiction layer (perfluorodecyltrichlorosilane) via a vapor deposition process. Polycarbonate films (Young Chang High Tech Materials Co., Ltd., Republic of South Korea), with glass transition temperature of 150 $^{\circ}\text{C}$, were imprinted with the nickel molds at a pressure of 40 bar, temperature of 160 $^{\circ}\text{C}$ for 300 s using an Eitre 6 nanoimprinting system (Obducat, Sweden). The system was then cooled to 25 $^{\circ}\text{C}$ before demolding, whereby the imprinted substrates were separated from the molds, was carried out manually.

Finally, a 70 nm thick layer of nickel was deposited onto the imprinted substrates using an electron beam evaporator (Denton Explorer, USA). The nickel-coated substrates, cut into 1 cm by 1 cm pieces, were used as nickel electrodes for subsequent experiments. Surface topographies of micro-structured nickel electrodes were examined using a microscope (Zeiss Axio Imager) and scanned using a scanning electron microscope (JEOL FESEM 6700). Figure 6.1.

6.2.2 Bacterial medium

A bacterial medium of *E. coli* K12 (DSMZ GmbH) was used for the experiment which was grown in Luria Bertani (LB) broth at low sodium chloride for 15 h at 37 °C in a shaker incubator at 200 rpm. Then, the biomass was separated by centrifugation at 6000 rpm for 5 min at room temperature. The pellet formed was washed with PBS three times to eliminate residual nutrients and impurities. The cells were resuspended in fresh LB from which a new culture was inoculated at 0.4 optical density at 600 nm wavelength (OD_{600}). The cells were incubated again for 2.5 h at 37 °C with fresh LB to ensure the homogeneity of the bacteria grown in the media^[23, 24]. Later, the biomass was centrifuged, and washed again as described earlier. The new biomass pellet was dissolved in 10 mL of 3-(N-morpholino)propanesulfonic acid (MOPS 1x) and the optical density was measured and adjusted to OD_{600} of 0.7. The bacterial suspension was placed into the electrochemical cells and 50 μ M of 2-hydroxy-1,4naphthoquinone (2-HNQ) was added as redox mediator.

6.2.3 Electrochemical characterization

The electrochemical testing was carried out in a three-electrode system, including an Ag/AgCl reference electrode (RE) (AIDA company, China), Platinum (Pt) wire as counter electrode (CE), and the customized Ni micropillar array as working electrode (WE), which was held by a PTFE jacketed electrode holder (AIDA company, China). The electrochemical cell used was a 11 mL glass vial with a Teflon lid, and sealed with Parafilm layer (Bemis company, Inc, USA). The electrodes were connected to a VSP multichannel potentiostat (Bio-Logic, France). The experiments were carried at 23 °C with magnetic stirrers (~150 rpm). Four independent biological replicates (n=4) were tested for each experimental condition, unless otherwise stated.

The electrochemical tests include cyclic voltammetry (CV), chronoamperometry (CA), and potentiostatic EIS. CV was tested at 0.4 V vs. Ag/AgCl immediately after inoculation ($t = 0$ h) and after 24 h ($t=24$ h). CA, current output, was measured continuously for 24 h under stirring conditions at 0.4 V vs. Ag/AgCl. And EIS was measured at 0.4 V vs. Ag/AgCl at time: 0, 2, 4, 19, 21, 23, 25 h. The stirring was paused during EIS and CV.

6.3 Result and Discussion

6.3.1 Electrode Fabrication

Lithography allows the fabrication of three-dimensional structures with rigorous size-controlled topography. Thus, it is an excellent technique to investigate the effect of micropillars on biofilm formation and electroactivity. Three types of electrodes were designed: a) Flat: smooth electrode without any modification serves as control, b) Ni10: hexagonal arrangement of 10 μm distance from pillar to pillar, and c) Ni100: similar arrangement of 100 μm distance from pillar to pillar. Ni10 and Ni100 present pillars of 10 μm diameter and 10 μm height differentiated by the distance among them, a schematic is included in Figure S6.1. The modified electrodes were fabricated using lithography nanoimprint, which allow the fabrication of several electrodes simultaneously with high quality standards so to serve as for the biological replicates.

Figure 6.1: Field emission scanning electron microscopy (FESEM) top view image of the bare electrodes (a) Ni10: 10 μm apart in a hexagonal arrangement. SEM 5kV 2000x magnification. (a) Ni100: 100 μm apart in a hexagonal arrangement. SEM 5kV 160x magnification.

The micropillared electrode enhanced the surface area to 0.95 cm² and 0.52 cm² for Ni10 and Ni100 respectively. The surface area was calculated considering there were 4 pillars per four-sided figure of 40 μm by 34.64 μm for Ni10 and 220 μm by 190.53 μm for Ni100 due to the hexagonal distribution of the pillars. The 34.64 μm and 190.53 μm corresponds to the projected coseno of the distance from pillar to pillar (See calculation in the supplementary information Chapter 6). Although, the pillar features were the same in both electrode types, the distance between pillars affected dramatically the surface area increased. The electrodes presented an increase of 91% for Ni10 while 3 % for Ni100 in comparison with the flat electrode of 0.5 cm². Increasing the 3 % of the surface area is connected to the previous Chapter 5 which also present a 3% increase on surface area while the pillars size and distribution differs. The morphology of the micropillars and full coverage was characterized by FESEM (Figure 6.1). The images confirmed the successful fabrication for both Ni10 and Ni100. The Ni electrodes presented a smooth conductive surface suited for the anodic electron transfer process from the biofilm. Previously, Nickel micropillars have been used for very different roles such as 30μm diameter pillar for cells microfluidic applications [25], and 200 μm diameter for liquid crystal thin film applications [26]. Also, Ni foam was also used for microbial fuel cells applications with good current output[27, 28].

In terms of microstructure, the literature shows that the efforts on increasing the surface area with very high aspect ratio pillars, returns a lower proportion of the current increased in comparison with the area gained. For example, a culture of *Geobacter sulfurreducens* was tested with a 500 μm height micropillar electrode which surface area increased on 600% and the current harvested increased only 56% of the surface area increased [21]. On the other hand, a case on *Shewanella oneidensis* with smaller nanofeatures enhanced the current harvested 40% when the surface was six times smaller[29]. Therefore, in search for an optimal height, this work presents 10 folds higher pillar for weak electroactive species. Also, the distance gap from pillar to pillar was assessed comparing 10 μm distance to 100 μm.

6.3.2 Bioelectrochemical characterization of the electrodes

The bioelectrochemical EET characterization for early biofilm attachment showed a higher electron transfer for the microstructured Ni10 electrode. The three electrodes

type were inoculated identically and characterized electrochemically by CV, CA, and EIS. Current output for Ni10 was higher than the other electrode tested under same conditions. The chronoamperometry (CA) was measured during the 24 h after an initial pulse of 0.4V vs Ag/AgCl (Figure 6.2). The CA curve presented a steady state through the 24h. The stochastic current showed a maximum point collected between 5 and 7 h after incubation for the average of the curve collected. The maximum values are 1.3, 1.8, and 1.5 μA for Flat, Ni10, and Ni100 accordingly. The large variability and poor reproducibility of CA for early biofilms underestimate the maximum reached information. As described in previous chapter, CA under stirring conditions may not present reliable information for early biofilm, however it is still a reference point to other literature. The overall performance was assessed. Ni10 showed a distinguished higher current measured which may indicate higher bacteria attached onto the electrode surface which metabolize the mediator enhancing the electron transfer. On the other hand, Ni100 performed very similar to the flat electrode and the surface increased in barely noticed. The low current is not suitable to complete the characterization of the early biofilm, and other electrochemical technique such as Electrochemical Impedance Spectroscopy (EIS) are required.

Figure 6.2: The micropillar structures Ni10 increase the current collection up to 40.1% higher current than the flat electrode. Four (4) independent replicates was presented for each electrode type: Flat, Ni10, and Ni100. 24 h chronoamperometric. Shaded area indicates standard deviation of the mean.

The CV were measured to determine the grouped electrochemical potential of the redox species in the solution and/or in the biofilm. Usually, in traditional electrochemical systems CV is a powerful electrochemical characterization tool, however biological reactions are more complex than other inorganics reactions. The CV curve was run for the customized electrodes in similar condition than previous chapter. Fix potential was kept at 0.4 V vs Ag/AgCl on inoculation time ($t=0$) and 24 h later for the electrodes Ni10, Ni100 and Flat (Figure S6.3). The dissolved oxygen available in the media was noticed as a reduction peak at potential of -0.2 V vs Ag/AgCl which is characteristic of bioelectrochemical systems [30]. It was expected that the CV curve would not present significant differences at inoculation time ($t=0$) among the electrodes (e.g. flat, Ni10, and Ni100) since all bacteria were in planktonic state. After 24 h, the oxygen was depleted by the bacteria therefore the reduction peak was not observed. Also, after 24 h, metabolic products of the bacteria were noticed with two oxidation peaks in the CV curve, at 0 and 0.2 V vs. Ag/AgCl which correspond to succinate (0.032 V) and cytochrome oxidations (0.25 V) [31]. Similar results have been previously observed in Chapter 5 in HNQ-mediated EET. Although, Ni10 present considerable higher surface area, the CV curve shows a slightly different curve which does not provide enough evidence to draw conclusion about the microstructure effect on the electrode. It may suggest that more bacteria were attached on the Ni10, hence more electrons were mediated. Ni100 does not show a significant difference with respect to the flat electrode which suggests that the surface area increased does not affect the bacteria attachment.

Electrochemical Impedance Spectroscopy (EIS) has been shown as a more adequate tool to assess the early biofilm on the electrode surface. For initial assessment, the monitored change was measured at inoculation time ($t=0$) and 24 h later. EIS excited the electrode's surface with low to high frequency and the experimental curves were read on a Nyquist plot (Figure S6.4). The impedance increased after 24 h which suggests that bacteria accumulated on the electrode surface increasing the impedance. The Nyquist curve shows a smaller semi-circle for Ni10 which demonstrate higher conductivity among the electrodes. Although the impedance increases after 24h, the same pattern is observed when Ni10 shows higher conductivity. It may suggest more bacteria on the electrode surface able to electron transfer due to micropillars.

After the bioelectrochemical characterization, the electrode Ni100 type have shown no significant difference in comparison with Flat electrode. Although, the surface area was increased a 3%, and used Ni as material like electrode in chapter 5, the performance was very different. We can conclude that the distance between pillars matters to the electron transfer and the distance of 100 μm between pillar does not enhance the bacteria attachment in comparison with the flat electrode. Hence, increasing the surface area is not enough to increase the signaling collection. The geometrical arrangement and topography of the surface were important factors. The research efforts should be focus on optimizing the distance among the features for futures designs. 10 μm gaps seems to be a better distance that influence the bacteria attachment to the surface and electron transfer. Therefore, the rest of the experiments only includes Ni10.

6.3.3 Impedance change at short intervals in 24 h

The impedance change was measured at short intervals of 2 h during the first 24 h after inoculation. The experimental data were fitted with a two times constant series model based on the software ISGP [32, 33] described in Chapter 4. The capacitance and charge transfer resistance at low and high frequencies were calculated from the experimental data using the following Eq (6.1, 6.2) [34]

$$Z(\omega) = R_1 + \frac{1}{(i\omega)^{\alpha_2}Q_2 + \frac{1}{R_2}} + \frac{1}{(i\omega)^{\alpha_3}Q_3 + \frac{1}{R_3}} = R_1 + \frac{R_2}{1 + (i\omega)^{\alpha_2}\tau_2} + \frac{R_3}{1 + (i\omega)^{\alpha_3}\tau_3} \quad (6.1)$$

$$\tau_1 = R_1Q_1 \quad , \quad \tau_2 = R_2Q_2 \quad (6.2)$$

in which R_1 is the ohmic resistance of the bulk solution, Q_2 and α_2 represent the constant phase element (CPE) that describes the pseudo-capacitance of the solution – biofilm interface, and R_2 is the resistance to charge transfer at the same interface. Similarly, Q_3 and α_3 represent the CPE that describe the pseudo-capacitance of the biofilm-electrode interface, and R_3 is the resistance to charge transfer at the same interface. The results include the biofilm – electrode interface which correspond to the low frequency component as well.

The electrode surface-biofilm interface was study at low frequencies of EIS for both electrodes type (e.g. Ni10 and Flat). The resistance at low frequencies (Figure 6.3) shows an increased resistance from inoculation time. However, on the first hours the resistance drops and before growing again. The resistance dropped on the first few hours, most probably due to the accumulated capacitance. Then, the resistance slowly increased as the early biofilm forms. The capacitance (Figure 6.4) corresponding to low frequency shows a constant increase from the inoculation time. These results suggest that capacitance changes with the number of electrons available.

Figure 6.3: Calculated resistance at low frequency (R_f) corresponds to the early biofilm. The microstructure electrode difference can be noticed only after 20 h of incubation. The bacteria attached onto the surface metabolize HNQ and reduce the resistance at lower frequencies.

The resistance at high frequency (Figure S6.5) represent the conductivity resistance for both electrodes type, also the results suggest that Ni thin film electrodes are fully covers in both type of electrodes (e.g. Ni10 and Flat) and present good conductivity. Also, the resistance decreases from the inoculation time which means that the electron flow rate on the electrode surface increase through time. The capacitance for high frequency shows the microstructured electrode present lower charge accumulation. It may suggest an initial local passivation, micro Ni dissolution enhancing bacterial growth, and re-passivation of the Ni by cell attachment and mediator diffusion. Followed by a prevention of the re-passivation of the surface by the growing biofilm, likely because of the low oxygen content. The passivation layer for Ni is a mixture of NiOx, so O₂

must diffuse within the biofilm to reform the passivation layer as observed previously on steel^[35]. In light of these considerations, the microstructured electrodes increase biofilm attachment

Figure 6.4: Capacitance at low frequency (Q LF) shows the electrode's surface-early biofilm attachment interaction for both Ni10 and Flat electrodes. Capacitance at high frequency (Q HF) represent to electrode's conductivity.

6.3.4 Electroactivity under starvation conditions.

The glucose concentration change of EET was measured for early biofilm. Glucose is the main carbon and energy source for *E. coli* inoculated in the bioelectrochemical reactor. The customized electrodes Ni10 and Flat were tested under equal conditions at different glucose concentration (e.g. 0.02%, 0.2% and 1.5 % glucose). The result shows that over 24 h, although, the current increase with the glucose concentration, the microstructured electrodes shows a higher current than flat in equal conditions (Figure 6.5) in all the cases. Glucose concentration and availability determine the growth rate and respiration rate. For concentrations of 1.5% glucose, the microstructured electrode curve shows a maximum at 5 h before the slope turns with negative slope. The same condition with flat electrode shows a lower current output constant at 1.23 μA throughout the 24 h. Both experiments have identical settings, therefore it suggests that the current output difference is due to the bacteria activity on the electrode surface. The current measured for very 0.02 % glucose concentrations over 24 h shows a decreasing

slope in the CA curve. Previous results claimed that low glucose input increased the growth rate in aerobic conditions for concentration of 0.1%^[36]. Therefore, the glucose concentration was not sufficient for the bacteria population to produce the fermentation cycle not to form biofilms. A constant decreasing slope was observed in previous experiments for abiotic/no-bacteria measured curves. Intermediate concentrations maintain an intermediate current with lower negative slope after reaching the maximum current at 4 h.

Figure 6.5: Amperometric current corresponding to different glucose concentration in the media. Glucose concentration of 0.02, 0.2, and 1.5%. Microstructured electrode Ni10 show higher current output. Three (3) replicates for each electrode type: Flat and Ni10 over 24 h.

The EET on early biofilm on the electrode surface was measured with EIS in order to explore the effect on glucose concentration. The EIS was measured at inoculation time ($t=0$) and 24 h later. The experiments were run for the three glucose concentrations previously stated (e.g. 0.02, 0.2, and 1.5%). The experimental Nyquist curves were used to calculate the analytical results of capacitance (Figure 6.6) and resistance (Figure S6.6) following the equations (1) and (2) previously described.

The results show that the starving condition at very low glucose concentration (0.02 %) the capacitance increased and resistance is decreased after 24 h, similar behavior was reported for reactor with mixed cultured bacteria^[37]. Knowing that a larger capacitor requires longer to charge with a high resistance. However, the resistance was reduced

after 24h therefore the charging rate increased. Therefore, although the resistance decrease, a larger capacitance explains the low current transfer observed in CA. The higher capacitance may indicate fewer electrons available from the anaerobic fermentation which is expected since the glucose concentration is lower. In the case of higher glucose concentration (1.5 %) the capacitance remains almost invariant after 24 h whereas the resistance increase. This result may suggest that the electrons product of the anaerobic fermentation was mostly transferred through the biofilm at a slower rate due to biofilm's resistance. The intermediate case, starvation condition at 0.2 % glucose concentration presents slightly higher capacitance, but vast increase of the resistance. Meaning, not many electrons available and a smaller biofilm able to transfer electrons. Microstructured electrodes showed the same behavior, only improving the electron flow presenting lower capacitance than the peer flat electrodes. Similar case for the resistance. Optical density at 600 nm wavelength (OD_{600}) was monitored in planktonic cells in the liquid face which would indicate that the current effect is only from the biofilm attached on the electrode's surface. EIS demonstrated to be more sensible to changes than CA therefore EIS should be preferred for future characterizations.

Figure 6.6: Capacitance increase on the electrode's surface after 24h. The higher capacitance indicate fewer electron available for transfer through the biofilm.

6.4 Conclusion

Understanding EET in thin biofilms of weak electroactive microorganisms is relevant to the development of bioelectrofermentation and other bioelectrochemical processes. Microstructured Ni electrode with 10 μm pillars were fabricated with soft lithography and used to grow early *E. coli* biofilms. Measurement of EET rate show that Ni microstructured surface favor bacteria attachment and biofilm formation. Interfacial resistance is maximum at intermediate glucose concentration, indicating enhanced biofilm formation. Interfacial resistance obtained from potentiostatic EIS results show faster accumulation of biofilms on microstructured electrodes. These results outline a strategy for rapid biofilm formation and detection of Ni-based electrodes, which may find application in biomedical devices and bioelectro-fermentation process.

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Chapter 7

Discussion and Future Work

In this chapter the main conclusions of this dissertation are highlighted. Overall, this dissertation advances the understanding of the bacteria-surface interaction in early biofilm formation using customized microstructured array electrodes. It further elaborates on the opportunities and strategies for future research.

7.1 Motivation, main findings and conclusions

The work done in this dissertation was motivated by three factors (i) the need of an improved understanding of the electron transfer mechanisms of early bacterial biofilm formation over an electrode's surface, (ii) a knowledge gap related to the role that metallic materials play on early biofilm formation and electrode charge transfer, and (iii) the lack of information about the effect of microstructured surface modifications and topographical arrangements in both early biofilm formation and electrode performance.

Three studies were carried using replicated bioelectrochemical setups out to address these motivations. Ordered array electrodes were fabricated with two different metallic materials and different surface array pattern arrangements. Surface area increments were designed and tested at different time points during the early bacterial biofilm formation stage, employing *E. coli* bacteria as a model organism. Electrochemical impedance spectroscopy (EIS) was used to analyze changes on the electrode's surface across all three studies, therefore factor (i) is assessed in chapters 4, 5 and 6. In chapter 4, a gold ordered array electrode was fabricated with two different microstructured patterns to examine factors (i) and (iii). Chapter 5 presents a similar microstructure arrangement on the electrodes, but using nickel as the base material to also evaluate factor (ii). This chapter also includes the analysis of other external factors that can affect electron transfer mechanisms such as electric potential, stirring and temperature, thus addressing factor (i). In chapter 6, the effect of a different feature arrangement presents a nickel electrode with micropillars of 10 μm diameter and 10 μm height arranged with gap distances of 10 and 100 μm , aimed to tackle factor (i). The main conclusions of these studies, implications and recommendations for future work are summarized in this chapter.

7.1.1 Increasing electron transfer with ordered array gold electrodes

This first study involved a replicated experimental design employing three different types of electrodes fabricated with photolithography. The electrodes were covered with a thin film of gold. Two of such electrode types had microstructured cylindrical pillars on their surface, of 4 and 8 μm diameter and 1 μm height, while the third type was a

flat electrode (without microstructures) for control. The current was measured continuously through 24 h, while other electrochemical tests such as voltammetry and impedance were measured at the beginning of the experiments and after 24 h. The redox mediator 2-HNQ was used for the model organism *E. coli*. The experiment had independent biological replicates to increase the chances of detecting significant effects. Scanning electron microscopy and confocal laser scanning microscopy images were used to observe the bacterial attachment on the electrode surface.

This study used chronocoulometric testing to measure the electron charge transfer which was calculated from the current output via numerical integration. The ordered array electrode enhanced the charge transfer up to 22% higher, compared to the control flat electrode. The characterization of the electrode surface using electrochemical impedance spectroscopy at low frequency showed a higher conductivity for the microstructured modified electrodes. Interestingly, the impedance lowered after 24 h when bacteria attached to the surface. Since *E. coli* does not produce current without a mediator, it is presumed that the mechanism follows a molecular diffusion of the mediator 2-HNQ into the biofilm, followed by a reduction of the mediator in the biofilm, and then a re-oxidation of the mediator. Images of scanning electron microscopy showed that cell accumulation occurred particularly at the base of the pillar microstructures. Confocal laser scanning microscopy images revealed that 41% of the cells in the electrode were alive, composing an early biofilm of 400 nm thickness.

The overarching conclusion from this study is that the increment in electrode's surface area via the use of short micropillar structures proportionally enhances the charge transfer to around three times higher than without any microstructure. Further, the combination of electrochemical tests and imaging shed light onto the electron transfer mechanisms occurring during early *E. coli* biofilm attachment on gold electrodes.

7.1.2 Early biofilm characterization on Ni electrodes

Finding that short microstructures over the electrode's surface enhanced the bacterial early biofilm electron transfer, motivated me to study the electrochemical response of another, cheaper metallic material (nickel) using the same bioelectrochemical setup. Further, the effect of external variables such as stirring, temperature, and electric

potential were also studied. Imaging via SEM was employed to further monitor the attachment of the bacterial cells onto the electrode. To the best of my knowledge, this is the first study of early bacterial biofilm using nickel electrodes.

The electrodes were fabricated with an ordered array pattern analog to that used previously on gold electrodes, in order to assess the effect of a different material under the same prior conditions. Using chronocoulometry methods, the microstructured nickel electrodes showed minimal enhancement on the charge transferred (3%) in comparison with the flat nickel electrodes, although the surface area was increased in 3 and 6% with the addition of the microstructures. To further investigate effects that might be missed by only focusing on charge transfer, the changes on the electrode's surface were also assessed using electrochemical impedance spectroscopy (EIS) at short intervals of time. The results showed a drop of the impedance during the first hours of the experimental runs, followed by a constant increase of the impedance with time. In terms of capacitance and resistance, the presence of microstructures enhanced the performance of the electrodes, with a higher increase in surface area resulting in a better relative performance. EIS was also used for the characterization of the extracellular electron transfer in biofilms under a range of electric potential, temperature and stirring. The EIS experimental data was fitted, while the charge transfer resistance and the capacitance were used for analysis. The results showed that non-stirring conditions favor reproducible results, rather than stirring conditions. Experiments at 37 °C displayed two EET features across the range of electric potentials, where one corresponds to 2-HNQ and the second may correspond to either an endogenous mediator from *E. coli* or otherwise to an unidentified polar compound. The nonideality of the capacitance showed no distinction with temperature.

From this study it was found that electrochemical impedance spectroscopy is a more responsive electrochemical method for early *E. coli* biofilm detection in nickel electrodes and it is therefore recommended over chronocoulometry. Again, the use of micropillar structures enhanced the performance of nickel electrodes under a bioelectrochemical system.

7.1.3 Topographic effect on electrodes

The effect of the topography was further assessed with the fabrication of nickel electrodes with a different ordered array pattern arrangement. The electrochemical results showed that the electron transfer and early biofilm growth depend on the electrode's topographic features.

A gap distance among microstructures of approximately 10 μm produced more current than 100 μm spaced one. Although, the surface area increases when using 10 μm rather than vs 100 μm gaps (91% vs 3%), the results did not correspond with the increase in current measured (38% vs. 15%). This implies that the surface available could be under-utilized during early bacterial biofilm detection therefore the use of shorter pillars may be more efficient. Thus, efforts should be put into optimizing gap distance rather than surface area. In addition, similar to previous study, the impedance characterization at short intervals showed a drop of the impedance during the first hours of the experimental runs, followed by a constant increase of the impedance with time. The electrodes performed similarly, being Ni10 more sensible. Additionally, amperometry revealed an increase in current with glucose concentration, while EIS analysis showed that capacitance increased when glucose concentration decreased. These results highlighted the sensibility of *E. coli* to the substrate concentrations in bioelectrochemical studies.

The main conclusion from this study is that the gap distance across topographical pattern arrangements on electrode surfaces has an important effect on the electrode performance during early biofilm attachment in the electrochemical setup. Additionally, the findings of this and the prior study using nickel electrodes aided into a better understanding of the electron transfer mechanisms taking place during early *E. coli* biofilm formation for this type of material.

7.2 Implications and recommendations for future work

7.2.1 Biofilms at the core of future biotechnological innovations

Biofilms study is an established research field that continues to grow as new technologies unveil new discoveries. Biofilms are found in every ecosystem on Earth^[1], and quoting HC Flemming: “we can’t escape from biofilms”. Hence, biofilms may hold an important key for novel discoveries, deserving more attention and studies. As previously defined, electroactive biofilms (EAB) correspond to the bacterial aggregation in a conductive electrode which hold the potential of power generation enough to self-sustain a wastewater treatment plant^[2]. However, the EAB technologies present a series of limitations that are currently holding back EAB breakthroughs.

EAB could be at the core of technological applications such as self-maintained or recovered electrical signals and potentially self-powered devices^[3]. The signals in EAB could measure the amount of current, electrical charge, cell voltage, power production or impedance. On the other hand, traditional electrochemical biosensing could enormously benefit from EAB attributes. Although electrochemical biosensing has a long record of inventions, the great majority of them are based on enzymatic sensors which present high selectivity, rapidity and specificity. However, enzymatic biosensors usually involve only one usage, together with high production costs which are not sustainable in practice for the long term. EAB have the potential to expand the biosensing field, but their performance not only depends on the microbial activity. EAB applications are limited by the biofilm or microbial community characteristics, as well as the electrode where the electron activities are measured. Therefore, more emphasis on the interface of the biofilm and electrode surface to understand their interaction is missing in the literature.

7.2.2 Early stage biofilms require more attention

Most EAB studies usually employ mature biofilms where the current production is achieved at a steady-state^[4]. It is a logical decision from the operational perspective of current harvesting when the BES is a microbial fuel cells (MFC) device. However, BES applications varies and other signals can be collected such as electrical charge, cell voltage, power production or impedance which may not require a mature biofilm. Also, the understanding of initial electron transfer mechanisms can derive on a better definition of requirement to fabricate high performance electrodes in BES applications.

The limitations on early biofilms present a lack of reproducibility and high variability. Future works on early stage biofilms must be designed with adequate biologically independent replications, so as to account for these limitations.

7.2.3 Overlooked use of EIS on BES

EAB applications are normally studied via direct measurement of current, electrical charge, voltage, and power production^[3]. However, there is a lot of potential on also measuring the impedance of the system. Impedance measures the effect of a perturbation on the electrode surface by using alternate current (AC) at different frequencies. Therefore, electrochemical impedance spectroscopy (EIS) is a powerful tool to monitor changes on an electrode surface through time, like during early biofilm formation. The experimental data obtained via EIS can be modelled to an equivalent electric circuit and parameters can be defined for further analysis. Such equivalent circuit idea was developed by the enthusiasm and interdisciplinary work of physicists and engineers^[5, 6]. However, impedance measurements are being neglected in the literature with only 135 citations until now compared to 4442 citations for the field of electrogenic bacteria, where the research is focused mostly on pure biological innovations. Therefore, there is a growing gap on interdisciplinary collaborations and device developments, which are not moving beyond the needs of biological advancements to consider relevant aspects derived from other disciplines, like impedance. This dissertation contributed to close both of these gaps by using EIS and involving an interdisciplinary approach for EAB studies. Future research efforts on EAB should continue to explore the advantages of impedance measurements.

7.2.4 Exploring more electrode's materials is encouraged

The electrode material is an important variable which has direct impact on the bioelectrochemical system performance. Attributes such as biocompatibility, low resistance, and high conductivity are basic requirements, yet the literature lacks specific material recommendations for high performance electrodes in bioelectrochemical systems. Gold is an excellent material by default and presents the advantage that many researchers have explored its applications in bioelectrochemical systems^[7-9]. In addition, experiments with gold electrodes present more literature resources to compare

the findings of EAB studies. Hence, future works on gold electrodes are recommended to continue to expand the fundamental research on early EAB formation. Particularly, the study of early biofilm formation at short intervals in gold electrodes was not addressed as part of this dissertation, making it an attractive avenue for future research. Industrial applications are encouraging the use of low-cost materials in electrodes. This dissertation explored two type of metallic materials: gold and nickel. Although both materials present metallic characteristics, they expressed a very different electrochemical output. Very little research was done on EAB using Ni before, and none on early biofilm stages. Therefore, this dissertation shed light on the behavior of early EAB interacting with Ni in bioelectrochemical systems, opening the field to more research questions to be addressed in the future. Nickel electrodes were previously shown to dissolve in the form of Ni^{2+} ions the medium^[10], while Ni^{2+} in low concentrations has been proven to promote bacterial growth^[11]. The work in this dissertation further suggests that Ni electrodes may present microlocalized effects on the extra micronutrients available for the biofilm, hence causing bacteria to grow more microlocally. Further experiments are needed to test this by measuring the Ni dissolution concentration/rate to assess if such conditions favor bacterial growth and actually enhance the electron transfer on the electrode. In addition, more research on other metallic materials on early EAB is needed.

7.2.5 Optimizing the microstructure size (bacterial ergonomics)

A main contribution of this dissertation is that small surface increases on electrodes, with the use of microstructures with approximately 10 μm distance in between them, enhance the extracellular electron transfer (EET) for early bacterial biofilms. The tempting idea of increasing the surface area when searching for an enhancement of the electron transfer, led me to define the criteria for surface increase as a controlled size approach. Here, the electrode design was focused on the bacteria cell size. This approach guided my research towards the use of short micropillars, which were able to promote an environment where the nutrients could diffuse and the shed forces from the media were reduced in order to facilitate bacterial cells attachment. Furthermore, the bacteria size-oriented design employed in this work prevented the use of porous materials since the apparent increase in surface area due to the pores would have been cancelled by the bacteria cell-size which is bigger than the average pore-size,

confounding the results. Future works should explore other different types of microstructures, while following the criteria adopted in this dissertation in order to optimize electrode design. Moreover, future research efforts would benefit from exploring the same topographical dispositions over the electrode's surface presented here, but using different types of bacteria other than *E. coli* as early biofilm forming organisms.

Smaller microstructures may produce a lower current in comparison with higher aspect ratio features. However, this dissertation showed that high aspect ratio microstructures underperform in terms on the theoretical capabilities they should offer. Therefore, future research questions should be formulated as either: *“Do we want an electrode with poor performance and high current?”*, or *“Do we want and electrode with high performance, which help us understand the electron transfer better and might lead to an important breakthrough?”* I strongly suggest that future research works aim for higher performance electrodes, since they might hold the key to understand the ways to better enhance the electron transfer mechanisms. In this regard, the bacteria size-oriented design is an attractive approach where the microstructure is optimized for the bacterial morphology, which increases the efficiency of the electrode. This scenario would also consequently lead to an increase of innovative applications as well. Based on the findings presented in this dissertation, I contend that the optimization of microstructures onto the electrode's surface is of paramount importance for future studies on early bacterial biofilms and their electron transfer mechanisms in bioelectrochemical systems.

7.3 Final remarks

Overall, this dissertation advances our understanding of the bacteria-surface interaction in early biofilm formation, for a weak electrogenic model organism, using microstructured array electrodes fabricated on Au and Ni. The findings in this dissertation should have implications for bacterial biofilm sensing developments, as well as for preventing microbially influenced corrosion in drinking water systems and biomedical devices. As the challenge of understanding complex biofilm systems remains, I expect that interdisciplinary approaches like the one presented in this

dissertation will continue to have an essential role in guiding our scientific hypotheses and interpretations.

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APPENDIX

Chapter 4: Supplementary Information

Figure S4.1: Schematic top view of the ordered structure array electrodes of (a) $8 \mu\text{m}$ and (b) $4 \mu\text{m}$ diameter pillars. (c) 3D schematic of the electrodes tilted view. Pillars are $1 \mu\text{m}$ high.

Figure S4.2: Schematic representation of the electrode fabrication process. Photolithography is used to create the pillar microstructures: 1. Deposition of gold thin film, 2. Coating with photoresist, 3. UV exposition through the specific glass photomask, 4. Development of the structure, 5. Final conductive thin film deposition.

Figure S4.3: CV curves response for ordered structure array gold electrodes of 8 μm pillar, 4 μm pillar, and flat electrode in MOPs media, *E. coli* bacteria, and HNQ. The electrode exposed area is 0.5 cm x 0.5 cm. The CV cycles are (a) before and (b) after performing 24h CA.

Figure S4.4: Charge-time response measured in logarithmic scale for the ordered structure array gold electrodes of 8 μm (red line) and 4 μm (blue line) pillars, with a potential step of 0.4 V versus a reference electrode (Ag/AgCl) during 24 h. The solution included MOPs buffer, *E. coli* bacteria $\text{OD}_{600} = 0.7$, and HNQ mediator. Electrode exposed area was 0.5 cm x 0.5 cm. Experimental controls included flat electrodes using media with (black line) and without (pink line) bacteria. Each line represents the average of four independent runs ($n = 4$) using new electrodes and bacteria preparation.

Figure S4.5: Equivalent electrical circuit used to fit impedance of an *E. coli* biofilm on top of the customized electrodes, together with physical interpretation of the circuit elements for a complementary understanding (*adapted from Babauta 2014*).

Figure S4.6: Resistance analysis of electrochemical impedance spectroscopy (EIS) qualitative equivalent circuit elements. Response measured for ordered structure array gold electrodes of 4 μm pillar, 8 μm pillar, and control experiment flat electrode in MOPs media, *E. coli* bacteria, and HNQ. The electrode exposed area was 0.5 cm x 0.5 cm. (a) Resistance (R2) increases after 24 h due to EET biofilm formation. (b) Pseudo-capacitance (Q2) decreases after 24 h due to EET biofilm formation.

Table S4.1: Fitting results of electrochemical impedance spectroscopy (EIS) measurements for different structures at the electrode's surface.

Electrode	Time	R1	Q2	a2	R2	Q3	a3	R3
	[h]	[ohm]	[F.s ^(a-1)]		[Ohm]	[F.s ^(a-1)]		[Ohm]
8 μ m pillars	0	54.5	3.91E-05	0.884	95,124.7	1.18E-04	0.618	1,185.20
	24	69.7	4.66E-05	0.866	59,870.5	6.63E-05	0.712	608.05
4 μ m pillars	0	63.1	3.50E-05	0.895	112,633.8	9.26E-05	0.624	826.04
	24	69.1	4.55E-05	0.853	64,616.2	5.68E-05	0.693	415.53
Flat	0	70.3	2.91E-05	0.856	144,304.0	7.10E-05	0.626	1,096.65
	24	76.1	3.89E-05	0.819	75,128.5	5.13E-05	0.691	614.30

Chapter 5: Supplementary Information

Figure S5.1: Schematic top view of the Nickel (Ni) ordered structure array electrodes of (a) 8 μm and (b) 4 μm diameter pillars. (c) 3D schematic of the electrodes tilted view. Pillars are 1 μm high.

Figure S5.2: Schematic representation of the electrode fabrication process. Photolithography is used to create the pillar microstructures. Step 1: Deposition of nickel thin film; Step 2: Coating with photoresist; Step 3: UV exposition through the specific glass photomask; Step 4: Development of the structure; Step 5: Final conductive thin film deposition.

Figure S5.3: (a) Current output over 24h on electrodes maintained at 0.4 V vs. Ag/AgCl at 23 °C under stirring conditions. The CA shows a higher average current output for micropillar Ni8 in comparison with micropillar Ni4 and flat. Abiotic control experiments show negligible current output. Slow capacitive discharge is observed in all electrodes, particularly on flat electrodes, in the first 2-4 h. (inset) Close-up of the current at 14-24 h. (b) Cumulative charge output over 24 h. All experiments were carried out at room temperature 23 °C under stirring conditions. The average of four independent biological replicates (n=4) is reported.

Figure S5.4: (a) Optical density measurement at 600nm wavelength at inoculation time vs 24 h after. The OD decrease which can be due to the nutrient exhaustion. (b) pH measured at the end of 24 h.

Figure S5.5: Cyclic Voltammetry curve for three types of electrodes Ni4, Ni8 and flat (a) CV at inoculation time ($t=0$) (b) CV after 24 h. After 24 h the curve shows two oxidation peaks corresponding to the metabolic activity of the bacteria.

Figure S5.6: FESEM images of the Ni micropillar array electrode. (a) Ni8: 8 μm diameter pillar: Top view, magnification 1200x, 5 KV, SEI (b) Ni4: 4 μm diameter pillar: Top view, magnification 1000x, 2KV, LEI.

Figure S5.7: R_{ct} comparison between stirring and non-stirring conditions at 23°C at 0h and 24 h. Data at all potential from -200 to 400mV in 50mV steps were binned. Results show that R_{ct} under stirring conditions was lower than under non-stirring conditions, due to change in biofilm assembly/structure.

Figure S5.8: R_{ct} from EIS at different potential and temperatures: (A) 23 °C; (B) 30 °C; (C) 37 °C under non-stirring conditions for Ni8. The EIS spectrum show two features at 0 and 350 mV, respectively. The earlier become more evident as the growth temperature **increases**. **Each measure is the average of at least two independent biological replicates (n=2).**

Table S5.1: Explanatory table of the experimental design for three type of electrodes (e.g. Ni8, Ni4, Flat)

Electrochemical Technique	Temperature	Stirring/ Non-Stirring	Time points	Potential (vs. Ag/AgCl)
CV	23 °C	Stirring	t=0, 24 h	0.4 V
CA	23 °C	Stirring	t=0-24 h	0.4 V
EIS	23 °C	Stirring	t= 0, 2, 4, 6, 21, 23 h	0.4 V
EIS	23 °C	Non-Stirring	t= 0, 8, 24 h	-0.2 to 0.4 V (50 mV steps)
EIS	30 °C	Non-Stirring	t= 0, 8, 24 h	-0.2 to 0.4 V (50 mV steps)
EIS	37 °C	Non-Stirring	t= 0, 8, 24 h	-0.2 to 0.4 V (50 mV steps)
EIS	23 °C	Stirring	t= 0, 8, 24 h	-0.2 to 0.4 V (50 mV steps)

Chapter 6: Supplementary Information

Ni10 and Ni100 Surface Area Calculation

Information known: Hexagonal distribution, pillars diameter is $10\ \mu\text{m}$ and distance from pillar to pillar is $10\ \mu\text{m}$ or $100\ \mu\text{m}$.

It's needed to determine a "unit" so to calculate how many units are there in the rest of the electrode.

A unit contents 4 pillars which are actually 6 half pillars and 1 full pillar.

For Ni10

Also, a unit would consist of a four-sided figure which sides are $40\ \mu\text{m}$ and 34.64 which correspond to the projected length of the cosine of the $20\ \mu\text{m}$ which is the diagonal distance from center to center ($5\ \mu\text{m}$ radius pillar A + $10\ \mu\text{m}$ distance in between + $5\ \mu\text{m}$ radius pillar B).

Each pillar increases the surface area by their sides walls which are calculated considering the diameter and height.

With all this consideration, the calculation area equation looks like:

$$\text{Total Area} = \text{Flat area} + \text{Pillar Area}$$

$$\text{Pillar area} = \text{number of pillars} * \text{surface area increased per pillar}$$

$$\text{surface area increased per pillar} = \text{diameter} * \text{height} * \pi$$

$$\text{number of pillars} = \frac{\text{surface area total}}{\text{one pillar by surface area}}$$

$$\text{one pillar by surface area} = 4 \text{ pillars in } 40\ \mu\text{m by } 34.64\ \mu\text{m}$$

$$34.64 = 2 * 20\ \mu\text{m} * \cos 30$$

For Ni100, the same logic is applied. There will be 4 pillars in a four-sided figure of $220\ \mu\text{m}$ by $190.53\ \mu\text{m}$

Figure S6.1: Schematic top view of the Nickel (Ni) ordered structure array electrodes of (a) Flat (b) Ni10. (c) Ni100. Pillars are 10 μm high.

Figure S6.2: Schematic of the electrode fabrication process. The mold is pressed against the polycarbonated film imprinting the pattern under 40bars, 160°C, for 300 s. After cooling down, the polycarbonate is ready for demolding. Following the Ni thin film deposition was done with an electron beam evaporator.

Figure S6.3: CV curves of the three electrode types: Flat, Ni10, and Ni100 with *E. coli* and 50 μM HNQ mediator at $t=0\text{h}$ and 24 h. After 24h, The early biofilm reduces the HNQ on the electrode. Planktonic cells do not participate into the HNQ reduction due to the distance to the electrode. Four replicates.

Figure S6.4: Impedance increase after 24h. The lower impedance was shown by the microstructured electrode Ni10. Average of four (4) replicates for each electrode type are shown. The experimental curves were measured at inoculation time ($t=0\text{h}$) and after 24h ($t=24\text{h}$).

Figure S6.5: Resistance increase on the electrode's surface after 24h. Higher resistance may indicate more electrons available that the electron processing capacity of the early biofilm, hence lower rate of electrons to charge the biofilm.

Figure S6.6: Measurements of OD600 in planktonic preserves approximately the same number of live cells which would indicate that the current effect is only from the biofilm attached on the electrode's surface.

Figure S6.7: Resistance change calculated at short intervals for high frequency correspond to the conductivity of the electrodes. Microstructured electrodes are slightly more conductive than flat electrodes therefore their resistance is lower.