Passive and active approaches to tackle antifouling

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2019

Gaw, S. L. (2019). Passive and active approaches to tackle antifouling. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/106677>

<https://doi.org/10.32657/10220/48934>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

האוניברסיטה העברית בירושלים
THE HEBREW UNIVERSITY OF JERUSALEM

**PASSIVE AND ACTIVE APPROACHES TO TACKLE
ANTIFOULING**

GAW SHENG LONG

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2018

PASSIVE AND ACTIVE APPROACHES TO TACKLE ANTIFOULING

GAW SHENG LONG

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2018

גישות פאסיביות ואקטיביות למניעת ביופאולינג

חיבור לקבלת תואר דוקטור לפילוסופיה

מאת

שאנג לונג גאו

הוגש לסנט האוניברסיטה העברית בירושלים

2018

Statement of Originality

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

03 August 2018

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Date

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Gaw Sheng Long

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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.

03 August 2018

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Date

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Professor Lee Pooi See

Authorship Attribution Statement

This thesis contains material from papers published in the following peer-reviewed journal(s) where I was the first and/or corresponding author.

Chapter 4 is published as Sheng Long Gaw, Sujoy Sarkar, Sivan Nir, Yafit Schnell, Daniel Mandler, Zhichuan J. Xu, Pooi See Lee, Meital Reches, ACS Applied Materials & Interfaces, 2017, 9 (31), 26503–26509.

The contributions of the co-authors are as follows:

- Prof Pooi See Lee, Prof Meital Reches and Associate Prof Zhichuan J. Xu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Prof Daniel Mandler.
- Dr Sujoy Sakar assisted in interpretation of the electrochemical data.
- Sivan Nir and Yafit Schnell provided guidance on the biological experiments.

Chapter 6 is published as Sheng Long Gaw, Gowripriya Sakala, Sivan Nir, Abhijit Saha, Zhichuan J. Xu, Pooi See Lee, Meital Reches, Rational design of amphiphilic peptides and its effect on antifouling performance, Biomacromolecules, 2018, 19 (9), 3620–3627.

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- I prepared the manuscript drafts.
- Gowripriya Sakala, Sivan Nir and Abhijit Saha provide guidance in the peptide synthesis and the biological experiments.

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A handwritten signature in blue ink, consisting of a large, stylized 'G' followed by 'S' and 'L'.

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Gaw Sheng Long

Abstract

Biofouling is the accumulation of unwanted organisms on a surface. Biomedical and marine industries are of are two main particular interest for this study. Biofouling in marine industry reduces the performance of the equipment, leading to an increase in operating cost and downtime. In the medical industry, it can lead to catastrophic effects on the patient and might lead to death in the event of bacterial infection. Till date, there is no absolute solution to this problem. Therefore, this study provides a valuable contribution to the community by providing three novel antifouling methods.

Biofouling is initiated with the formation of biofilms and other micro-organisms before large organisms started to adhere. Biofilms are usually formed with the colonization of bacteria. The current state of the art has provided solutions to either preventing (antifouling) or destroys (antimicrobial) micro-organism adhering on the surface. This thesis has also reviewed the current state of the art and categories the prior works to either active or passive approaches for the solution to prevent biofouling.

An active approach via electrochemical reaction for generating antifouling surfaces through the formation of a hydrogen gas bubble layer was achieved. It attained through the application of a low voltage square-waveform pulse to the conductive surface. This electrochemically generated gas bubble layer serves as a separation barrier between the surrounding and the target surface where the adhesion of bacteria can be deterred. It has proven to prevent 99.5% of *Escherichia coli* from adhering onto the surface.

In order to put this system into a practical application, the need to reduce the energy consumption is required. A multilayer polyvinyl butyral scaffold film on the stainless steel substrate has reduced the amount of energy required by 96x to a single pulse in 16 hours. This polyvinyl butyral film will reduce the *E.coli* binding strength on the surface. During the electrochemical reduction process, the *Escherichia coli* will be released from the surface. This method has been proven effective for accelerated bacteria study and the

polyvinyl butyral is stable in Lysogeny broth after prolonged cycling. This study also serves as a new viable antifouling solution which is applicable to all conductive substrate.

Due functionality of the system, the active approach via electrochemical reaction might not be suitable. For instance, this method does not cater to biomedical devices as it requires tissue growth around the implants to achieve stability and functionality. Passive approach such as coating is a feasible choice. One of the environmentally friendly highly sought after biocompatible antifouling materials is peptide. It is made up of amino acids and it can be easily synthesized by the combination of different amino acids in random or alternate arrangements to attain the desired functionality. This work has provided insights on the different antifouling properties of the peptides when manipulating the positions of the amino acids within the peptide chains. It offers an important milestone for future peptide antifouling research. The antifouling performance of the peptides chain was not solely dependent on the properties of each amino acids but the sequencing of the amino acids within the peptide chain.

תקציר

ביופאולינג הוא תהליך לא רצוי שבו מצטברים אורגניזמים על משטחים שונים. תעשיות ימיות ורפואיות סובלות מהתופעה. בתעשייה הימית, ביופאולינג פוגע בציד וגורם להשבתתו. בתעשייה הביורפואית, ביופאולינג יכול להוביל לזיהומים חידקים אשר עלולים להוביל למותו של המטופל. למרות ההשלכות ההרסניות של התהליך אין עד היום פתרון אבסולוטי לבעיה. מחקר זה התמקד במציאת פתרונות לבעיה ולכן יש לו השלכות רבות על הקהילה.

ביופאולינג מתחיל ביצירת ביופילם על ידי מיקרואורגניזמים ולאחר מכן התיישבות של אורגניזמים גדולים יותר. כיום הפתרונות מתמקדים במניעת ההצמדות (אנטיביופאולינג) או בהרג המיקרואורגניזמים (אנטימירוביאלי). תיזה זו מציעה פתרונות בתחום בשתי קטגוריות: פאסיבית ואקטיבית.

גישה אקטיבית אשר פותחה היא שיטה אלקטרוכימית ליצירת משטחים המונעים ביופאולינג באמצעות יצירת שכבת בועות מימן. השכבה נוצרת על ידי הפעלת מתח נמוך המופעל על משטח מוליך. שכבת הבועות שנוצרת אלקטרוכימית מייצרת שכבת הפרדה בין הסביבה והמשטח. השכבה מורידה את כמות חיידקי ה-*Escherichia coli* על המשטח ב-99.5%. בכדי להביא את המערכת הזו ליישומים שימושיים, היה צורך להוריד את צריכת האנרגיה של המערכת. לכן, פותחה שכבה של פולימר על פלדת אל-חלד אשר הורידה את צריכת האנרגיה פי 96. שכבה זו של הפולימר אף הורידה את הצמדות החיידקים למשטח.

לבסוף, נחקרה גם מערכת אנטיפאולינג פאסיבית. מערכת זו בנויה מפפטידים אשר עוברים הרכבה עצמית ליצירת ציפוי. בעבודה נבדקה השפעת חומצות האמינו השונות וסדר הופעתן ברצף הפפטידי. נראה כי לסדר יש תפקיד חשוב ביצירת ציפוי כנגד חיידקים.

Lay Summary

Biofouling is the accumulation of unwanted organisms on a surface. Biomedical and marine industries are of are two main particular interest for this study. Biofouling in marine industry reduces the performance of the equipment, leading to an increase in operating cost and downtime. In the medical industry, it can lead to catastrophic effects on the patient and might lead to death in the event of bacterial infection. Till date, there is no absolute solution to this problem. Therefore, this study provides a valuable contribution to the community by providing three novel antifouling methods.

Biofouling is initiated with the formation of biofilms and other micro-organisms before large organisms started to adhere. Biofilms are usually formed with the settlement of bacteria. Previous work has provided solutions to either preventing (antifouling) or destroys (antimicrobial) micro-organism adhering on the surface. This thesis has also reviewed also categories the prior works to either active or passive approaches for the solution to prevent biofouling.

An active approach is via applying voltage on surfaces to form of a gas bubble layer. It attained through periodically pulsing on conductive surface. This gas bubble layer serves as a separation barrier between the surrounding and the target surface where the adhesion of bacteria can be deterred. It has proven to prevent 99.5% of *Escherichia coli* from adhering onto the surface.

In order to put this system into a practical application, the need to reduce the energy consumption is required. A multilayer polyvinyl butyral scaffold film on the stainless steel substrate has reduced the amount of energy required by 96x to a single pulse in 16 hours. This polyvinyl butyral film will reduce the *Escherichia coli* binding strength on the surface. During bubbles generation process, the *Escherichia coli* will be removed from the surface. This method has been proven effective with accelerated bacteria study and the polyvinyl butyral is stable in LB medium after numerous cycling. This study also

serves as a new workable antifouling solution which is applicable to all conductive substrate.

Due functionality of the system, the active approach via electrochemical reaction might not be suitable. For instance, this method does not cater to biomedical devices as it requires tissue growth around the implants to achieve stability and functionality. Passive approach such as coating is a feasible choice. One of the environmentally friendly highly sought after biocompatible antifouling materials is peptide. It is made up of amino acids and it can be easily synthesized by the combination of different amino acids in random or alternate arrangements to attain the desired functionality. This work has provided insights on the different antifouling properties of the peptides when manipulating the positions of the amino acids within the peptide chains. It offers an important milestone for future peptide antifouling research. The antifouling performance of the peptides chain was not solely dependent on the properties of each amino acids but the sequencing of the amino acids within the peptide chain.

Acknowledgements

I would like to extend my sincerest gratitude and appreciation to the following persons, who have made invaluable contribution during these 4 years of PhD journey.

Firstly, I would like to sincerest gratitude to my supervisor Professor Lee Pooi See for giving me the valuably opportunity to be in the CREATE Program. She has also been giving valuable comments and her patience guidance throughout the PhD study.

Next, I would like to express my gratitude to my co-supervisors Associate Professor Xu Zhichuan Jason, Professor Meital Reches for the guidance, coaching and valuable inputs in the project discussion. I would like to thank Professor Daniel Mandler, all my fellow groupmates, Technical Staff in Nanyang Technological University and The Hebrew University of Jerusalem for their kind support throughout the research period.

Lastly, I would like to thank the Singapore National Research Foundation, Campus for Research Excellence And Technological Enterprise (CREATE) Program (NTU-HUJ) CREATE Program.

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Figure 4.9 Normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on control substrate (without electrochemical treatment) and sample substrate (with electrochemical treatment at -0.8V) immersed for 16 hours in *E. coli* inoculum [n=9; p=0.03].

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Figure A6.2 High-performance Liquid Chromatography [HPLC] chromatograms of DOPA – Phe(4-F) – Lys [B] peptide.

Figure A6.3 High-performance Liquid Chromatography [HPLC] chromatograms of Lys – DOPA – Phe(4-F) [C] peptide.

Figure A6.4 High-performance Liquid Chromatography [HPLC] chromatograms of Phe(4-F) – DOPA – Lys [D] peptide.

Figure A6.5 High-performance Liquid Chromatography [HPLC] chromatograms of Phe(4-F) – Lys – DOPA [E] peptide.

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Figure A6.11 ^1H NMR (DMSO- d_6 , 400 MHz, δppm) Phe(4-F) – Lys – DOPA [E].

Figure A6.12 ^1H NMR (DMSO- d_6 , 400 MHz, δppm) DOPA – Lys – Phe(4-F) [F].

Figure A6.13 ^{13}C NMR (DMSO- d_6 , 125 MHz, δppm) Lys – Phe(4-F) – DOPA [A].

Figure A6.14 ^{13}C NMR (DMSO- d_6 , 125 MHz, δppm) DOPA – Phe(4-F) – Lys [B].

Figure A6.15 ^{13}C NMR (DMSO- d_6 , 125 MHz, δppm) Lys – DOPA – Phe(4-F) [C].

Figure A6.16 ^{13}C NMR (DMSO- d_6 , 125 MHz, δppm) Phe(4-F) – DOPA – Lys [D].

Figure A6.17 ^{13}C NMR (DMSO- d_6 , 125 MHz, δppm) Phe(4-F) – Lys – DOPA [E].

Figure A6.18 ^{13}C NMR (DMSO- d_6 , 125 MHz, δppm) DOPA – Lys – Phe(4-F) [F].

Abbreviations

CFUs	Colony Forming Units
CV	Cyclic Voltammetry
DIPEA	N,N-Diisopropylethylamine
DLVO	Derjaguin-Landau-Verwey-Overbeek
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DOPA	3, 4-dihydroxy-L-phenylalanine
EPS	Exopolymeric Substances
FITC	Fluorescein-5-Isothiocyanate
Fmoc	Fluorenylmethyloxycarbonyl protecting group
HATU	Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
HPLC	High-Performance Liquid Chromatography
IMO	International Maritime Organization
LB	Lysogeny Broth
LCMS	Liquid Chromatography-Mass Spectrometry
LYS	Lysine
NMR	Nuclear Magnetic Resonance
OD	Optical Density
OEG	Oligo (ethylene glycol
PBS	Phosphate Buffer Solution
PCM	Plate Count Method
PDMS	Polydimethylsiloxane
PEG	Polyethylene Glycol
PHE(4F)	4-fluoro-phenylalanine
PVB	Polyvinyl Butyral
QCM	Quartz Crystal Microbalance
SAM	Self-Assembly Monolayer
SEM	Scanning Electron Microscopy
SFE	Surface Free Energy

SS	Stainless Steel
TBT	Tributyltin
UV-Vis	Ultraviolet-Visible
XPS	X-ray Photoelectron Spectroscopy

Chapter 1

Introduction

The main objective of this thesis is to establish novel methods to deter biofouling. Biofouling is the accumulation of the unwanted micro-organisms on the surface. Biomedical and marine industries are of our particular interest for this study. Biofouling in marine industry reduces the performance of the equipment, leading to an increase in operating cost and downtime. In the medical industry, it can lead to catastrophic effects on the patient and might lead to death in the event of bacterial infection. Till date, there is no absolute solution to this problem. Therefore there is a need to seek alternative methods to deter biofouling. In this chapter, the potential impact to the community and the rationale for the work is discussed. The overview and the hypothesis of the thesis are also summarized.

1.1 Hypothesis/Problem Statement

Biofouling is the accumulation of unwanted living organisms such as microorganisms, plants, algae on a wetted surface. [1] The process can be separated into 2 stages. The first stage is the micro-fouling stage where bacteria will adhere and biofilm formation will occur. The second stage is the macro-fouling where larger organisms such as barnacles, algae and plants will start to attach and colonies on the surface. Figure 1.1 is the illustration of the whole process of the development of biofouling.

Figure 1.1 Development of Marine Biofouling [2]

Marine equipment is one of the oldest modes of transportation across continents since prehistorical time. Till date, it is still a popular mode of transportation due to its low operating cost. However, there has been a serious problem in this industry termed biofouling where there is no absolute solution to solve it. There are currently more than 4000 marine fouling species reported worldwide. [1] Fouling will increase the surface roughness, hydrodynamic drag, fuel consumption and reduction in operating speed and maneuverability. [3] These have costs the US Navy approximately US\$1 billion per year. [4]

Biomedical implants are becoming a popular method of replacement and substitution of the dis-functional biological parts of the human body due to the improvements in the implant technologies and the high success rate. It has been forecasted that the total dental and prosthetic market to reach an estimated \$9.8 billion by 2022 with an annual growth

of 4.7% per year. [5] When these synthetic materials implants are introduced into the body, it will trigger foreign body response. This will lead to the formation of a conditioning layer which consists of lipids and glycoproteins onto the surface. This film is required for the biological interface between the body and the implants. However, this is also a suitable platform which promotes bacteria adhesion and colonies which lead to biofouling that limits its lifespan. Furthermore, the procedures for implantation are usually invasive which also increase the risk of device-related infections. In most biofouling device failure situations, the need for expensive invasive procedures for the removal and replacements of the device is required.

Although the exact mechanisms for biofouling have not been fully understood, physical and chemical surface properties are known to influence the type and extent of surface fouling. [6, 7] The change to the surface properties can be categorized by active and passive approach. Active approaches require external stimuli such as power, antibiotics or biocides to attain the performance, whereas passive approach does not require any external continuous input to attain the desired outcome.

One of the most effective active approaches to deter biofouling in the marine industries is the tributyltin (TBT) self-polishing copolymer paints. It has proven to be the most effective antifouling method.[1, 8] However it was banned worldwide in 2008 due to the leaching of TBT into the marine eco-habitat which causes a very damaging impact on the marine organisms. [9] Various studies have shown the effects of TBT on aquatic life. It has been shown that TBT will affect the endocrine glands of marine mammals and upset the hormone level in pituitary, gonad and thyroid glands. It has also reported that TBT will damage the reproductive and central nervous systems, bone structure, and the gastrointestinal tract of mammals. [10] Over the years, scientists have researched on passive approaches such as changing the surface topography, superhydrophobic coating or coating natural antifoulants to deter biofouling. [11-13] These methods are usually expensive and suffer from scale-up or sustainable issues.

There are a lot of alternatives that have been explored, however, none of the alternatives

is as effective as the TBT based paint. Currently, most the antifouling paints in the market consist of biocides. It contains polymer blended with biocide or metals. Biocide and metals will leach out over the time which has the same reaction mechanism as TBT self-polishing paint which was banned. The self-polishing paints have a controllable hydration and dissolution rate which can last up to 36 months. [14] It is widely used due to its better performance as compared to others. Recent studies have shown the accumulation of the biocides and metals accumulated in the marine life. This will be harmful to human as they are part of the food chain. [15] It is expected that legislation regulation will be soon imposed on the banned of metal biocides. It has been a stricter regulation on the biocide paints. Only approved biocides by the authority can be used as the coating. [16] There has been a raising awareness on the environmental impact of the biocides released into the marine eco-system. Researchers have diverted their attention on methods to prevent fouling without the use of toxic biocides and metal. Therefore, it is of a great interest to develop a new environmentally friendly method of preventing bacteria from adhering and colonizing on the surface.

In this dissertation, it is hypothesized that sustaining a continuous layer of air bubbles by electrochemical gas generation at the substrate/medium interface will lead to effective resistance to biofouling. This gas layer serves as an effective separation barrier between the substrate and the medium which can effectively prevent the anchoring of micro-organisms. This method is applicable to any conductive substrates and it requires a low power consumption. It also does not have negative impact on the environment.

This active approach requires continuous periodically pulsing of potential in order to maintain the continuous gas bubbles which act as the effective separation barrier. In order to make this method more feasible for application, the total cost required to maintain the system has to be kept as low. Therefore, there is a motivation to reduce the amount of energy required to maintain the system. It is hypothesized that a sub-micron multilayer scaffold has the entrapment capability of preventing the gas bubbles from detaching from the surface. This will reduce the frequency of potential application to maintain the separation barrier between the surface and the medium, and therefore deemed as an

energy saving approach.

In most situations in the biomedical industry, adhesion of the replacement devices needs to have strong adhesion with the existing biological parts for ideal functionality. Passive approach such as having a coating to alter the surface properties has been a practical method of preventing micro-organisms from adhering on the surface of the implant. Various methods such as hydrophilic low-fouling and non-fouling surface would have the same chemical and structural properties as the electrical neutrality and have the ability to form a hydrating layer through hydrogen bonds between the surface and the water molecules.[17] Various passive methods such as having a coating with hydrophobic, surfaces with biomolecular have also been explored. [18, 19]

One of the environmental biocompatible passive coatings that will be explored is peptides. It can be synthesized by easily combining different properties of individual amino acids to attain the desired functionality. Till date, several peptides have been designed specifically to deter biofouling. [20-23] The arrangements of the amino acids reported are usually in alternate or random arrangement. [23] Therefore, it is of great interest to determine the effects of positioning of the individual amino acids within the peptide chain to strategically manipulate and enhance its performance to deter biofouling. It is hypothesized that the position of the amino acids in the peptide affects the ability to deter biofouling.

1.2 Objectives and Scope

This thesis aims to explore novel environmental friendly methods to prevent fouling. In order to prevent micro-organisms from adhering to the surface, surface properties play an important role. There are several methods of changing the surface properties. We have identified two environmental friendly active and passive approaches to change the surface properties for our study. The active approach is done through electrochemistry. A layer of gas bubbles is being generated by pulsing potential periodically. The purpose is to maintain the solid-gas-liquid interface which will prevent the adhesion of micro-

organism. The energy consumption can be further reduced by having a sub-micron multilayer polymer scaffold which might have the entrapment capability of preventing the bubbles generated from detaching from the surface. The passive approach will be by coating a peptide which has the ability to deter fouling. The biofouling deterrence that is affected by the position of individual amino acids has been carefully studied. An ideal position of individual amino acids within a peptide chain is established.

1.3 Dissertation Overview

The thesis focuses on deriving novel environmentally friendly methods of preventing micro-organisms from attaching to the surface through altering the surface properties in active and passive approaches. The active approach is the electrochemical reduction reaction of water producing gas bubbles through potential pulsing that created a separation barrier between the surface and its surrounding. A sub-micron polymer scaffold has been established to achieve energy efficiency or energy savings by reducing the frequency of the application of potential pulses. The passive approach stems from a peptide-based coating which has the ability to deter biofouling. The arrangement of the individual amino acid within the peptide chain is established through careful analyses of its biofouling deterrence performance. The research overview is summarized as follows:

Chapter 1 provides the introduction, rationale for the research and outlines the goals and scope.

Chapter 2 reviews the literature on the different antifouling approaches and developments which supports the investigated scope of the thesis.

Chapter 3 discusses the rationale and the principles underlying the synthesis, characterization techniques used in this thesis.

Chapter 4 discusses the active approach through periodically applying short potential pulse on a conductive surface to generate gas. This chapter explains the mechanism of the

biofouling deterrence ability.

Chapter 5 elaborates the reduction of the energy consumption used by periodically applying short potential pulse to generate gas. This chapter elaborates the preparation of a sub-micron multilayer structure polymer film which is hypothesized to have gas bubbles entrapment capability that will reduce the frequency of application of potential pulses.

Chapter 6 presents the passive approach through having a new amphiphilic peptide coating. This chapter presents the detailed study on the biofouling deterrence effect when the position of individual amino acids changes within an amphiphilic peptide chain.

Chapter 7 summarizes the work and provides the future research direction based on the proposed mechanism presented in this thesis.

1.4 Findings and Outcomes/Originality

In this dissertation, active and passive approaches of changing the surface properties to effectively prevent biofouling are presented. The following are the summary of the scientific contribution of this thesis:

1. The active approach of using electrochemical reduction of water via application of potential to produce hydrogen gas bubbles on the surface. These gas bubbles will serve as a separation barrier to prevent the micro-organism from anchoring on to the surface. This method has also been proven to be effective.
2. In order to reduce the energy consumption of the electrochemical system, a sub-micron polymer scaffold has been coated on the conductive substrate. This sub-micron polymer has the ability to release the bacteria adhered on the surfaces during the gas formation reaction. This has greatly reduced the need for frequent pulse potential required to maintain the effectiveness of the system.

3. The passive approach uses an amphiphilic peptide to deter the micro-organism from adhering on the surface. The effect between the biofouling deterrence capability and the position of the amino acids has been carefully studied. We have judiciously tailored the peptide configurations to achieve the best antifouling performance by i) positioning the amino acid lysine adjacent to DOPA moiety in the linear peptide chain for better adhesion, ii) having linear fluorinated N-terminal to improve the packing density of the film by straightening the peptide chain, and iii) placing DOPA at the C-terminal.

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Chapter 2

Literature Review

Biofouling is the accumulation of unwanted organisms on surfaces. It is initiated with the formation of biofilms and other micro-organisms before large organisms started to adhere. Biofilms are usually formed with the colonization of bacteria. In this chapter, the bacteria adhesion theory is explained followed by the elaboration of the current state of the art on the solutions to prevent biofouling. There are two major ways of preventing biofouling, namely, antifouling or antimicrobial. Antifouling is preventing micro-organisms from adhering on the substrate. It can further sub-categorized into prevention of adhesion or the release of the adhered micro-organisms. Antimicrobial is the disruption or inhibition of the growth of bacteria. The last section of this chapter will be discussing the motivation of the thesis after reviewing all the reported works.

2.1 Bacteria adhesion theory

The main component that forms the biofilms are the bacteria which are in micron size. This is similar to the range of the colloidal particles. Scientists have correlated the adhesion of bacteria on the surface to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory which is first discovered to explain the interaction between a colloidal particle with a surface. [1] The interaction is the summation of the van der Waals and Coulomb interaction between the particles and the surface.[2] Van der Waals attraction force becomes dominant around the vicinity of the surface which would lead to irreversible adhesion whereas this force will be weakened as the distance increases further away from the surface. In contrast, coulombic interaction becomes the dominating force.

When the system consists of charged particles, counter ions opposite the surface will be attracted towards the vicinity of the surface forming a double layer. Both bacteria and natural surface are usually negatively charged in aqueous medium. [3] This will incur the formation of a repulsive electrostatic energy due to the overlapping of the electrical double layers. [1, 3-6] This energy is greatly dependent on the ionic strength of the medium. Bacteria cannot overcome the energy barrier when the ionic strength is low due to the shielding of the surface charges by the ions in the double layers that lead to the increase the repulsive energy [Figure 2.1]. [1, 7, 8]

Figure 2.1 Schematic drawing of interaction energy between a bacteria and surface when varying the medium ionic strength. [9]

There are mainly 2 steps to the cell adhesion. Bacteria will come into a position near the surface by motility or Brownian motion and adhere on the surface reversibly (Figure 2.2). Next, the bacteria will use its pili and flagella or produce exopolymeric substances (EPS) which will form a bridging between the surface and the bacteria. The pili and flagella are able to overcome the energy barrier due to its small radii. When the energy barrier is too high, this will increase the difficulties of the anchoring of the pili and flagella on the surface which will prevent the bacteria from adhering on the surface.

Figure 2.2 Schematics drawing of the two-step bacterial adhesion process. [9]

The actual bacteria adhesion is a very complicated process where it often deviates from the proposed mechanisms. This is due to the various different organic and inorganic substances in the environments forming the conditioning films before the adhesion of the bacteria. [4, 10] The actual physicochemical properties of the initial bare substrates are different from the substrates with conditioning films. [11] Therefore it is necessary to consider all aspects before conducting further studies and indeed the surface properties of the bare substrate is the most crucial factor.

2.2 State of the art of solutions to biofouling

Biofouling is the accumulation of unwanted species on the surface substrate which is initiated by the formation of biofilms. [12] Over the years, there has been extensive study on this issue however there are still no absolute solutions to tackle this problem. Solutions to prevent biofouling can be categorized into two categories. The first category

is called antifouling. Antifouling is preventing the adhesion of micro-organisms from adhering on the substrate. It can be further sub-categorized into prevention of adhesion or the release of the adhered micro-organisms. The second category is termed antimicrobial. It is to destroy or inhibit the growth of bacteria. Figure 2.3 illustrates the breakdown and some of the examples of the possible solutions to prevent biofouling.

Figure 2.3 Schematic illustrations of the solutions to prevent biofouling.

2.2.1 Antifouling

Biofouling is an accumulation of organism from a series of process discussed in chapter 1.1. Antifouling is described as the ability to prevent the growth of organisms on a surface. As biofouling is a series of process, and there are various methods that can be employed in deterring it at different stages of the process. In this thesis, approaches taken to achieve antifouling are classified into two sections. The first section will give details on the prevention of adhesion of organism while the second section will explain the

release of adhered organism.

2.2.1.1 Prevention of adhesion of organisms

The best solution to prevent biofouling is to prevent micro-organism from adhering on the surface and start to proliferate. Over the years, there are many research works focusing on the prevention of anchoring of micro-organism. This will prevent the formation of biofilms which cease the initial stage of biofouling. There are two main approaches where the solutions can be divided into passive or active. The passive approach changes the surfaces properties of the substrate, physical micro-engineered topography or through the application of synthetic coating.

In nature, marine mammals such as sharks, whales and mollusk shells living in the aquatic environments do not suffer from biofouling issues on their skin surface due to their special micro-topography. [13-18] While micro-topography has the ability to prevent the micro-organisms from attaching onto the surface, this mechanism is still not clearly understood. Various studies have attributed the prevention of micro-organism to various mechanisms such as the change in wettability and influence of surface near-fluid dynamics, and most explanations attribute the antifouling performance to the adhesion strength. [19-22] Different organisms have different shapes and sizes. Micro-organisms have size range in $\sim 1\mu\text{m}$, diatoms ranges in size between $3\text{-}15\mu\text{m}$ while macro-organism such as barnacles larvae and tubeworms ranges between $120\text{-}500\mu\text{m}$. There is a common explanation which correlates the adhesion site and the adhesion strength. Scardino et al have reported the reduction in adhesion strength was due to the decrease in the number of attachment sites on the micro-engineered topography as compared to a smooth surface (Figure 2.4). [21]

Figure 2.4 Schematic illustration of theoretical attachment points by (a) diatoms on smooth surface (multiple attachment points), (b) *F. carpentariae* on 2 mm ripples (multiple attachment points), (c) *N. jeffreyi* settling on 2 mm ripples (3 attachment points), (d) *Amphora* sp. settling on 4 mm ripples (2 attachment points). [21]

Over the years, researchers have tried to mimic the surface of those marine organisms. Anthony et al have reported the inhibition of settlement of algae. It is inspired by the Sharklet AFTM which is a bio-mimic inspired surface for antifouling. Various different surface topographies have been fabricated using Polydimethylsiloxane (PDMS). [15] Figure 2.5 illustrates the different engineered topographic. All the spacing between the features are kept at $2\mu\text{m}$ apart and some of the engineering sizes are kept at $2\mu\text{m}$ as it has been previously suggested that feature size of $5\mu\text{m}$ can deter the spores of the algae from settling. Sharklet AFTM surface lateral resolution shows the largest reduction in spore settlement. This has concluded that both topographic surface lateral resolution and topographic surface design are important factors for antifouling topography design.

Figure 2.5 SEM images of engineered topographies on a PDMS surface. (a) 2 μm ribs of lengths 4, 8, 12, and 16 μm combined to create the Sharklet AFTM. (b) 10 μm equilateral triangles combined with 2 μm diameter circular pillars. (c) Hexagonally packed 2 μm diameter circular pillars. (d) 2 μm wide ridges separated by 2 μm wide channels. [15]

The next passive approach is the application of a synthetic coating. Self-Assembly Monolayer (SAM) polyethylene glycol (PEG) or (oligo (ethylene glycol) (OEG) coating have been reported to have a significant reduction in the non-specific absorption of protein. [23-25] It is suggested that the PEG-based coating provides the following three characteristics : (i) hydrophilic repeating unit, (ii) well solvated and form hydrogen bond with water and (iii) an oligomer that has conformational freedom in water. Other alternatives such as zwitterionic coating, amphiphilic polymer coating, peptide and peptoid based coating have also been reported. [26-31] As most of the synthetic coating does not adhere well in aquatic environment, therefore, there is a need to improve this adhesion capability so as to increase in the reliability and lifespan.

Some of the organisms such as mussels in the aquatic environment have robust attachment capability which can attach and within harsh condition. Extensive research on the mechanism found that the strong adhesion properties are due to the mussel adhesive

proteins that contain an unusual amino acid, 3, 4-dihydroxy-L-phenylalanine (DOPA). [32-34] One of the environmentally friendly coatings will be the peptide-based coating. It can be easily synthesized based on the ease of combining different properties of individual amino acids to attain their desired outcome. [29, 35, 36] Therefore there is an increased interest in using a combination of peptide and DOPA to prevent biofouling. [35, 37]

The next approach of preventing adhesion of organisms will be via active approach where external stimuli are required in order to achieve the desired outcome. One of the most effective and well reported methods will be by releasing biocides into the environment which will prevent the micro-organism from adhesion on attaching in the surface. [12, 38, 39] Releasing of biocides has caused adverse effects to the marine aquatic environments due to its active ingredients which will accumulate in other organisms and pass on to human in the food chain. [40-42] International Maritime Organization (IMO) had banned the use Tributyltin (TBT) which is reported to be the most effective biocides till date, this is due to the adverse environmental impact other organisms in the aquatic habitat. [40, 41] There has been increasing awareness in releasing active metallic nanoparticles into the environment as it might also pose a same threat as the TBT. [43, 44] Other environmental friendly alternatives include alternating the pH, surface charge and using electric field to prevent the adhesion or attachment of organism on the surface. [45-48]

2.2.1.2 Release adhered organism (Foul Release)

Foul release is the second sub-category for antifouling. There is a minimum adhesion strength required for the organism before it will proliferate and have irreversible binding on surfaces. Therefore the objective of the foul release coating is to weaken or reduce the adhesion points between the surface and the organism so as to prevent irreversible adhesion. These organisms will easily detach off due to the stress caused by its weight or the hydrodynamic flow caused by the ship's navigation (Figure 2.6). [49, 50]

Figure 2.6 Schematic drawing of the mechanism of foul release coating. [50]

Passive approaches of changing the surface properties can be conducted through physical and chemical methods. Physical method of changing the surface properties can be achieved by decreasing the modulus of the surface. Hydrogels and xerogel have proven to be an effective foul release coating. This is due to the low modulus of the substrate which prevents strong anchoring of organisms (Figure 2.7). [51-53] Other materials such as polydimethylsiloxane (PDMS) have also been extensively researched due to low surface energy and low modulus properties. [53-56]

Figure 2.7 Illustration of the growing process of a barnacle. (a) Normal Surface (b) Hydrogel [51]

One of the drawbacks of the low modulus materials used is the poor durability and easily damaged. It is also prone to brown slimes dominated by diatoms as they attach more strongly to hydrophobic surfaces. [53, 57] One of the conditions for foul release technology will be the need of fast-moving (> 15 knots) vessels so as to create enough hydrodynamic force to release the organism adheres on the surface. Therefore it is less suitable for vessels that spend long periods in port or those that cruise at lower speeds. [53]

This problem is addressed by modifying the surface chemically. The main objective is to change or reduce the surface energy. Polymer brushes, fluorinated polymers, amphiphilic polymers have been reported to be effective release of biofouling. [58-61] Combination of both physical and chemical methods are reported to have a synergistic effect. [61] Amphiphilic polymers consist of both hydrophilic and hydrophobic properties. The hydrophilic properties show a good deterrence of preventing attachment of proteins and bacteria whereas the hydrophobic properties possess good foul release properties. [62, 63]

The release of adhered organism could also be done through active approach where the use of enzyme or self-polishing coating is employed. During this process, the adhered organism will be released from the surface. The enzymatic coatings usually consist of catalytically active proteins and are present in nature. The enzyme will either degrade the fouling organism or the bio-adhesive or produce other biocidal compounds (Figure 2.8). [64, 65] The degradation of adhesive enzyme coating is the most favorable approaches where it uses proteases and glycosylases to break down the bio-adhesive based on protein and other polysaccharide components. [65]

Figure 2.8 The proposed mechanism of enzymatic antifouling [64]

Self-polishing polymer coatings are usually acrylic or methacrylic copolymers which are easily hydrolyzed in seawater. [66] Organisms attached on the surface of the coatings will be removed together with the binder or matrix during the hydrolysis of the side groups of copolymer chains (Figure 2.9). Seawater penetrates through the coating and dissolves the soluble pigment. The copolymer will prevent further penetration as it is hydrophobic. Seawater will then fill the pores created by the soluble pigment particles. The methacrylic ester group which is not stable in water will hydrolysis and erode off by the seawater. [50, 67]

Figure 2.9 Schematic drawing of typical self-polishing copolymer coatings. [50]

2.2.2 Antimicrobial

Antimicrobial is termed as to destroy or inhibit the growth of microorganisms. It can be executed through active chemical compounds or via physically. Antimicrobial is a very crucial property, especially in the medical industries. Preventing the bacterial colonization will limit the spread of infections. This chapter is also sub-categorized into two categories (passive/active approach) which are similar to the current strategy of antimicrobial.

The passive approach is performed by changing the surface properties chemically via coating. This approach is similar to the contact killing coating strategy. [68, 69] A wide range of antimicrobial materials has been employed to various surfaces. Antimicrobial materials such as polyamines, chitosan, enzymes and peptides have been reported to be effective. [70-75] Positively charged peptides have also been reported to have antimicrobial effect. [76]

The active approach is achieved through the release of the antibiotics or biocides. [68] The slow release of antibiotics and biocides often incorporate together with the self-polishing copolymer or soluble polymer matrix which will release once the polymer is hydrolyzed. [50, 68, 69] This method is considered effective, however, it has detrimental environmental impact due to the non-target biocides/antibiotics.

2.3 PhD Studies in Contest with Literature

In this thesis, the main focus is to devise new environmental friendly antifouling/antimicrobial solutions. As both antifouling and antimicrobial current approaches can be separated into active and passive approaches, therefore it is necessary to look into the respective solutions.

When a super-hydrophobic coated surface is immersed in the water, a film of air (air plastron) will be formed. This creates a solid-gas-liquid interface between the surface and

the water which serves as a barrier to prevent adhesion of organisms. However, this barrier will diminish with increasing immersion time. This is due to the displacement of gas into the medium or displacement by the organisms. [32, 77] Various works have reported the use of alternating potential to alter the surface charges or surface vicinity charges. [48, 78] Electrical potential can also be used to generate gas such as hydrogen evolution reaction in energy related studies. Therefore, combining the use of electrochemical reaction to produce the air plastron and to create the solid-gas-liquid interface can be an opportunity for realizing a new active approach for antifouling.

Furthermore, in order to make this system more sustainable and lower energy consumption, an alternative or improvement to this system is needed. Due to the complex mechanism of bubbles detachment and displacement from the surface, a multilayer scaffold which serves as the barrier to prevent the detachment of the bubbles from the surface might aid in the reduction of the potential required to maintain the gas plastron.

Some applications such as medical implants can only cater to passive approach such as coating. Amphiphilic polymer has shown good antifouling properties due to its dual hydrophilic and hydrophobic properties. The hydrophilic properties show a good deterrence of preventing attachment of proteins and bacteria whereas the hydrophobic properties possess good foul release properties. One of the drawbacks is the toxicity and biocompatibility of the polymer. Peptide is a class of materials which is non-toxic and biocompatible. Peptides can be easily designed with the combination of different properties of individual amino acids to attain the desired functionality. In most of the peptide research, the arrangements of the amino acids reported are usually in alternate or random arrangements. [79] Therefore, it is of great interest to determine the effects of positions of the individual amino acids within the amphiphilic peptide chain to strategically manipulate and enhance the antifouling performances.

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Chapter 3

Experimental Methodology

Biofouling is a series of processes which started with the formation of biofilms. Bacteria colonization causes the formation of the biofilm. In this thesis, the antifouling performance test conducted will be via the incubation of the substrates in bacteria inoculum and uses the plate count method for the quantitative analysis. Several microscopy techniques are employed to determine the antifouling mechanism. Peptide syntheses are elaborated and the characterization techniques are also reviewed. The details explanation for the principles of individual techniques are discussed in this chapter.

3.1 Bacteria quantification

3.1.1 Optical density (OD) Measurement

Bacteria composed of a variety of organic compounds such as DNA, proteins, lipids and carbohydrates. When suspended in a medium such as Lysogeny broth (LB), it will interact with light similar to the Beer-Lambert law. Beer-Lambert law is the correlation between the attenuation of light to the properties of the material through which the light is travelling. A spectrophotometer has a light source which generates a specific wavelength. The light path passes through the cuvette where it is absorbed by the specimen in the cuvette and read by a detector. [1].

Absorbance A_λ at wavelength λ is defined as:

$$A_\lambda = \log[I_0(\lambda) / I(\lambda)] \quad [\text{Equation 3.1}]$$

And is equal according to the Lambert-Beer law to:

$$A_\lambda = \epsilon(\lambda) c d \quad [\text{Equation 3.2}]$$

Where $\epsilon(\lambda)$ is the extinction coefficient at wavelength λ for the particular molecule, c is the concentration and d the path length of the cuvette.

In this thesis, this technique is used to measure the concentration of the bacteria in the medium at the start of the experiment. A spectrometer is used to measure the OD at a wavelength of 600nm of the bacteria suspension of the starter and dilute it to reach achieve an OD of 0.3 so as to attain a concentration of 5×10^7 cells/mL. [2] This is to ensure uniform concentration of the number of cells introduced in order to test the antifouling properties in each experiment.

3.1.2 Plate Count Method (PCM)

Plate count method is a quantitative method for counting the number of viable microorganisms of interest. It is by diluting the original sample in a serial dilution configuration. 10% of the original sample will be diluted with 90% of the Phosphate Buffer Solution (PBS). Figure 3.1 shows the schematic drawing of the dilution method. The sequence continues on until the desired dilution ratio. The aliquots of the different dilutions are then plated onto individual agar plate. The agar plates are optimally incubated. The number of colony forming units (CFUs) are assumed to be separated as a single viable microbial cell. The number of colonies is counted and combined to attain the original number of microorganism in the sample. [3]

In this thesis, this method has been used to quantify the number of viable bacteria adhere to different samples. The results obtained are then normalized with respect to the control for easy comparison.

Figure 3.1 Schematic Drawing of Plate Count Serial Dilution.

3.1.3 Crystal Violet Staining Microscopy

Crystal Violet is a kind of dye which stains the cell wall when applied. The color contrast between the stained cell walls and the surrounding can be easily differentiated. This method is ideal for understanding the distribution of micro-organism on the surface of interest under an optical microscope.

In this thesis, crystal violet staining microscopy is used to understand the fouling mechanism and to reaffirm the analysis from the results obtained from the plate count method.

3.1.4 Live Dead Kit Staining Florescent Microscopy

Live Dead Kit staining uses a cell-permeable green fluorescent Cyto-dye (Ex. max.: 488 nm; Em. max.: 518 nm) to have the live cells stained while it uses propidium iodide (Ex. max.: 488 nm; Em. max.: 615 nm) to stain the dead cells. The stained live and dead cells can be visualized by fluorescence microscopy using a band-pass filter which detects Fluorescein-5-Isothiocyanate (FITC) and rhodamine. The live cells will only be stained by the Cyto-dye which will appear in fluorescing green. On the other hand, the dead cell will be stained with propidium iodide and the cyto-dye. This will results in red/yellow under fluorescence. This method is used to differentiate the viability of different cells. [4, 5]

In this thesis, live dead kit staining microscopy is used to understand the fouling mechanism and reconfirm the analysis from the results obtained from the plate count method.

3.2 Morphology Characterization

3.2.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a technique which uses a high-energy beam of electrons to scan and interact with the sample to obtain secondary electrons, backscattered electrons and characteristic X-rays. Electrons are produced at the top of the column and accelerated down through a combination of lenses and apertures to produce a focused beam of electrons which interact with the surface of the sample (Figure 3.2).

Figure 3.2 Schematic drawing of the electron path in SEM

Depending on the depth of penetration of the electron beam based on the different accelerating voltage and the density of the sample, various signals can be obtained (Figure 3.3). The secondary electrons signals are then collected to form the images.

Figure 3.3 Schematic drawing of the electron beam interaction.

In this thesis, due to the sub-micron structure of the multilayer polymer scaffold formed on the stainless steel substrate, SEM is used to characterize the morphology, stability of the polymer film and to study the mechanism of the antifouling function of the scaffold.

3.3 Electrical Characterization

3.3.1 Cyclic Voltammetry

Cyclic Voltammetry is a technique which is used to investigate the reduction and the oxidation process of the molecular species. The setup component consists of a potentiostat, working electrode, counter electrode and reference electrode. All the three electrodes are submerged in the electrolyte. The reference electrode (known electrochemical potential) is used to measure the potential at the working electrode. The counter electrode is used to complete the circuit. In this thesis, this technique is used to measure the reduction/oxidation potential of water to form the hydrogen/oxygen gas bubbles.

3.4 Peptide Fabrication

3.4.1 Solid Phase Synthesis

Peptide synthesis is a repeated amidation reaction between an amino group of one amino acid and the carboxylic group of a second amino acid. This is called the peptide bond. It was first demonstrated by Fischer. [6] Bruce Merrifield then reported on the solid phase peptide synthesis in 1963 which have drastically changed the process of peptide synthesis. [7] The synthesis process was simplified and less tedious. This development also led to the development of automated peptide synthesis.

Peptide synthesis involves numerous repeated steps coupling desired number of amino acids. Solid phase peptide synthesis uses an excessive amount of reagents at high concentration which drive coupling reactions. The excess reagents and side products are then separated from the growing and insoluble peptide through filtration and washings. The whole process can be conducted in one vessel. Figure 3.4 is the schematic drawing of the principle of the synthesis. [8] Amino resin is used as the support for the synthesis where the N terminal protected C-terminal amino-acid residue is first anchored. Before the additional of subsequence amino acids, the N terminal protected group has to be removed. Amino Acids are loaded in a linear fashion from the C-terminus to the N-terminus. After the desired coupling is completed, the terminal protected group is removed before the final process. Cleavage which is the final process of the synthesis is the process whereby the peptide is released from the resin support. Tert-Butyl methyl ether is then added to the supernatant to precipitate out the peptide. The precipitate is then centrifuged down and re-dissolves acetonitrile and water and lyophilized to obtain the raw peptide. In chapter 6 of the thesis, solid phase peptide synthesis is used to synthesize all the proposed designed peptide.

Figure 3.4 Schematic drawing of the principle of solid phase peptide synthesis. [8]

3.5 Peptide Quantification

3.5.1 High-Performance Liquid Chromatography (HPLC)

HPLC is an analytical chemistry technique used to quantify, identify and separate individual component in a mixture solvent. It uses high pressured pumps to passes pressurized liquid solvent containing the mixture through a column filled with solid adsorbent material. Due to the interaction between each component and the adsorbent

material, it will cause the differences in the elution timing when it flows out of the column. This allows the separation of the components. In chapter 6, HPLC is employed to determine the purity of the synthesized peptide.

3.5.2 Liquid Chromatography-Mass Spectrometry (LCMS)

LC-MS is an analytical chemistry technique which is a combination of liquid chromatography and a mass spectrometry (MS). This technique is useful as it has the ability to analyse individual component in a mixture. As mentioned in the previous section, liquid chromatography separates mixtures with multiple components whereas mass spectrometry provides the structural identity of the individual components with higher molecular specificity and detection sensitivity. Mass spectrometry converts the sample into gas phase ions by electron ionization. [9] This molecular ion undergoes fragmentation where individual primary product ion is derived from the molecular ion. The ions are separated in the mass spectrometer according to their mass-to-charge ratio and are detected in proportion to their abundance where a mass spectrum of the molecule is thus produced. The peak which appears the highest value m/z gives the molecular mass of the compound. In chapter 6, LCMS is employed to determine the mass of the synthesized peptide.

3.5.3 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) is a chemical analytical technique to determine the content, purity and its molecular structure. The principle is based on the spins of atomic nuclei. It measures the magnetic effect caused by the spin of protons and neutrons. Both these nucleons have intrinsic angular momenta or spins and hence act as an elementary magnet. The results are reported in hyperfine structure of spectral lines. When the nucleus with a certain magnetic moment is placed in the magnetic field, due to space quantization, for each allowed direction there will be a slightly different energy level. In chapter 6, NMR is used to determine the content and overall structure of the synthesized peptides.

3.6 Peptide Coating Characterization

3.6.1 Quartz Crystal Microbalance

Quartz crystal microbalance (QCM) is a high-resolution mass-sensing technique. It is based on the piezoelectric effect. The piezoelectric effect was first discovered by Jacques and Pierre Curie in 1880 and QCM was then established by Günter Sauerbrey in 1959. [10, 11] He has demonstrated the direct change in the quartz oscillation frequency with respect to the change in surface mass. [11] This is a real-time measuring technique which allows the close monitoring of the changes in mass in a precise manner.

Two electrodes are sandwiched on both sides of a piezoelectric crystal. An alternating voltage is applied to the electrodes which will oscillate the piezoelectric crystal at its resonance frequency. This resonance frequency depends on the total oscillating mass of the sensor and sensor surface adhering layers. It will decrease when a thin film attaches to the piezoelectric crystal.

The mass of rigid films can be calculated by using the following equation:

$$\Delta m = - (C \cdot \Delta f) / n \quad [\text{Equation 3.3}]$$

In chapter 6 of the thesis, QCM is employed to determine the binding of the peptide on the titanium coated substrate. The change in frequency leads to a conclusion that the peptides are attached to the titanium surface once it is introduced. Pure methanol is introduced to stimulate the removal of excess peptide which does not bind on the surface. The comparison between the original and end frequency will allow the confirmation of the peptide coating.

3.6.2 X-ray Photoelectron Spectroscopy

X-ray Photoelectron Spectroscopy (XPS) is a surface characterization technique which is used to analyze the chemical element and the chemical state present on the surface of the specimen. It can detect various elements except for hydrogen and helium due to its low photoelectron cross sections.

The basic principle of XPS is a photoelectric effect where the photons from the X-rays source interact with the electrons in atoms of the specimens. Due to the differences in the binding energy between different elements and different chemical states, the energy required to break the electrons away from the atoms differs. Electrons ejected out from the atoms will be collected by the detector where the kinetic energy of these electrons are measured and processed (Figure 3.5). The basic equation is as follows:

$$E_b = h\nu - E_k \quad [\text{Equation 3.4}]$$

E_b is the electron binding energy, E_k is the electron kinetic energy measured by the instruments and $h\nu$ is the photons energy from the X-ray source.

Figure 3.5 Schematic drawing of the interaction of photon ($h\nu$) with an atomic orbital electron. [12]

The data collected is processed to produce a spectrum of photoelectron intensity as a

function of binding energy. Each binding energy peak position corresponds to an element. The spectrum requires further peak fitting via software such as CasaXPS before it can be analyzed.

In chapter 6 of this thesis, XPS analysis is used to determine the binding of the peptide onto the Titanium coated surface. Peptides are coated on the surface via self-assembly to form the coating, therefore, amount present will be very little. Due to the high sensitivity of this technique, by analyzing both F and N, we can confirm the presence of peptides on the substrate. This is due to both N and F are present within the molecular chain of the synthesized peptide. Furthermore, as the anchoring sites between the peptide and the substrate are through the DOPA (catechol binding), the chemical states of the Ti and O will also be affected when peptides are anchored on it. By analyzing these 4 elements, it can confirm the presence of peptide on the Titanium coated surface and its binding site. This will enable the confirmation of the different molecular chain arrangements for the various coated surfaces with the different synthesized peptide.

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Chapter 4

Active antifouling approach via electrochemical reaction to sustain a solid-gas-liquid interface

Biofouling, the adsorption of organisms to surface, is a major problem today in many areas, especially in the marine industry. It is first initiated with the formation of biofilms via colonization of bacteria followed by the adhesion of macro-organism. Macrofouling leads to heavy load accumulating on marine vessels and causes larger fuel consumption. The process of removal usually requires huge effort and long downtime of the equipment. In this chapter, an effective electrochemical approach for generating antifouling surfaces will be discussed. The formation of a hydrogen gas bubble layer was achieved through the application of a low voltage square-waveform pulse to the conductive surface. This electrochemically generated gas bubble layer serves as a separation barrier between the surrounding and the target surface where the adhesion of bacteria can be deterred. This study serves as a basis for self-sustainable antifouling techniques such as incorporating it with photocatalytic and photoelectrochemical reactions.

*This section published substantially as Sheng Long Gaw, Sujoy Sarkar, Sivan Nir, Yafit Schnell, Daniel Mandler, Zhichuan J. Xu, Pooi See Lee, Meital Reches, ACS Applied Materials & Interfaces, 2017, 9 (31), 26503–26509.

4.1 Introduction

Biofouling in marine industries is usually initiated by colonization of bacteria leading to biofilms formation followed by the attachment of macro-organisms. [1] It often leads to the reduction in system performance. For instance, it will lead to the increase of surface roughness of the marine vessels hull, resulting in higher frictional drag and fuel consumption. One possible solution is the incorporation or release of antimicrobial compound from the surface. However, most of the biocides are environmentally toxic, having a negative effect on non-target species. [2-4] Numerous antifouling methods have been attempted including surface modification through coating with chemically active compounds, mimicking natural antifouling surfaces, and engineering different surface topography. [5-18] One of the most effective ways of prevention is to deter the formation of biofilms. Great efforts have been developed in preventing bacterial adhesion/growth via embedding nanoparticles, surface grafting of polymeric brushes, hydrogels, and antifouling self-assembly monolayer. [19-24] One approach is to create superhydrophobic/hydrophobic surfaces by entrapment of air in micro- and nano-sized pores on the surface when the surface is immersed in liquids. This solid-gas interface has the ability to prevent microorganisms from adhering onto the surface albeit for a short period as organisms will eventually displace the gas bubbles layer. [7]

In the last decade, applications of electrical potential for preventing the formation of biofilms have gained much attention. Various possible mechanisms such as the electrochemical production of hydrogen peroxide, radical generation, alternating pH, temperature changes, and electroosmotic flow have been proposed. [25-28] Results from the applications of high intensity [kV] electric field have also shown changes in biological membranes organization, disruptions of metabolic processes, deformations of the shape of the cell, and production of toxic substances where cells are damaged. [29-34] On the contrary, low-intensity [V] electric field has the ability to override the inherent resistance of bacteria to biocides, decrease the concentration of attached bacteria, decreased viability of bacterial cells and attachment on charged surfaces. [27, 28, 34-40]

Recently, Zhu et al. have reported a biocide-free antifouling method. [41] The method is based on the disturbance of charge around the vicinity of the protected surface via triboelectrification-induced oscillating electric potential. [41] However, one of the drawbacks of this system is its inability to enlarge the protected area and duration. The triboelectric oscillator was limited in the charge that it provides as the protected area increases. This leads to the reduction of the electric potential between the electrodes.

Today, most of the antifouling solutions are still facing the issues of large surface area coverage and sustainability. In this work, a unique method through the incorporation of electrochemical reaction by the application of low voltage square waveform pulses on the surface to create a sustainable solid-gas interface for antifouling will be discussed. This solution has the ability to prevent the adhesion of 99.5% bacteria on the surface. This method has also antimicrobial activity. It killed the bacteria (*Escherichia coli*) that pre-adhered on the surface. The solid-gas interface has the ability to create a dry environment that retards the growth of bacteria.

4.2 Experimental Section

4.2.1 Bacteria Quantification

E. coli starter (ATCC#25922) was prepared by incubating a colony of *E. coli* in 15ml LB Broth (Merck Millipore) shaking at 120 rpm at 37 °C for 6 hours. The *E. coli* starter was then diluted in LB Broth to achieve 5×10^7 cells/mL by the UV-Vis absorbance reading of 0.3 at a wavelength of 600 nm. [42] UV-vis absorption was measured by 1650PC UV-vis spectrophotometer (Shimadzu, Kyoto, Japan). The LB medium served as the electrolyte for the electrochemical experiment. To access the antifouling properties on the surface, *E. coli* biofilms were scrapped off from the substrate using a sterilized cotton swab. Serial dilutions were performed before performing the Plate Count Methods (PCM). [43]

4.2.2 Bacteria Staining

4.2.2.1 Crystal Violet

The substrates were immersed in 0.2% crystal violet (Merck, Darmstadt, Germany) for 15 minutes. They were rinsed three times with ultra-pure distilled water. The samples were dried at room temperature and placed under the optical microscope (Carl Zeiss, Axio Vision) for imaging.

4.2.2.2 Live/Dead® Assay Kit

QIA76 Live/Dead Double Staining assay kit (Merck Millipore) was used. Substrates were stained in accordance with the manufacturer protocols provided. Fluorescent microscopy images were obtained by fluorescence microscopy (Carl Zeiss, Axio Vision).

4.2.3 Electrochemical Reaction Setup

A three-electrode setup controlled by a CH Instruments-750B potentiostat was used with Ag wire as the reference and a platinum wire as counter electrode. 316 stainless steel (SS) sheet was used as the working electrode.

Electrochemical Procedure: Electric potential (-3 V , -0.8 V) was first applied for 5 seconds (on). It was then turned-off for a duration of 600 seconds (off). This on/off procedure was repeated throughout the duration of the whole experiment. All potentials applied were referenced to Ag wire.

4.3 Results and Discussion

A solid-gas interface can be formed when a superhydrophobic/hydrophobic surface is immersed in an aqueous solution. [44] This interface has the ability to prevent microorganisms from adhering onto the surface. [7] It is hypothesized that by sustaining a continuous layer of dry air bubbles by electrochemical gas generation at the substrate/medium interface, it will lead to effective resistance to biofouling (Figure 4.1).

Figure 4.1 Schematic illustration of the proposed antifouling mechanism. When the Stainless Steel (SS) substrate is immersed in a medium of *E. coli*, a layer of air bubbles is formed on the SS substrate via electrochemical gas generation (sample on the left) and a bare SS substrate (control on the right).

To determine the range of electrochemical reaction potential of the SS substrate in LB medium, a linear cyclic voltammetry test was performed. Generally, hydrogen evolution reaction undergoes two reaction steps on stainless steel. [45, 46] When a negative potential is applied to the working electrode, Volmer step [Equation 4.1] is the initial adsorption of hydrogen protons on the surface. The adsorbed hydrogen will either react with the solvated hydrogen proton (Heyrovsky step) [Equation 4.2] or combined with adjacent adsorbed surface hydrogens to form the hydrogen molecule (Tafel step) [Equation 4.3]. [45-49]

The resulted cyclic voltammogram (Figure 4.2) shows the hydrogen evolution reaction at the electrical potential range below -1 V with reference to Ag. Hydrogen proton adsorption ranges between -0.2 V and -0.8 V, where low current is observed. This is in accordance to previous studies on stainless steel. [46] To create a solid-gas interface on the stainless steel surface, an electrical potential of -3 V was applied as hydrogen evolution reaction occurs for stainless steel when the potential is lower than -1 V (as observed from CV curve in Figure 4.2). Due to the complex mechanism of bubbles formation/detachment and displacement of gas bubbles by micro-organism, a square waveform potential pulse was maintained throughout the experiment. This is to ensure that new gas bubbles are generated to fill up the area where the existing bubbles have detached off so as to maintain the gas bubbles layer. Gas bubbles (Figure 4.3) could be visually observed on the surface of the clean/bare SS substrate after applying an electric potential of -3 V for 5 seconds.

Figure 4.2 (a) Linear Sweep Cyclic Voltammetry (CV) curves of SS substrate in LB at 50mV/s scan rate.

Figure 4.3 Microscopy image of the clean/bare SS substrate surface after applying an electric potential of -3 V for five seconds.

Two experimental setups were prepared. The first setup consisted of a SS substrate (sample), Pt (counter electrode) and Ag wire (reference electrode) are connected to a potentiostat. The other setup consisted of only SS substrate (control). Both substrates

were immersed in a medium containing *E. coli* for 16 hours to allow substantial biofilm formation at 37°C. After the biofilm formation, One of the SS substrates underwent the square waveform pulsed electrochemical treatment cycle using the setup with potentiostat as shown in Figure 4.4. Figure 4.5 shows the chronoamperometry during the electrochemical treatment of the different substrates under the experimental conditions. The sample or substrate with a biofilm substrate exhibited higher current when compared to the clean substrate (bare SS). This was due to the presence of *E. coli* on the surface which enhanced the HER performance. [50] This might be due to the formation of the biofilm where it contains extracellular polymeric substance (EPS) which has elements that improve the HER performance. [50] To assess the antifouling properties of the sample, Plate Count Methods (PCM) were performed (see experimental section).

Figure 4.4 Voltage-time curves of the of the square-waveform potential pulses on the SS substrate. (Pink represents the experiment conducted at -3V while blue represents the experiments conducted at -0.8V). 5 seconds “ON” state, 600 seconds “OFF” state.

Figure 4.5 Chronoamperometry curves of the electrochemical treatment cycle on the different sample substrates under different experimental conditions. 5 seconds “ON” state, 600 seconds “OFF” state.

The graph in Figure 4.6 reports the normalized bacterial growth (*E. coli*) obtained from PCM under an electrical potential of -3 V. The plot shows a reduction of 99.5% in the bacterial growth on the sample substrate when compared to the control.

Figure 4.6 Normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on control substrate (without electrochemical treatment) and sample substrate (with electrochemical treatment at -3V) immersed for 16 hours in *E. coli* inoculum [$n=9$; $p=0.0003$].

Optical microscope images of the crystal violet stained bacteria on the surface in Figure 4.7 (A) and (B) display minute amount of *E. coli* adhered on the sample substrate when compared to the control substrate. Figure 4.8 (A) and (B) are fluorescent microscopy images of the Live/Dead® assay stained of the sample and control substrates (without electrochemical treatment) respectively. The green fluorescent dye indicates the presence of live *E. coli*, whereas the red fluorescent dye represents dead *E. coli*. The control substrate displayed a higher intensity of green fluorescent dye than the sample substrate (Figure 4.8 (A) and (B)).

Figure 4.7 Optical microscopy image (100x) of crystal violet stained (A) control substrate (without electrochemical treatment), (B) sample substrate (with electrochemical treatment at -3V) immersed for 16 hours in *E. coli* inoculum.

Figure 4.8 Fluorescence microscopy image (100x) of Live/Dead® assay stained SS control substrate (A) control substrate (without electrochemical treatment), (B) sample substrate (with electrochemical treatment at -3V) immersed for 16 hours in *E. coli* inoculum.

Overall, the results from the three different experiments are in agreement with each other and are consistent. From these three experimental systems, it is evident that the gas bubbles obtained through the electrochemical reaction (by applying -3V for 5 seconds (ON) at an interval of 600 seconds (OFF)) have successfully created a solid-gas interface, which has antifouling ability of reducing extensive amount of *E. coli* adhering onto the surface.

Hydrogen evolution reaction consists of two reactions. In order to ascertain that the antifouling activity is due to the solid-gas interface, a replicate experiment was conducted at -0.8 V potential. At this potential, only Volmer step reaction [Equation 4.1] occurs. Hydrogen is adsorbed on the surface of the substrate when a potential is applied and therefore gas bubbles cannot be visually observed at this potential.

Figure 4.9 shows the normalized bacterial growth obtained from PCM under an electrical potential of -0.8 V. A greater number of *E. coli* was attached to the surface of the sample when compared to the control.

Figure 4.9 Normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on control substrate (without electrochemical treatment) and sample substrate (with electrochemical treatment at -0.8 V) immersed for 16 hours in *E. coli* inoculum [$n=9$; $p=0.03$].

Both optical (Figure 4.10 (A) and (B)) and fluorescent (Figure 4.11 (A) and (B)) microscopy images from crystal violet and Live/Dead® assay stained surfaces show higher amounts of *E. coli* (normalized bacteria growth count) adhered on the substrate when subjected to potential of -0.8 V when compared to the control.

Figure 4.10 Optical microscopy image (100x) of crystal violet stained (A) control substrate (without electrochemical treatment), (B) sample substrate (with electrochemical treatment at - 0.8V) immersed for 16 hours in *E. coli* inoculum.

Figure 4.11 Fluorescence microscopy image (100x) of Live/Dead® assay stained SS control substrate (A) control substrate (without electrochemical treatment), (B) sample substrate (with electrochemical treatment at -0.8V) immersed for 16 hours in *E. coli* inoculum.

The increase in the bacterial counts may be due to the change in the surface free energy and the increase in the concentration of positive ions in the diffuse layer. When -0.8 V is applied to the sample, hydronium ions adsorb onto the surface and are reduced to hydrogen (Volmer step) [Equation 4.1]. Once the voltage is turned-off, adsorbed hydrogen may desorb from the surface. This process will increase the surface free energy

of the substrate. The increase of surface free energy may have caused the E. coli to adhere on the surface, as E.coli favor adherence onto high surface energy surface. [51,52]

Thermodynamic adhesion energy (ΔF_{adh}) is generally use to qualitatively predict the bacterial adhesion. [53] Negative adhesion energy will lead to the bacterial adhesion while a positive does not. Thermodynamic adhesion energy (ΔF_{adh}) comprise of:

$$\Delta F_{adh} = \gamma^{bs} - \gamma^{bl} - \gamma^{sl} - \text{Equation 4.4}$$

γ^{bs} : Interfacial free energy of bacteria– solid interface

γ^{bl} : Interfacial free energy of bacteria– liquid interface

γ^{sl} : Interfacial free energy of solid– liquid interface

Bacteria adhesion onto substrate is dependent on both substrate surface free energy (SFE) and SFE of bacterial cell. Zuo et al has reported on higher adhesion of E. coli to high SFE surface as compare to a low SFE surface. Based on calculation, the thermodynamic adhesion energy was found out to be negative for E.coli on high SFE surface which is consistent with various studies. [51, 52] In addition, as E.coli are gram-negative bacteria. During the application of negative potential, hydronium ions will adsorb on the surface forming a positively charged stern layer. This will increase the concentration of positive ions in the double layer region which might be an additional driving force to attract more E. coli into the double layer region after the potential pulse. [53-55]

The generation of hydrogen peroxide for antimicrobial and bacterial detachment by low voltage electrochemical reaction has been previously reported. [56] In this reported experiment, 1.5 V potential was applied to a conductive polymer membrane for a prolonged time. This method reduced the number of bacteria only by 80% and the formation of biofilm resumed when the potential was removed. A repeated identical condition experiment was carried out with a positive bias potential of 5V. Under this condition, oxygen gas bubbles are likely generated. A physical bubble layer is observed. The results in Figure 4.12 show that there were no detectable bacteria on the sample

substrate when compared to the control. This shows the effectiveness of the gas bubble layer, however, it is not advisable to apply positive bias potential on the substrate as it will oxidize the substrate and corrosion will occur.

Figure 4.12 Normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on control SS substrate (without electrochemical treatment) and sample SS substrate (with electrochemical treatment at 5V) immersed for 16 hours in *E. coli* inoculum [$n=3$; $p=0.000001$].

To make this technique accessible to a large variety of applications, an experiment with the identical condition is repeated with a nickel foil. The results from this experiment (Figure 4.13) show a reduction of 95% in the bacterial count as compared to the control.

Figure 4.13 (a) Normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on control Ni substrate (without potential) and sample Ni substrate (with potential @ -3V) immersed for 16 hours in *E. coli* inoculum [$n=3$; $p=0.000002$].

This proves the effectiveness of this technique and the fact that it is applicable to other conductive surfaces. This technique can also be incorporated with solar panels in tropical environment to achieve self-sustainable capability. It could also be applied to non-conducting surfaces via coating of photocatalytic catalysts where photocatalytic/photoelectrochemical reaction occurs when the surface is exposed to light.

4.4 Conclusion

In this chapter, a new approach with antifouling functionality has been successfully demonstrated. It has an antifouling performance that prevents 99.5% of *E. coli* from adhering onto the surface through application of short square-waveform pulses to induce water reduction. It is proposed that gas bubbles that remain on the surface serve as an effective barrier that separates the substrate surface and the liquid medium. This study also serves as a new viable approach for self-sustainable antifouling and antimicrobial solution due to the renewable gas layer generation with pulse potential applications.

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Chapter 5

Porous polymer scaffold to reduce the energy expenditure for antifouling

In the previous chapter, an effective antifouling solution via an electrochemical approach has been reported. The electrochemically generated gas bubble layer serves as a separation barrier between the surrounding and the target surface where the adhesion of bacteria can be deterred. This hydrogen gas bubble layer was achieved through the application of a low voltage square-waveform pulses to the conductive surface. However, one of the drawbacks is the requirement of periodically pulsing to maintain this gas bubble layer. In order to put this system into a practical application, the need to reduce the energy consumption is required. A multilayer PVB scaffold film is fabricated on the stainless steel substrate where it is hypothesized that it has entrapment capability of preventing the gas bubbles from detaching from the surface. This is aimed at reducing the need for frequent application of potential pulses to maintain the separation barrier between the surface and the medium. In this chapter, the process of fabrication of the multilayer PVB scaffold film, the antifouling performance of different pulsing interval, mechanism of the antifouling properties, accelerated bacteria test and prolong polymer cycling stability are discussed.

*This section has filed for a patent.

5.1 Introduction

In chapter 4, a unique method through the incorporation of electrochemical reaction via application of low voltage square waveform pulses on the surface to create a sustainable solid-gas-liquid interface for antifouling has been established. The solution has the ability to prevent the adhesion of 99.5% bacteria on the surface. However, the drawback is the requirement of constant periodically application of pulses to maintain the system antifouling performance. To put this system into a practical application, the need to reduce the energy consumption is required.

In chapter 4, it is proposed that the gas bubbles generated by the electrochemical reduction of water form a solid-gas interface barrier which prevents the bacteria from adhering on the surface. Due to the complex mechanism of bubbles formation/detachment and displacement of gas bubbles by micro-organism, a square waveform potential pulse was maintained throughout the experiment. In order to decrease the energy consumption, the frequency or the voltage applied has to be reduced. It is hypothesized that a multilayer scaffold has the entrapment capability of preventing the gas bubbles from detaching from the surface. This might reduce the need for frequent application of potential pulses to maintain the separation barrier between the surface and the medium.

Porous materials have been used as antimicrobial material due to its topology or trapping active materials/chemical modification which changes the surface properties. [1-5] Levkin et al has reported on the liquid-infused porous polymer (poly(butyl methacrylate-co-ethylene dimethacrylate) which changes the surface properties leading to low biofilm formation or growth [1]. Rodriguez-Hernandez et al has worked on antimicrobial 3D porous scaffold which is a combination of chemically active surface and micro-size topology. [2] There are also reports fabricating superhydrophobic porous network which has antimicrobial function. [3-5] Our proposed idea of entrapping electrochemically generated gas bubbles in a porous scaffold to realize efficient antimicrobial function is unprecedented.

There are several considerations that need to be considered before the fabrication of the multilayer scaffold. Firstly, it must be a scaffold able to withstand and would not detach off the conductive surface during the application of potential. Secondly, the morphology of the scaffold must be uniform. Lastly, the scaffold must allow the electrolyte to seep through to reach the conductive substrate where electrochemical reduction will occur.

Breath figure refers to the fog that forms when water vapor contacts with a cold surface. This water condensation process is commonly observed in the daily life. Breath figure was first investigated by John Aitken in 1895. [6] Lord Rayleigh revealed the morphology of water droplets on cold surfaces. [7] In 1994, Bernard François first reported self-organized honeycomb morphology of star-polymer polystyrene films. [8] The micrometer-sized pores exhibited a highly regular hexagonal arrangement on the top of the surface was correlated with the breath figure mechanism which was established by Lord Rayleigh and John Aitken. The process of formation of the highly ordered honeycomb polymer films via breath figure method was explained by Sanjay Patel et al in 2001.[9] Figure 5.1 illustrate the proposed model for the formation of the structure in polymer films. Water molecules in the environment will condense and form a close ordered packed array of water droplets. It will cool and sink onto the polymer solvent. Due to the differences evaporation time and the immiscibility of the polymer solvent and the water droplets, a highly ordered honeycomb polymer film will be formed after both solvents are evaporated. [9] Over the last few decades, there are various reports on ordered honeycomb structure polymer formed by the breath figure method for various polymers such as polystyrene, PS/polyparaphenylene, polysulfone and polyvinyl butyral. [10-13]

Figure 5.1 Schematic drawing of the proposed model for the formation of the structure in polymer films. [9]

Polyvinyl Butyral (PVB) is a polymer with high optical clarity, toughness, flexibility properties which adhere to most surfaces. It is currently widely used as an interlayer material for laminated glass for safety glass which will mitigate the hazard from shattered glass fragments. [14] Yedong et al have reported the fabrication of honeycomb structure PVB using breath figure method. [13]

In this chapter, a multilayer scaffold PVB film was prepared on the stainless steel substrate to create the entrapment capability to prevent the gas bubbles from detaching off the surface. It has retained its antifouling performance of more than 90% with a single pulse over a period of 16 hours. The actual antifouling mechanism was also studied.

5.2 Experimental Section

5.2.1 Multilayer PVB Scaffold Film

PVB polymer solution was prepared by dissolving PVB powder (Sigma-Aldrich) in chloroform (Tedia). The mixture was stirred overnight. Stainless Steel (SS) substrate was dip coated into the PVB solution and removed. It was then placed it in a humidity chamber maintained at 70% to dry for 30 minutes. The substrate was then heated to 60°C for 3 hours before testing for its antifouling properties. Control experiments are includes for samples without electrochemical treatment.

5.2.2 Bacteria Quantification

E. coli starter (ATCC#25922) was prepared by incubating a colony of E. coli in 15ml LB Broth (Merck Millipore) shaking at 120 rpm at 37 °C for 6 hours. The E. coli starter was then diluted in LB Broth to achieve 5×10^7 cells/mL by the UV-Vis absorbance reading of 0.3 at a wavelength of 600 nm. [15] UV-vis absorption was measured by 1650PC UV-vis spectrophotometer (Shimadzu, Kyoto, Japan). The LB medium served as the electrolyte for the electrochemical experiment. To access the antifouling properties on the surface, E. coli biofilms were scrapped off from the substrate using a sterilized cotton swab. Serial dilutions were performed before performing the Plate Count Methods (PCM). [16]

5.2.3 Electrochemical Reaction Setup

A three-electrode setup controlled by a potentiostat was used with Ag wire as the reference and a platinum wire as counter electrode. 316 stainless steel (SS) sheet was used as the working electrode.

Electrochemical Procedure: Electric potential (-1.7 V) was first applied for 5 seconds (on). It was then turned-off for a 1, 3, 5, 16 hours (off). This on/off procedure was repeated throughout the duration of the whole experiment. All potentials applied were referenced to Ag wire.

5.3 Results and Discussion

In order to make this newly established antifouling method in chapter 4 more attractive for practical application, the reduction of energy consumption is necessary. [17] It is hypothesized that a multilayer scaffold has the entrapment capability of preventing the gas bubbles from detaching from the surface (Figure 5.2).

Figure 5.2 Schematic illustration of the proposed antifouling mechanism of the multilayer PVB scaffold coated stainless Steel (SS) substrate immersed in a medium of E.coli before and after potential application.

Bare SS substrate is immersed in a PVB/Chloroform solution with a concentration of 10mg/ml. This concentration is optimised to ensure the electrolyte seeps through the PVB film to reach the conductive substrate where electrochemical reduction will occur. The top and cross-section morphology of the as-prepared PVB film is characterised by scanning electron microscope (SEM).

Figure 5.3 is the top view of the as-prepared PVB film after 3 hours of heat treatment in the oven at 60°C to improve the adhesion strength and the mechanical strength. Image J software was used to determine the size of the structure. A histogram (Figure 5.4) ($n=100$) is plotted and a mean of 1100 ± 100 nm is attained. The mean size of the pores is slightly smaller by ~ 500 nm when compared with the results reported by He et al. [13] This might be due to the different concentration of the polymer and humidity used between the two experiments. Figure 5.5 shows the cross-section of the film. The thickness is approximately 2 μ m. The cross-section shows that the polymer network is relatively open and well inter-connected from the surface to the SS substrate. This will allow the electrolyte to seep through the scaffold to the surface of the SS substrate for electrochemical reduction reaction.

Figure 5.3 Scanning Electron Microscopy (SEM) image of the fabricated PVB film after 3 hours of heat treatment at 60°C. (a) Low Magnification (b) High Magnification.

Figure 5.4 Histogram of the pore size distribution of the fabricated PVB film. (n=100)

Figure 5.5 Scanning Electron Microscopy (SEM) image of the cross-section of the fabricated PVB film after 3 hours of heat treatment at 60°C.

To determine the range of electrochemical reduction reaction potential of the fabricated PVB film on the SS substrate in LB medium, a linear cyclic voltammetry test was performed. Cyclic voltammogram (Figure 5.6) shows the hydrogen evolution reaction at

the electric potential range below -1.3 V. To generate the gas bubbles, -1.7 V potential for 5 seconds was applied to the substrate in all the experiments.

Figure 5.6 Linear Sweep Cyclic Voltammetry (CV) curves of fabricated PVB on SS substrate in LB at 50mV/s scan rate.

Similar to the experimental procedure in chapter 4, two experimental setups were prepared for each experiment. The first setup consisted of a fabricated PVB film on SS substrate (sample), Pt and Ag wires were connected to a potentiostat. The other setup consisted of only fabricated PVB film on the SS substrate (control). Both substrates were immersed in a medium containing *E. coli* for 16 hours to allow substantial biofilm formation at 37°C .

The sample underwent the square waveform pulsed electrochemical treatment cycle at different interval of 1, 3, 5, 16 hour(s). To assess the antifouling properties of the sample, Plate Count Methods (PCM) were performed (see experimental section). Figure 5.7 reports the % bacterial reduction (*E. coli*) obtained from PCM when compared between the sample and the control at a different interval. All the samples show a bacterial reduction of more than 95%. The results are similar to the antifouling performance obtained in chapter 4. The energy required to maintain the same performance as chapter 4 has been reduced by 98%.

Energy required for the bare SS substrate to maintain antifouling performance (reducing 95% of E.Coli):

$$Power^* = Voltage \times Current - Equation 5.1$$

* [This should be the same as the voltage and current of the pulse is the same

$$Energy = Power \times Time - Equation 5.2$$

$$Energy = Power \times (5 \text{ seconds} \times 6 \text{ times/hour} \times 16 \text{ hours})$$

$$Energy = Power \times 480 \text{ seconds}$$

Energy required for fabricated PVB film on the SS substrate to maintain antifouling performance (reducing 95% of E. Coli):

$$Energy = Power \times Time$$

$$Energy = Power \times (5 \text{ seconds} \times 1 \text{ times}/16 \text{ hours})$$

$$Energy = Power \times 5 \text{ seconds}$$

Energy Reduction:

$$\left[1 - \frac{Power \times 5 \text{ seconds}}{Power \times 480 \text{ seconds}} \right] \times 100\% = 98.95\%$$

Figure 5.7 Summary of the % bacteria reduction (E. coli) (%) obtained from Plate Count Methods (PCM) between the control substrate (without electrochemical treatment) and sample substrate (with electrochemical treatment at -1.7V) at an interval of 1, 3, 5, 16 hour(s). [n=9].

There is a common explanation which correlates the adhesion site and the adhesion strength. Scardino et al have reported the reduction in the adhesion strength due to the decrease in the number of attachment site on the micro-engineered topography as compared to the smooth surface. [18] E.coli has a structure of $\sim 1 \times 3 \mu\text{m}$ whereas the fabricated PVB films have pore size morphology of 1100 nm (or 1.1 μm). [19] This might lead to the reduction in the binding strength of the E.coli adhere onto the PVB. In gas bubbles growth and their release from the surface, there are several forces [Equation 5.3] which determine the detachments of the bubbles.[20-22] Bubbles will detach off the surface if $\sum F_x > 0$. During electrochemical reduction process, bubbles growth occur continuously due to the reduction of water under potential where gas bubbles will coalescence, forming larger bubbles and eventually detach off the surface. [20, 23] It is hypothesized that during gas bubbles detachment where $\sum F_x > 0$, it is able to overcome the binding strength of the E.coli leading to the release of E.coli from the surface.

$$\sum F_x = F_{du,x} + F_{\sigma,x} + F_h + F_L + F_{cp} - \text{Equation 5.3}$$

$F_{du,x}$: Unsteady growth force which acts as drag force

$F_{\sigma,x}$: Surface tension force between the bubble and electrode

F_h : Hydrodynamic force due to hydrodynamic pressure

F_L : Shear lift force

F_{cp} : Contact pressure force

To determine the mechanism of the antifouling properties, a new experiment with the new parameter is proposed. Similar to the previous experiment, two experimental setups were prepared for each experiment. The first setup consisted of a fabricated porous PVB film on SS substrate (sample), Pt and Ag wires were connected to a potentiostat. The other setup consisted of only the fabricated porous PVB film on the SS substrate without electrochemical pulsing (control). Both substrates were first immersed in a medium containing E. coli for 16 hours to allow substantial biofilm formation at 37°C. The sample substrate then undergoes a single potential pulse at -1.7V for 5 seconds. Plate Count Methods (PCM) were performed to assess the antifouling performance. Figure 5.8

shows the % bacterial reduction (*E. coli*) obtained from PCM when compared between the sample and the control. There is a bacteria reduction of more than 95% when compared. Scanning electron microscopy (SEM) images in figure 5.9 show a significant reduction of the amount *E. coli* adhere on the sample as compared to the control. Both SEM and PCM show consistent results.

Figure 5.8 Normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on control fabricated PVB on SS substrate (without electrochemical treatment) and sample fabricated PVB on SS substrate (with single pulse electrochemical treatment at -1.7V) after immersing in *E. coli* inoculum for 16 hours [n=9].

Figure 5.9 Scanning electron microscopy (SEM) image of (A) Control fabricated PVB on SS substrate (without electrochemical treatment) (1) Low magnification (2) High magnification. (B) Fabricated PVB on SS substrate (with single pulse electrochemical treatment at -1.7V) (Insert is higher magnification) after immersing in *E. coli* inoculum for 16 hours (1) Low magnification (2) High magnification.

To eliminate the possibility of the release of the *E. coli* from the surface via electrochemical reduction, a repeated experiment is conducted on bare stainless steel. Figure 5.10 shows the results. There was approximately 20% of the *E. coli* detached off the bare SS substrate when compared with the control under the same potential application. The detachment of *E. coli* when an electric potential is applied is consistent with previous reports. [17, 24-26] This might be due to the electrostatic and electrophoretic repulsive forces generated when a potential is applied as there are movements of ions/charges during electrochemical reduction process. (Figure 5.11) [17, 24-26] However, the degree of detachment is not significant as reported previously. This might be due to the short duration (5 seconds) of the potential pulses applied in this

experiment. Scanning electron microscopy images in figure 5.12 show a similar amount of bacteria adhered to both the control bare SS substrate (without electrochemical treatment) and the bare SS substrate with single pulse electrochemical treatment at -1.7V, after immersing in *E. coli* inoculum for 16 hours. The results indicate that the PVB polymeric scaffold is an essential component to achieve the antifouling property.

Figure 5.10 Normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on control bare SS substrate (without electrochemical treatment) and sample bare SS substrate (with single pulse electrochemical treatment at -1.7V) after immersing in *E. coli* inoculum for 16 hours[n=9]

Figure 5.11 Schematic drawing of forces resulting from an applied current on adhering negative charge bacteria. [26]

Figure 5.12 Scanning electron microscopy (SEM) image of (A) Control bare SS substrate (without electrochemical treatment). (B) Sample bare SS substrate (with single pulse electrochemical treatment at -1.7V) after immersing in *E. coli* inoculum for 16 hours.

In the state of art, there is a common explanation which correlates the adhesion site and the adhesion strength. The binding strength onto the surface will be reduced with the decrease in the number of attachment sites. [18] *E. coli* has a structure of $\sim 1 \times 3 \mu\text{m}$ whereas the fabricated PVB films have pore size morphology of $\sim 1100 \text{ nm}$. In figure 5.9A, it is observed that individual *E. coli* is adhered between several pores due to the

topography of the films. The area of adhesion for the E.coli has been reduced due to the empty voids of the pores. This reduces the number of attachment sites where it leads to the reduction in binding strength. During electrochemical reduction reaction, the hydrogen gas generated on the surface of stainless steel by the electrochemical reduction is able to overcome the binding strength of the E.coli leading to the release of E.coli from the surface (Figure 5.9B).

In order to put this method to practical application, an accelerated bacteria test and polymer stability test were carried out. Accelerated bacteria test was carried for 48 hours with the potential pulse of -1.7V at an interval of 1 hour. Bacteria were renewed every 16 hours through the replacement with new bacteria inoculum. Figure 5.13 reports the results obtained after the end of the experiment. The result is consistent with the previous results of having bacteria reduction of more than 95%.

Figure 5.13 Normalized bacteria growth (E. coli) (%) obtained from Plate Count Methods (PCM) on control fabricated PVB on SS substrate (without electrochemical treatment) and sample fabricated PVB on SS substrate (with pulse electrochemical treatment at -1.7V at interval of 1 hour) after immersing in E. coli inoculum for 48 hours. [n=9]

Polymer stability test was carried out in the fabricated PVB on SS substrate in LB medium. It was electrochemically cycled for more than 1000 hours with the potential

pulse of -1.7V for 5 seconds at an interval of 16 hours. Figure 5.14 shows the SEM images obtained after the test. There was no morphology change to the structure of PVB film between the control and sample substrate after prolonged testings, indicating the robustness and stability of the coating.

Figure 5.14 Scanning electron microscopy (SEM) image of (A) Control fabricated PVB on SS substrate (without electrochemical treatment) (1) Low magnification (2) High magnification. (Insert is higher magnification). (B) Sample fabricated PVB on SS substrate (with electrochemical treatment interval pulses of 16 hours at -1.7V) (Insert is higher magnification) after immersing in LB for 1000 hours (1) Low magnification (2) High magnification.

5.4 Conclusion

In this chapter, the energy required to achieve an antifouling fouling performance has been reduced by 96x to a single pulse in 16 hours. This is achieved by fabricating a multilayer porous PVB scaffold film on the SS substrate. This PVB film will reduce the E.coli binding strength on the surface. During the electrochemical reduction process, the hydrogen gas generated on the surface of stainless steel will be able to overcome the binding strength of the E.coli leading to the release of E.coli from the surface. This method has been proven effective for accelerated bacteria growth study and the PVB is stable in LB medium after prolonged cycling. This study also serves as a new viable antifouling solution which is applicable to all conductive substrates.

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Chapter 6

Passive antifouling approach via designing and rationalizing a new amphiphilic tripeptide coating

One of the environmentally friendly highly sought after biocompatible antifouling materials is peptide. It is made up of amino acids and it can be easily synthesized by the combination of different amino acids in random or alternate arrangements to attain the desired functionality. Therefore, it is of great interest to improve the antifouling performance of peptides. It is hypothesized that by judiciously tailoring the amino acids configurations in the peptide, the antifouling performances of the peptides can be enhanced. Results obtained show that antifouling properties can be enhanced by 30% with an amphiphilic peptide-based coating of Phe(4-F)-Lys-DOPA. This chapter provides important strategic design guidelines for peptide-related coating with effective antifouling properties.

*This section submitted substantially as Sheng Long Gaw, Gowripriya Sakala, Sivan Nir, Abhijit Saha, Zhichuan J. Xu, Pooi See Lee, Meital Rechtes, Rational design of amphiphilic peptides and its effect on antifouling performance, *Biomacromolecules*, 2018, 19 (9), 3620–3627.

6.1 Introduction

For medical devices such as implants, biofouling will cause inflammation and lead to failure. In most circumstances, invasive medical surgery is often required to resolve the issue. Interactions between microorganisms and the substrates often play a critical role in this process. [1] Surface property is one of the factors that will affect biofouling. It has been proven to be effective by changing the surface chemistry. [2-5] Environmental-friendly antifouling solutions have been extensively studied due to stringent regulations nowadays. [6-11] Amphiphilic polymer coatings result from the combination of hydrophobic and hydrophilic monomers could serve as an effective antifouling coating. [2, 5, 12-15] Most of the proposed amphiphilic polymers consist of long repeating monomer units with either random, block or alternate sequencing of the two properties that increase the complexity and unpredictable properties of the polymer. This often leads to uncertainty in the antifouling mechanism. [2, 5, 12-15]

One of the environmentally friendly and highly sought after biocompatible materials is peptide. It is made up of amino acids and it can be easily synthesized by the combination of different properties amino acids to attain the desired functionality. Till date, several peptides have been designed specifically for antifouling applications. [6, 16-18] The arrangements of the amino acids reported are usually in alternate or random arrangement. [18] Therefore, it is of great interest to determine the effects of positioning of the individual amino acids within the peptide chain to strategically manipulate and enhance the antifouling performances.

The selected amino acids of interest are DOPA, Phe and Lys. 3, 4-dihydroxy-L-phenylalanine (DOPA) is an amino acid with adhesive properties. It is the main constituent of mussel adhesive protein (MAPs) that binds to different materials. [19, 20] DOPA binds different types of materials as it can form various bonds with the surface. [20-22] With the incorporation of DOPA in a peptide sequence, the peptide can be attached to the surface. [23, 24] Meital et al have shown that anchoring two (ii) 4-fluoro-phenylalanine residues (Phe(4-F)) to a surface using DOPA provides the surface with

hydrophobicity that leads to the formation an antifouling coating. [6] Furthermore, the positively-charged Lysine (Lys) is of hydrophilic nature and has also been well reported for its antibacterial effect. [25, 26] The sequence of amino acids within a peptide chain can be arranged based the desired outcome properties. It is hypothesized that the position of the amino acids affects the antifouling performances of the peptides.

In this chapter, the investigation was carried out on the amphiphilic peptide that consists of three amino acids, namely, DOPA that serves as a binding unit to the surface, a hydrophobic Phe(4-F) moiety and a hydrophilic, positively charged lysine (Lys) residue. The peptide provides a self-assembly capability and is of particular interest for antifouling application. This work shed light on the importance of rationalizing and positioning of individual amino acids in the peptide chain for optimum antifouling performance. The antifouling performance of the same chemical composition peptide has improved by 30% through strategic positioning of the amino acids within the peptide.

6.2 Experimental Section

6.2.1 Peptide Synthesis and Characterization

Peptides were manually synthesized using manual solid-phase peptide chemistry synthesis. Fmoc-DOPA(acetonide)-OH (GL Biochem (Shanghai)), L-4-fluoro phenylalanine-OH (GL Biochem (Shanghai)), Fmoc-L-Lys(Boc)-OH (Matrix Innovation (Canada)), HATU (Matrix Innovation (Canada)), Fmoc-Rink Amide resin (Matrix Innovation (Canada)) were purchased and used. Coupling conditions of using Amino Acids/HATU/ N,N-Diisopropylethylamine (DIPEA) (Bio-lab (Israel)) were employed to obtain the desired peptides. Removal of Fmoc group was performed using 20% Piperidine in Dimethylformamide (DMF) (Bio-lab (Israel)) for 15 minutes for three times followed by rinsing thrice with DMF to remove any residuals. Amino acids were coupled in excess of 5 fold in all reactions. The coupling reaction lasted for 2 hours. The peptide was cleaved off from the resin support by using Trifluoroacetic acid (Bio-lab (Israel)), Triisopropylsilane (Tokyo Chemical Industry), Deionized water in a ratio of 95/2.5/2.5

for 3 hours and a precipitation in cold diethyl ether (Bio-lab (Israel)). The precipitate was centrifuged, re-dissolved in a mixture (50% acetonitrile and 50% water) and lyophilized to obtain the peptide. The purity of the peptide was confirmed using an analytical high-performance liquid chromatography (HPLC) (Merck Hitachi). The peptide was then characterized using high-resolution liquid chromatography mass spectrometry (LCMS) (Agilent) and nuclear magnetic resonance spectroscopy (NMR) (Bruker).

6.2.2 Bacteria Quantification

E. coli bacteria starter (ATCC#25922) was prepared by incubating a colony of E. coli at 37 °C for 6 hours in 15ml LB Broth (Merck Millipore) with shaking at 120 rpm. The E. coli starter was then diluted with LB Broth to a UV-Vis absorbance reading of 0.3 at a wavelength of 600 nm to attain bacterial concentration of 5×10^7 cells/mL. [27] The absorption was measured by 1650PC UV-vis spectrophotometer (Shimadzu, Kyoto, Japan). To assess the antifouling properties on the surface, E. coli biofilms were scrapped off from the substrate using a sterilized cotton swab. Serial dilutions were performed before carrying out the Plate Count Methods (PCM). [28]

6.2.3 Bacteria Staining

6.2.3.1 Crystal Violet

The substrates were immersed in 0.2% crystal violet (Merck, Darmstadt, Germany) for 15 minutes. They were rinsed three times with ultra-pure distilled water. The samples were dried at room temperature and placed under the optical microscope (Carl Zeiss, Axio Vision) for imaging.

6.2.3.2 Live/Dead® Assay Kit

QIA76 Live/Dead Double Staining assay kit (Merck Millipore) was used. Substrates were stained in accordance with the manufacturer protocols provided. Fluorescent microscopy images were obtained by fluorescence microscopy (Carl Zeiss, Axio Vision).

6.2.4 X-ray Photoelectron Spectroscopy (XPS)

The measurements were performed using a Kratos Axis Ultra X-ray photoelectron spectrometer (Kratos Analytical Ltd., Manchester, UK) using an Al K α monochromatic radiation source (1,486.7 eV) with 90° takeoff angle (normal to analyzer). The high-resolution XPS spectra were collected with pass energy 20 eV and step 0.1 eV. The binding energies were calibrated relative to C 1s peak energy position as 285.0 eV. Data analyses were performed using Casa XPS (Casa Software Ltd.).

6.2.5 Quartz Crystal Microbalance (QCM)

The measurements were performed using a quartz crystal microbalance (Q-Sense AB, Biolin Scientific, Gothenburg, Sweden) in a flow module system. Titanium surface sensor was purchased and used. Prior to the experiments, the sensor was rinsed with ethanol and DI water followed by nitrogen air dry and 3 minutes of oxygen plasma (Harrick Plasma Cleaner, NY). Acquisition of the data starts when the system stabilizes. 0.5mg/ml of the peptide-methanol mixture was introduced into the system for 45 minutes [Point 1]. It was then switched to pure methanol for 10 minutes to determine the binding of the peptide onto the surface [Point 2].

6.3 Results and Discussion

In order to complete the full combination of the tripeptide sequencing, a total of six different peptides (Figure 6.1) using solid phase synthesis method were synthesized. The synthesized peptides characterized by analytical HPLC (Figure A6.1 - A6.6), ^1H NMR (Figure A6.7 - A6.12), ^{13}C NMR (Figure A6.13 - A6.18) and LCMS (Table 6.1) respectively.

Figure 6.1 The molecular structures of the designed peptides.

Table 6.1 Liquid Chromatography Mass Spectrometry Results of peptide A-F.

	Calculated	Observed
Peptide A	490.25	490.24
Peptide B	490.25	490.24
Peptide C	490.25	490.24
Peptide D	490.25	490.24
Peptide E	490.25	490.24
Peptide F	490.25	490.26

Each peptide was dissolved in methanol to a concentration of 0.5 mg/mL. Titanium coated silicon substrates were used as the surface for the study. The substrate was immersed in the peptide solution for 8 hours at 23°C to form the coating. The coated substrate was rinsed twice with methanol and dried with nitrogen. The contact angle was measured 3 times for each sample. Table 6.2 showed the mean contact angles measured on the different samples. The control sample which was immersed in methanol showed a contact angle of $22^{\circ} \pm 1$ while peptide coated samples showed a higher contact angle for all the different samples. The increase in the hydrophobicity was due to the fluorinated side chain present in the peptides.

Table 6.2 Contact angle of the Ti coated silicon substrate after dip-coating of peptide A-F.

	Contact Angle
Control	$22^{\circ} \pm 1$
Peptide A	$33^{\circ} \pm 1$
Peptide B	$36^{\circ} \pm 2$
Peptide C	$37^{\circ} \pm 2$
Peptide D	$29^{\circ} \pm 1$
Peptide E	$34^{\circ} \pm 2$
Peptide F	$34^{\circ} \pm 1$

In order to ascertain the binding of the peptides on the titanium surface, quartz crystal microbalance with dissipation monitoring (QCM-D) and X-ray photoelectron spectroscopy (XPS) measurements were used. Figure 6.2-6.7 shows the QCM-D results of all peptides. From the results, there was a change in resonance frequency observed between the initial and end of the experiment. This indicated the binding of peptides on the titanium surface despite washing with pure methanol at the end of the experiment. The mass of peptide adhered area calculated based on the formula in equation 6.1.

$$\Delta m = -\frac{(C \times \Delta f)}{n} \text{ [Equation 6.1]}$$

$C = 17.7 \text{ ng Hz}^{-1}\text{cm}^{-2}$ for a 5 MHz quartz crystal, $n = 5$

Figure 6.2 Quartz Crystal Microbalance (QCM) measurement of Lys – Phe(4-F) – DOPA [A] peptide.

Figure 6.3 Quartz Crystal Microbalance (QCM) measurement of DOPA – Phe(4-F) – Lys [B] peptide.

Figure 6.4 Quartz Crystal Microbalance (QCM) measurement of Lys – DOPA – Phe(4-F) [C] peptide.

Figure 6.5 Quartz Crystal Microbalance (QCM) measurement of Phe(4-F) – DOPA – Lys [D] peptide.

Figure 6.6 Quartz Crystal Microbalance (QCM) measurement of Phe(4-F) – Lys – DOPA [E] peptide.

Figure 6.7 Quartz Crystal Microbalance (QCM) measurement of DOPA – Lys – Phe(4-F) [F] peptide.

The XPS measurements obtained from the coatings of the 6 different peptides and the control sample (bare Ti on silicon) were fitted with Lorentzian-Gaussian peaks fitting for all the spectra obtained. In the nitrogen spectra (Figure 6.8), two peaks could be fitted. The presence of the 400 eV peak in the spectra of samples corresponds to the C-N bonding due to the organic molecules adsorbed on the surface. [29-31] The second peak at ~401.9eV is the N-H bonding presence in the chemical structure of peptides. [29-31] This peak could only be detected in the samples coated with the peptides. In the fluorine spectra (Figure 6.9), two peaks could also be fitted. The ~684.5eV peak was consistently found on all samples, including the control sample, and was identified to be Ti-F bonding. [32] The presence of the peak attributes to the contamination of the titanium coated silicon substrates during its preparation or ions implanted into the silicon substrates. Another peak at ~687.4eV was the C-F bonding. [33] The presence of the C-F bonding was due to the fluorine terminated group in the Phe(4-F) amino acid. Therefore, it was present in all the peptide coated sample spectra only.

Figure 6.8 X-ray photoelectron spectroscopy (XPS) analysis of nitrogen spectra of sample coated with peptide A-F.

Figure 6.9 X-ray photoelectron spectroscopy (XPS) analysis of fluorine spectra of sample coated with peptide A-F.

DOPA had been extensively reported before for its adhesive properties. [23, 24, 34] The proposed adhesive mechanism between DOPA and a titanium surface is through the catechol binding. [20] The hydroxide terminal at the DOPA will bind to the surface forming Ti-O linkage between the peptide and titanium surface. This will lead to the change in the Ti-O binding energy between a coated and a non-coated peptide titanium surface. Therefore, systematic studies are required for the Ti and O XPS in this work.

There are 8 peaks that could be fitted in the titanium spectra (Figure 6.10). [35, 36] There was no significant peak shift to the doublets peaks assigned to Ti, $Ti3^+$ and $Ti4^+$. In contrast, a consistent peak shift was observed in the doublet peaks of $Ti2^+$ on the peptide-coated samples when compared to control. [35, 36] $Ti2^+$ peak for the control sample had a binding energy of $\sim 455.04\text{eV}$ whereas there was a consistent positive shift of $\sim 0.2\text{-}0.3\text{ eV}$ in the $Ti2^+$ peak positions for all the six peptide coated samples. This indicated a change in the chemical bonding of the Ti-O bonding on the surface of control and the peptide coated substrate.

Figure 6.10 X-ray photoelectron spectroscopy (XPS) analysis of titanium spectra of sample coated with peptide A-F.

In the oxygen XPS spectra (Figure 6.11), TiO_2 , Ti-O and C=O peaks could be fitted. [35, 37] There were no significant shifts observed in the TiO_2 and C=O peaks among the samples. However, a significant peak shift for the Ti-O binding peak could be detected when compared. The Ti-O binding peak for the control sample showed a binding energy of 531.07eV, while there was a consistent $\sim 0.1\text{-}0.2\text{eV}$ positive shift of binding energy for all the peptide-coated samples. A change in the chemical bonding of the elements will cause deviation to the binding energy of a core electron of elements. Due to the change in chemical bonding of the bare titanium surface and a strong binding self-assembled peptide surface, the binding energy has been altered when the peptide was chemically anchored on to the surface. Indeed, both oxygen and titanium XPS results showed a consistent positive shift in the binding energy which supported the hypothesis of DOPA and titanium catechol binding through the formation of Ti-O bonding.

Figure 6.11 X-ray photoelectron spectroscopy (XPS) analysis of oxygen spectra of sample coated with peptide A-F.

Based on the XPS results, different chemical arrangement for each peptide anchored on the surface was proposed due to the different position of the DOPA moiety (Figure 6.12) in the peptide chain. Peptide A had DOPA at the C-terminal of the peptide with adjacent Phe(4-F) and Lys at the N-terminal whereas peptide B had the opposite arrangement. Based on the molecular arrangement of peptides A and B, both peptides would form a monolayer of linear chains where Lys would be exposed to the surrounding in either C-terminal (Peptide A) or N-terminal (Peptide B) (see Figure 6.12). Both Peptide C and D contain DOPA in the middle of the peptide between Phe(4-F) and Lys. Peptide C had Phe(4-F) at the C-terminal while Lys at the N-terminal and peptide D had the opposite structure. Therefore, the formation of a “Y” branched peptide layer for these two peptides would be expected. In peptides E and F, Lys was adjacent to the DOPA moiety. This would anticipate a monolayer linear chain formed where its Phe(4-F) moiety at the N-terminal (Peptide E) or C-terminal (Peptide F) exposing to the surrounding.

Figure 6.12 Schematic drawing of the proposed chemical arrangement of the peptides on the surface.

To test the antifouling performance of the peptide-coated surfaces, the substrates were immersed in a medium containing *E. coli* at 37°C for 16 hours for substantial biofilm formation. [38] Plate Count Method (PCM) was performed for each surface. Figure 6.13 shows the summary results from the three triplicate experiments. Peptide A reduced the amount of bacteria by an average of 30% while peptide B reduced it by an average of 25% when compared to a bare substrate. Peptide C and D reduced the amount of adhered bacteria by 43% on average. Peptide E and peptide F reduced the amount of bacteria by

56% and 51% respectively. From these results, the following findings were summarized. First, peptide C, D, E, and F performed better than peptide A and B. This might be due to the enhanced adhesion properties of the peptides to the surface due to DOPA and the lower surface energy owing to the exposure of the fluorinated terminal of phe(4-F). [6, 39] Second, the linear chain tripeptide (peptide E and F) had better antifouling performance than the “Y” branched (peptide C and D) (Figure 6.12). It was noted that peptide E and F have the best antifouling properties. This was probably due to the exposed fluorinated side chain located at the end of the chemical chain leading the ability to drag the chain toward the surface that enabled the formation of better packing density film (Figure 6.12). [9] Lastly, a consistent trend between peptide A/B and peptide E/F for a ~5% mean improvement in the antifouling performance when DOPA was on the C-terminal as compared to N-terminal. However, the ~5% mean improvement is insignificant due to the variation between repeated experiments as shown the T-test result (Table 6.3).

Figure 6.13 Normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on Ti coated silicon substrates. (n=9; p=Table 4.3).

Table 6.3 P-values of the normalized bacteria growth (*E. coli*) (%) obtained from Plate Count Methods (PCM) on Ti coated silicon substrates from Figure 6.13.

	Control	Peptide A	Peptide B	Peptide C	Peptide D	Peptide E	Peptide F
Control	1.000	<0.001	0.003	<0.001	<0.001	<0.001	<0.001
Peptide A	<0.001	1.000	0.387	0.018	0.020	0.002	0.007
Peptide B	0.003	0.387	1.000	0.010	0.011	0.002	0.005
Peptide C	<0.001	0.018	0.010	1.000	0.868	0.007	0.084
Peptide D	<0.001	0.020	0.011	0.868	1.000	0.007	0.074
Peptide E	<0.001	0.002	0.002	0.007	0.007	1.000	0.258
Peptide F	<0.001	0.007	0.005	0.084	0.074	0.258	1.000

These results revealed that the following peptide configuration achieved the best antifouling performance, namely: (1) linear chain peptide monolayer with Lys adjacent to the DOPA for better adhesion, (2) linear fluorinated N-terminal to improve the packing density of the film by straightening the peptide chain and (3) DOPA positioned at the C-terminal.

In order to have a better understand the antifouling mechanism, the best antifouling performance surface coated with peptide E is stained with crystal violet and a live/dead kit. The tests were conducted on the control substrate and substrate coated with peptide E. Figure 6.14(A) - 6.15(A) showed the optical microscopic images of the crystal violet stained surface. From the images, it was observed that the amount of the bacteria adhered to the surfaces indeed consistently reduced on the coated samples as compared to the control. This result correlates with the bacterial count obtained from the PCM (Figure 6.13). Figure 6.14(B) - 6.15(B) are the fluorescent microscope images of the live dead kit stained surface. These results were consistent with the optical microscope images and the bacterial counts. The peptide E coated surface shows a substantial decrease in the bacteria

adhered on the surface as compared to the control. Results have also indicated that the amounts of dead bacteria on all peptide-coated surfaces were not substantially high. Therefore, with these optical microscope images, it can be concluded that the reduction in the bacterial count was due to the antifouling properties and not because of the antibacterial properties of the coating. One of the possible explanations for the absence of antibacterial properties in peptide single Lys amino acids within the peptide chain, whereas the antibacterial properties of Lys-based peptides reported usually consist of numerous lysine amino acids repeating units. [25]

Figure 6.14 Optical microscopy images (50x) of crystal violet stained substrate (A) Bare titanium substrate. Fluorescence microscopy image (50x) of Live/Dead® assay stained substrate (B) Bare titanium substrate. (Fluorescence Microscopy Image: Red indicate dead E. coli; Green indicate live E. coli)

Figure 6.15 Optical microscopy images (50x) of crystal violet stained substrate (A) Phe(4-F)-Lys-DOPA [E] coated titanium substrate. Fluorescence microscopy image (50x) of Live/Dead® assay stained substrate (B) Phe(4-F)-Lys-DOPA [E] coated titanium substrate. (Fluorescence Microscopy Image: Red indicate dead *E. coli*; Green indicate live *E. coli*)

6.4 Conclusion

This work has provided insights on the different antifouling properties of the peptides when manipulating the positions of the amino acids within the peptide chains. It offers an important milestone for future peptide antifouling research. The antifouling performance of the peptides chain was not solely dependent on the properties of each amino acids but the sequencing of the amino acids within the peptide chain. Based on the results obtained, the following are the suggested configuration for optimum antifouling by i) positioning lysine adjacent to the DOPA moiety in the linear chain peptide for better adhesion, ii) linear fluorinated end terminal to improve the packing density of the film by straightening the peptide chain, and iii) positioning DOPA at the C-terminal.

A new method of improving the antifouling performance by 30% has been devised by manipulating the position of the amino acids of the same chemical composition peptide chain. This chapter highlighted the importance of rationale design and position of the individual amino acids within a peptide chain for which is useful in future peptide research studies.

This passive approach of preventing biofouling through self-assembly coating is more suitable for biomedical implants application as tissue growth around the implants is desired to achieve stability and functionality. Chapter 4 and 5 reports on the active approach where such applications might not be suitable due to the separation gas layer induced by the electrochemical reaction which physically prevents the adhesion of molecules.

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Chapter 7

Thesis Conclusion and General Outlook

In this chapter, the conclusion, general outlook and future work are presented. Three novel methods in both active and passive approaches of deterring biofouling have been investigated. The novel active approach of using electrochemical reaction has given the community a new approach of deterring biofouling. The investigation on the importance of the position of amino acid within a peptide has also provided a guideline for future peptide studies. Future works based the plans for industrial commercialization for the active electrochemical approach is proposed. Future works based on longer peptides with proposed positioning guidelines and random/alternate arranged are also suggested.

7.1 Thesis Conclusion

This thesis is dedicated to address the issue of biofouling. Biofouling is termed as the accumulation of unwanted living organism on a wetted surface. The process is separated into 2 stages where the first stage is micro-fouling stage where bacteria will adhere and biofilm formation will occur and the second stage is the macro-fouling where larger organisms will adhere. This thesis focuses mainly on deterring of biofilm formation which prevents the first stage of biofouling so that subsequent stages will not occur. Due to the different areas of applications, this thesis provides both active and passive approaches which deter the colonization of bacteria. Appropriate techniques were used to characterize and analyze the results to support the proposed mechanisms.

7.1.1 Active antifouling approach via electrochemical reaction to sustain a solid-gas-liquid interface

The objective of this study is to create a sustainable solid-gas-liquid interface which prevents the micro-organism from adhering onto the surface. Super-hydrophobic or hydrophobic surfaces have the ability to entrap air in micro- and nano-sized pores on the surface when immersed in liquids. This thin air film which creates the solid-gas interface has a short sustainable period as it will eventually be displaced off by the micro-organism. [1] Electrochemical reaction has the ability to generate either hydrogen or oxygen gas bubbles through hydrolysis of water molecules on the surface of the conductive substrate.

In this study, a unique method of deterring biofouling has been realised. It is through the incorporation of electrochemical reaction with the application of low voltage square waveform pulses on the surface to create a sustainable solid-gas-liquid interface for antifouling. This solution has the ability to prevent the adhesion of 99.5% bacteria on the surface. Plate count method has been used to confirm antifouling performance. Optical and fluorescents microscopy were conducted to confirm the proposed antifouling mechanism. Counter experiments to rule out the possibility of antifouling performance

via adhered ions have been performed. This method has been proven effective on various conductive substrates. This solid-gas-liquid interface via electrochemical reaction method can also be extended to oxygen evolution reaction. However, it is not advisable as the process will oxidize the substrate and corrosion will occur. In comparison with the state of the art, this is the first work that reports the creating of sustainable solid-gas-liquid interface through electrochemical reaction.

7.1.2 Porous polymer scaffold to reduce the energy expenditure for antifouling

The objective of this study is to reduce the amount of energy required to sustain the solid-liquid-gas interface. Due to the complex mechanism of bubbles formation/detachment and displacement of gas bubbles by micro-organism, a square waveform potential pulse was maintained throughout the experiment in the previous study. In order to decrease the energy consumption, the frequency or the voltage applied has to be reduced. A multilayer scaffold is proposed to have the entrapment capability of preventing the gas bubbles from detaching from the surface. This might reduce the need for frequent application of potential pulse to maintain the separation barrier between the surface and the medium.

In this study, a multilayer scaffold PVB film was fabricated on the stainless steel substrate. It has retained its antifouling performance of more than 90% with a single pulse over a period of 16 hours. It was concluded that the microstructure morphology of the fabricated PVB film has reduced the number attachment site of between the E.coli and the surface which reduces the adhesion strength. During hydrogen gas generation process on the surface of stainless steel by the electrochemical reduction, it overcomes the binding strength of the E.coli leading to the release of E.coli from the surface. An accelerated bacteria test and polymer stability test were performed to determine the prolong stability and performance of the coating. Results obtained shown no morphology change to the fabricated PVB films and the antifouling performance is consistent with previous results which have proved the effectiveness and robustness of this technique.

7.1.3 Passive antifouling approach via designing and rationalizing a new amphiphilic tripeptide coating

The objective of the study is to understand the importance of positioning of individual amino acids in the peptide chain for antifouling. Due to different applications such as biomedical implants, passive approach through the application of a coating has been extensively used to deter biofouling from occurring. Peptide is an environmentally friendly, biocompatible antifouling material that is highly sought. It is made up of amino acids and it can be easily be synthesized by the combination of different properties amino acids in an alternate or random arrangement to attain a desired functionality.

In this study, the investigation of the amphiphilic peptide that consists of three amino acids was studied. The peptide provides a self-assembly capability and is of particular interest for antifouling application. All the permutation of the different peptide combination was synthesized. The antifouling performance of the same chemical composition peptide has improved by 30% through strategic positioning of the amino acids within the peptide. The following are the suggested configuration for optimum antifouling by i) positioning lysine adjacent to the DOPA moiety in the linear chain peptide for better adhesion, ii) linear fluorinated end terminal to improve the packing density of the film by straightening the peptide chain, and iii) positioning DOPA at the C-terminal. This study offered an important milestone for future peptide antifouling research. The antifouling performance of the peptides chain was not solely dependent on the properties of each amino acids but the sequencing of the amino acids within the peptide chain.

7.1.4 General Outlook

Biofouling has been a problematic issue and till date there is no absolute solution to solve this issue.

In this thesis, both active and passive approaches studied are environmentally friendly

solutions which do not release or cause any harmful effect to the eco-systems. In chapter 4, the use of potential to create the solid-liquid-gas interface has successfully prevented 99.5% bacteria from adhering on the surface. This method is proven to be effective on other conductive substrates. In chapter 5, the multilayer polymer scaffold has successfully reduced the binding energy of the bacteria adhered on the surface. These adhered bacteria will detach off the surface during the generation of gas bubbles through electrochemical reaction. This method has been proven to be effective throughout an accelerated bacteria test and polymer stability study. This novel method could be an ideal solution to solve biofouling issues. In chapter 6, the positioning of the individual amino acid plays a crucial role in the antifouling performance. In most of the prior works, the arrangements of the amino acids used for antifouling are usually random or alternate. This report serves as the guidelines for future peptide studies.

Lastly, this thesis has contributed three novel solutions to the community where it aids in further antifouling researches.

7.2 Future Work

In chapter 5, the multilayer polymer scaffold has successfully reduced the binding energy of the bacteria adhered on the surface. These adhered bacteria will detach off the surface during the generation of gas bubbles through electrochemical reaction. Results have shown a bacteria reduction of more than 90% with one pulse of potential at the end of 16-hour incubation with bacteria.

This method might be more attractive to the industries if the following proposed further works are conducted. Firstly, biofouling is a series of process in which the formation of the biofilms might take hours to days. [2] The energy consumed can be lowered if optimization is conducted to study on the maximum number of days where the formation of biofilms can be removed by the single pulse of potential. Secondly, repeating the experiments in the actual aquatic environment is necessary as there are currently more than 4000 micro-organism in the marine habitat. [3] This study will allow scientists to

evaluate the performance in the real aquatic environment to evaluate the feasibility of commercialization. Thirdly, due to the big surface area of the marina equipment, large protection area is required. Although the formation of the multilayer polymer scaffold PVB films is simple through breath beneath method, enlarging to larger surface area might be challenging as most reported works are conducted in laboratories scale. [4-7] In order to increase the surface area of the coating, current dip-coating technique might not be ideal for the fabrication process. An alternative method is required. Lastly, due to the large surface protection area, the design of the setup has to be carefully evaluated. Although this method can be easily changed from three electrodes setup to two electrode setup, there are still various challenges which need to be addressed. The distance between both electrodes, surface area and materials of the counter electrode are potential factors which limit the performance due to the kinetic of electrochemical reaction. Further extensive researches are required to address such issues.

In chapter 6, the positioning of the individual amino acid plays a crucial role in the antifouling performance has been reported. Tripeptide which consists of three amino acids is used in the study. Therefore, an experiment could be conducted to compare the antifouling performance between the proposed guidelines with random/alternate arranged long sequence peptides. This will emphasize the importance of the positioning of individual amino acids within a peptide chain.

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APPENDIX

Figure A6.1 High-performance Liquid Chromatography [HPLC] chromatograms of Lys – Phe(4-F) – DOPA [A] peptide.

Figure A6.2 High-performance Liquid Chromatography [HPLC] chromatograms of DOPA – Phe(4-F) – Lys [B] peptide.

Figure A6.3 High-performance Liquid Chromatography [HPLC] chromatograms of Lys – DOPA – Phe(4-F) [C] peptide.

Figure A6.4 High-performance Liquid Chromatography [HPLC] chromatograms of Phe(4-F) – DOPA – Lys [D] peptide.

Figure A6.5 High-performance Liquid Chromatography [HPLC] chromatograms of Phe(4-F) – Lys – DOPA [E] peptide.

Figure A6.6 High-performance Liquid Chromatography [HPLC] chromatograms of DOPA – Lys – Phe(4-F) [F] peptide.

Figure A6.7 ¹H NMR (DMSO-d₆, 400 MHz, δppm) Lys – Phe(4-F) – DOPA [A].

¹H NMR (DMSO-d₆, 400 MHz, δppm): 8.74-8.71 (d, 2H), 8.58-8.56 (d, 1H), 8.71-8.15 (d, 1H), 8.04(bs, 3H), 7.7 (bs, 3H), 7.36 (s, 1H), 7.29-7.25 (t, 2H), 7.1-7.06 (t, 3H), 6.63-6.59 (m, 2H), 6.49-6.47 (d, 1H), 4.56-4.41 (m, 1H), 4.35-4.3 (m, 1H), 3.7 (bs, 1H), 3.04-2.99 (m, 1H), 2.83-2.62 (m, 6H), 1.69-1.64 (m, 2H), 1.54-1.46 (m, 2H), 1.32-1.27 (m, 2H)

Figure A6.8 ¹H NMR (DMSO-d₆, 400 MHz, δppm) DOPA – Phe(4-F) – Lys [B].

¹H NMR (DMSO-d₆, 400 MHz, δppm): 8.95 (s, 1H), 8.83 (s, 1H), 8.76-8.74 (d, 1H), 8.24-8.22 (d, 1H), 7.91 (bs, 3H), 7.7 (bs, 3H), 7.33-7.28 (m, 3H), 7.11-7.07 (m, 3H), 6.68-6.64 (m, 2H), 6.51-6.49 (d, 1H), 4.62-4.57 (m, 1H), 4.22-4.16 (q, 1H), 3.86 (bs, 1H), 3.08-2.95 (m, 2H), 2.85-2.74 (m, 6H), 2.67-2.57 (m, 1H), 1.7-1.63 (m, 1H), 1.55-1.52 (m, 3H), 1.33-1.27 (m, 2H)

Figure A6.9 ¹H NMR (DMSO-d₆, 400 MHz, δppm) Lys – DOPA – Phe(4-F) [C].

¹H NMR (DMSO-d₆, 400 MHz, δppm): 8.79 (s, 1H), 8.7 (s, 1H), 8.51-8.49 (d, 1H), 8.25-8.23 (d, 1H), 8.03 (bs, 3H), 7.74 (bs, 3H), 7.36 (bs, 1H), 7.26-7.23 (dd, 2H), 7.12-7.05 (dd, 2H), 6.68-6.67 (1, 2H), 6.61-6.59 (d, 1H), 6.52-6.49 (dd, 1H), 4.5-4.44 (m, 1H), 4.42-4.37 (m, 1H), 3.67 (bs, 1H), 3.01-2.96 (m, 1H), 2.87-2.78 (m, 2H), 2.78-2.67 (m, 2H), 2.57-2.52 (m, 1H), 1.67-1.62 (m, 2H), 1.51-1.44 (m, 2H), 1.28-1.22 (m, 2H)

Figure A6.10 ¹H NMR (DMSO-d₆, 400 MHz, δppm) Phe(4-F) – DOPA – Lys [D].

¹H NMR (DMSO-d₆, 400 MHz, δppm): 8.81 (s, 1H), 8.71 (s, 2H), 8.2-8.18 (d, 1H), 8.25-8.23 (d, 1H), 8.01 (bs, 3H), 7.73 (bs, 3H), 7.31-7.28 (m, 3H), 7.15-7.1 (t, 2H), 7.08 (s, 1H), 6.7-6.69 (m, 1H), 6.63-6.61 (d, 1H), 6.55-6.52 (dd, 1H), 4.54-4.48 (m, 1H), 4.22-4.16 (m, 1H), 3.98-3.94 (m, 1H), 3.15-3.11 (dd, 1H), 2.93-2.85 (m, 2H), 2.75-2.73 (m, 2H), 2.67-2.55 (m, 1H), 1.7-1.65 (m, 1H), 1.57-1.5 (m, 3H), 1.32-1.28 (m, 2H)

Figure A6.11 ¹H NMR (DMSO-d₆, 400 MHz, δppm) Phe(4-F) – Lys – DOPA [E].

¹H NMR (DMSO-d₆, 400 MHz, δppm): 8.73-8.68 (d, 2H), 8.61-8.59 (d, 1H), 8.11 (bs, 3H), 8.04-8.02 (d, 1H), 7.76 (bs, 3H), 7.45 (s, 1H), 7.25-7.22 (t, 2H), 7.09-7.04 (t, 3H), 6.64 (s, 1H), 6.61-6.59 (d, 1H), 6.5-6.48 (d, 1H), 4.37-4.29 (m, 2H), 4.02 (bs, 1H), 3.08-3.04 (dd, 1H), 2.9-2.73 (m, 4H), 1.62 (bs, 1H), 1.6-1.5 (m, 3H), 1.31-1.25 (m, 2H).

Figure A6.12 ¹H NMR (DMSO-d₆, 400 MHz, δppm) DOPA – Lys – Phe(4-F) [F].

¹H NMR (DMSO-d₆, 400 MHz, δppm): 8.94 (s, 1H), 8.84 (s, 1H), 8.63-8.61 (d, 1H), 8.12-8.1 (d, 1H), 7.95 (bs, 3H), 7.76 (bs, 3H), 7.52 (s, 1H), 7.09-7.04 (t, 3H), 7.27-7.24 (dd, 2H), 7.13 (bs, 1H), 7.08-7.03 (t, 2H), 6.68-6.64 (m, 2H), 6.5-6.47 (dd, 1H), 4.48-4.43 (m, 1H), 4.31-4.28 (m, 1H), 3.89 (bs, 1H), 3.02-2.97 (dd, 1H), 2.91-2.73 (m, 4H), 2.61-2.55 (m, 1H), 1.66-1.57 (m, 1H), 1.54-1.49 (m, 3H), 1.3-1.25 (m, 2H).

Figure A6.13 ¹³C NMR (DMSO-d₆, 125 MHz, δppm) Lys – Phe(4-F) – DOPA [A].

¹³C NMR (DMSO-d₆, 125 MHz, δppm): 172.8, 170.3, 168.5, 159.8, 158.1, 157.8, 144.8, 143.7, 131, 128.4, 120, 114.9, 54.3, 51.8, 26.5, 21

Figure A6.14 ¹³C NMR (DMSO-d6, 125 MHz, δppm) DOPA – Phe(4-F) – Lys [B].

¹³C NMR (DMSO-d6, 125 MHz, δppm): 173.1, 170.1, 168.2, 162.3, 159.9, 157.7, 145.2, 144.6, 131.8, 125.3, 114.9, 52.2, 35.8, 30.7, 26.7, 22.2

Figure A6.15 ¹³C NMR (DMSO-d₆, 125 MHz, δppm) Lys – DOPA – Phe(4-F) [C].

¹³C NMR (DMSO-d₆, 125 MHz, δppm): 172.3, 170.8, 159.9, 158, 157.8, 144.9, 143.8, 131, 128.2, 119.8, 116.6, 114.7, 114.6, 51.7, 28.4, 20.8

Figure A6.16 ¹³C NMR (DMSO-d₆, 125 MHz, δppm) Phe(4-F) – DOPA – Lys [D].

¹³C NMR (DMSO-d₆, 125 MHz, δppm): 173.1, 170.7, 160.5, 158, 157.8, 143.8, 131.6, 128.2, 118.5, 116.6, 115.2, 115.1, 54.6, 52.2, 35.7, 31.5, 26.7, 22.2

Figure A6.17 ¹³C NMR (DMSO-d₆, 125 MHz, δppm) Phe(4-F) – Lys – DOPA [E].

¹³C NMR (DMSO-d₆, 125 MHz, δppm): 172.9, 170.4, 167.7, 160.3, 158.1, 157.8, 144.8, 131.5, 128.4, 119.8, 116.7, 115.3, 52.4, 26.6, 21.9

Figure A6.18 ¹³C NMR (DMSO-d₆, 125 MHz, δppm) DOPA – Lys – Phe(4-F) [F].

¹³C NMR (DMSO-d₆, 125 MHz, δppm): 172.5, 170.6, 168.1, 159.7, 145.2, 144.5, 133.8, 130.9, 125.5, 120.2, 116.8, 114.8, 114.6, 53.6, 52.5, 31.7, 26.7, 22