Construction of controllable and reproducible biofilms through hydrogel based biofilm mimics

Zhang, Yingdan

2016

Zhang, Y. (2016). Construction of controllable and reproducible biofilms through hydrogel based biofilm mimics. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/69406>

<https://doi.org/10.32657/10356/69406>

**CONSTRUCTION OF CONTROLLABLE AND
REPRODUCIBLE BIOFILMS THROUGH
HYDROGEL BASED BIOFILM MIMICS**

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**Interdisciplinary Graduate School
Singapore Centre for Environmental Life Sciences Engineering**

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

2016

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor Prof Yehuda Cohen and co-supervisor Cao Bin for their mentorship and guidance throughout my research. Their knowledge and expertise in the field of biofilms and environmental microbial biotechnology has rendered me great support. I must also thank them for the freedom they have given me during the course of my research to explore concepts and hypotheses. I would also like to express my deepest appreciation to them for introducing me to the cross disciplinary research. Their encouragement and enthusiasms for my work have inspired me to work towards the various goals of my project.

I would like to take this opportunity to express my deep gratitude towards my mentor Prof Stefan Wuerztz for his continuous support in the long journey.

I am also extremely grateful to Assist Prof Yang Liang and his group in SCELSE, Singapore for their valuable contribution to the work, especially in transcriptomic analysis and genetic engineering related work. It is also a great pleasure to extend my sincere gratitude to Assist Prof Kang Yuejun and Assist Prof Zhang Yilei for the valuable guidance and collaboration, especially in the microfluidic related research.

A very special thanks to the group members Krishnakumar, Anee, Yichao, Manisha, Calvin, Yidan, Ding, Daphne, Amit, Sudha, Dr. Liu Weijie and other fellow colleagues for their immense support in my research. I could not have carried out this research smoothly without their suggestions and assistance.

Many thanks must also be given to all my colleagues in Singapore Centre on Environmental Life Sciences Engineering (SCELSE) for their friendships, constructive suggestions and endless supports. The assistance provided by the technical and administration staff in SCELSE is also well appreciated.

Heartful thanks to my family for always being there for me and listening to my issues. Without their encouragement and support, it would not have been possible for me to make it this far in my education.

Finally, I would like to acknowledge the financial support from National Research Foundation and Ministry of Education Singapore under its Research Centre of Excellence Program, and Nanyang research scholarship for the year 2012-2016.

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
µM	Micromolar concentration
µm	Micrometre
µmoles	Micromoles
3-CA	3-chloroaniline
AHL	Acyl homoserine lactone
BCA	Bicinchoninic acid
c-di-GMP	Cyclic di-guanylate monophosphate
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
CV	Crystal violet
d	Time in days
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
eDNA	Extracellular deoxyribonucleic acid
EPS	Extracellular polymeric substances
g	Gram
GFP	Green fluorescent protein
h	Time in hour
kDa	Kilo Dalton
L	Litre
LB	Luria Bertani medium
M	Molar concentration
M9	M9 minimal medium
min	Time in minute
mg	Milligram
mL	Millilitre
mM	Millimolar concentration

mm	Millimetre
M1	Modified M1 medium
mmoles	Milimole
NCBI	National Center for Biotechnology Information
nm	Nanometer
OD	Optical density
°C	Degree celcius
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acids
RPKM	Reads Per Kilobase of transcript per Million mapped reads
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acids
s	Time in seconds
v/v	Volume by volume
w/v	Weight by volume

LIST OF PUBLICATIONS

Journal papers

Zhang Y, Ng CK, Cohen Y, Cao B* (2014) Cell growth and protein expression of *Shewanella oneidensis* in biofilms and hydrogel-entrapped cultures. *Molecular BioSystems* 10(5): 1035-1042

Huang S, Liu S, Wang K, Yang C, Luo Y, **Zhang Y**, Cao B, Kang Y, Wang M (2015) Highly fluorescent and bioresorbable polymeric nanoparticles with enhanced photostability for cell imaging. *Nanoscale* 7(3): 889-895.

Zhao C, Wu J, Ding Y, Wang VB, **Zhang Y**, Kjelleberg S, Loo J, Cao B*, Zhang Q* (2015) Hybrid conducting biofilm with built-in bacteria for high-performance microbial fuel cells. *ChemElectroChem* 2(5): 654-658.

Sivakumar K, Mukherjee M, Cheng H-I, **Zhang Y**, Ji L, Cao B* (2015) Surface display of roGFP for monitoring redox status of extracellular microenvironments in *Shewanella oneidensis* biofilms. *Biotechnology and Bioengineering* 112(3): 512-520.

Yang C, Liu H, **Zhang Y**, Xu Z, Wang C, Cao B, Wang M (2016) Hydrophobic-sheath segregated macromolecular fluorophores: Colloidal nanoparticles of polycaprolactone-grafted conjugated polymers with bright far-red/near-infrared emission for biological imaging. *Biomacromolecules* 17: 1673-1683.

Manuscripts in preparation

Zhang Y, Devi S, Mohanty A, Cohen Y, Cao B* “Comparison of High Quality RNA Extraction Methods from Ca-alginate Entrapped Bacterial Cultures”

Zhang Y, Devi S, Ding Y, Cohen Y, Yang L, Cao B* “Global profiling of acetyl-HSL quorum sensing target genes of *Pseudomonas aeruginosa* mimetic biofilm”

Zhang Y, Li C, Wu Y, Zhang Y, Kang Y, Cohen Y, Cao B* “Microfluidic Gradient Generator-Flow Chamber as a Novel Tool to Study Biofilm Development in Defined Chemical Gradients”

Conference presentations

Zhang Y, Cohen Y, Cao B* (2014). Engineering Artificial Extracellular Matrix for Biofilm-based Biocatalysis. 14th AIChE Annual Meeting, Atlanta, GA, USA (Oral presentation)

Zhang Y, Cohen Y, Cao B* (2016). Microfluidic gradient generator-flow cell system as a novel tool to study biofilm development and control. 251th ACS National Meeting & Exposition, San Diego, CA, USA (Oral presentation).

Zhang Y, Cohen Y, Cao B* (2016). A novel microfluidic gradient-generation flow cell for biofilm study. Biofilms7 Conference, Porto, Portugal (Poster presentation).

ABSTRACT

The surface-associated growth of microbial populations / communities has been revealed as the most ubiquitous lifestyle of microorganisms, which is usually referred to as biofilm. In biofilms, cells are encased within a self-produced matrix of extracellular polymeric substances (EPS). Biofilm matrix is highly structurally and physicochemically heterogeneous. The EPS matrix enhances bacterial tolerance to environmental stresses and mechanical and metabolic stability, rendering biofilms a promising workhorse in industrial applications. However, the heterogeneity of biofilm structures and metabolic activities together with uncontrollable biofilm dispersal poses a great challenge to the industrial application of biofilm-based bioprocesses. To enable the control of biofilm-based processes, this project is designed and organized to develop a platform towards controllable and reproducible biofilm development and biofilm-based bioprocesses. In this work, we demonstrated the concept of biofilm mimics supported by hydrogel matrix and evaluated the effects on cells by the artificial matrix in protein level. And we explored the potential of matrix functionalization by introducing signaling or functional molecules in the hydrogel matrix. Further, we designed a new device which was able to generate controllable and reproducible chemical gradients for biofilm development. Taken together, we successfully established the platform towards the reproducible and controllable biofilms and biofilm-based bioprocesses.

First, by using hydrogel as artificial matrix, we developed biofilm mimics and elucidated the responses of bacterial cultures to the artificial entrapment. Using calcium-alginate as a model hydrogel matrix and *Shewanella oneidensis* as a model organism, bacterial colonies were developed within the artificial matrix. The growth, surface properties and protein expression of the biofilm mimics were evaluated, in comparison with the flow cell biofilms developed by the same strain. Briefly, there were no significant differences regarding both growth and surface properties. The proteomic analysis provided basic

information on bacterial activities, such as ion uptake, energy metabolism and eDNA synthesis, advancing the understanding on bacterial responses to the artificial matrix and to the microenvironments provided by the matrix. This study showed the great potential to utilize the bacterial populations / communities developed within the artificial matrix as an alternative to flow cell biofilms.

To better regulate the performance of hydrogel matrix supported biofilm mimics, we explored two approaches: (1) regulation through cell communication systems and (2) mediation through environmental chemical cues. And to better understand biofilm mimics-based responses to the regulation approaches, we explored and optimized the method of high quality RNA extraction from biofilm mimics for downstream transcriptomic analysis. Further, we investigated the responses of biofilm mimics to synthesized quorum sensing molecules using *Pseudomonas aeruginosa* and its mutated strain as model organism through transcriptomic analysis. We compared the quorum sensing regulated genes in biofilm mimics to those reported in biofilms, facilitating the understanding of QS regulation in the artificial matrix.

To study biofilm mimics responses to highly dynamic environmental cues, we developed a high throughput platform of fluidic chamber which can generate defined and reproducible chemical gradients. We explored the application of this device in the biofilm development study using *S. oneidensis* as model organism, showing the biofilm formation trend along chemical gradients in the chamber. Meanwhile, we showed the great potential to study biofilm communities by using this gradient-generator flow cell system with two strains of *Comamonas testosteroni* as model organisms. Furthermore, this chamber can be integrated with the artificial matrix to generate reproducible external signals for biofilm mimics regulation, which will be explored in future work.

1.1 Background

The surface-associated growth mode of microorganisms is revealed to be the most ubiquitous microbial lifestyle, where cells physically cluster in populations within a self-produced complex matrix. This growth mode is commonly referred to as biofilm, which is of great importance to ecosystems, clinical and industrial settings. Biofilm contributes substantially to energy flow, geochemical cycling of nutrients and organic matter, surface conditioning and the health of natural environments (Battin et al. 2003). On one hand, biofilm is found to be a major source of environmental contaminant, such as in potable water systems (Flemming 2002; Viau et al. 2012). Biofilm-associated infections have been reported as one of the major challenges in clinical settings (del Pozo and Patel 2007; Donlan and Costerton 2002). On the other hand, biofilms have shown great potential in industrial applications as biocatalysts for biocatalysis and wastewater treatment, due to their robustness and ability to sustain metabolic activities even in harsh environments (Gross et al. 2013; Gross et al. 2010; Venkata Mohan et al. 2007).

The significance of biofilms originates from its surface-associated growth manner, where microbial cells are encased within a highly structured matrix (Stoodley et al. 2002). The biofilm matrix is mainly composed of self-produced extracellular polymeric substances (EPS), in which exopolysaccharides, proteins and nucleotides are the major components (Flemming and Wingender 2010). The EPS matrix supports the biofilm architecture and has a profound impact on the exchange of nutrients and wastes in and out of biofilm (Dirk de Beer 1994; Mann and Wozniak 2012). Moreover, the EPS matrix contributes to the immediate microenvironments of encased cells, which show highly physicochemical heterogeneity. Hence, cells in biofilm differ significantly from their planktonic counterparts with respect to lifecycle and physiological properties (Pamp et al. 2009). The

biofilm cells collaborate metabolically and respond to the heterogeneous environmental conditions. The structural and metabolic heterogeneity within a biofilm advances the application of biofilm-based bioprocesses, such as biocatalysis and wastewater treatment (Costerton et al. 1994; Halan et al. 2012). Meanwhile, the self-immobilized lifestyle of biofilm protects the encased cells from external stresses such as exposure to antibiotics, high shear forces and extreme pH and temperature conditions (Pamp et al. 2009). The biofilms are thereafter proposed to be a promising candidate in engineering industry, for biocatalysis and bioremediation.

1.2 Research gaps and challenges

Though biofilms have great potential in industrial applications, commercialization of biofilm-based bioprocesses is still at an infant stage. There are several major issues that impede biofilms to be widely applied in industries, especially in water and wastewater treatment.

The biofilm-based metabolic activities are regulated both through intrinsic genetic systems and by sensing and responding to external signals / cues (Boles et al. 2004; Forde et al. 2004). The efficiencies of some metabolic activities are time-dependent, which are tightly associated with biofilm developmental stages. Especially, the growth stages of single microcolonies in a biofilm community are highly dynamic, resulting in the vastly heterogeneous metabolic activities within the community. As a consequence, the genetically regulated lifecycle limits the long-term operation and efficiency of biofilm-based bioprocesses (Kerr et al. 2002). Meanwhile, the biofilm-based processes are affected by the physicochemical heterogeneity of the EPS matrix. For instance, the diffusion limitation and adsorption of substrates within the EPS matrix are significantly affected by the physicochemical properties of the EPS components, which further influence the biofilm microenvironments. Moreover, biofilm dispersal upon exposure to

harsh environment significantly influences the biofilm architecture and hence, biofilm-based bioprocesses (Gross et al. 2007). The high degree of structural and metabolic heterogeneity coupled with chances of biofilm dispersal when exposed to unfavorable environments, render the control of biofilm-based applications challenging.

The facts outlined here encourage the broader investigation of approaches to understand and control biofilm-based bioprocesses. Among these issues, the structural heterogeneity of the EPS matrix, as well as biofilm metabolic heterogeneity, is considered to be the key challenge toward long-term industrial application. However, the regulation of the EPS heterogeneity, in order to control biofilms activities, remains the critical knowledge gap.

1.3 Objectives

To address the key question outlined in the previous section, this study is designed and organized with the objective to develop a platform to regulate biofilm and biofilm-based bioprocesses. This study was inspired by tissue engineering which utilizes artificial matrix instead of self-generated extracellular matrix to limit the heterogeneity of tissue developing conditions. The study has focused on biofilm development through the approach of biofilm mimics in hydrogel based artificial matrix. In addition, this study has explored the strategy of artificial matrix functionalization for enhanced regulation of biofilm-based activities (**Figure 1.1**). The central hypothesis of this work is that we can develop controllable and reproducible biofilms and biofilm-based bioprocesses by using hydrogel as artificial matrix and by regulating the environment.

The specific tasks are described as follows:

- To apply hydrogel matrix for biofilm mimics, and to evaluate the development of biofilm mimics in the matrix;
- To establish proper and standardized method for biofilm mimics analysis;

- To functionalize the hydrogel matrix with bioactive molecules, regulating biofilm mimics-based activities;
- To generate controllable and defined heterogeneous environment for biofilm mimics, investigating and mediating their responses to environmental cues.

Figure 1.1 Illustration of the objective and specific tasks of this study

In this study, we utilized *Shewanella oneidensis*, *Pseudomonas aeruginosa* and *Comamonas testosteroni* as model organisms. The biofilm of *S. oneidensis* has been well studied as model organism for environmental related research such as bio-reduction of heavy metals and solid electrodes (Cao et al. 2011, Fredrickson et al. 2008); *P. aeruginosa* is a well characterized opportunistic pathogen in plants and human, whose biofilm is used as a model for infection studies (Barraud et al. 2006, de Kievit 2009); and *C. testosteroni* has been isolated and identified in wastewater treatment systems and contaminated environment, indicating its important environmental implications (Wu et al. 2015). These three microorganisms are well studied in our lab and their genome sequences have been well annotated.

1.4 Scope

Chapter 1: A brief background of biofilm and its heterogeneity are outlined in this chapter. It lays emphasis on the importance of controllable biofilm and biofilm-based bioprocesses. The objective and scope of the work are also discussed in detail.

Chapter 2: Literature review is described for the whole thesis. Composition and function of EPS matrix is firstly outlined in detail. Heterogeneity of biofilms in terms of intrinsic, metabolic and structural dynamics is then described. Two major regulatory networks of biofilm in terms of quorum sensing and c-di-GMP networks are also discussed. The commonly applied devices for biofilm study are included. Moreover, this chapter outlines the relevant development and application of hydrogels.

Chapter 3: Biofilms mimics by encapsulating and developing *S. oneidensis* MR1 colonies within calcium-alginate hydrogel matrix is illustrated and compared with flow-cell-biofilms. We studied the morphology, growth rate, and the protein expression of biofilm mimics for a better understanding of biological impacts from artificial matrix.

Chapter 4: Method optimization of high quality RNA extraction from biofilm mimics in artificial matrix. We compared three commonly applied methods in biofilm study, and optimized the protocol for high quality RNA extraction.

Chapter 5: Regulation of hydrogel-based biofilm mimics is presented through the quorum sensing system in *P. aeruginosa*. We profiled the QS target genes in the artificial matrix-encased biofilm mimics, to better understand the QS regulation networks in our system. In addition, we identified the impact of external QS signals on bacterial gene expressions in hydrogel matrix.

Chapter 6: Regulation of biofilm development is investigated by controlling the environmental chemical conditions. We developed a microfluidic gradient-generation flow cell system, enabling the generation of defined and reproducible chemical gradients for biofilm study.

Chapter 7: Summary of this thesis work is provided along with proposed future recommendations.

2.1 Background on biofilm

The bacterial ability to physically cluster in populations has been noted for decades in aquatic systems (Edwards and Kjellerup 2013, Hall-Stoodley et al. 2004), such as marine environment, river and drinking water systems, and also on tissue surfaces (Costerton et al. 1987; Costerton et al. 1999; Edwards and Kjellerup 2013; Hall-Stoodley et al. 2004). Only recently, these multicellular bacterial communities have gained large scale attention as functional and collaborative clusters on interfaces, where cells communicate with neighbours and respond to environmental conditions (Stanley and Lazazzera 2004). This lifestyle of microbial cells has been referred to as biofilm, which is considered to be the most resilient and ubiquitous lifestyle on earth (Cao et al. 2012; Kjelleberg and Givskov 2007).

In general, biofilm development involves four typical stages—initial attachment, early maturation, maturation and dispersion (**Figure 2.1**) (Hall-Stoodley et al. 2004; O'Toole et al. 2000). The biofilm formation is initiated by the attachment of free living cells onto an interface, where cells aggregate and produce extracellular polymers to form a permanent bonding with the substratum (Stoodley et al. 2002). Mature biofilm with stratified and complex architecture is developed on the attached cell aggregates. Further, detachment of cells, both active and passive, occurs to release free living cells for new life cycles (Costerton et al. 1994).

Figure 2.1 Schematic illustration of biofilm lifecycle (Stoodley, Sauer et al. 2002). A. Adhesion of planktonic cells onto interfaces is regarded as initiation of a biofilm lifecycle; B. Early development of biofilm from initial adhered cell aggregates; C. Mature biofilm architecture; D. Active and passive dispersion of cells from biofilm structure.

The biofilms show great resistance and tolerance to antimicrobials, toxins and harsh physical conditions, which contribute to its ubiquitousness in natural, industrial, and clinical systems (Hall-Stoodley et al. 2004). For instance, biofilm developed by pathogenic microorganisms on tissues or clinical devices is a major concern due to its strong antibiotic resistance (Kjelleberg and Givskov 2007). Besides, biofilm-based biofouling on industrial systems is a bottleneck for equipment maintenance (Simoes et al. 2007). On the other hand, the self-immobilized growth mode with enhanced tolerance to toxic chemicals benefit the biofilms to serve as biocatalysts (Halan et al. 2012). Meanwhile, biofilm is a critical element in ecosystem, which has profound impact on geochemical cycling of carbon and inorganic nutrients (Hall-Stoodley et al. 2004).

The strong resistance and tolerance of biofilms originate from the presence of self-generated matrix of extracellular polymeric substances (EPS) (Branda et al. 2005; Cao et al. 2011c; Flemming and Wingender 2010). The EPS matrix provides the microhabitat, where cells can differentiate and collaborate among each other. The biofilm heterogeneity

is observed as a result of the stratified distribution of cells within the EPS matrix (Flemming and Wingender 2010).

2.1.1 Biofilm matrix

Biofilm matrix is one major component of biofilm, accounting for over 90% of the overall biofilm dry mass, whereas microbial cells encased within the matrix only account for less than 10% (Flemming and Wingender 2010). Biofilm matrix (**Figure 2.2**) is mainly composed of bacterial secreted EPS, in which exopolysaccharides, proteins, and extracellular DNA (eDNAs) are the three major components (Cao et al. 2011; Flemming and Wingender 2010). The EPS matrix immobilize cells within the hydrated microenvironments (Flemming et al. 2007).

Figure 2.2 **a**, “A model of a bacterial biofilm attached to a solid surface”. **b**, “The major matrix components — polysaccharides, proteins and DNA — are distributed between the cells in a non-homogeneous pattern, setting up differences between regions of the matrix.” **c**, “The classes of weak physicochemical interactions and the entanglement of biopolymers that dominate the stability of the EPS matrix.” **d**, “A molecular modelling simulation of the interaction between the exopolysaccharide alginate (right) and the extracellular enzyme lipase (left) of *Pseudomonas aeruginosa* in aqueous solution.” The figure is reprinted from (Flemming, Hans-Curt, and Jost Wingender. *Nature Reviews Microbiology* 8.9 (2010): 623-633.) with permission from Nature Publishing Group.

- ***Exopolysaccharides***

Extracellular polysaccharides have been reported to be one major component of biofilm matrix, which are essentially very long chain molecules with a molecular mass between 0.5 to 2×10^6 Da (Flemming and Wingender 2010; Wingender et al. 2000). Most of the exopolysaccharides in EPS matrix are heteropolysaccharides with a mixture of different sugar residues, and are polyanionic due to the presence of uronic acids or ketal linked pyruvate (Flemming et al. 2007; Xiao and Koo 2010). The composition and physicochemical properties of exopolysaccharides vary greatly, which are supposed to be dependent on strains, nutrient supply, surface properties, growth stages, etc. (Ghafoor et al. 2011).

The exopolysaccharides can interact with a wide range of other molecules in different ways, such as non-covalent interactions or through multivalent cationic bridges (Flemming et al. 2000). The highly heterogeneous nature of exopolysaccharides as well as these intermolecular interactions contribute to the highly variant physical nature of EPS matrix (Sutherland 2001).

The production of exopolysaccharides is an important strategy for bacterial survival, even though it is not synonymous with bacterial adhesion and does not advance bacterial growth in some circumstances (Vuong et al. 2004). The exopolysaccharides are the fundamental structural component of biofilm matrix, and are responsible for the mechanical stability of biofilms (Flemming, Neu et al. 2007). The long chain exopolysaccharides promote the adhesion of cells onto a wide range of substratum. The highly hydrated exopolysaccharide network facilitates the maintenance of cells under moist environment (Flemming et al. 2007; Flemming and Wingender 2010). Exopolysaccharides are also regarded as a nutrient reservoir, where low-molecular weight

products from exopolysaccharide degradation serve as potential carbon and energy source for the encased cells (Branda et al. 2005).

One example of bacterial exopolysaccharides that has been well investigated is the bacterial alginate, which is also an important virulence factor. The bacterial alginate is an unbranched heteropolymer with a high molecular weight, composed of randomly arranged β -D-mannuronate and α -L-guluronate (Franklin et al. 2011). The alginate polymers have been reported to be involved in the initiation of biofilm formation, virulence and antibiotic resistance. The biofilms impaired with alginate production are more sensitive to antibiotic treatment and host immune clearance (Franklin et al. 2011; Gordon et al. 1988). Overproduction of alginate polymers are responsible for the mucoid phenotype of biofilms, where alginate layers on top of the whole matrix protect the encased cells (Hentzer et al. 2001; Simpson et al. 1988). Mechanism of alginate synthesis and degradation involves the enzymatic conversion of D-fructose-6-phosphate into D-mannuronic acid chains, which are translocated across the periplasm (May et al. 1991; Rehman et al. 2013). The biosynthesis of alginate polymers is regulated by the secondary messenger c-di-GMP, a key regulation system in biofilm. For example, the alginate biosynthesis related genes, such as *algD*, *algK*, *algA*, *algEFG*, are encoded by the *algD* operon, which are the c-di-GMP effectors (Maleki et al. 2016).

- ***Extracellular proteins***

Extracellular proteins constitute a considerable portion of dry biofilm biomass. The extracellular proteins enhance matrix stability by interacting with cellular surfaces and other EPS components within the matrix (Flemming and Wingender 2010). For instance, the cell surface protein CdrA in *Pseudomonas aeruginosa* has been reported to crosslink with Psl polysaccharides, thereby strengthening the EPS matrix (Borlee et al. 2010). The outer membrane protein AggA in *S. oneidensis* is reported to be significantly upregulated

in biofilm-developing cells in comparison with their planktonic counterparts (Liang et al. 2010). Also, the AggA has been reported to be responsible for the secretion of biofilm matrix-associated proteins (Bap-family) (De Windt et al. 2006). Another important biofilm formation protein in *S. oneidensis* is BpfA, a large protein anchored on the membrane with a number of repeats at 100 amino acids for each repeat (Cao et al. 2011). BpfA participates in cell-to-cell and cell-to-matrix interactions as a surface adhesin, whose export to the matrix has been proposed to be under the regulation of c-di-GMP system (Wu et al. 2013).

The extracellular proteins, in natural cell aggregates such as sludge flocs, are found to be more abundant than exopolysaccharides (Flemming et al. 2007). Enzymes, which degrade both soluble polymers and insoluble particles are mainly responsible for a considerable amount of extracellular proteins (Flemming et al. 2007). These extracellular enzymes enhance nutrient supply during biofilm development by degrading large polymers into low molecular size products (Cao et al. 2011). The degradation of EPS structural components also promote the cell detachment from the biofilm matrix even if the degradation rates are usually slow and require a series of enzymes (Flemming and Wingender 2010; McDougald et al. 2011).

- ***Extracellular DNA***

Extracellular DNA (eDNA) fragments have gained interest as critical component in biofilm matrix rather than being residual materials from lysed cells (Das et al. 2010). The origin of eDNA is not clear yet, since the genetic information of eDNA is not as same as genomic DNA (Steinberger and Holden 2005). The concentration and distribution of eDNA varies significantly in biofilms of different strains. For instance, eDNA is found to be a major matrix component in *Staphylococcus aureus* biofilms, whereas a minor

component in *Staphylococcus epidermidis* biofilm matrix (Flemming and Wingender 2010). Meanwhile, the eDNA can form grid-like structures and filamentous networks. In general, the amount of eDNA and its respective localization are both highly time-dependent and biofilm growth stage related (Das et al. 2010; Vilain et al. 2009).

The most studied functions of eDNA in biofilm matrix encompass its contribution to the sticky and viscous feature of matrix and its role as intercellular connectors. The addition of DNase has been reported to limit biofilm formation and development (Whitchurch et al. 2002). Meanwhile, the extracellular DNA disseminates genetic information among different microorganisms in biofilm matrix, facilitating horizontal gene transfer (Martins et al. 2010). Moreover, evidence has been provided that eDNA enhances bacterial resistance to antimicrobial peptides by inducing the expression of related genes and by binding cations to create a cation-limited environment (Chiang et al. 2013). The addition of exogenous DNA fragments into biofilm matrix is found to increase the biofilm tolerance towards antibiotics (Harmsen et al. 2010).

- ***Functions of EPS***

The EPS matrix has been widely explored for its importance on biofilm formation and biofilm-based activities. The most significant and apparent function of biofilm matrix is to encase and support cells in a three-dimensional scaffold, maintaining the mechanical stability of biofilm (Branda et al. 2005; Hall-Stoodley et al. 2004). This function is achieved by the presence of exopolymeric networks, which mediates cell-to-cell and cell-to-surface interactions.

The EPS matrix supports the exchange of nutrients and wastes in and out of biofilms. The mass transfer of molecules depends primarily on the composition and physicochemical properties of the matrix. The studies on the diffusion of macromolecules within biofilm matrix have shown that the transport is hindered by matrix porosity and adsorption of

solutes (Peulen and Wilkinson 2011). In biofilms, the penetration of particles with sizes larger than the effective pore size of the EPS matrix is highly restricted (Bryers and Drummond 1998). The adsorption and accumulation of molecules are also affected by the charges and hydrophobicity of both matrix and molecules (Habimana et al. 2011). However, limitation in the transport of unfavorable reagents, such as antibiotics, oxidizing compounds, and ultraviolet radiation, allow the EPS matrix to shield the cells effectively (Branda et al. 2005).

Meanwhile, the entrapment of cells by biofilm matrix keep them in close proximity, hence maintaining high cell density in biofilm. As a consequence, the population dependent intercellular communication system is enhanced by the EPS matrix. Chemical gradients within the matrix is expected due to high cell density related chemical secretion, consumption and adsorption, further advancing the formation of synergistic bacterial consortia (Pamp et al. 2009). Hence, the EPS matrix ecologically advances bacterial cooperation and competition, resulting in consistent adaptation of biofilm to the environment (Mann and Wozniak 2012).

Moreover, the EPS matrix facilitates biofilm colonization and adhesion onto interfaces. The exopolysacchrides anchor cells firmly onto numerous types of substratum; the extracellular proteins bind cell surface with the polymeric matrix; and the eDNA is involved in the generation of filamentous networks and contributes to matrix adhesion (Flemming and Wingender 2010). Besides, the viscous biofilm matrix is able to maintain a high water content, facilitating the biofilm to survive water-deficient conditions and in maintaining a hydrated environment for enzymatic activities (Flemming et al. 2007).

Another critical function of biofilm matrix is its role as a rich pool of numerous nutrients and resources, such as carbon, nitrogen, genes, etc. The EPS matrix is able to adsorb

organic and inorganic compounds, which can be utilized as metabolic substrates for encased cells. These adsorbed chemicals are potentially used for bacterial growth and ion exchanges, which facilitates biofilm application in environmental engineering for detoxification (Flemming and Wingender 2010). Further, the extracellular polymers of EPS matrix serve as excess carbon and energy sources, which can be degraded by extracellular enzymes to generate short chain molecules (Mann and Wozniak 2012). And the eDNAs in the EPS matrix promotes the horizontal gene transfer among the encased cells, improving community diversity (Steinberger and Holden 2005).

Furthermore, the EPS matrix is an important biocatalytic center, benefiting from the presence of diverse extracellular enzymes (Gross et al. 2013). The chemical compounds are catalyzed by the extracellular enzymes directly without encountering membrane barrier, which improves the biocatalytic efficiency. Interaction of enzymes and polysaccharide chains promotes the accumulation of extracellular enzymes and stabilization of enzymatic activities (Wu et al. 2015).

2.1.2 Heterogeneity of biofilm

The biofilm has been revealed to be a heterogeneous microbial consortium, through approaches based on direct observation of biofilm structures, chemical profiling of biofilm matrix and transcriptomic analysis of gene expressions. The biofilm heterogeneity, with respect to both morphological features and intrinsic gene expressions, is highly strain and time-dependent. Besides, microenvironments within the EPS matrix, especially the physicochemical properties as described in previous section, are highly heterogeneous.

- ***Physicochemical heterogeneity***

The biofilm architectures, in most cases, are highly structured where cells are spatially distributed within the EPS matrix. The biofilm structure is growth-associated with significant changes being observed during the transition between different growth stages (**Figure 2.1**). The changes in biofilm architecture during development are exhibited not only in thickness, but also in composition and distribution of EPS components and chemical molecules (McDougald et al. 2011). For instance, the concentration of overall exopolysaccharides and the ratio of different exopolysaccharide compounds influence biofilm growth as well as the supply of carbon and nitrogen sources (Flemming and Wingender 2010). Moreover, the interaction and distribution of EPS components in biofilm architecture is tightly associated with the dynamic environments as mentioned previously. Furthermore, complex water channels have been reported in many biofilms, which elevates the overall biofilm heterogeneity (Hall-Stoodley et al. 2004).

The diverse physicochemical properties of biofilm matrix result in a situation where chemical distribution within the matrix also becomes heterogeneous (Mann and Wozniak 2012; Petroff et al. 2011). Chemical compounds in biofilms are transported through diffusion and convection simultaneously, which is highly associated with the nature of EPS matrix as described in the previous section. The dynamic presence of different EPS compounds, in particular the functional groups such as carboxyl and hydroxyl groups, have significant effects on chemical adsorption and diffusion (Singh et al. 2006). The interaction of EPS structural components as well as the overall biofilm thickness, also impacts chemical transport and distribution significantly (Schorer and Eisele 1997). Meanwhile, bacterial consumption or production of the chemical molecules, coupled with the dynamic transport processes, lead to various distributions in the EPS matrix. This type

of microscale heterogeneity of chemicals is prevalent, where concentration of both nutrients and metabolic products are characterized by spatial gradients within biofilm matrix (**Figure 2.3**).

Figure 2.3 Schematic illustration of chemical heterogeneity in biofilms owing to reaction-diffusion interactions: metabolic substrate diffuse from fluid into biofilms, and metabolic product are concentrated inside biofilm (Stewart and Franklin 2008).

For instance, oxygen penetration within biofilm matrix has been investigated by electrochemical oxygen probes, which has reported a decreasing trend from biofilm surface to the bottom close to substratum (Ning et al. 2012). The oxygen gradients through biofilm are found in both gaseous and submerged aqueous conditions, while the gradient patterns may vary among different types of biofilms (Borriello et al. 2004; de Beer et al. 1994). Similarly, the metabolic waste molecules tend to accumulate in the centre of the matrix, due to ineffective diffusive process. In denitrifying biofilms, the products of denitrification have been observed to accumulate in biofilm matrix, hence impeding the development of biofilm (Barraud et al. 2006).

- ***Intrinsic heterogeneity***

The adaptation strategy of biofilm-encased cells to heterogeneous local chemical environment is reflected by the alteration in intrinsic gene expressions (Xu et al. 1998). It

has been shown that bacteria in biofilms alter genetic expressions in growth-associated fashion (Hall-Stoodley and Stoodley 2002). During the shift from planktonic mode to initial attachment phase of biofilms, the transcriptomic analysis revealed a significant decrease in expression levels of motility-related genes (Waite et al. 2005). Expression levels of genes associated with biofilm formation were shown to be elevated during the transition from initial reversible attachment to irreversible attachment and maturation stage (Yeater et al. 2007). Meanwhile, a number of metabolic pathways have also been reported to be growth-associated, with differential expression levels at various developmental stages of biofilm development (Grande et al. 2014).

Moreover, dynamic gene expressions and bacterial differentiation and cooperation are expected within the same biofilm structures at different locations, though little has been investigated due to the lack of proper techniques. The DNA replication within biofilm has been reported to be of stratified pattern by using BrdU labelling (Rani et al. 2007). The highly stratified DNA synthesis indicated a spatially distributed differential growth of cells within the matrix. Another study demonstrated the generation of variants within biofilms through genetic changes and formation of distinct subpopulations (Boles et al. 2004). In this study, researchers picked biofilm colonies at different growth stages and observed the formation of different morphological colonies. The variants were shown to be heritable, suggesting occurrence of genetic changes during the formation of subpopulations. They proposed that the biofilm dynamics is in compliance with “insurance effects”, which supports the theory by ecologists that increased diversity enhances the thriving of a community.

2.2 Biofilm regulatory networks

As mentioned in the previous section, biofilm is not only a physically clustered bacterial aggregate, but a collaborative consortium, where cells interact with neighboring environment and communicate with other cells. The bacterial communication and signaling regulatory systems play a critical role in biofilm formation, virulent activities and metabolic activities. The biofilm regulatory networks in terms of intercellular communication system and intracellular signaling network will be described here in detail.

2.2.1 Quorum sensing

Quorum sensing (QS) is defined as the phenomenon of intercellular communication in response to cell density, which broadly exists in most microorganisms enabling synchronized population behavior (Parsek and Greenberg 2000; Sperandio et al. 2001). The QS system is one typical microbial intercellular communication system, through which microbial cells coordinate their gene expressions and respective biological processes in response to environmental QS signals (Rutherford and Bassler 2012; Waters and Bassler 2005). Generally, the QS signals are synthesized in bacteria, and are secreted to the extracellular environment, where signals are accumulated with proliferation of bacterial populations and increase in cell densities (Bassler and Miller 2013). At a certain threshold concentration, quorum signals are detected by cells that regulates the expression of downstream target genes (Waters and Bassler 2005).

The class of diffusible acyl-homoserine-lactones (AHL) is identified as the QS signal in more than hundreds of Gram-negative bacteria (Case et al. 2008; Chugani et al. 2012). The AHL molecules are generally synthesized by the LuxI family of synthases, while different AHLs are generated by different LuxI homologs (Parsek and Greenberg 2000). The AHL signal recognition is specific to the length of acyl side chains, ranging from C4

to C18, and the substitutions on the side chain, which also impacts the diffusivity and transport of the molecules (Fuqua et al. 2001; Ng and Bassler 2009). Short chain AHL signals are able to diffuse through bacterial membrane freely, while the export of longer chain molecules, C12-HSL as an example, requires the presence of efflux pumps such as *mexAB-oprM* (Evans et al. 1998; Pearson et al. 1999). The accumulated AHL signals act by binding to LuxR protein, which is specific and cognate to AHL synthases (Bassler and Miller 2013). Two domains are reported to be present in LuxR family members; an N-terminal AHL-binding domain and a C-terminal DNA-binding domain, enabling LuxR to interact with specific AHL signals and direct downstream gene expressions (Fuqua et al. 2001).

Figure 2.4 Schematic illustration of LasIR and RhIR QS regulations (Whiteley, Lee et al. 1999). C12-HSLs / C4-HSLs are synthesized by LasI / RhI and secreted into extracellular environment, and are able to be accepted by LasR / RhIR in receptor initiating downstream target genes. A hierarchical relationship is found between LasIR and RhIR systems.

One most studied model organism for the AHL regulation system is *Pseudomonas aeruginosa*, an opportunistic pathogen for different hosts including plants, insect, human and other mammals (Hoiby et al. 2010; K Bhardwaj et al. 2013). Two AHL QS circuits present in *P. aeruginosa* are LasI-LasR and RhlI-RhlR systems, between which a hierarchical relation exists (**Figure 2.4**). The LasIR system regulates the expression of the transcriptional activator in RhlIR system (Schuster et al. 2003; Whiteley et al. 1999).

In the LasIR-regulated QS system, product of *lasI* is responsible for the synthesis of signal N-(3-oxododecanoyl)-L-HSL (3OC12-HSL), a diffusible extracellular signal in conjunction with LasR to form LasR-3OC12HSL complex (Ng and Bassler 2009; Yong et al. 2015). The secretion of 3OC12-HSL into the external environments depends both on the molecular diffusivity and bacterial efflux pump (Case et al. 2008; Evans et al. 1998). The LasR-3OC12HSL complex further activates a series of downstream genes, including genes for virulence factors such as *lasAB*, *apr*, *toxA* and *lasI* itself (de Kievit 2009; Erickson et al. 2002). Activation of *lasI* establishes the auto inducing feed-forward loop, which regulates the 3OC12HSL synthesis (Erickson et al. 2002). Another important target of LasIR system is the *rhlI*, the other *luxI* homolog, which synthesizes butanoyl homoserine lactone (C4HSL) (Williams and Camara 2009). Complex of C4HSL and its receptor RhlR activates target genes including *rhlI* leading to another auto inducing feed-forward loop of the QS circuit (Rutherford and Bassler 2012). Other genes under the mediation of RhlI include those under *rhlAB* operon coding for rhamnosyltransferase and those responsible for virulence factors (McKnight et al. 2000; Sharma et al. 2014). Both LasIR and RhlIR QS systems in *P. aeruginosa* are reported to be responsible for inducing antibiotic resistances, whereas *luxI* deficient mutants have shown more antibiotic susceptibility than wild types (Shih and Huang 2002). The enhanced susceptibility has correlated with lower EPS production by cells with deficient QS systems in *luxI* mutants.

More recent studies have shown antibiotic resistant genes might be under regulation of QS pathways (Brooun et al. 2000; Rice et al. 2005; Shih and Huang 2002).

Transcriptomic studies have revealed the involvement of QS systems in global gene regulations of microbial communities, for example, the LuxRI QS systems in *P. aeruginosa* regulate expression levels of up to 10% of the whole genome directly or indirectly (Schuster and Greenberg 2006). Biological processes regulated by QS systems include biofilm formation, virulence, antibiotic resistance, aromatic compound degradation and denitrification (Rutherford and Bassler 2012). Numerous Gram-negative pathogens have been identified that control virulence factors by LuxIR QS circuits, for instance, *P. aeruginosa* with LasRI and RhlRI circuits, *Chromobacterium violaceum* with CviRI circuits and *Brucella melitensis* with VjbR (Bassler and Miller 2013; Schuster and Greenberg 2006). Meanwhile, a series of bacterial strains isolated from the activated sludge deployed for water and wastewater treatment plant also harbor the AHL-based QS circuits (Jiang et al. 2013; Rutherford and Bassler 2012). For instance, more than 11% of the documented aromatic-degrading bacterial populations were reported to harbor QS genes together with aromatic ring-hydroxylating dioxygenase genes (Valle et al. 2004). The denitrification activity of *P. aeruginosa* was found to be affected by *rhRI* mutations, as compared to wild type strains (Toyofuku et al. 2007).

The prevalence and fundamental roles of QS circuits in virulence and other biological processes make it possible to manipulate QS for novel antimicrobial activity and/or enhanced degradation or transformation of chemical compounds (Huang et al. 2013; Rutherford and Bassler 2012; Yong et al. 2015). Generally, two engineering strategies are designed and applied towards manipulating QS in target microbial organisms—exogenous addition/depletion of QS signal molecules and genetic engineering of the QS

regulons. Exogenous addition or depletion of QS signals can be achieved by applying synthetic AHL signals or by utilizing QS inhibitors or quorum quenching agents.

QS inhibitors (QSI) are proposed to be a novel class of antimicrobial drugs, towards which bacteria are likely to exhibit less resistance (K Bhardwaj et al. 2013). Generally, several guidelines are considered for QSI design, such as high specificity to the QS-regulators, nontoxic to the host cells, chemically stable and lack of interference with bacterial basal metabolic processes (Fulghesu et al. 2007). The inhibition of QS is performed by QSI through influencing either one or more of the following processes: AHL signal generation by synthases, detection of AHL by receptors, or activation of respective regulons, etc. (Khan et al. 2015). Recent studies have shown that, in the infection model by *P. aeruginosa*, the lethal effect on *Caenorhabditis elegans* is associated with hydrogen cyanide and phenazines, which are reported to be under the control of QS regulators (Papaioannou et al. 2009). QSI-like garlic extracts were supplied into the *P. aeruginosa* infected *C. elegans* and the killing efficacy of *C. elegans* was evaluated by slow-killing assay. The results showed that only 10% of *C. elegans* were killed by infection, whereas almost 100% lethal effects were observed in non-QSI-treated samples and samples infected by QS-overexpression mutants. Moreover, in vivo model of *Pseudomonas* infected mice, furanone was introduced as QSI into the blood to suppress the QS system (Hentzer et al. 2003). The QS level in the infected mouse lungs which was determined through fluorescent signal sensor strains reported a significant reduction of fluorescence in the presence of furanone. The results demonstrated the possibility of utilizing QSI to specifically block cell-to-cell communication in order to control bacterial virulence. The inhibition of QS levels in biofilms by QSI has shown its great potential in clinical application, since QS is a strong target for bacterial infections for therapeutic intervention (K Bhardwaj et al. 2013).

Quorum quenching agents have also gained attention for their capability to inhibit industrial biofilms, especially in biofilm-based biofouling control (Xu and Liu 2011; Yeon et al. 2009). Biofouling is one of the critical issues in industry caused by unwanted biofilm formation on membranes and equipment surfaces, which decreases membrane permeability, limits equipment lifespan, and leads to elevated energy demand (Flemming et al. 1996; Jiang et al. 2013). Researchers have used acylase as a quorum quenching agent by encapsulating the enzyme within sodium alginate hydrogel capsules to maintain better enzymatic activity (Yong et al. 2015). The supplementation of immobilized acylase in membrane bioreactors inhibited biofilm formation and enhanced membrane permeability, with no apparent side effects on performance of reactor. These observations have implicated an attractive approach for biofilm control through QS-manipulation.

Exogenous supplementation of synthetic AHLs has also been explored, aiming to enhance QS regulated bioactivities. Researchers have proposed the aromatic degradation in *Pseudomonas*-based wastewater treatment plant by introducing synthetic AHLs (Choudhary and Schmidt-Dannert 2010; Simoes et al. 2007). By adding AHLs into the activated sludge for phenol degradation, more stable degradation efficiency was achieved, when compared with the non-treated controls. Meanwhile, a significant composition shift occurred in the AHL-treated sludge community during phenol degradation. These results suggest that QS system could be utilized to enhance aromatic degradation activity as well as compositional rearrangement of biofilm communities (Valle et al. 2004).

Genetic engineering of QS-circuits in biofilms has been investigated as the other promising approach to regulate QS systems. Researchers profiled the aromatic degradation related genes under AHL regulation and observed that transcription of several genes including *catB* (the catechol-1,2-dioxygenase coding gene) are regulated by

AHL molecules (Chugani and Greenberg 2010). Thereafter, genetically engineered *Pseudomonas* strains with overexpression of the *rhl-QS* components were constructed, which exhibited enhanced efficiency in degrading phenol and benzoate (Yong and Zhong 2010; Yong and Zhong 2013). The regulation of denitrification in *P. aeruginosa* PAO1 under anaerobic conditions was also investigated. Engineered QS mutation strains were constructed to study the enzyme activities involved in denitrification pathways. The results indicated that *rhl-QS* system mediated the transcriptional regulation of a series of genes involved in denitrification such as *narkI*, *nirS*, *norC* and *nosR* (Toyofuku et al. 2007; Toyofuku et al. 2008; Yoon et al. 2002). These results have shown how manipulating QS systems facilitate the regulation of bacterial growth and denitrification activity.

2.2.2 C-di-GMP

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), a soluble molecule, has emerged as a secondary messenger for intracellular signaling system that is broadly conserved in many Gram-negative bacterial species (Hengge 2009; Schirmer and Jenal 2009). C-di-GMP was first discovered as the activator for membrane bound cellulose synthesis in *Gluconacetobacter xylinus* by Benziman's group (Romling et al. 2005). Until recently, it has been widely reported that c-di-GMP signaling system is also involved in the regulation of complex bioprocesses, such as biosynthesis of adhesion factors and exopolysaccharides, the inhibition of various forms of motility, and virulence factors in plants and animals, etc. (Boyd and O'Toole 2012; Cotter and Stibitz 2007; Fang and Gomelsky 2010). Particularly, c-di-GMP has been revealed to be a critical factor for the transition from motile planktonic lifestyle to surface-associated biofilm lifestyle in response to fluctuating environmental conditions such as pH, iron availability, oxygen

tension, light, temperature, etc. (Jenal and Malone 2006; Kalia et al. 2013; Tuckerman et al. 2009).

Figure 2.5 Schematic illustration of c-di-GMP system (McDougald, Rice et al. 2011). The c-di-GMP is synthesized from 2-GTP molecules by diguanylate cyclase (with GGDEF domain), and is degraded into pGpG and GTP by phosphodiesterase (with EAL or HD-GYP domain). The c-di-GMP regulates downstream biofilm activities, such as motility, virulence and biofilm formation. The level of c-di-GMP in bacterial cells is mediated by external signals.

In general, the c-di-GMP signaling system consists of four components (**Figure 2.5**): two enzymes that synthesize and degrade the secondary messenger, effectors that are regulated by the second messenger, the targets of the effectors that generate output (D'Argenio and Miller 2004; Sondermann et al. 2012).

C-di-GMP is produced by diguanylate cyclases (DGCs) from two GTP molecules, and it is degraded by phosphodiesterases (PDEs) into 5'-phosphoguanylyl-(3'-5')-guanine (pGpG) which is subsequently broken into two GMP molecules (Romling et al. 2005). The DGCs are featured by the GGDEF domain which is termed after the amino acid sequence motifs essential for the enzymatic activity; similarly, c-di-GMP specific PDEs

have two core amino acid motifs—EAL domain for degrading c-di-GMP to pGpG and HD-GYP domain for degrading c-di-GMP into GMP (Hengge 2009; Schirmer and Jenal 2009). Generally, the active DGC has been found to be a dimer of two subunits with GGDEF domains for binding GTP molecules. The active site is located at the interface between the two subunits (Jenal and Malone 2006; Ryjenkov et al. 2005). An active and c-di-GMP specific PDE with EAL domain is a monomeric enzyme which can linearize c-di-GMP molecules to 5'-pGpG. The activity of PDEs containing EAL domain requires the presence of Mg^{2+} or Mn^{2+} , and are inhibited by Ca^{2+} and Zn^{2+} (Charbonneau et al. 1990; Schmidt et al. 2005). The other group of PDEs, which are unrelated to the EAL containing PDEs, belong to HD superfamily of metal-dependent phosphohydrolases. This type of PDEs contain HD-GYP domain which are c-di-GMP specific, responsible for degrading c-di-GMP into GMP molecules (Ryan et al. 2006; Slater et al. 2000).

In many cases, a c-di-GMP associated protein can contain both GGDEF and EAL / HD-GYP domains. These proteins usually exhibit either DGC or PDE activity response to certain environmental and growth conditions (Hengge 2009). The expressions and activities of the DGCs and PDEs are subjected to a wide range of environmental or cellular input signals (Mills et al. 2011). Many GGDEF and EAL domains containing proteins are found to be associated with various sensory domains, suggesting the highly heterogeneous signal integration in the c-di-GMP signaling networks (Kalia et al. 2013). Such fine-tuned sensory systems facilitate microorganisms to respond and adapt to changes in environments and in metabolic and physiological outputs. The GGDEF proteins and EAL proteins have been utilized to study c-di-GMP regulation mechanisms in biofilm development and bioprocesses. For examples, YedQ is identified as a DGC in *E. coli* containing GGDEF domain only. The inactivation of YedQ leads to less cellulose production whereas the overproduction of YedQ results in decreased swimming motility (Sanchez-Torres et al. 2011). YhjH, a potent phosphodiesterase, is also revealed to be

involved in the motility cascade through interfering with the curli control (Povolotsky and Hengge 2012).

The effectors of c-di-GMP signaling systems bind to c-di-GMP molecules, exerting functions on cellular processes, motility, transcription and post-translational modifications (Mills et al. 2011). The effectors have c-di-GMP sensing or binding domain, which are also highly heterogeneous. Usually, the c-di-GMP effectors cannot be identified or recognized by a single domain or binding site (Jonas et al. 2009; Krasteva et al. 2012). Currently, there are several types of proteins categorized as c-di-GMP effectors in the secondary intracellular signaling system, including PilZ family proteins, transcriptional regulator FleQ, and a class of riboswitches (Amikam and Galperin 2006; Hickman and Harwood 2008; Smith et al. 2009).

A broad spectrum of target genes of the c-di-GMP binding effects has been identified, modulating a wide range of biofilm activities from biofilm formation to the expression of virulence factors (Bush et al. 2015; Krasteva et al. 2010). Several operons that are under the regulation of c-di-GMP signaling system have been well investigated. For instance, the *pel* and *psl* operons, which are responsible for the synthesis of Pel and Psl exopolysaccharides, are well studied to be repressed by FleQ in the absence of c-di-GMP (Baraquet et al. 2012; Colvin et al. 2012; Ha and O'Toole 2015). Moreover, the *cdrA* gene encoding an adhesion factor for Psl exopolysaccharides is also reported to be repressed by FleQ effector (Chua et al. 2015; Pawar et al. 2016). Expression of *flhA* and *fliLMNOPQ* genes involved in flagellar export is activated by FleQ effector (Baraquet et al. 2012).

The secondary messenger c-di-GMP is involved in the regulation of numerous biofilm processes, in response to neighbouring environmental conditions. As discussed in the previous section, target genes of c-di-GMP binding effectors include those responsible for

exopolysaccharide synthesis, motility and virulence. Meanwhile, better resistance to harsh and toxic environments, and increased biodegradation efficacy of biofilms with elevated c-di-GMP levels have been discovered (Harrison et al. 2007). These findings suggest the possible application of c-di-GMP system in manipulating biofilm-based bioprocesses. Strategy that are usually applied in regulating c-di-GMP levels includes engineering GGDEF or EAL domain encoding genes into target organisms in which the engineered c-di-GMP synthesis or depletion takes over endogenous levels. Moreover, regulation of the c-di-GMP levels can be engineered to be under control of specific exogenous chemical compounds or physical conditions (Benedetti et al. 2016).

2.3 Hydrogel matrix

Hydrogels are usually regarded as a group of cross-linked polymeric materials with high water swollen capability, which makes it insoluble in water (**Figure 2.6**) (Billiet et al. 2012; Verhulsel et al. 2014). The most common hydrogel material in daily life is the starch granule, although it is usually not regarded as a hydrogel, because of its natural state in powder form (Athawale and Lele 1998). The starch granules are hard without water content, while upon heating in solvent, it becomes soft. They are comprised of crosslinked carbohydrate polymers, which give them features that are consistent under hydrogel definitions and specifications (Athawale and Lele 2000). Gelatin, another classic hydrogel material, also has a long history of being a food ingredient for gelling and foaming processes (Djagny et al. 2001). Gelatins are commonly obtained from partial hydrolysis of collagen which is derived from pig or cow skins and bones. Gelatins are normally used in food preparation, especially in dessert preparation to give a solid configuration (Wallace et al. 2000). Despite these two common examples, hydrogels are also widely applied in agriculture, industry, clinical treatment, and daily life as well (Das 2013; Ganji et al. 2010).

Figure 2.6 “Hydrogels can be cast into practically any shape, size, and form. Polymer powder (far left) added to a drop of water (left) and cross-linked results in the formation of a hydrogel (middle). Even if the hydrogel network is dehydrated (right), it still retains its overall shape. (Insert) The stages of the dehydration process are shown from left to right.” *Figure is reproduced from (Seliktar, Dror. Science 336.6085 (2012): 1124-1128.) with permission from The American Association for the Advanced of Science.*

2.3.1 Classification of hydrogels

The term hydrogel represents a variety of crosslinked polymeric materials with various modifications for different applications. Different hydrogels share the basic characteristics, such as high water swollen capability, highly crosslinked polymeric networks, and stability in water (Peled et al. 2007). Hydrogels differ from each other based on many aspects, including sources, polymeric compositions, types of crosslinking and physical appearance (Ganji et al. 2010; Verhulsel et al. 2014). These distinct features of hydrogels are considered to be the primary criteria for its categorization.

Hydrogel materials can be derived from natural products, alginate and cellulose being typical examples (**Figure 2.7**); and they can be synthesized based on its origin, such as polyethylene glycol based products (Pawar and Edgar 2012; Peled et al. 2007). The sources of naturally extracted hydrogel materials are usually easy to collect, hence advancing their mass production in industry. The hydrogel from natural extracts show good biocompatibility, promising their application in medicine and bioengineering. The

synthetic polymeric hydrogels are characterized for their relative stability in structural and physicochemical properties and good capability for reproduction.

Figure 2.7 Illustration of hydrogels. (I) “SEM image and fluorescence microscopy images of the protamine-silica microbeads (a) SEM image of the whole bead; (b) edge-on view of the bead; (c) fluorescence microscopy image of the bead; (d) fluorescence microscope image of the inset of the bead.” *Figure is reprinted from (Jiang, Yanjun, et al. Journal of Materials Chemistry 19.47 (2009): 9068-9074) with permission from Royal Society of Chemistry.* (II) “Optical microscope images of calcium alginate beads before and after swelling at different pH conditions; pH=1 for A₁ and A₁', pH=3 for A₂ and A₂', pH=5 for A₃ and A₃'.” *Figure is reproduced from (Gogoi, Neelam, and Devasish Chowdhury. Journal of Materials Chemistry B 2.26 (2014): 4089-4099) with permission from Royal Society of Chemistry.*

Hydrogels can be generated from homopolymers, whose structures are constructed by single species monomers. Hydrogels can possess copolymeric and multipolymeric networks in which different polymers crosslink together (Daniele et al. 2014; Frisman et al. 2012). Crosslinking of the hydrogel network is either based on chemical junction or physical junction. The chemically crosslinked junctions are permanent, whereas physically crosslinked junctions are transient (Berger et al. 2004). Physical interactions are either in the form of ionic interactions, hydrophobic interactions and hydrogen bonding, or entanglements of polymeric chains (Berger et al. 2004).

Generally, hydrogels are prepared as matrices, films or microspheres, depending on the manufacturing processes and the nature and mechanisms of crosslinking networks. For example, alginate-based hydrogels are usually prepared as spherical beads through micro-pipetting which promotes the batch production of hydrogel beads (Moyer et al. 2010). These beads have been used in biocatalysis for enzymes and/or whole cells encapsulation, allowing efficient exchanges of metabolites. Cellulose-based hydrogels can be prepared as films or membranes by electroporation, providing large surface area for cell attachment and biofilms formation (Rodriguez et al. 2014). The light-initiated PEG-based hydrogel materials can be prepared to predesigned structures and patterns using photomasks (Frisman et al. 2011).

The selection of hydrogel materials from the pool of various choices is experimental dependent which involves the consideration of both experimental objectives and operational conditions. The selected hydrogels should have standard mechanical properties, either soft-stretchable or stiff-rigid, consistent with experimental setup (Mathur et al. 1996). Porosity, hydrophobicity and charge are also critical to enhance cell encapsulation within the matrix to promote cell adhesion on the surface. Moreover, the nature and strength of crosslinking is also an important criteria for applying the hydrogels in diverse operational conditions.

2.3.2 Biological application of hydrogels

Biological application of synthetic hydrogel was first reported in 1960 as hydrophilic material in place of plastics to allow desirable water content and permeability to metabolites (Wichterle and Lim 1960). After that, numerous types of hydrogels from natural to synthetic polymers have been developed and studied for cell adhesion, cell encapsulation, chemical encapsulation and release. The major advantages of hydrogel in

biological studies are its good biocompatibility and ability to impart mild physicochemical environments (Mathur et al. 1996; Satarkar et al. 2010).

Hydrogels are applied in emerging clinical approaches in tissue engineering, drug delivery, wound dressing, etc. The hydrogels are designed to support cells and mimic native tissue environment in tissue engineering, as either degradable or non-degradable artificial matrix (Drury and Mooney 2003; Lee and Mooney 2001). Meanwhile, bioactive molecules, such as growth factors and peptides can be introduced or grafted into the hydrogel matrix to regulate specific cell activities (Censi et al. 2012). Hydrogels are also advancing the controllable drug delivery system, in which drugs are protected within the hydrogel matrix (Hoare and Kohane 2008). This approach enables drugs to be delivered continuously to the target at a predetermined releasing rate for a predefined period of time, which overcomes the shortcomings of traditional drug treatment (Xing et al. 2015). Wound dressing is another important biological and clinical application of hydrogel, where its aspects such as nontoxicity, high water content, porous structure for air exchange, and programmable drug releasing efficacy is of great advantage (Fan et al. 2014; Sudheesh Kumar et al. 2012). The hydrogel materials also provide a humid environment for wound repair, keeping skin cool to mitigate the pain (De Cicco et al. 2014).

Hydrogels are also promising materials in industrial application, where they are utilized to encapsulate enzymes or whole cells for biocatalysis or bioremediation. Enzyme immobilization is usually carried out by inert or inorganic hydrogel polymers, which decrease the challenges of non-specific adsorption and contamination, aiming to improve enzyme specificity and activity (Datta et al. 2013). The alginate-gelatin-calcium hydrogel matrix has been broadly applied to encapsulate enzymes, with advantages such as prevention of enzyme leakages, enhancement of mechanical stabilities and improvement

in recyclability (Shen et al. 2011). Whole cell encapsulation in hydrogel material was proposed as a promising alternative of enzyme immobilization to further enhance biocatalytic efficacy. This strategy facilitates long-term retention and recyclability of biocatalysts by enhancing its stability under harsh environments in bioreactors (Nassif et al. 2003). For instance, silica-alginate, a non-biodegradable hydrogel with good mechanical and chemical stability, has been used to encapsulate *Stereum hirsutum*, an environmental microorganism, for water pollutant remediation (Perullini et al. 2010). The porosity of hydrogel structure allows diffusion and accumulation of pollutant compounds in the matrix. Moreover, the hydrogel matrix enhances bacterial viability and enzyme activity, hence improving the recyclability for more than 5 cycles of reaction. Another study using PVA-based hydrogel to encapsulate yeast cells for xylose-to-xylitol bioconversions also yielded good results with a recyclability for up to 6 cycles (Cunha et al. 2007). Interestingly, the conversion rate and productivity was shown to be higher when entrapped cells were recycled in comparison with first batch, suggesting an adaptation trend of cells to harsh environment.

2.4 Devices for biofilm studies

Biofilm development and activities are highly heterogeneous, which are tightly affected by the environmental conditions. In nature, the physicochemical properties are in spatial and temporal heterogeneity, which makes it difficult to predict. In industry, the environmental conditions are generally more stressful for biofilm development with respect to operational conditions such as pH, toxins and shear forces. To better understand the impacts of numerous environmental conditions on biofilm development and biofilm-based bioprocesses, several devices have been developed. The designs are in

accordance with specific objectives, aiming to develop biofilms under specific environmental conditions with limited unexpected heterogeneity (**Table 2.1**).

Table 2.1 List of different devices for biofilm studies.

		Advantages	Major limitation	References
Fluid-based chambers / reactors	3-channel flow cell	<ul style="list-style-type: none"> • Compatible with non-destructive imaging; • Easy to be assembled and disassembled • Applicable to relatively high-shear turbulence 	Less controllable in physicochemical environments	(Tolker-Nielsen and Sternberg 2011, Tolker-Nielsen and Sternberg 2014)
	Flat plate flow cell	<ul style="list-style-type: none"> • Compatible with non-destructive imaging; • Applicable with microelectrode 		(Yang and Lewandowski 1995, Babauta, Atci et al. 2014)
	Capillary flow cell	<ul style="list-style-type: none"> • Relative high shear rate • Compatible with non-destructive imaging 	Less control on dynamic environment generation, with only uniform physicochemical conditions	(Stoodley, Dodds et al. 1998)
	CDC biofilm reactor	<ul style="list-style-type: none"> • Relative high shear rate • Relative large scale 		(Goeres, Loetterle et al. 2005)
	Rotating disc reactor	<ul style="list-style-type: none"> • Moderate shear rate • Relative large scale 		(Goeres, Hamilton et al. 2009)
	Drip flow reactor	<ul style="list-style-type: none"> • Relative low shear rate / laminar flow • Compatible with imaging 		(Goeres, Hamilton et al. 2009)
	Robbins devices	<ul style="list-style-type: none"> • Able to generate mechanical pressures at different rates • Multiple sampling sites for attachment and dispersal studies 		(Coenye, De Prijck et al. 2008)
	Planar flow chamber	<ul style="list-style-type: none"> • Various patterns of shear forces • Compatible with imaging 	Less control on chemical environments	(Zhang, Sileika et al. 2011)
Microfluidic devices	Double inlet microfluidic flow cell	<ul style="list-style-type: none"> • Able to create chemical gradients through chemical diffusion 	Limited growth chamber for biofilm development study	(De La Fuente, Montanes et al. 2007)
	Microfluidic flow cell with gradient mixer	<ul style="list-style-type: none"> • Able to create chemical gradients through chemical diffusion 		(Kim, Kim et al. 2010, Song, Au et al. 2014)

2.4.1 Fluid-based chambers and reactors

Various types of flow chambers and reactors have been developed and broadly applied in biofilm studies, aiming to mimic the aquatic systems for biofilm development (**Figure 2.8**). The 3-channel flow cell developed at Technical University of Denmark (DTU) (**Figure 2.8A**) enables biofilm cultivation under continuous flow with relatively high-shear turbulence rather than laminar flow condition (Tolker-Nielsen and Sternberg 2011). Meanwhile, the device is easy to be assembled and disassembled, with good compatibility towards imaging systems and allows in situ non-destructive observation of biofilm growth and distributions. This device has been applied to investigate biofilm development between mutated strains and their wild type counterparts, and it also has been utilized to study the effect of diverse environmental conditions on biofilm development (Tolker-Nielsen and Sternberg 2014).

Similarly, flat plate flow cell developed in Montana (**Figure 2.8B**) is also commercially available to cultivate biofilms under aquatic environment, allowing biofilm observation with microscopy imaging facilities. Further modification of flow cell has been carried out to facilitate the integration of microelectrode into biofilm investigation under fluidic conditions (Babauta et al. 2014). The biofilm study with this flow cell system by coupling oxygen and pH microprobes provided critical information on the mass-transfer and bacterial activities within the biofilm matrix (Yang and Lewandowski 1995). These fluid based flow cell systems provide basic cultivating environment for biofilm development, whereas they are less controllable in physicochemical properties.

Figure 2.8 Fluid based flow cells and reactors for biofilm cultivation. **A**, 3-channel flow cells developed in DTU (Weiss Nielsen et al. 2011); **B**, Flat plate flow cell (*reprinted with permission from Center for Biofilm Engineering, Montana State University, Bozeman*); **C**, Capillary flow cell (*reprinted with permission from BioSurface Technologies, Montana, USA*); **D**, Drip flow reactor. *This figure is reprinted from (Goeres, Darla M., et al. Nature protocols 4.5 (2009): 783-788) with permission from Nature Publishing Group*; **E**, CDC (the centers for disease control) biofilm reactor (Gomes et al. 2014); **F**, Rotating disc reactor (Gomes et al. 2014); **G**, Robbins device (Gomes et al. 2014). *The figure E,F,G are reprinted from (Gomes, I. B., M. Simões, and Lúcia C. Simões. Water research 62 (2014): 63-87.) with permission from Elsevier*

To better understand the biofilm formation and development under different physicochemical environments, capillary flow cells and CDC biofilm reactor were used to simulate relative high shear rate condition (**Figure 2.8C, E**). Rotating disc reactor allows moderate shear rate (**Figure 2.8F**) and drip flow reactors provide low shear forces / laminar flow (**Figure 2.8D**) (Goeres et al. 2009). The Robbins devices have been

developed and modified to generate mechanical pressures at different rates with multiple sampling sites for biofilm attachment and dispersal studies (**Figure 2.8G**) (Coenye et al. 2008). The reproducibility of these reactors in biofilm development has been reported, which advances downstream biofilm-based applications, such as biocatalysis and bioremediation (Goeres et al. 2005; Williams et al. 2011). However, these biofilm reactors can only support relatively uniform physicochemical environments for biofilm study, with less control on dynamic environment generation.

Figure 2.9 Illustration of the planar flow cell construction; four inlets/outlets are arranged on each side of the chamber. The fluid within the flow cell is adjustable by regulating inlet/outlet arrangement. *Figure is reprinted from (Zhang, Wei, et al. Biotechnology and bioengineering 108.11 (2011): 2571-2582.) with permission from John Wiley and Sons.*

A planar flow chamber has been developed, which is able to precisely control the distribution of fluid physical conditions within the chamber (**Figure 2.9**) (Zhang et al. 2011). This device was able to generate various patterns of shear forces by switching the inlet and outlet of 16 connecting channels (**Figure 2.9**). This flow cell has been successfully applied to investigate biofilm development under flow velocity gradient.

Meanwhile, this device also has been utilized to demonstrate the impacts of dynamic physical conditions on the interactions of multispecies biofilms.

2.4.2 Microfluidic devices

The study of biofilms under defined, controllable and dynamic environments is advanced by the emerging microfluidic techniques, which enable precise control of device design and fabrication in micro or nano scale. A microfluidic chamber (**Figure 2.10**) was designed to study the bacterial adhesion onto surfaces during biofilm initiation by assessing the drag forces (De La Fuente et al. 2007).

Figure 2.10 “Schematic illustration of the microfluidic chamber design. The channels were 80 μm in width, 50 μm in depth and 3.7 cm in length.” *Figure is reprinted from (De La Fuente, Leonardo, et al. Applied and environmental microbiology 73.8 (2007): 2690-2696.) with permission from American Society for Microbiology.*

Several devices have been designed to generate chemical gradients, which are utilized to study bacterial chemotaxis and biofilm formation under dynamic environments (Kim et al. 2010). The fluid-based microfluidic chamber was designed to create chemical gradients through chemical diffusion, which has been utilized to investigate biofilm formation under nutrient gradients (**Figure 2.11A**) (Song et al. 2014). More recently, a microfluidic device containing multiple separate channels was developed, aiming to precisely control chemical environments (**Figure 2.11B**). Researchers have demonstrated the application of this device in biofilm studies for its convenient and high-throughput screening of biofilm

responses to different chemical concentrations (Hong et al. 2012; Kim et al. 2012). Another microfluidic device (**Figure 2.11C**) based on similar chemical gradient mixer has been developed to study biofilm susceptibility to antibiotic gradients (Kim et al. 2010). In general, these microfluidic based devices are mainly applied to study of biofilm initial attachment under heterogeneous physicochemical environments.

Figure 2.11 Microfluidic devices developed for biofilm studies. **A**, Double inlet microfluidic flow cell; Figure is reprinted from (Song, Jisun L., et al. *Biotechnology and bioengineering* 111.3 (2014): 597-607.) with permission from John Wiley and Sons **B**, Schematic illustration of the microfluidic flow cell for creating multiple chemical conditions for biofilm study; the device contains a gradient mixer and eight separate flow cell channels. Figure is reproduced from (Kim, Keun Pil, et al. *Lab on a Chip* 10.23 (2010): 3296-3299.) with permission from Royal Society of Chemistry. **C**, Schematic diagram of the device containing the gradient generator and detection microchannel; Figure is reprinted from (Hong, Seok Hoon, et al. *Nature communications* 3 (2012): 613.) with permission from Nature Publishing Group.

Cell Growth and Protein Expression of *Shewanella oneidensis* in Biofilms and Hydrogel-entrapped Cultures

This chapter has been published as Zhang, Y., C. K. Ng, Y. Cohen and B. Cao (2014). "Cell growth and protein expression of Shewanella oneidensis in biofilms and hydrogel-entrapped cultures." Mol Biosyst 10(5): 1035-1042. Permission has been granted by the Centre National de la Recherche Scientifique (CNRS) and the Royal Society of Chemistry.

3.1 Introduction

Biofilms, structured surface-associated microbial communities, are a predominant microbial growth mode in natural and engineered habitats (Halan et al. 2012; Hall-Stoodley et al. 2004). Cells in biofilms are embedded in a matrix formed by self-produced EPS that provide a sticky framework to hold cells within the complex structure (Flemming and Wingender 2010). The structural and metabolic complexity governed by both genetics and environmental conditions provides biofilms with advantages for survival in various harsh environments (Hall-Stoodley and Stoodley 2002). The application of biofilms to bioprocesses such as bioremediation, used water treatment, and industrial biocatalysis has been studied for decades with the goal of maximizing biofilm performance (Gross et al. 2007; Gross et al. 2010; Hwang et al. 2009). However, the intrinsic heterogeneous and dynamic properties of biofilms render the performance of biofilm-based bioprocesses difficult to predict and control. As a result, the long-term robust and efficient biofilm performance remains one of the main bottlenecks (Gross et al. 2007).

To achieve robust biofilm-based bioprocesses, biofilm mimics, *i.e.*, microbial cells entrapped in scaffolds that are permeable for nutrients, have been shown to be an efficient and key culturing method for cells being controlled and reproducible (Avnir et al. 2006). Entrapped cell systems have been shown to be a highly relevant mimic of cells in nature

or *in vivo* in waste water treatment and tissue engineering (Khetan and Burdick 2009). How the physically entrapped cells differ from the cells in biofilms in terms of cellular functions remain elusive, which becomes a bottleneck in predicting the activities of bioprocesses employing entrapped cells as biofilm mimics.

The goal of this study was to elucidate physiological differences between cells that are physically entrapped in a synthetic matrix and biofilm cells that are entrapped in a self-produced polymeric matrix. Specifically, we used *Shewanella oneidensis* MR-1 as a model organism and alginate hydrogel as an entrapment scaffold and compared the entrapped cultures with biofilms in terms of cell growth, viability, surface characteristics, and protein expression. *S. oneidensis* MR-1 was used as a model organism because its genome sequence is available and its biofilms have a wide range of environmental and biotechnological applications (Heidelberg et al. 2002). Alginate hydrogel was used as a synthetic matrix because i) alginate is a highly biocompatible polysaccharide abundant in various organisms including bacteria (Sun and Tan 2013), and ii) cell entrapment in alginate hydrogel can be achieved at mild pH and temperature conditions (Pawar and Edgar 2012). Cell growth in hydrogel-entrapped cultures and biofilms were quantified. Viability, surface charge, and hydrophobicity of the cells from both hydrogel-entrapped cultures and biofilms were also quantitatively compared. Further, protein expression in cells growing in these two conditions was analysed using quantitative proteomics.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Growth Conditions

S. oneidensis MR-1 wild type (WT) and a green fluorescent protein (GFP)-tagged MR-1 strain were used throughout this study. Fluorescence-tagging of *S. oneidensis* MR-1 was carried out through filter mating with *E. coli* harboring *gfp* using the helper strain

containing RK600 (Kessler et al. 1992) and another helper strain containing pBF13 (Bao et al. 1991) following previous protocols (Lambertsen et al. 2004; Stretton et al. 1998). LB broth was used for bacterial growth and, when necessary, the medium was supplemented with 50 µg/mL of ampicillin, 10 µg/mL of gentamicin, and/or 6 µg/mL of chloramphenicol.

3.2.2 Preparation of Bacterial Cultures

Biofilms. Biofilms of MR-1 WT were grown in tubular biofilm reactors (silicon tubings of 20 cm long with an internal diameter of 3.2 mm). Diluted LB broth (10× dilution) was used as growth medium and was delivered at a flow rate of 2 mL/h using a high precision peristaltic pump (ISMATEC IPC-N 12, Wertheim, Germany). Each reactor was inoculated using 300 µl of diluted MR-1 WT culture (OD₆₀₀ ~0.1). After 4 days, the biofilms were harvested, washed with PBS buffer (pH 7.0).

Entrapped culture in alginate hydrogel. One mL of a diluted MR-1 WT overnight culture with an OD₆₀₀ of 0.1 was mixed with 49 mL of 1.2% sodium alginate liquid solution (Khalil 2005), which was then dropped into 0.1 M CaCl₂ with a volume of 10µl for each drop to form Ca-alginate hydrogel beads. The beads were collected and washed with sterile water to remove remaining calcium ions (Konsoula and Liakopoulou-Kyriakides 2006; Pramanik et al. 2011). The alginate hydrogel beads with MR-1 WT entrapped in them were saturated in LB broth and were cultured under aerobic condition in a 30°C incubation shaker. The LB broth was changed twice a day. After 3 days of growth, the cells were liberated from the hydrogel by dissolving the Ca-alginate beads in a 50 mM sodium citrate solution (pH 7.5) at 4°C (Khalil 2005). To rule out the effects of citrate treatment on protein composition, both the hydrogel-entrapped cultures and biofilms were treated with 50 mM sodium citrate for the same amount of time (~20 min)

before protein extraction. PBS buffer (pH 7.0) was used to wash and resuspend the cells for further use.

3.2.3 Protein Extraction and Quantification

Protein extraction and quantification were carried out on ice. Cells were lysed in 1.5 mL of lysis buffer (0.1% SDS, 0.5 M TEAB) with vigorous vortex and ultrasonication (Sonics & Materials Vibra-Cell VC505, Newtown, USA) for 15 min. The lysates were centrifuged at 20,000g for 1 h and the supernatants were kept at -20°C for further analysis. Proteins were quantified using bicinchoninic acid (BCA) assay (Walker 1994) with bovine serum albumin (BSA) as a standard.

3.2.4 iTRAQ-based Proteomic Analysis

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. For each growth condition, one biological sample (pool of three independent protein samples) and three technical replicates for each biological sample were used in iTRAQ experiments. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to desalt the protein samples. The SDS-PAGE gel with protein bands for each sample was cut and kept in pure water. Proteins in the gels were reduced in 5 mM Tris-(2-carboxyethyl) phosphine (TCEP) for 1 h at 60°C, followed by blocking cysteine residues in 10 mM methyl methanethiosulfate (MMTS) for 30 min at room temperature in the dark. Trypsin was added at a ratio of 1:50 (trypsin/sample) and was then incubated at 37°C overnight. The tryptic peptides were extracted using 50%ACN/5%acetic acid from the gels for 3 times and were desalted using Sep-Pak C18 cartridges (Waters, Milford, MA) and dried in a SpeedVac (Thermo Electron, Waltham, MA). The iTRAQ labelling of the tryptic peptides was performed using a 4-plex iTRAQ reagent kit (Applied Biosystems, Foster City, CA) following the protocols provided by the manufacturer.

Protein samples from biofilms and hydrogel- entrapped cells were labelled as “115” and “116”, respectively. The iTRAQ labelled peptides were reconstituted in Buffer A (10 mM ammonium acetate, 85% acetonitrile, 0.1% formic acid) and fractionated using ERLIC column (200 x 4.6 mm, 5µm particle size, 200 Å pore size) by HPLC system (Shimadzu, Japan) at flow rate of 1.0 mL/min using our previously optimized protocol (Afkarian et al. 2010). Mobile phase buffer A (0.1% formic acid in H₂O) and buffer B (0.1% formic acid in 80% acetonitrile) were used to establish the 90-min gradient, which began with a ramp from 13 to 40% B over 70 min, followed by a ramp from 40 to 90% B in 8 min. The gradient was maintained at 90% B for 2 min before re-equilibrating the column at 13% B for 10 min (Adav et al. 2010; Guo et al. 2010). The HPLC chromatograms were recorded at 280 nm and fractions were collected online using automated fraction collector. Twenty fractions were collected and concentrated using vacuum centrifuge and reconstituted in 3% ACN with 0.1% formic acid for LC-MS/MS analysis. The peptides were separated and analysed on a home-packed nanobore C18 column (15 cm x 75 µm; Reprosil-Pur C18-AQ, 3 µm, Dr Maisch, Germany) with a Picofritnanospray tip (New Objectives, Woburn, MA, USA) on a TempoTMnano-MDLC system coupled with a QSTAR® Elite Hybrid LC-MS/MS system (Applied Biosystems). Peptides from each fraction were analyzed in triplicate by LC-MS/MS over a gradient of 90 min. The flow rate of the LC system was set to a constant 300 nL/min. Data acquisition in QSTAR Elite was set to positive ion mode using Analyst® QS 2.0 software (Applied Biosystems). MS data was acquired in positive ion mode with a mass range of 300–1600 m/z. Peptides with +2 to +4 charge states were selected for MS/MS. For each MS spectrum, the three most abundant peptides above a five-count threshold were selected for MS/MS and dynamically excluded for 30 s with a mass tolerance of 0.03 Da. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS

accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2 s. Each MS/MS spectrum was searched for species *S. oneidensis* against the NCBI protein database. The following criteria were required to consider a protein for further statistical analysis: i) two or more distinct peptides had to be identified (Brown et al. 2010); ii) the fold change had to be greater than 1.5 or less than 0.67; and iii) the statistical significance of the comparison using Student's t-test (p value) had to be lower than 0.05.

3.2.5 Quantitative Analysis of Cell Growth

Cell growth in both hydrogel-entrapped and planktonic conditions was evaluated by measuring OD₆₀₀ and cell viability. To evaluate cell growth inside the hydrogel beads, approximately 200 beads (~2 mL) were dissolved in 50 mM sodium citrate to release the entrapped cells. The liberated cells were washed with PBS for twice, and then were resuspended in 2 mL PBS buffer. As a control, planktonic cells were treated with sodium citrate in the same way. About 100 µl of the cell suspension was mixed with 100 µl BacLight Live/Dead staining solution (Invitrogen, USA), and was incubated in dark at room temperature for 15 min. The OD₆₀₀ and fluorescence were measured using a microplate reader (TECAN M200, Männedorf, Switzerland). Cell viability was estimated based on a standard curve prepared as described elsewhere (Liu et al. 2013). To compare cell growth in hydrogel-entrapped cultures and biofilms, biovolume of GFP-tagged *S. oneidensis* MR-1 strain was estimated (Weiss Nielsen et al. 2011). The biovolumes were determined using a confocal laser scanning microscopy (CLSM) (ZEISS LSM780, Oberkochen, Germany) with a scientific 3D/4D image processing and analysing software IMARIS (Bitplane, Belfast, UK).

3.2.6 Microscopy Imaging of Hydrogel-entrapped Cells

Overnight culture of the GFP-tagged *S. oneidensis* MR-1 was diluted to OD₆₀₀ ~0.1 with fresh growth medium and then mixed with alginate solution at a ratio of 1:50 (v/v). The hydrogel beads (~10 µl each) were prepared as described earlier and 30 beads were submerged in 5 mL LB medium and the supernatant was changed to fresh LB medium once every 12 h. A portion of the beads containing GFP-tagged *S. oneidensis* were taken out every day and were washed with PBS buffer twice. The beads were then transferred to the wells in a 24-well microtiter plate (NuncTM Multidishes, Non-treated, ThermoScientific, Denmark) and were immersed in 20 µl PBS buffer. 3-D images of the alginate beads were taken using a CLSM with an excitation of 488 nm and an emission of 513 nm. The alginate beads were also observed with a stereo microscopy (ZEISS SteREO Discovery V8, Oberkochen, Germany) every 24 hours.

3.2.7 Physicochemical Properties of Cell Surface

S. oneidensis cells from overnight planktonic cultures and liberated from alginate beads (3 days) were harvested and washed with PBS buffer. The cells were then resuspended in PBS buffer. The hydrophobicity was determined using previous protocols (Whitchurch et al. 2002). Zeta potential was measured by using the Malvern Zetasizer Nano Z Dedicated zeta potential analyzer (Nano ZS, Malvern Instruments, Worcestershire, UK). Each experiment was performed three times with two independent cultures for each condition.

3.2.8 Extracellular DNA (eDNA) Quantification

The extraction and quantification of eDNA were performed on ice for both biofilms and the hydrogel-entrapped cultures following the method described elsewhere with some modifications (Wu and Xi 2009). Biofilms from a tubular biofilm reactor were harvested, homogenized and centrifuged at 8,000g to separate the supernatant and cell pellets.

Hydrogel-entrapped cells were liberated and centrifuged at 8,000g to separate supernatant from cell pellets. The supernatants containing eDNA were lyophilized and dissolved in an appropriate amount of MilliQ water. The eDNA concentration in the supernatant was quantified with dsDNA BR assay (Qubit® assay kit, Invitrogen, USA) using a Qubit 2.0 Fluorometer (Invitrogen, USA). Cell pellets were lysed by boiling in 0.2 M NaOH for 20 min. Then, total protein concentrations were quantified using BCA assay (Walker 1994) with BSA as a protein standard.

3.3 Results and Discussion

3.3.1 Cell Growth and Behaviour in Hydrogel Beads

Cell growth and behaviour (represented by GFP expression) of *S. oneidensis* entrapped in the alginate hydrogel beads was monitored by stereo microscopy and CLSM imaging (**Figure 3.1A and B**). Cell growth in the hydrogel beads were also quantified by measuring OD₆₀₀ of the liberated cells (**Figure 3.1C**). The hydrogel beads containing *S. oneidensis* constitutively expressing a green fluorescent protein (GFP) were cultured for 72 h. The microscopic images showed the proliferation of cells in the alginate beads as indicated by the increasing number of colonies and the enlarging volume of the colonies over the course of time. The specific growth rate quantified using biovolumes was estimated to be $(3.28 \pm 0.80) \times 10^5 \mu\text{m}^3 \text{mm}^{-2} \text{h}^{-1}$, which was comparable with the growth of biofilms in flow cells $((2.10 \pm 1.30) \times 10^5 \mu\text{m}^3 \text{mm}^{-2} \text{h}^{-1})$ (**Table 3.1**).

The cells entrapped in hydrogel exhibited a growth trend that follows standard bacterial growth curves in batch cultures, while the growth rate was substantially lower than that of the planktonic cultures (0.04 ± 0.02 vs. $0.27 \pm 0.12 \text{ CFU} \cdot \text{h}^{-1}$) likely due to nutrient diffusion limitations intrinsic to such entrapment environments. The growth curve reached a plateau after 48 h (OD₆₀₀ ~1.20), suggesting that a maximum cell density that could be

held within the hydrogel beads was obtained. This was supported by the microscopic observation that cells started escaping the beads on day 3 (**Figure 3.1A**).

Table 3.1 Growth and physicochemical characteristics of *S. oneidensis* growing in biofilms, hydrogel-entrapped cultures, and planktonic cultures.

Characteristics	Biofilms	Hydrogel-entrapped cultures	Planktonic cultures
Growth rate (biovolume) ($\mu\text{m}^3 \text{ mm}^{-2} \text{ h}^{-1}$)	$(2.1 \pm 1.3) \times 10^5$	$(3.28 \pm 0.8) \times 10^5$	n.d. ^a
Growth rate (CFU h^{-1})	n.d.	0.04 ± 0.02	0.27 ± 0.12
Viability (%)	42.7 ± 0.5	42.0 ± 1.8	44.6 ± 0.3
Zeta potential (mV)	-6.22 ± 0.53	-4.72 ± 0.60	-6.41 ± 0.58
Surface hydrophobicity (%)	30.7 ± 1.1	31.4 ± 0.8	34.0 ± 2.8

^a n.d., not determined

To investigate whether the gel entrapment negatively influences cell viability, we quantified the ratio of live cells in the liberated cells from the hydrogel beads at the late exponential growth phase (~60 h). Our results showed that about $42.0 \pm 1.8\%$ of all the liberated cells were live cells based on the BacLight Live/Dead staining assay. Similarly, cell viability in 3-day biofilms was also determined. Interestingly, the ratio of live cells ($42.7 \pm 0.5\%$) was comparable with that of the liberated cells from hydrogel beads (**Table 3.1**). We further examined the effect of citrate treatment on cell viability and found that the treatment with citrate in the same way as in the cell liberation process does not affect cell viability.

The surface charge (zeta potential) and hydrophobicity of *S. oneidensis* cells liberated from the hydrogel beads at the stationary growth phase (~60 h) were determined to be -4.72 ± 0.60 mV and $31.4 \pm 0.8\%$, respectively. Cells from planktonic cultures (~16 h) and flow cell biofilms (~72 h) were also examined. There was no difference in surface charge and hydrophobicity among the cells from these three cultivation conditions (**Table 3.1**).

Figure 3.1 (A) Stereo microscopic images and (B) CLSM images of alginate hydrogel beads containing GFP-tagged *S. oneidensis* MR-1; (C) Cell growth curve of *S. oneidensis* MR-1 in the alginate hydrogel beads. The error bars represent standard deviations (n=3).

Taken together, our results demonstrated that i) although the hydrogel-entrapped cells exhibit a substantially slower growth than cells in planktonic cultures, the growth rate of the entrapped culture is comparable with biofilms; and ii) cells growing in hydrogel beads and in biofilms have comparable cell viability, surface charge, and hydrophobicity. However, whether and how the hydrogel-entrapped cells differ from biofilm cells in terms of cell physiology remains unknown. To address this research question, we carried out a quantitative proteomic comparison between gel-entrapped cells and biofilms.

3.3.2 Comparison of Proteomes

The iTRAQ-based quantitative proteomic analysis was used to investigate the physiological difference between cells growing in alginate beads and in biofilms. A total of 1,712 proteins were positively identified, covering 38.3% of the whole proteome of *S. oneidensis* (~4,798 proteins) (Brown et al. 2006; Chourey et al. 2009; Elias et al. 2005), which is a typical proteome coverage that can be achieved and is in agreement with previous studies (Brown et al. 2006; Chourey et al. 2009; Kolker et al. 2005). Among the identified proteins, 43 proteins were found to have significantly different expression levels in alginate-entrapped cells compared to biofilm cells (fold change > 1.5 or < 0.67 with p-value ≤ 0.05 (Hare et al. 2012)). Specifically, in alginate-entrapped cells, 28 and 15 proteins showed increased and decreased expression levels (vs. in biofilm cells), respectively. These differentially expressed proteins include proteins involved in energy metabolism, iron uptake, matrix-associated proteins, and purine biosynthesis.

- ***Energy metabolism***

Compared with biofilms, cells growing in alginate beads were found to have a higher expression level of 7 proteins involved in different metabolic components in energy metabolism (**Table 3.2**).

Eno (enolase), PykA (pyruvate kinase II), PpsA (phosphoenolpyruvate synthase) are proteins involved in energy generation through glycolysis during respiration and fermentation. AcnB (aconitate hydratase), SO0970 (fumarate reductase flavoprotein subunit) are enzymes catalyzing reactions in the TCA cycle and SO1521 (respiratory FAD-dependent D-lactate dehydrogenase) is an electron carrier in the electron transport chain. Both TCA cycle and the ETC are essential components in the respiratory pathway. AdhB (alcohol dehydrogenase) is a key enzyme in the fermentative pathway. All the 7

proteins showed a higher expression level in the gel-entrapped cells (vs. biofilm cells) with a protein abundance ratio of 2.05-6.43 (**Figure 3.2**).

Table 3.2 Differentially expressed proteins involved in energy metabolism.

Accession no. ^a	Locus tag ^b	Protein	Description	Ratio ^c
gi 24372027	SO0432	AcnB	Aconitate hydratase	3.28
gi 25452947	SO0970	-	Fumarate reductase flavoprotein subunit	3.73
gi 24373064	SO1490	AdhB	Alcohol dehydrogenase	6.43
gi 24373095	SO1521	-	FAD-dependent D-lactate dehydrogenase	2.05
gi 24374035	SO2491	PykA	Pyruvate kinase II	3.87
gi 24374185	SO2644	PpsA	Phosphoenolpyruvate synthase	3.77
gi 30315823	SO3440	Eno	Enolase	2.99

^a Accession number in NCBI protein database; ^b Locus tag in *S. oneidensis* MR-1; ^c Ratio indicates the abundance of a protein expressed in gel-entrapped cells relative to biofilm cells.

Figure 3.2 Differentially expressed proteins involved in energy metabolism. The fold-change (increased expression in entrapped cells vs. biofilms) for each protein is shown in parentheses. Other identified proteins with no significant changes in their expression are also listed.

S. oneidensis is a facultative bacterium (Yuan et al. 2012) and has highly versatile metabolic capabilities (Fredrickson et al. 2008). Cells of *S. oneidensis* growing in biofilms are subjected to nutrient gradients because of the presence of EPS matrix. As a result, proteins functioning in respiratory and fermentative pathways are often observed in biofilms (McLean et al. 2008). In alginate beads, the Ca-alginate gel forms a polymeric

matrix for cells to colonize, and thus, gradients of nutrient, such as dissolved oxygen (Elias et al. 2008), are also expected. Hence, it is not surprising to identify proteins in both respiratory and fermentative pathways in the gel-entrapped *S. oneidensis* cells. Interestingly, cells growing in alginate gel were found to exhibit a higher energy metabolic activity as evidenced by the higher expression level of the proteins involved in energy metabolism, suggesting a higher energy requirement for the gel-entrapped cells (vs. biofilms).

- ***Iron uptake***

Of the 43 proteins identified by proteomic analysis with differential expression in gel-entrapped cells compared with that in biofilm cells, 5 were involved in iron uptake (**Table 3.3**). PubA (putrescine monooxygenase) and PubC (NTP-dependent putrebactin synthetase) are required for siderophore biosynthesis; PutA (TonB-dependent putrebactin siderophore receptor) and SO3914 (TonB-dependent siderophore receptor) are outer membrane receptors for siderophores; FbpA (ABC-type Fe³⁺ uptake system substrate-binding component) is required to bind and uptake iron.

Table 3.3 Differentially expressed proteins involved in iron uptake.

Accession no. ^a	Locus tag ^b	Protein	Description	Ratio ^c
gi 24372333	SO0744	FbpA	Iron(III) ABC transporter periplasmic iron(III)-binding protein	3.22
gi 24374555	SO3030	PubA	Siderophore biosynthesis protein	0.27
gi 24374557	SO3032	PubC	Siderophore biosynthesis protein	0.17
gi 24374558	SO3033	PutA	Ferric alcaliginsiderophore receptor	0.18
gi 24375402	SO3914	-	TonB-dependent receptor	0.14

^a Accession number in NCBI protein database; ^b Locus tag in *S. oneidensis* MR-1; ^c Ratio indicates the abundance of a protein expressed in gel-entrapped cells relative to biofilm cells.

Importance of iron in bacterial growth and biofilm formation has been well documented (Avidan et al. 2010). Bacterial siderophore is a group of organic compounds that have

high affinity to bind iron and facilitates iron uptake by the cells during cell growth and biofilm development. Siderophore biosynthesis proteins (PubA and PubC) and siderophore receptors (PutA and SO3914) were found less abundant (~3.7- to 7.2-fold decrease) in gel-entrapped *S. oneidensis* cells, suggesting that siderophore-mediated iron uptake is depressed in the gel-entrapped cells. The ABC-type Fe^{3+} uptake system (FbpA) showed a 3.2-fold increase in the gel-entrapped cells. The ABC-type of iron uptake is energy intensive (Sandy and Butler 2009) and the up-regulation of FbpA is consistent with the higher demand of metabolic energy in the entrapped cells. These data implicated that i) the siderophore-mediated iron uptake may play a more important role in biofilms than in the gel-entrapped cells, and ii) the iron uptake in the gel-entrapped cells could be more energy intensive than that in biofilms.

- ***Proteins Involved in Biofilm Formation and Extracellular DNA (eDNA)***

Matrix-associated proteins and proteins involved in purine ribonucleotide biosynthesis also showed altered expression profiles in the gel-entrapped cells (vs. biofilms) (**Table 3.4**). AggA (type I protein secretion system secretin component) and BpfA (biofilm-promoting protein) exhibited a 2.8- and 3.2-fold decrease, respectively, in expression in the gel-entrapped cells. PurB (adenylosuccinate lyase), PurH (bifunctional purine biosynthesis protein), and PurL (phosphoribosylformylglycinamidine synthase) showed an increased expression (~2.0- to 2.6-fold) in the gel-entrapped cells.

Both AggA and BpfA are important proteins with functions in biofilm formation of *Shewanella* cells (Cao et al. 2011; Theunissen et al. 2009). AggA encoded by gene *aggA* in the putative RTX toxin operon has been shown to play a critical role in auto-aggregation and pellicle formation through enhancing cell to cell and/or cell to abiotic surface attachment (De Vriendt et al. 2005; De Windt et al. 2006; Liang et al. 2010).

BpfA is a Bap/RTX hybrid cell surface protein secreted by type I secretion system and has been reported to be a key mediator of biofilm formation in *S. oneidensis* (Theunissen et al. 2010a). Since AggA and BpfA play an important role in surface attachment and biofilm formation, a decrease in the expression of AggA and BpfA in the gel-entrapped cells implicated that the presence of a gel matrix could reduce the need of extracellular proteins for stabilizing the structure of the microbial population.

Table 3.4 Differentially expressed matrix-associated proteins and proteins involved in purine biosynthesis.

Accession no. ^a	Locus tag ^b	Protein	Description	Ratio ^c
gi 32171650	SO0442	PurH	Bifunctional purine biosynthesis protein	2.61
gi 24374176	SO2635	PurB	Adenylosuccinatelyase	2.31
gi 32171649	SO3287	PurL	Phosphoribosylformylglycinamide synthase	1.96
gi 24375799	SO4317	BpfA	Biofilm-promoting protein	0.31
gi 24375802	SO4320	AggA	Type I protein secretion system secretin component	0.36

^a Accession number in NCBI protein database; ^b Locus tag in *S. oneidensis* MR-1; ^c Ratio indicates the abundance of a protein expressed in gel-entrapped cells relative to biofilm cells.

Proteins PurB, PurH, and PurL are enzymes responsible for purine biosynthesis, which has been shown to play an essential role in the production of eDNA (Otto et al. 2010; Vilain et al. 2009). eDNA has previously been shown to be an important component in biofilms of several bacterial species, including *Staphylococcus aureus*, *S. epidermidis*, *S. pneumoniae*, *B. cereus*, *P. aeruginosa*, and *S. oneidensis* (Dominiak et al. 2011), functioning as an adhesion (Das et al. 2010; Whitchurch et al. 2002). Surprisingly, although a gel matrix was present in the gel-entrapped cultures and production of additional adhesins might not be able to benefit the cells in terms of maintaining a structural stability, PurB, PurH, and PurL showed an increased expression (~2.0- to 2.6-fold) in the gel-entrapped *S. oneidensis* cells (vs. biofilms).

We further quantified the eDNA content in both the gel-entrapped cultures and the biofilms and found that the eDNA content in gel-entrapped *S. oneidensis* cultures was substantially higher than that in biofilms (**Figure 3.3**). This observation implies that, in

addition to be serving as an adhesion, eDNA may play other important functional roles that remain unknown and warrant further investigations.

Figure 3.3 The eDNA content in 3-day biofilms and gel-entrapped cultures of *S. oneidensis*; * $p < 0.01$ by Student two-tailed *t*-test.

3.4 Conclusions

In this study, using *S. oneidensis* as a model organism, we compared cell growth and protein expression in hydrogel-entrapped cultures with biofilms and found that i) the hydrogel-entrapped cells exhibit a comparable growth rate with the cells in biofilms; ii) there is no substantial difference in cell viability, surface charge, and hydrophobicity between the cells growing in alginate hydrogel beads and in biofilms; iii) the growth of gel-entrapped cells has a higher demand in metabolic energy than the biofilms; iv) siderophore-mediated iron uptake in the gel-entrapped cells is down-regulated; v) the presence of a hydrogel matrix reduces the need of matrix-associated proteins for stabilizing the structure of the microbial population in the gel-entrapped cultures, while induces the production of eDNA, suggesting that, other than serving as an adhesion, eDNA may play certain alternative important roles that remain elusive. Our findings advance the fundamental understanding of the physiology of hydrogel-entrapped cells, which can lead to strategies for constructing more efficient biofilm mimics in various applications.

High Quality RNA Extraction Method from Biofilm mimics

4.1 Introduction

The biofilm mimics has been proposed as a promising alternative to naturally developed biofilm, where cells are encased within the hydrogel matrix instead of the self-produced biofilm matrix (Strathmann et al. 2000; Zhang et al. 2014). The hydrogel was employed to mimic the EPS matrix, providing structural and mechanical support to the encased cells, where immobilized cells show enhanced operational stability (Coquet et al. 1998). Meanwhile, the hydrogel provides protection on cells against harsh environments, improving the local cell density and enhancing their tolerance towards antimicrobials, toxins and physical stresses (Gonen-Wadmany et al. 2011; Khetan and Burdick 2009; Seliktar 2012). Moreover, the hydrogel matrix is proposed to simplify and define the microenvironment of biofilm mimics, towards controllable and reproducible biofilm construction (Coquet et al. 1998).

The alginate-based hydrogel has been widely applied for bacteria immobilization, which is a promising technique in chemical conversion, waste bioremediation and investigation of bacterial mechanisms (Cheng et al. 2012; Choi et al. 2012; Degiorgi et al. 2002). Alginate, a copolymer composing of β -D-mannuronic acid and α -L-guluronic acid, is able to crosslink in the presence of divalent Ca^{2+} cations (Pawar and Edgar 2012; Sun and Tan 2013; Sun et al. 2012). The gelation of Ca-alginate hydrogel is a non-toxic process under mild conditions, promising the biocompatibility of Ca-alginate matrix (Pawar and Edgar 2012; Sun and Tan 2013; Sun et al. 2012). The Ca-alginate encapsulated bacterial cultures have been employed in biofilm mimics, aiming to construct the robust and reproducible biofilm in biocatalysis engineering (Zhang et al. 2014). While, mechanical immobilizations by Ca-alginate impose physicochemical stresses on cells, which differ

from the stresses by self-produced biofilm matrix. The stress-response of biofilm mimics in Ca-alginate matrix is supposed to play critical interference on biofilm-mimics-based activities and on the function-prediction.

To better understand the bioprocesses of Ca-alginate-entrapped bacterial cultures, profiling of bacterial responses at genetic level is required. Transcriptomic analysis has been employed as a powerful tool to investigate bacterial responses in exposure to the dynamic microenvironments (Dickson et al. 2012; Kruckeberg et al. 2009). The expression profiling of intact RNA is supposed to provide direct and reliable information on the differences between immobilized cells and naturally generated biofilms. While, the expression profiling relies on reproducible and high quality RNA isolated from the biofilm mimics in Ca-alginate scaffold.

However, highly quality RNA isolation from cells encased in polysaccharide hydrogel matrix is an issue, due to the interferences by these synthetic materials. The highly crosslinked polymers of the Ca-alginate matrix blocks the RNA isolation. The presence of large polymers from partially degraded Ca-alginate matrix decreases the extraction and purification efficiency of RNA samples (Kruckeberg et al. 2009; Wang and Stegemann 2010). Meanwhile, the short fragments of the partially degraded polymers are able to be co-extracted and precipitated together with RNA fragments, contaminating the isolated RNA samples (MacRae 2007). As a consequence, thorough profiling of biofilm mimics-based gene expressions are significantly impacted by the poor RNA quality and quantity.

Therefore, this study was motivated by the need of extracting significantly higher quantity and quality of RNA from alginate hydrogels for downstream transcriptomic analysis. A comparative assessment and optimization of commonly applied RNA extraction techniques using Qiagen, Trizol and CTAB with post purification were

performed. Briefly, TRIzol reagent and Qiagen RNeasy® Mini kit are based on the application of guanidinium thiocyanate buffer for cell lysis, whereas CTAB method deploys the cationic extraction buffer cetyl trimethylammonium bromide, a strong detergent, for isolation (Yu et al. 2013). The model organism for biofilm mimics is *Pseudomonas aeruginosa* PAO1, a Gram negative bacteria, which is broadly applied in biofilm studies. Our results showed that protocol modification based on Qiagen extraction kit gave the best RNA quality and quantity for downstream sequencing.

4.2 Material and methods

4.2.1 Bacterial strain and cultivation in hydrogel matrix

Pseudomonas aeruginosa PAO1 was used in this study as model organism. Ca-alginate hydrogel was applied as artificial matrix for bacterial encapsulation and development following the modified protocols (Khalil 2005; Zhang et al. 2014). Briefly, initial inoculation of PAO1 cultures in 1.3% sodium alginate (w/v in water) was maintained at a density equal to OD₆₀₀ at 1.0 in water solution. Crosslinking of alginate hydrogel was achieved by pipetting 10 uL of bacterial inoculated sodium alginate solution into 0.1M CaCl₂ bath and non-encapsulated PAO1 cells were washed off by sterilized water (Konsoula and Liakopoulou-Kyriakides 2006; Pramanik et al. 2011). PAO1 cells were cultivated within the hydrogel matrix in LB broth, which was replaced on a daily basis.

4.2.2 RNA extraction

Biofilm mimics cultures of PAO1 within Ca-alginate matrix were protected by RNA protect reagent (Qiagen, Hilden, Germany), and were maintained in -80°C before RNA extraction processes. Bacterial cells were liberated from the artificial matrix to enhance RNA extraction efficiency by modified Ca-alginate dissolving buffer consisting of 55mM sodium citrate, 30mM Na₂EDTA and 150mM NaCl. (Ueng et al. 2007). Liberated PAO1

cells were proceed to downstream RNA extractions by modified methods prior to quality and quantity assessment (**Figure 4.1**).

Figure 4.1 Flowchart of RNA extraction protocols based on three different commonly applied extraction methodologies: Qiagen, CTAB and TRIzol. Pretreatment of Ca-alginate encapsulated PAO1 cultures was carried out following the same procedures, releasing cells from the artificial matrix.

Method 1: modified from Qiagen RNA extraction kit (RNeasy Protect bacteria mini kit, Qiagen, Hilden, Germany).

The extraction procedures were in accordance to the instructions of the manufacturer provided along with the kit. In short, cell pellets were treated with lysis buffer containing 4 mg/mL lysozyme at room temperature for 5min. After treatment, appropriate volume of buffer containing 2-mercaptoethanol was added and mixed well by vortexing, followed by addition of 100% ethanol to precipitate the RNA. Subsequently, the precipitated RNA samples were purified on column via RNeasy[®] kit according to manufacturer`s instructions.

Method 2: TRIzol extraction modified from Wang et al. (Wang and Stegemann 2010)

Cell lysis was modified by using TRIzol[®] reagent (Life technology, Carlsbad, CA, USA), followed by phase separation with addition of chloroform. The upper clear aqueous phase containing RNA fragments were further treated with 100% isopropanol for RNA precipitation. Purification was carried out with 75% ethanol and RNA Clean & Concentrator[™] (Zymol, Irvin, CA, USA) following manufacturer`s instructions. Briefly, this column binding-based purification also utilized ethanol and organic solvent to remove contaminants and residue extraction buffer.

Method 3: CTAB extraction modified from Sim et al. (Sim et al. 2013)

Disruption of cells was conducted by pre-warmed (65°C) cetyl-trimethylammonium bromide CTAB buffer (3% CTAB, 3% Poly-vinylpyrrolidone (PVP 40), 28% NaCl, 100mM Tris-HCl pH8.0, 20mM EDTA and 0.2% 2-mercaptoethanol in diethylpyrocarbonate (DEPC)-treated water). Chloroform-isoamyl alcohol (24:1) (CI) was applied for phase separation, where upper aqueous phase was treated with 100%

isopropanol for RNA precipitation. The same purification procedures as those for TRIzol RNA extraction method was applied after RNA precipitation.

4.2.3 Protein quantification

Total protein quantification was carried out following modified protocol (Ding et al. 2014). Cell pellets liberated from the Ca-alginate hydrogel were lysed in 0.2M NaOH and heated for 20 min. The quantification was performed using Qubit® Protein Assay Kit and Qubit fluorometer (Life technologies, Carlsbad, CA, USA).

4.2.4 RNA assessment

Purity, quantity and integrity RNA assessment in terms of purity and quantity was measured by a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA) and Qubit® fluorometer (Life Technology, Carlsbad, CA, USA). The extraction efficiency of each extraction method was estimated based on total RNA per mg protein (Ling et al.). RNA purity was evaluated according to ratios of A260/A280 and A260/A230, with ideal ratio ranges of 2.0-2.1 and 2.1-2.2 respectively. DNA contamination was also quantified using Qubit® fluorometer using DNA High Sensitivity (HS) Assay Kit (Life Technology, Carlsbad, CA, USA). RNA integrity for each sample was examined by Agilent 2200 TapeStation according to manufacturer's instructions (Agilent Technologies, Santa Clara CA, USA).

RNA sequencing The next-generation sequencing library was generated by TruSeq RNA sample preparation protocol (Illumina, San Diego, CA) with modifications. The Invitrogen Picogreen assay and Bioanalyzer DNA 1000 chip (Agilent) were applied to evaluate library quantitation and average size. The library sequencing using Illumina HiSeq2500 rapid run was accomplished following suggested protocols. Briefly, the

library concentration was normalized to 2nM and validated, before the libraries were assembled for sequencing.

The raw data of Illumina RNA sequencing were deposited into the NCBI Sequence Read Archive database (<http://www.ncbi.nlm.nih.gov/sra>) with an accession number SRR1642858. The sequence reads were assembled and analyzed in RNA-Seq with the *P. aeruginosa* PAO1 genome (<http://www.ncbi.nlm.nih.gov/nucore/110645304>) as reference genome. The expression analysis was accomplished by CLC genomics workbench 6.0 (CLC Bio, Aarhus, Denmark). The criteria used to filter the unique sequence reads were following the CLC instructions: maximum of 2 mismatches, minimum length fraction of 0.9 and minimum similarity fraction of 0.8. The data obtained for every gene were normalized by calculating the reads per kilobase per million (RPKM). The results were annotated using databases in www.pseudomonas.com and in PseudoCAP (www.geneontology.org/GO.current.annotations.shtml).

4.3 Results and discussion

Three broadly applied RNA extraction methods in biofilm study were modified and used for RNA extraction from Ca-alginate hydrogel encapsulated *P. aeruginosa* PAO1 cultures. The extraction efficiency and quality was evaluated and compared, aiming to establish the standardized methodology for hydrogel-based biofilm mimic study.

RNA extractions based on Qiagen RNasy mini kit and TRIzol reagent are commercially available and have been applied for numerous biological studies. Both methods utilize guanidine thiocyanate for cell disruption and RNase inactivation, whereas each product contains specific proprietary components to enhance RNA isolation efficiency. The Qiagen kit utilizes silica-based membrane for RNA binding and isolation, while TRIzol reagent uses phase separation by chloroform to remove DNA and protein fragments. The CTAB reagent is a cationic surfactant that has been established for DNA or RNA

isolation from highly polysaccharide containing materials such as plants (Holmes et al. 2014). The removal of polysaccharides and secondary metabolites is also enhanced by the addition of poly-vinylpyrrolidone (PVP) (Azmat et al. 2012). Post-purification process was carried out for TRIzol reagent and CTAB buffer based RNA samples to further remove DNA fragments and organic polymers.

Table 4.1 Purity, quantity and integrity of total RNA obtained with the three different extraction methods: Qiagen, CTAB and TRIzol along with as required post-purification using Zymo kit.

Methods	Total RNA μg	Extraction efficiency μg RNA/mg Protein	A260/A280 ^a	A260/A230 ^b	DNA contamination ^c (%)	RIN ^d
Qiagen	9.95 ± 1.31	4.29 ± 0.004	2.11 ± 0.02	2.06 ± 0.05	11.10 ± 1.01	9.8
CTAB	22.17 ± 1.86	9.58 ± 0.001	1.96 ± 0.02	1.30 ± 0.08	>50	1.0
CTAB + Zymo	13.10	5.66	2.06	1.81	21.8	1.0
TRIzol	28.03 ± 3.80	12.11 ± 0.003	1.92 ± 0.03	0.50 ± 0.11	14.25 ± 0.64	1.0
TRIzol +Zymo	15.31	6.61	1.99	1.79	14.7	1.0

^a the ratio within 1.9-2.1 indicates a pure RNA sample (QIAGEN 2012)

^b the ratio within 2.0-2.2 indicates a pure RNA sample (Nanodrop 2012)

^c percentage of DNA contamination less than 15% indicates a relative pure RNA sample

^d RIN (RNA integrity number) value of ≥7 indicates an intact RNA sample, but also depends on electropherogram analysis (Mueller et al., 2004)

The efficiency of RNA extraction for each method was evaluated as total RNA (μg) per mg protein, and the quality was examined by purity and integrity (**Table 4.1**). TRIzol extraction reagent showed the highest efficiency at 12.11 ± 0.003 μg RNA/mg Protein before post purification processes. The efficiency by CTAB reagent before post purification, at 9.58 ± 0.001 μg RNA/mg Protein, was also higher than that by Qiagen kit, which was only 4.29 ± 0.004 μg RNA/mg Protein. Post purification compromised the extraction efficiencies for both TRIzol and CTAB extractions, though efficiencies after purification were still a little bit higher than the Qiagen extracted sample. The low yield of RNA by Qiagen extraction kit was explained by the manufacturer for several reasons—insufficient disruption and homogenization of bacterial cultures; too much

starting material exceeding column loading capacity; the low eluting efficiency from column membrane; incomplete removal of ethanol containing buffer and cell culture medium. Considering our experimental processes, the low RNA extraction efficiency by Qiagen kit was likely due to the RNA binding and eluting efficiency of the silica-based column membrane. Incomplete cell lysis and residues of cell-culturing medium may be responsible for hindering the RNA binding conditions created by addition of ethanol containing buffers. Moreover, the eluting efficiency might be limited by incubation time and residual ethanol.

RNA purity in terms of A260/A280, A260/A230 and DNA contamination was evaluated and compared among RNA samples extracted by three different methodologies. Qiagen extraction kit showed the best purity in comparison with TRIzol and CTAB reagents, with both A260/A280 and A260/A230 within ideal ranges (1.9-2.2) (Nanodrop 2012; QIAGEN 2012), as well as a relatively low DNA contamination (11.10 ± 1.01 %). Contamination by organic compounds in TRIzol reagent-extracted RNA samples before post purification was the major issue (A260/A280 at 0.50 ± 0.11), whereas DNA removal was challenging (>50%) in CTAB extracted samples without post purification. A lower value of A260/A230 ratio indicates a higher chance of chaotropic salts or organic contamination in RNA samples (Nanodrop 2012), which might be due to remaining guanidine/phenol, isopropanol and ethanol during RNA precipitation and washing steps as well as short strand fragments of polysaccharides in samples (Eldh et al. 2012). It was hypothesized that, alginate-polymers are not eliminated totally even after dissolving procedures, with short polymers interspersed with bacteria thereby interfering with RNA extraction and quality (Kruckeberg et al. 2009). After post purification, TRIzol and CTAB based RNA samples significantly much lower organic contaminations. However, DNA contamination of purified CTAB extracted sample was lower by half than that before purification at 21.8%, whereas for TRIzol based extraction no apparent

improvement in DNA removal was observed. The CTAB buffer based extraction was established as a standardized protocol for DNA extraction from plant tissues and was modified for RNA extraction, which may be responsible for the presence of large amount of DNAs (Peng et al. 2014). TRIzol reagent based extraction methods have been reported to be efficient for DNA, RNA and protein extractions, where the isolation of each components has to be carried out by phase separation and downstream processes (Jordon-Thaden et al. 2015).

Integrity evaluation by Tapestation bioanalyzer shows RIN (RNA integrity number) values of 9.8, 1.0 and 1.0 for RNA extracted with Qiagen, TRIzol and CTAB respectively (**Table 4.1**). RIN evaluation is based on algorithm considering informative features to determine the integrity of RNA samples, with a range of 1 (totally degraded sample) to 10 (intact sample) (Mueller et al. 2004). An ideal RIN data should be above 7.0 when compared to the ladder, which is considered as good quality for RNA sequencing (Copoïs et al. 2007; Fleige and Pfaffl 2006). Usually a lower RIN value indicates either the presence of more small RNA fragments in testing samples or highly degraded RNA fragments during extraction. However, the RIN value is not representative when the electropherogram figure is unusual with peaks appearing at shorter migration time (Imbeaud et al. 2005). As observed in the gel image and electropherogram of total RNA in **Figure 4.2B**, RNA obtained from Qiagen shows two sharp peaks and corresponding clear bands at 16s and 23s rRNA. On the other hand, RIN values from CTAB and TRIzol RNA extracts were not as good, due to the presence of non-significant peaks (other than 16s or 23s) (**Figure 4.2 C-F**). According to electropherogram image, small RNA fragments appearing at shorter migration times found in the extracted RNA samples were the major issue hindering the RIN values. Three possible explanations were proposed for the attained results: CTAB and TRIzol methods are not as efficient as Qiagen RNasey

mini kit in removing small RNAs during RNA isolation; RNA samples got fragmented during the extraction procedures for CTAB and TRIzol methods; relatively higher amount of shorter polymers of alginate remain in RNA samples obtained from CTAB and TRIzol methods than from the Qiagen kit. According to the manufacturer's instruction of Qiagen extraction kit, the selective RNA binding silica-based membrane is efficient to remove small RNA fragments with nucleotides less than 200, such as 5S rRNA and tRNA fragments. These short fragmented RNAs comprises almost 15% to 20% of the total RNA, which may hinder the precision of downstream transcriptomic analysis if not eliminated from RNA samples (He et al. 2010; Peano et al. 2013).

Figure 4.2 Electropherogram of RNA samples analyzed by 2200 TapeStation system. A: RNA Ladder; B: RNA obtained by Qiagen RNase mini kit; C: RNA obtained by CTAB method; D: RNA obtained by CTAB and purified by Zymo; E: RNA obtained by TRIzol reagent; F: RNA obtained by TRIzol and purified with Zymo.

Based on the comparison of RNA quality between RNA samples extracted with different methodologies, modified Qiagen extraction kit was proposed to be the best choice for

preparation of high quality RNA samples for transcriptomic analysis. Though the yield of RNA was lower for Qiagen extracted sample, which may hinder the transcriptome profiling and quantification, the relatively high RNA integrity and purity, especially low DNA contamination can significantly reduce interferences during sequencing analysis. RNA-Seq reads were then assembled and mapped into *P. aeruginosa* PAO1 genome, followed by corresponding genes being annotated of gene product description and functions.

In total, 4826 genes were identified from RNA-Seq reads, which accounts for 90.78% of the whole genome of PAO1 (Stover et al. 2000), indicating a good coverage of the PAO1 transcriptome. All the identified genes were categorized into functional groups according to clusters of orthologous groups (COGs). Meanwhile, organismal functions of Kyoto Encyclopedia of Genes and Genomes (KEGG) were considered when a gene was involved in two or more COG functions. In general 3427 genes were categorized by functions, while 1399 genes were not found in PAO1 COG database (**Figure 4.4**). Coverage of identified genes in each functional category (**Figure 4.3**) ranged from 33.5% (Intracellular trafficking, secretion, and vesicular transport) to 84.8% (cell cycle, cell division, chromosome partitioning). Variation in coverages is most likely due to the highly heterogeneity present in the hydrogel-entrapped PAO1 cultures, especially the colonies at stationary stage with highly heterogeneous microenvironments. Gene expression levels are highly dependent with interaction of environmental conditions with the neighboring cells (Sauer et al. 2002; Waite et al. 2005). Alginate polymer is one of the major extracellular polysaccharides of PAO1 biofilm matrix, where alginate mainly acts as a factor for adhesion and virulence (Leid et al. 2005; Sarkisova et al. 2005). The presence of alginate hydrogel-based artificial matrix imposed both physical and chemical stresses for encapsulated PAO1 cultures, which in response alter gene expressions

accordingly. Moreover, loss of RNA information during extraction processes may also be responsible for the variances occurred in the coverages and expression levels.

Figure 4.3 Coverage of identified genes from RNA-Seq reads in each COGs functional categories; RNA sample are extracted by Qiagen RNeasy mini kit from Ca-alginate immobilized *P. aeruginosa* PAO1; annotated RNA fragments are classified based on COG classes. Percentages are calculated as the number of corresponding genes dived by total number of genes in that category

Figure 4.4 COG categories of identified genes by RNA-Seq Analysis; RNA sample are extracted by Qiagen RNeasy mini kit from Ca-alginate immobilized *P. aeruginosa* PAO1; annotated RNA fragments are classified based on COG classes. Number for each category represents the number of corresponding genes in each class

4.4. Conclusions

In this study, using *P. aeruginosa* as a model organism, we compared the RNA extraction quality and efficiency from three commonly reported methods—Qiagen RNeasy Protect bacteria mini kit, TRIzol reagent and CTAB buffer. We found that the Qiagen extraction process, modified with Ca-alginate lysis and RNA stabilization, gave the best RNA samples for downstream sequencing analysis. Our comparative results and optimizations advance the handling of high quality RNA extraction from Ca-alginate entrapped bacterial cultures, facilitating the investigation of bacterial immobilization for biofilms mimic studies.

Transcriptomic Analysis of QS-regulated Genes in Biofilm mimics

5.1 Introduction

Biofilm mimics in artificial matrix has been proposed as a promising approach towards the controllable and reproducible biofilm development and biofilm-based bioprocesses (Zhang et al. 2014). In naturally generated biofilms, cells are encased within the self-produced extracellular polymeric substance (EPS) matrix which show highly structural and physicochemical heterogeneity (Haagensen et al. 2015; Hall-Stoodley et al. 2004). The biofilm-based bioprocesses are tightly associated with the EPS matrix supported microenvironments and the genetically regulated lifecycles, which render the control and application challenging (Xu et al. 1998). In biofilm mimics, cells reside within the hydrogel-based artificial matrix, which is applied to mimic the self-generated biofilm matrix. The regulation of biofilm mimics-based bioprocesses are supposed to be accomplished by bioactive molecules supplemented in the hydrogel matrix, which is inspired by the strategy in tissue engineering (Lee and Mooney 2001).

Cells in biofilm collaborate with other cells and interact with neighboring environments through complex signaling networks, instead of physically clustering together. The biofilm-based bioactivities are regulated by numerous external signals, ranging from physical properties to chemical molecules. The quorum sensing (QS) signals, which are involved in the intercellular communication system, regulate global gene expressions including those responsible for virulence and survivability (Ng and Bassler 2009; Rutherford and Bassler 2012). The QS regulation systems have been investigated before, revealing the importance of these systems in control of biofilm formation and biofilm-based processes (Hong et al. 2012). Hence, QS signals are proposed to be a promising

candidate as the bioactive molecules to functionalize the artificial matrix for biofilm mimics regulation.

However, the QS regulation system is highly strain specific, and timing- and environment dependent, and shows significant differences under variant situations (Chugani et al. 2012; Schuster et al. 2003). The physicochemical properties of biofilm mimics differ from EPS matrix in naturally generated biofilm, which is hypothesized to impact QS regulation systems significantly. To better regulate biofilm mimics-based bioprocesses through QS signals functionalized matrix, it is crucial to understand the QS regulation systems in biofilm mimics.

In this study, we utilized *Pseudomonas aeruginosa* PAO1 as model organism, in which two QS regulation circuits—LasIR and RhlIR—are widely studied. In LasIR circuit, *lasI* is responsible for *N*-(3-oxododecanoyl)-*L*-homoserine lactone (C12-HSL) synthesase, and *rhlI* for the coding of *N*-butanoyl-*L*-homoserine lactone (C4-HSL) synthesase in RhlIR circuit. We developed biofilm mimics of PAO1 wild type and PAO1 $\Delta lasI \Delta rhlI$ double deletion mutant in calcium-alginate hydrogel with and without the C12-HSL and C4-HSL signals. The C4-HSL and C12-HSL target genes in biofilm mimics of *P. aeruginosa* in stationary growth stage were identified by comparing the transcriptomic profiling among these biofilm mimics samples. This global profiling of QS target genes provided a hint on the controllable bioactivities of PAO1 biofilm mimics with QS signal functionalized matrix.

5.2 Material and methods

5.2.1 Bacterial strains and biofilm mimics preparation

Pseudomonas aeruginosa PAO1 wild type and its homologous double deletion mutant PAO1 $\Delta lasI\Delta rhII$ were used in this study. Ca-alginate hydrogel immobilization of *P. aeruginosa* PAO1 and PAO1 $\Delta lasI\Delta rhII$ cultures (**Table 5.1**) induced and non-induced by QS signals, *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL or C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), was performed following the modified protocols (Khalil 2005; Zhang et al. 2014). Briefly, 1.3% sodium alginate (w/v in water) was used with initial bacterial inoculation for each culture at a density equal to 1.0 at OD₆₀₀ in water. 0.1M CaCl₂ bath was applied for crosslinking of alginate hydrogel, and sterilized water was utilized to wash off the non-encapsulated cells. The encapsulated bacteria in the calcium-alginate hydrogel beads were cultivated in LB broth with/without C12-HSL and C4-HSL molecules, which was replaced on a daily basis (**Table 5.1**).

Table 5.1 Bacterial strains and cultivations

Samples	Strains	Acetyl-HSL signals generation	QS signals introduced in the Ca-alginate matrix	
			<i>N</i> -(3-oxododecanoyl)-L-homoserine lactone	<i>N</i> -butanoyl-L-homoserine lactone
WT	<i>P. aeruginosa</i> PAO1 wild type	+	-	-
MU	<i>P. aeruginosa</i> PAO1 $\Delta lasI\Delta rhII$ ^a	-	-	-
MUS	<i>P. aeruginosa</i> PAO1 $\Delta lasI\Delta rhII$ ^a	-	2 μ M	2 μ M

^a Construction of the strains was conducted according to methods elsewhere (Hansen et al. 2012; Hentzer et al. 2002; Yang et al. 2009)

5.2.2 Microscopic imaging of biofilm mimics

Fluorescent staining of biofilm mimics samples was prepared with different types of fluorescent dyes (**Table 5.2**) according to manufacturers' instructions. Confocal laser scanning microscopy (CLSM, ZEISS 780, Oberkochen, Germany) was utilized to observe

biofilm mimics in hydrogel matrix at respective excitation/emission wavelength (**Table 5.2**).

Table 5.2 Parameters for each fluorescent dye

Fluorescent	Excitation/emission (Peled et al.)	Manufacturer
SYTO [®] 9, Live staining (green)	480/500	Invitrogen, Carlsbad, California
PI, dead staining, (Sperandio et al.)	490/635	Invitrogen, Carlsbad, California
RedoxSensor Red (Sperandio et al.)	540/600	Invitrogen, Carlsbad, California

5.2.3 RNA extraction

RNA extraction and purification from biofilm mimics cultures at maturation stage was carried out based on modified RNasy Protect bacteria mini kit (Qiagen, Hilden, Germany). RNA protect reagent (Qiagen, Hilden, Germany) was applied upon the collection of biofilm mimics in the hydrogel matrix immediately. Bacterial cells were liberated from calcium-alginate matrix by modified dissolving buffer, consisting of 55mM sodium citrate, 30mM Na₂EDTA and 150mM NaCl. (Ueng et al. 2007). Cell pellets were proceeded to on-column RNA isolation and purification processes.

RNA assessment in terms of purity and quantity was measured by NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA) and Qubit[®] fluorometer (Life technology, Carlsbad, CA, USA). RNA integrity was evaluated by Agilent 2200 TapeStation system according to manufacturer's instructions (Agilent Technologies, Santa Clara CA, USA).

5.2.4 Hi-Seq RNA sequencing

The isolated RNA for each sample was sequenced according to Illumina RNA sequencing protocol with some modifications. Briefly, TruSeq RNA sample preparation protocol

(Illumina, San Diego, CA) with modifications was used to generate the next-generation sequencing library. After standard quantitation, normalization and validation, the libraries were then assembled at equal volumes and sequenced in two lanes of an Illumina HiSeq2500 rapid run at a final concentration of 7.2 pM and a read-length of 101 bp paired-end.

The raw data obtained from Illumina RNA sequencing were deposited into the NCBI Sequence Read Archive database (<http://www.ncbi.nlm.nih.gov/sra>) with accession numbers as SRR1642858, SRR1642862 and SRR1642863 for WT, MU and MUS. The sequence reads were assembled and analyzed in RNA-Seq and expression analysis application of CLC genomics Work-bench 6.0 (CLC Bio, Aarhus, Denmark). The *P. aeruginosa* PAO1 genome was used as the reference genome (<http://www.ncbi.nlm.nih.gov/nuccore/110645304>) for the assembly. Criteria to filter the unique sequence reads were set as: maximum of 2 mismatches, minimum length fraction of 0.9, and minimum similarity fraction of 0.8. Data obtained for each gene was normalized by calculating the reads per kilo-base per million (RPKM). Annotation was performed for all identified genes using databases in www.pseudomonas.com and in PseudoCAP (<http://www.geneontology.org/GO.current.annotations.shtml>).

5.3 Results and discussion

5.3.1 Growth characterization

Bacterial growth for each sample was monitored by confocal laser scanning microscopy (CLSM) by staining cells with SYTO9/PI or Redoxsensor red/SYTO9 as shown in **Figure 5.1**. *P. aeruginosa* PAO1 *lasIrhlI* double deletion mutant grown in Ca-alginate beads in the absence of acetyl-HSL signals showed better growth with larger total biovolume and higher ratio of respiring cells than PAO1 wild type culture. The PAO1

lasIrhlI double deletion mutant cultivated with artificial supply of acetyl-HSL signals showed similar growth and respiration rate with the wild type culture.

Figure 5.1 Growth characterization of WT, MU and MUS. **A.** Confocal laser scanning microscopy (CLSM) imaging of Live/Dead cells immobilized in Ca-alginate for three days; scale bar at 50μm. **B.** Biovolume of live/dead cells for each sample; calculation based on imaging processing of CLSM 3D images. **C.** CLSM of respiring/all cells immobilized in Ca-alginate beads; scale bar at 50μm. **D.** Percentage of respiring cells out of total cells; calculation based on image processing of CLSM 3D images. (*p < 0.05 by Student two-tailed *t*-test)

5.3.2 Transcriptomic data analysis

Illumina Hi-Seq RNA sequencing-based transcriptomic analysis was used to profile QS-regulated genes of *P. aeruginosa* PAO1 entrapped within the artificial Ca-alginate hydrogel matrix at stationary phase, and to investigate the effects of engineered matrix with acetyl-HSL signals on biofilms-based performances. Three groups of transcriptome were extracted from WT (*P. aeruginosa* PAO1 wild type), MU (*P. aeruginosa* PAO1

$\Delta lasI\Delta rhII$ acetyl-HSL generation mutant, non-induced by acetyl-HSL signals) and MUS (*P. aeruginosa* PAO1 $\Delta lasI\Delta rhII$ acetyl-HSL generation mutant, induced by acetyl-HSL signals in the matrix) respectively. Raw data collected from the Hi-Seq RNA sequencing for three groups of RNA were analyzed utilizing CLC genomics Work-bench 6.0, and compared.

5.3.3 Profiling of AHL-QS regulated genes in Ca-alginate entrapped PAO1

We suppose those genes are regulated by acetyl-HSL QS systems that show different expression-levels in MU compared to WT, with filtration criteria as $|\log_2(\text{fold change})| \geq 1.0$ and $p < 0.05$. In general, there were 1093 significant genes identified by comparison, which might be directly or indirectly regulated by C12-HSL and C4-HSL QS signals in the artificial matrix-immobilized *P. aeruginosa* PAO1 at stationary phase, covering 19.25% of total genome. The identified genes were categorized through PseudoCAP functional classes (**Figure 5.2**). Though the coverage of acetyl-HSL QS regulated genes was relatively higher than those reported (Schuster and Peter Greenberg 2006), it was more surprising to note that most of these genes were shown be to repressed by the presence of C4 and C12 acetyl-HSL QS signals in the artificial matrix entrapped cultures. Consistent with the reported analysis, these genes we found to be re involved in various functions, including environmental adaptions and secretion pathways, especially secretion of virulent factors (Schuster et al. 2003; Wagner et al. 2003).

Figure 5.2 Functional classifications of the 1093 genes that were identified to be acetyl-HSL QS regulated in *P. aeruginosa* PAO1 immobilized in Ca-alginate matrix in the stationary phase.

Percentage of each class is calculated as the number of genes in each class, which show either higher or lower expression levels in MU compared to WT, divided by the total number of genes in that class.

Energy metabolism Compared to the wild type, PAO1 $\Delta lasI\Delta rhII$ immobilized in Ca-alginate beads were found to have a higher expression levels of 56 genes out of total 62 genes involved in different energy metabolisms pathways, at stationary phase (**Table 5.3**). These genes include those involved in glycolysis and electron transportation during respiration and fermentation, such as *lpdVG* (lipoamide dehydrogenase), PA3416, PA3417, *ace* (pyruvate dehydrogenase), and *nuoEFGH* (NADH dehydrogenase subunit EFGH). Moreover, genes involved in nitrogen metabolism including *napB* (PA1173, cytochrome C protein NapB), *napA* (PA1174, nitrate reductase catalytic subunit), *nirS*

(PA0519, nitrite reductase) and *norCB* (PA0523, PA0524 nitric-oxide reductase subunits) were identified with significant ($p \leq 0.05$) expression differences.

Table 5.3 MU vs. WT: Energy metabolism (pseudoCAP)

Locus tag ^a	Gene	Product Description	Log ₂ Ratio ^b	p-value
PA2297		Ferredoxin	+ ∞	
PA2694		thioredoxin	+ ∞	
PA2715		Ferredoxin	+ ∞	
PA2996	<i>nqrD</i>	Na(+)-translocating NADH-quinone reductase subunit D	+ ∞	
PA1931		Ferredoxin	+ ∞	
PA3193	<i>glk</i>	Glucokinase	+ ∞	
PA2997	<i>nqrC</i>	Na(+)-translocating NADH-quinone reductase subunit C	4.00	0.029
PA4587	<i>ccpR</i>	cytochrome c551 peroxidase precursor	3.64	0.001
PA1173	<i>napB</i>	cytochrome C protein NapB	3.44	0.021
PA1483	<i>cycH</i>	cytochrome C biogenesis protein	3.18	0.016
PA2994	<i>nqrF</i>	Na(+)-translocating NADH-quinone reductase subunit F	2.89	0.016
PA4571		cytochrome C	2.83	0.018
PA1557	<i>ccoN2</i>	Cytochrome c oxidase, cbb3-type, CcoN subunit	2.59	0.000
PA1555	<i>ccoP2</i>	Cytochrome c oxidase, cbb3-type, CcoP subunit	2.52	0.000
PA2995	<i>nqrE</i>	Na(+)-translocating NADH-quinone reductase subunit E	2.43	0.011
PA1589	<i>sucD</i>	succinyl-CoA synthetase alpha chain	2.38	0.032
PA3879	<i>narL</i>	transcriptional regulator NarL	2.34	0.001
PA4721		sugar fermentation stimulation protein A	+ ∞	
PA5439		glucose-6-phosphate 1-dehydrogenase	2.33	0.046
PA1586	<i>sucB</i>	dihydrolipoamide succinyltransferase (E2 subunit)	2.25	0.002
PA4329	<i>pykA</i>	pyruvate kinase II	2.13	0.017
PA3878	<i>narX</i>	two-component sensor NarX	2.03	0.036
PA5427	<i>adhA</i>	alcohol dehydrogenase	1.99	0.003
PA5491		cytochrome	1.94	0.039
PA2999	<i>nqrA</i>	Na(+)-translocating NADH-quinone reductase subunit A	1.93	0.027
PA4430		cytochrome b	1.93	0.012
PA1556	<i>ccoO2</i>	Cytochrome c oxidase, cbb3-type, CcoO subunit	1.91	0.000
PA1174	<i>napA</i>	nitrate reductase catalytic subunit	1.84	0.000
PA4971	<i>aspP</i>	adenosine diphosphate sugar pyrophosphatase	1.82	0.007
PA3415		branched-chain alpha-keto acid dehydrogenase subunit E2	1.77	0.005
PA1587	<i>lpdG</i>	lipoamide dehydrogenase-glc	1.74	0.002
PA0854	<i>fumC2</i>	fumarate hydratase	1.71	0.006
PA4812	<i>fdnG</i>	formate dehydrogenase-O, major subunit	1.70	0.000
PA0548	<i>tktA</i>	transketolase	1.69	0.042
PA5016	<i>aceF</i>	dihydrolipoamide acetyltransferase	1.69	0.031
PA1588	<i>sucC</i>	succinyl-CoA synthetase beta chain	1.65	0.005
PA5015	<i>aceE</i>	pyruvate dehydrogenase subunit E1	1.62	0.020
PA2643	<i>nuoH</i>	NADH dehydrogenase subunit H	1.55	0.035
PA4922	<i>azu</i>	azurin precursor	1.51	0.000

PA2642	<i>nuoG</i>	NADH dehydrogenase subunit G	1.50	0.000
PA2952	<i>etfB</i>	electron transfer flavoprotein subunit beta	1.50	0.002
PA1787	<i>acnB</i>	aconitate hydratase 2	1.48	0.003
PA3416		pyruvate dehydrogenase E1 component, subunit beta	1.46	0.003
PA3397	<i>fpr</i>	NADP+-dependent ferredoxin reductase	1.45	0.009
PA4732	<i>pgi</i>	glucose-6-phosphate isomerase	1.36	0.006
PA4811	<i>fdnH</i>	nitrate-inducible formate dehydrogenase, beta subunit	1.34	0.041
PA3417		pyruvate dehydrogenase E1 component subunit alpha	1.32	0.010
PA1585	<i>sucA</i>	2-oxoglutarate dehydrogenase (E1 subunit)	1.32	0.002
PA2641	<i>nuoF</i>	NADH dehydrogenase I subunit F	1.29	0.011
PA2640	<i>nuoE</i>	NADH dehydrogenase subunit E	1.25	0.007
PA1562	<i>acnA</i>	aconitate hydratase 1	1.20	0.003
PA2624	<i>idh</i>	isocitrate dehydrogenase	1.17	0.017
PA3930	<i>cioA</i>	cyanide insensitive terminal oxidase	1.15	0.000
PA2250	<i>lpdV</i>	lipoamide dehydrogenase-Val	1.13	0.029
PA1583	<i>sdhA</i>	succinate dehydrogenase (A subunit)	1.10	0.001
PA1175	<i>napD</i>	NapD protein of periplasmic nitrate reductase	1.03	0.003
PA4061		thioredoxin	1.02	0.006
PA4133		cytochrome c oxidase subunit (cbb3-type)	-1.35	0.026
PA0518	<i>nirM</i>	cytochrome C-551	-1.46	0.025
PA2664	<i>fhp</i>	nitric oxide dioxygenase	-1.87	0.020
PA0524	<i>norB</i>	nitric-oxide reductase subunit B	-2.02	0.033
PA0519	<i>nirS</i>	nitrite reductase	-2.02	0.000
PA0523	<i>norC</i>	nitric-oxide reductase subunit C	-3.19	0.001

^a Locus tag in *Pseudomonas aeruginosa* PAO1

^b The ratio indicates the abundance of a gene expressed in MU cells relative to WT cells

In the Ca-alginate immobilized biofilm mimics, cells were habituated in polymeric matrix, where developed bacterial colonies were observed as separate spheres. Hence, gradients of nutrients and oxygen are expected within each hydrogel matrix and within the immobilized biofilm mimics due to diffusion and consumptions. *P. aeruginosa* possesses diverse energy generation pathways, including both respiration and fermentation. In the presence of plenty of oxygen, cells will consume oxygen as terminal electron acceptors, while in case of limited or no oxygen, cells use nitrate or nitrite instead (Hamada et al. 2014; Hassett et al. 2002; Yoon et al. 2002). Arginine is another alternative in the paucity of nitrate or under various stresses (Alvarez-Ortega and Harwood 2007; Eschbach et al. 2004).

In the absence of acetyl-HSL QS signals, the immobilized PAO1 culture showed more abundant expression of aerobic respiration and pyruvate-fermentative related genes, leading to the speculation that the MU samples required more energy for survival and proliferation in comparison with the WT sample. The ratio of respiring cells in the MU samples was significantly higher compared to that in the WT culture, which was consistent with the RNA sequencing data (**Figure 5.1 C, D**). Furthermore, we looked into the gene expressions in two energy consuming metabolic categories, cell division and central intermediary metabolisms, and identified that 6 and 22 genes in each respective category showed higher expression levels in MU than in WT cultures, representing almost 25% of total genes in these two categories (**Table 5.4, 5.5**). The higher expression levels of genes responsible for energy consuming metabolic activities was in accordance with the higher expression levels of energy producing genes in the MU samples.

Table 5.4 MU vs. WT: Cell division (pseudoCAP)

Locus tag ^a	Gene	Product Description	Log ₂ Ratio ^b	p-value
PA0375	<i>ftsX</i>	cell division protein FtsX	1.92	0.003
PA1528	<i>zipA</i>	cell division protein ZipA	1.22	0.002
PA3244	<i>minD</i>	cell division inhibitor MinD	1.17	0.005
PA4000		hypothetical protein	1.52	0.025
PA4419	<i>ftsL</i>	cell division protein FtsL	3.23	0.007
PA1800	<i>tig</i>	trigger factor	1.18	0.042

^a Locus tag in *Pseudomonas aeruginosa* PAO1
^b The ratio indicates the abundance of a gene expressed in MU cells relative to WT cells

Table 5.5 MU vs. WT: Central intermediary metabolism (pseudoCAP)

Locus tag ^a	Gene	Product Description	Log ₂ Ratio ^b	p-value
PA0363	<i>coaD</i>	phosphopantetheine adenylyltransferase	1.97	0.026
PA0710	<i>gloA2</i>	lactoylglutathione lyase	+ ∞	
PA1555.1	<i>ccoQ2</i>	Cytochrome c oxidase, cbb3-type, CcoQ subunit	1.54	0.000
PA2140		metallothionein	+ ∞	
PA2193	<i>hcnA</i>	hydrogen cyanide synthase HcnA	- ∞	
PA3182	<i>pgl</i>	6-phosphogluconolactonase	+ ∞	
PA4204	<i>ppgL</i>	periplasmic gluconolactonase, PpgL	1.05	0.022
PA3471		malate dehydrogenase	1.40	0.042
PA4867	<i>ureB</i>	urease subunit beta	+ ∞	
PA4880		bacterioferritin	1.90	0.027
PA5046		malic enzyme	1.31	0.008
PA5111	<i>gloA3</i>	lactoylglutathione lyase	2.30	0.017
PA5435		pyruvate carboxylase subunit B	1.12	0.006
PA4130		sulfite or nitrite reductase	-1.97	0.005
PA2445	<i>gcvP2</i>	glycine dehydrogenase	1.52	0.006
PA0266	<i>gabT</i>	4-aminobutyrate aminotransferase	1.14	0.038
PA0482	<i>glcB</i>	malate synthase G	1.50	0.007
PA0555	<i>fda</i>	fructose-1,6-bisphosphate aldolase	1.04	0.018
PA0795	<i>prpC</i>	citrate synthase 2	1.13	0.049
PA3181		keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase	2.18	0.019
PA1614	<i>gpsA</i>	glycerol-3-phosphate dehydrogenase, biosynthetic	2.15	0.017
PA4563	<i>rpsT</i>	30S ribosomal protein S20	3.45	0.004
PA5570	<i>rpmH</i>	50S ribosomal protein L34	+ ∞	

^a Locus tag in *Pseudomonas aeruginosa* PAO1

^b The ratio indicates the abundance of a gene expressed in MU cells relative to WT cells

Among the energy related genes, those involved in NO_3^- respiration and denitrification particularly gained our interest, as the nitrogen-related metabolic activities are important in pathogen infections and in ecological applications. It has been reported that a QS mutant with less QS signals is enhanced in NO_3^- reduction and is decreased in production of N_2 , whereas extra addition of C4/C12 signals in QS mutant represses the NO_3^- respiring activity (Toyofuku et al. 2007). Our transcriptomic observation (**Figure 5.3**) is consistent with the reported work that *napA* and *napB*, responsible for the conversion of nitrate to nitrite in PAO1, showed higher expression levels in MU in comparison with WT cultures. However, genes for the subsequent steps in denitrification that converts

nitrite to nitric oxide and nitrous oxide, including *nirS*, *norB* and *norC*, were observed with lower expression levels in MU, indicating a decreased production of nitrogen. The conversion of NO_2^- has been reported to consume ATP produced for anaerobic growth in the lack of oxygen availability, where inactivation of NIR enhances bacterial growth. Moreover, the presence of *nirS* leads to an accumulation of toxic NO inhibiting the growth of biofilm biomasses. Hence, the lower expression levels of *nirS* and *napAB* is the likely reason for the enhanced biofilm development in MU samples.

Figure 5.3 Genes identified involving in denitrification pathway (MU vs. WT)

Therefore, we speculated that acetyl-HSL QS signals represses the energy generation, which is utilized for biofilm development in the artificial matrix, especially under oxygen limited condition. And the bacterial growth of PAO1 QS mutant in the artificial matrix is protected by the reduced generation of NO.

Secreted factors and protein secretion There were 13 genes involved in secreted factors and 28 genes for protein secretion identified among the acetyl-AHL QS dependent genes by transcriptomic analysis, which account for 18.6% and 24.8% in each functional group (**Table 5.6, 5.7**). Most of the protein secretion related genes showed higher abundance ($\log_2\text{Ratio} > 1.20$) in MU cultures in the absence of acetyl-HSL signals, indicating an increased expression level of secreted factors in MU. These identified protein secretion coding genes were revealed to mainly focus on the type III secretion system (T3SS), including *pscO* (PA1696, translocation protein in type III secretion), *pscB* (PA1715, type III export apparatus protein), *pseEFG* (PA1718, PA1719, PA1720, type III export proteins PscEFG) and *pcrG* (PA1705, regulator in type III secretion). Moreover, *exsCE* (PA1710, PA1711, exoenzyme S synthesis protein precursors) involved in T3SS

regulation and *exoT* (PA0044 exoenzyme T), which is one of the four commonly known Type III secretion-effectors in *P. aeruginosa* were identified with higher abundance in MU (Diaz et al. 2011). The ExsC and ExsE are two critical proteins in a regulatory cascade directing the DNA binding efficiency of ExsA in T3SS regulation (Zheng et al., 2012). Briefly, in the absence of host cells, where Ca^{2+} is lower in environment, ExsA binds with ExsD while ExsE binds with ExsC, forming two complexes. While, in contact with the host cell in the presence of calcium, ExsE is released out of cells, facilitating the formation of ExsD-ExsC complexes with ExsA, which binds to the promoters of T3SS genes (Dasgupta et al. 2004; Zheng et al. 2007; Zheng et al. 2012). The T3SS has been revealed to be responsible for translocation of toxins and/or effectors directly into host by PAO1, leading to effective infections (Vance et al. 2005). The identified genes that code T3SS structural components, regulator and effectors all showed higher abundance in MU than in WT, suggesting that, acetyl-HSL QS represses T3SS in the artificial matrix at stationary phase of PAO1 culture. A previous work connecting QS regulation to T3SS in *E. coli* has reported a positive activation of QS on T3SS, whereas the repression of T3SS by QS at higher cell density was reported in *Vibrio* strains (Henke, et al. 2004).

Though the T3SS was observed with enhanced expression levels in MU in the absence of acetyl-HSL signals, more than half of the identified genes coding for secreted factors were found with lower abundance in MU when compared to WT samples. These secreted factor coding genes were reported to be the direct target of LasIR and RhlIR QS regulation systems, such as *lasA* (PA1871, LasA protease precursor), *lasB* (PA3724, elastase LasB) and *rhlAB* (PA3479, PA3478, rhamnosyltransferase subunits) (Cowell et al. 2003; Schuster et al. 2013; Toder et al. 1994). Hence, the observation of lower expression levels of these genes in MU samples are consistent with the absence of acetyl-HSL signals.

Based on the transcriptomic analysis on secretion related genes, we propose the hypothesis that secretion systems are repressed by acetyl-HSL QS regulation in calcium-alginate matrix encapsulated PAO1 cultures, while secreted factors are not broadly and significantly affected. Further investigation on QS regulated virulent factors in biofilm mimics is in need, especially T3SS related secretion molecules.

Table 5.6 MU vs. WT: Secreted factors (pseudoCAP)

Locus tag ^a	Gene	Product Description	Log ₂ Ratio ^b	p-value
PA0044	<i>exoT</i>	exoenzyme T	2.04	0.047
PA0423	<i>pasP</i>	hypothetical protein	1.64	0.035
PA0843	<i>plcR</i>	phospholipase accessory protein PlcR	+ ∞	
PA0852	<i>cbpD</i>	chitin-binding protein CbpD	-3.13	0.006
PA1249	<i>aprA</i>	alkaline metalloproteinase precursor	-1.67	0.003
PA2939		aminopeptidase	-1.78	0.013
PA3478	<i>rhlB</i>	rhamnosyltransferase subunit B	-2.28	0.010
PA3479	<i>rhlA</i>	rhamnosyltransferase subunit A	-5.42	0.003
PA3842		chaperone	+ ∞	
PA5112	<i>estA</i>	esterase EstA	1.13	0.005
PA4528	<i>pilD</i>	type 4 prepilin peptidase PilD	2.17	0.015
PA1871	<i>lasA</i>	LasA protease precursor	-3.35	0.001
PA3724	<i>lasB</i>	elastase LasB	-3.11	0.019

^a Locus tag in *Pseudomonas aeruginosa* PAO1

^b The ratio indicates the abundance of a gene expressed in MU cells relative to WT cells

Table 5.7 MU vs. WT: Protein secretion (pseudoCAP)

Locus tag ^a	Gene	Product Description	Log ₂ Ratio ^b	p-value
PA0081	<i>fhaI</i>	Fha domain-containing protein	1.56	0.041
PA0678		HxcU pseudopilin	+ ∞	
PA0687		type II secretion system protein	+ ∞	
PA0943		hypothetical protein	1.34	0.003
PA1659		hypothetical protein	+ ∞	
PA1664		hypothetical protein	- ∞	
PA1665		hypothetical protein	+ ∞	
PA1666		hypothetical protein	+ ∞	
PA1692		hypothetical protein	+ ∞	
PA1696	<i>pscO</i>	translocation protein in type III secretion	+ ∞	
PA1701		hypothetical protein	+ ∞	
PA1705	<i>pcrG</i>	regulator in type III secretion	+ ∞	
PA1715	<i>pscB</i>	type III export apparatus protein	+ ∞	
PA1719	<i>pscF</i>	type III export protein PscF	+ ∞	
PA2673		type II secretion system protein	+ ∞	
PA4276	<i>secE</i>	secretion protein SecE	1.20	0.002
PA4403	<i>secA</i>	secretion protein SecA	1.69	0.034
PA5210		secretion pathway ATPase	1.15	0.016
PA1711	<i>exsE</i>	ExsE	+ ∞	
PA1670	<i>stpI</i>	StpI	+ ∞	
PA1710	<i>exsC</i>	ExsC, exoenzyme S synthesis protein C precursor.	+ ∞	
PA1718	<i>pscE</i>	type III export protein PscE	+ ∞	
PA1720	<i>pscG</i>	type III export protein PscG	+ ∞	
PA4143		toxin transporter	2.42	0.036
PA2950		trans-2-enoyl-CoA reductase	2.71	0.046
PA1699		hypothetical protein	+ ∞	
PA1700		hypothetical protein	+ ∞	
PA1702		hypothetical protein	+ ∞	

^a Locus tag in *Pseudomonas aeruginosa* PAO1

^b The ratio indicates the abundance of a gene expressed in MU cells relative to WT cells

5.3.4 Gene regulation by functionalized artificial matrix

To investigate the regulation of biofilm mimics-based bioprocesses by engineered hydrogel matrix, we compared the transcriptomes between sample MUS and sample MU. In MUS, *P. aeruginosa* PAO1 $\Delta lasI\Delta rhII$ acetyl-HSL generation mutant was cultivated under induction of acetyl-HSL in the engineered matrix; while in MU, PAO1 $\Delta lasI\Delta rhII$ was cultivated without acetyl-HSL.

In total, we identified 723 significant genes, and categorized based on PseudoCAP functional classes as shown in **Figure 5.4**. Less than 5% (31 genes) of these significant genes showed higher expression levels by the induction of acetyl-HSL signals in the matrix, while most other genes were down regulated. The trend of engineered matrix-induction was similar to those regulated by self-generated signals, though the overlap of these significant genes was less than 50%. Two hypotheses are proposed to explain the differences, (1) different levels of total acetyl-HSL present in MUS and WT, (2) different heterogeneities of acetyl-HSL distribution between MUS and WT. The engineered matrix with introduced acetyl-HSL signals is speculated to regulate global gene expression which differ from those induced by self-generated signals. The responses of biofilm mimics to the engineered artificial matrix remain unclear, which shall be a critical challenge for application of the engineered matrix in biofilm study.

Figure 5.4 Functional classifications of the significant genes compared between MUS and MU. Percentage of each class is calculated as the number of genes in each class divided by the total number of genes in that class.

According to the transcriptomic analysis comparing MUS and MU samples, the identified significant genes included 19 (11%) involved in energy metabolism, 18 (14%) for carbon compounds metabolism, 9 (10%) in central intermediary metabolism, and 9 (13%) responsible for motility and attachment. Almost all of these genes showed decreased expression levels in the presence of acetyl-HSL induction by engineered matrix in MUS than the non-induced MU culture, which is consistent with our QS-dependent genes identified in the Ca-alginate immobilized cultures. Moreover, we looked into those genes playing a role in the secretion and adaptation in the following section.

Protein secretion and secreted factors There were 33 genes involved in protein secretion system and 7 genes responsible for secreted factors, which were identified to be differentially expressed by comparing MUS and MU transcriptomes (**Table 5.8, 5.9**). Not surprisingly, protein-secretion related genes, especially those in T3SS and T2SS, were either not detectable or showed lower expression levels in MUS sample, which is consistent with the previous results of QS-regulation in the artificial matrix. Repressed expression of T3SS related genes by introducing C12 and C4 into the artificial matrix further suggested that biofilm mimics developed in the engineered matrix with acetyl-HSL signals has a lower capability of toxin secretion.

Table 5.8 MUS vs. MU: Secreted factors (pseudoCAP)

Locus tag ^a	Gene	Product Description	Log ₂ Ratio ^b	p-value
PA2303	<i>ambD</i>	Secreted Factors (toxins, enzymes, alginate)	- ∞	
PA2774		hypothetical protein	- ∞	
PA4213	<i>phzD1</i>	phenazine biosynthesis protein PhzD	- ∞	
PA4216	<i>phzG1</i>	probable pyridoxamine 5'-phosphate oxidase	- ∞	
PA2255	<i>pvcB</i>	paerucumarin biosynthesis protein PvcB	- ∞	
PA4225	<i>pchF</i>	pyochelin synthetase	-1.067	0.037

^a Locus tag in *Pseudomonas aeruginosa* PAO1
^b The ratio indicates the abundance of a gene expressed in MUS cells relative to MU cells

Table 5.9 MUS vs. MU: Protein secretion (pseudoCAP)

Locus tag ^a	Gene	Product Description	Log ₂ Ratio ^b	p-value
PA0076		hypothetical protein	- ∞	
PA0087		hypothetical protein	- ∞	
PA0680		HxcV pseudopilin	- ∞	
PA1659		hypothetical protein	- ∞	
PA1666		hypothetical protein	- ∞	
PA1668		hypothetical protein	- ∞	
PA1691	<i>pscT</i>	translocation protein in type III secretion	- ∞	
PA1692		translocation protein in type III secretion	- ∞	
PA1693	<i>pscR</i>	type III secretion system protein	-3.123	0.009
PA1698	<i>popN</i>	Type III secretion outer membrane protein PopN precursor	- ∞	
PA1701		hypothetical protein	- ∞	
PA1705	<i>pcrG</i>	regulator in type III secretion	- ∞	
PA1711	<i>exsE</i>	ExsE	- ∞	
PA1715	<i>pscB</i>	type III export apparatus protein	- ∞	
PA1719	<i>pscF</i>	type III export protein PscF	- ∞	
PA1722	<i>pscI</i>	type III export protein PscI	- ∞	
PA1723	<i>pscJ</i>	type III export protein PscJ	- ∞	
PA1724	<i>pscK</i>	type III export protein PscK	- ∞	
PA1725	<i>pscL</i>	type III export protein PscL	- ∞	
PA1867	<i>xphA</i>	Protein secretion/export apparatus	- ∞	
PA2672		type II secretion system protein	- ∞	
PA2673		type II secretion system protein	- ∞	
PA2674		type II secretion system protein	- ∞	
PA2676		type II secretion system protein	- ∞	
PA2866	<i>mttC</i>	secretion protein MttC	- ∞	
PA3099	<i>xcpV</i>	general secretion pathway protein I	- ∞	
PA3100	<i>xcpU</i>	General secretion pathway outer membrane protein H precursor	- ∞	
PA1720	<i>pscG</i>	type III export protein PscG	- ∞	
PA4143		toxin transporter	-2.544	0.042
PA1699		hypothetical protein	- ∞	
PA1700		hypothetical protein	- ∞	
PA1702		hypothetical protein	- ∞	
PA3406	<i>hasD</i>	transport protein HasD	- ∞	

^a Locus tag in *Pseudomonas aeruginosa* PAO1

^b The ratio indicates the abundance of a gene expressed in MUS cells relative to MU cells

Adaptation, protection, and antibiotic resistance

There were 12 (9%) genes for adaptation and protection, and 4 (24%) genes for antibiotic resistance and susceptibility identified to be differentially expressed by comparing MUS to MU cultures (**Table 5.10, 5.11**). Most of these genes were absent or showed lower expression levels (with log₂Ratio

< -1.6) in MUS cultures in the presence of acetyl-HSL induction than in MU. Interestingly, most of these genes are responsible for transportation of either lipopolysaccharides (LPS) or proteins. *arnT* (PA3556, inner membrane L-Ara4N transferase ArnT), and *arnEF* (PA3556, PA3557, ArnEF) are genes coding the inner membrane protein that are involved in modification of LPS to outer membrane for adaptive resistance (Delucia et al. 2011; Werneburg et al. 2012). *oprN* (PA2495, multidrug efflux outer membrane protein OprN), which is involved in conferring resistance to antibiotic compounds, has also been reported to be responsible for exportation of 2-heptyl-3-hydroxy-4-quinolone (PQS) precursors. PQS is regarded as the third QS pathway in PAO1, correlating with *las* and *rhl* systems (Yang et al. 2009). As such, *oprN* involved MexEF-OprN efflux pump shows impacts in many QS-dependent virulence phenotypes (Wang et al. 2013). Decreased expression level of *oprN* in MUS cultures is consistent with the speculation that, the requirement of PQS complementation reduces in the presence of acetyl-HSL induced MUS culture than in non-induced MU culture. Therefore, we hypothesize that, in the engineered artificial matrix, PAO1 requires less adaptive mechanisms, especially secretive procedures, for protection and antibiotic resistance.

Table 5.10 MUS vs. MU: Antibiotic resistance and susceptibility; Adaptation; protection (pseudoCAP)

Locus tag ^a	Gene	Product Description	Log ₂ Ratio ^b	p-value
PA0177		purine-binding chemotaxis protein	- ∞	
PA1159		cold-shock protein	1.095	0.019
PA1782		serine/threonine-protein kinase	- ∞	
PA1858	<i>str</i>	streptomycin 3"-phosphotransferase	- ∞	
PA2055		major facilitator superfamily (MFS) transporter	- ∞	
PA2185	<i>katN</i>	non-heme catalase KatN	- ∞	
PA2495	<i>oprN</i>	Multidrug efflux outer membrane protein OprN	- ∞	
PA2832	<i>tpm</i>	thiopurine S-methyltransferase	- ∞	
PA3331		cytochrome P450	- ∞	
PA3556	<i>arnT</i>	inner membrane L-Ara4N transferase ArnT	- ∞	
PA3557	<i>arnE</i>	ArnE	- ∞	
PA3558	<i>arnF</i>	ArnF	- ∞	
PA3719	<i>armR</i>	antirepressor for MexR, ArmR	+ ∞	
PA4557	<i>lytB</i>	LytB protein	-1.632	0.027
PA4612		hypothetical protein	-2.762	0.041
PA5536		hypothetical protein	- ∞	

^a Locus tag in *Pseudomonas aeruginosa* PAO1
^b The ratio indicates the abundance of a gene expressed in MUS cells relative to MU cells

5.4 Conclusions

In this study, using *P. aeruginosa* as a model organism, we compared the transcriptomes of Ca-alginate immobilized cultures of wild type and *lasIrhII* double deletion mutant, which cannot synthesize acetyl-HSL signals. We globally identified acetyl-HSL QS-dependent genes of PAO1 in biofilm mimics and found that (i) aerobic and fermentative related energy generation is repressed in artificial matrix-immobilized PAO1 cultures and cells require less energy in the matrix under the regulation of quorum-sensing.; (ii) Ca-alginate matrix-immobilized PAO1 cultures requires higher extracellular secretion to enhance the survivability in the absence of acetyl-HSL QS regulation.

Furthermore, by comparing transcriptomes between acetyl-HSL induced and non-induced PAO1 *lasIrhII* double deletion mutant grown in the artificial matrix, we aimed to investigate how engineered matrix can regulate biofilm mimics-based bio-performances.

We found that expression of secretion related genes, both virulence- and adaptation/antibiotic resistance related were downregulated.

Our finding advances the fundamental understanding of the acetyl-HSL QS regulation in hydrogel-entrapped biofilm mimics, and biofilms-based performances in engineered matrix by introducing signals, which can lead to strategies for more efficient biofilm-mimic engineering in various applications.

Microfluidic Gradient Generator-Flow Cell System as a Novel Tool to Study Biofilm Development under Defined Chemical Gradients

6.1 Introduction

Biofilms, surface-associated microbial communities encased within a self-produced matrix of extracellular polymeric substances (EPS), are the most ubiquitous and resilient form of microbial lifestyle on earth (Hansen et al. 2007). The robustness, structural stability, and tolerance to harsh environmental conditions make biofilms an attractive choice for industrial applications, such as biocatalysis, bioremediation and wastewater treatment (Gross et al. 2007). However, highly dynamic and heterogeneous microenvironments in the EPS matrix and an intrinsically complex life cycle of biofilms as well as the interactions between them pose a great challenge for predicting and controlling the performance of biofilm-mediated bioprocesses (Zhang et al. 2011). Therefore, there is a critical need to understand the influence of environmental conditions including hydrodynamic and chemical conditions on biofilm development.

Devices that are widely applied to study biofilm development are mainly designed as continuous flow through chambers. One most commonly used flow chamber is a 3-channel flow cell, which is configured to be assembled and disassembled with ease (Tolker-Nielsen and Sternberg 2011). This flow chamber enables non-destructive, direct microscopic observation of biofilm structures under hydrodynamic conditions (Tolker-Nielsen and Sternberg 2011). This 3-channel flow cell and other similar biofilm flow chambers have been extensively applied to investigate biofilm development under simple, whereas uncontrolled fluidic conditions. Zhang et al. (2011) developed a novel planar flow cell capable of achieving precise control of flow velocity and shear force distribution within the chamber. This planar flow cell has been applied to investigate the influence of

flow velocity on biofilm growth and interspecific interaction in multispecies biofilms (Zhang et al. 2013; Zhang et al. 2011). Other designs of biofilm growth devices capable of controlling hydrodynamic conditions have also been reported. For instance, De La Fuente et al. (2007) designed a microfluidic chamber to examine the impact of fluid drag forces and wall stresses on initial attachment of bacteria on surfaces (De La Fuente et al. 2007). Englert et al. (2010) constructed a flow-based microfluidic device (μ Flow) capable of generating concentration-gradients of chemoeffectors. The μ Flow device has been used to study bacterial migration in response to the gradients of chemoeffectors. Kim et al. (2010) developed a microfluidic system enabling the generation of stable antibiotic concentration gradients within the system. This microfluidic system was utilized to quantitatively and physiologically evaluate bacterial susceptibility to antibiotics in biofilm, whereas the chamber with antibiotic gradient was only fabricated as 300 μ m in width. In contrast, biofilm growth chambers capable of generating defined chemical gradients within a proper dimension for biofilm development are lacking.

The goal of this study was to develop a novel fluidic system that enables us to investigate biofilm development under defined chemical gradients. Specifically, we designed and fabricated a novel fluidic device by coupling a microfluidic gradient generator to a confocal laser scanning microscopy (CLSM)-compatible biofilm growth chamber, which allows non-destructive, continuous monitoring of biofilm development under defined chemical gradients. The chemical gradients generated in the biofilm growth chamber were simulated and validated experimentally. By using *Shewanella oneidensis* and *Comamonas testosteroni* as model organisms, we then demonstrated the application of this fluidic system in quantifying biofilms development under defined calcium and nitrate gradients.

6.2 Materials and methods

6.2.1 Design and fabrication of microfluidic gradient generator-flow chamber

The microfluidic gradient generator-flow chamber was designed by coupling a microfluidic chemical mixer to a biofilm flow chamber (**Figure 6.1A**). The fluidic system consists of three layers: the top layer, which is a microfluidic chemical mixer; the middle layer, which is a biofilm growth chamber; and the bottom layer, which is a glass slide substratum for biofilm growth (**Figure 6.1B**). The top and middle layers were made of polydimethylsiloxane (PDMS). The fabrication protocols were modified based on commonly applied PDMS fabrication procedures (Eddings et al. 2008; Guo et al. 2014; Sia and Whitesides 2003).

Figure 6.1 A. Design and specifications of the gradient-generation flow cell. The system consists of one gradient generator with two medium inlets and seven levels of chemical mixer, one flow cell chamber with two cell inoculation inlets and two inoculation outlets, and 9 main outlets at the end of the chamber as well. B. The microfluidic gradient-generation flow cell consists of three layers. Bottom is a microscope cover glass for direct observation by imaging facility; middle layer is a 2 mm PDMS sheet of open biofilm chamber and bacterial inoculation and effluent channels; top layer is the chemical mixer made of PDMS.

6.2.2 Simulation of chemical gradients

Simulation of chemical gradients was conducted using COMSOL Multiphysics 4.3b (COMSOL, Inc.) (Hashim et al. 2012). The geometry was designed in AutoCAD 2007 (Autodesk), processed as *stl* file, and imported into COMSOL. The modules of *Laminar Flow* (**Equations 1 and 2**) and *Transport of Diluted Species* (**Equation 3**) in COMSOL were applied to simulate the fluid flow, chemical mixing and diffusion inside the biofilm growth chamber:

$$\frac{\partial \rho}{\partial t} + \rho \cdot \nabla \vec{u} = 0 \quad (\text{Equation 1})$$

$$\rho \frac{\partial \vec{u}}{\partial t} + \rho \vec{u} \cdot \nabla \vec{u} = -\nabla p + \nabla \cdot \left(\mu (\nabla \vec{u} + (\nabla \vec{u})^T) - \frac{2}{3} \mu (\nabla \cdot \vec{u}) \vec{I} \right) + \vec{F} \quad (\text{Equation 2})$$

$$\frac{\partial c}{\partial t} = D \nabla^2 c - \vec{u} \cdot \nabla c + R \quad (\text{Equation 3})$$

where ρ is the flow density, $\vec{u}$ is the flow velocity, p is the fluid pressure, μ is the fluid dynamic viscosity, $\vec{F}$ is the external force, D is the diffusion coefficient, c is the chemical concentration, and R is the chemical reaction rate.

Chemical concentration at respective mixing output at each mixing level in the microfluidic gradient generator can be calculated based on the chemical dilution equation shown as **Equation 4** (Lee et al. 2009):

$$C_i = \frac{V_{L,i}}{V_{L,i} + V_{R,i}} C_0 + \frac{V_{L,i}}{V_{L,i} + V_{R,i}} C_{i+1} \quad (\text{Equation 4})$$

where C_0 represents the original chemical concentration at the input, C_i is the chemical concentration at mixing output, V is the flow rate that is tightly linked with the channel length L and flow resistance R .

6.2.3 Experimental validation of chemical gradients

The simulation results of the chemical gradients were validated by experimental results using bovine serum albumin (BSA), CaCl_2 , and KNO_3 as the model chemicals with sterilized water as a solvent. Generation of chemical gradient was achieved by introducing chemical containing solution at inlet 1 and water at inlet 2, where the inlet concentrations were 2 mg/L, 0.68 mM, and 4 mM for BAS, CaCl_2 and KNO_3 respectively. The solution / water was introduced into the gradient generator chamber at a flow rate of 3 mL/h by a syringe pump (NE-1000, New Era Pump Systems Inc., NY, USA). A duration of 30 min was set for equilibrium of the chemical gradients after the chamber was fully-filled, followed with 1 mL of effluent sample from each individual outlets of the chamber was collected and quantified. BSA was quantified using BCA assay following the protocol described elsewhere (Zhang et al. 2014). Ca^{2+} concentration was quantified using Ion Chromatography (Rieman and Walton 2013). Nitrate concentration was determined by using HACH kits with a HACH DR 3900 photometer based on manufacturer's instructions (HACH, Loveland, Co) (Wu et al. 2015).

Comparison of the experimental validation and simulation was evaluated by the goodness of fit as shown in **Equation 5**:

$$R^2 = 1 - \frac{\sum_{i=1}^9 (C_{s(i)} - C_{e(i)})^2}{\sum_{i=1}^9 (C_{s(i)} - C_{s\text{ ave}})^2} \quad (\text{Equation 5})$$

Where R^2 is the goodness of fit, i represents the i^{th} effluent position, C_s is the simulated concentration and C_e is the experimental concentration.

6.2.4 Bacterial strains and growth conditions

S. oneidensis MR-1 tagged with a green fluorescent protein (MR-1-gfp) (Zhang et al. 2014), *C. testosteroni* WDL7 wild-type (WDL7-wt), and *C. testosteroni* I2 tagged with a red fluorescent protein (I2-rfp) (Boon et al. 2002) were used in this study. The MM1 medium and M9 medium were used for the growth of *S. oneidensis* and *C. testosteroni*, respectively (Ding et al. 2014; Wu et al. 2015).

6.2.5 Biofilms development under defined chemical gradients

Biofilms development of *S. oneidensis*: MM1 media with and without 0.68 mM CaCl_2 were supplied at a flow rate of 3 mL/h to the fluidic system through inlets 1 and 2 of the microfluidic generator, respectively, using a syringe pump (NE-1000, New Era Pump Systems Inc., NY, USA). After 3 h to fully-fill the whole fluid system, the media flow was stopped and *S. oneidensis* MR-1-gfp was introduced into the biofilm growth chamber through the cell inoculation inlets. The inocula were prepared by diluting an overnight culture of MR-1-gfp using fresh MM1 medium without CaCl_2 to a final OD_{600} of 0.1. After 5 mL of the inocula being injected into the growth chamber, all flows were stopped for 2 h to allow the bacteria to attach onto the glass slide. Then the media flows was resumed at a rate of 3 mL/h.

Biofilms development of *C. testosteroni*: *C. testosteroni* WDL7-wt and I2-rfp single-species biofilm was cultivated in the gradient-generator-flow chamber following a similar protocol. Briefly, M9 minimal medium with and without 4 mM KNO_3 was delivered through inlets 1 and 2 of the microfluidic generator, respectively, at a flow rate of 3 mL/h for biofilm growth. Inocula of WDL7-wt / I2-rfp by diluting the overnight culture with fresh M9 medium without additive nitrate to a final OD_{600} of 0.1 were injected in the

growth chamber. All flows were stopped for 2 h to allow bacteria to attach onto the glass slide, before the medium flow was resumed at a rate of 3 mL/h.

Similarly, the same protocol for chemical gradient generation and biofilm development was applied to develop WDL7 and I2 co-organism biofilms, as the single-species biofilm within this microfluidic device. The overnight cultures of WDL7 and I2 were both adjusted to a density of OD₆₀₀ at 1, before a mixture of the two species were prepared at a volumetric ratio of 1:1. The mixture was adjusted to a density of OD₆₀₀ at 0.1 with fresh M9 medium for inoculation in the growth chamber.

6.2.6 CLSM image acquisition and processing

Biofilm development in the growth chamber was monitored using a confocal laser scanning microscope (CLSM, ZEISS LSM780, Oberkochen, Germany) following the modified method described previously (Wu et al. 2015). Briefly, nucleic acid specific fluorescent dyes, SYTO9 (stains all cells) and propidium iodide (PI stains membrane compromised cells) (ThermoFisher Scientific, Massachusetts, US), were used whenever necessary. The acquisition of CLSM images taken at specific x locations were set with a size of 425 μm x 425 μm x 100 μm , and 5 replicates were applied for one location. The size of CLSM tile-image was set as 425 μm x 3.5 mm with a scaling of 0.42 μm x 0.42 μm . Images were further processed by image processing and analysing software IMARIS (Bitplane, Belfast, UK) and Comstat (Heydorn et al. 2000).

6.3 Results

6.3.1 Chemical gradients in the microfluidic gradient generator-flow chamber

Simulation results of chemical gradients in the microfluidic gradient generator-flow chamber are shown in **Figure 6.2**. To better illustrate the positions within the chamber,

x -axis was defined as the direction normal to the flow and y -axis was the direction parallel to the flow (**Figure 6.2A**). The chemical gradient was generated along the x -axis from $x = 0$ mm to $x = 20$ mm, while no significant gradient was observed along the y -axis from $y = 0$ mm to $y = 35$ mm (**Figure 6.2B**). Moreover, different diffusion coefficients (D) within a wide range of 10^{-9} to 10^{-13} m²/s showed no apparent impacts on the chemical gradient generated in the chamber effluent side where $y = 35$ mm (**Figure 6.2C**), suggesting the applicability of the fluidic system to generate defined gradients for various commonly used chemicals ranging from macromolecules to low-molecular weight organic compounds and inorganic ions.

To validate the simulated results, we used BSA, nitrate, CaCl₂ as model chemicals and quantified the chemical gradients at the outlets (equal to positions at $y = 35$ mm) of the growth chamber operated with the same parameters set for the simulation. The chemical gradients generated in our experiments for BSA and calcium chloride were in good agreement with the simulated results that R^2 were above 0.9 (**Figure 6.2D**). However, the goodness of fit of experimental nitrate concentration gradient to simulation was 0.763, that discrepancy occurred especially at positions where concentrations were relatively low.

Figure 6.2 A. Schematic illustration of the positions in the chamber. The x-axis is defined normal to the fluid flow, and y-axis is defined parallel to the fluid flow. Positions at the edge close to inlet 1 were defined as $(x, y) = (0 \text{ mm}, y)$, and those at edges close to inlet 2 were defined as $(x, y) = (20 \text{ mm}, y)$. Similarly, positions at the inlets were termed as $(x, y) = (x, 0 \text{ mm})$, and positions at outputs were termed as $(x, y) = (x, 35 \text{ mm})$. B. Simulated chemical gradients along x-axis at various y positions in the chamber. C. Simulated chemical gradients with a series of diffusion coefficient (D) along x-axis at $y = 35 \text{ mm}$. D. Experimental validation results (shown as data points and respective trend lines) and comparison with simulation results. Goodness of fit of each model chemical molecule was calculated and shown as well.

6.3.2 Development of single-species biofilms under ion-gradients

To study the impact of substrate-gradients on biofilm development, we developed *S. oneidensis* MR-1-gfp biofilms under a defined gradient of CaCl_2 in MM1 medium in the microfluidic chamber. The biofilm development was observed by CLSM tile imaging at $y = 3.5\text{mm}$ along the x -direction of the chamber from $x = 0\text{ mm}$ to $x = 20\text{ mm}$. The representative images at day 3, 5 and 7 are shown in **Figure 6.3A** for elucidation, and the quantitative analysis of biofilm biovolume, surface coverage and roughness was carried out based on day 7 tile image(**Figure 6.3B, C**). The biovolume showed an increasing trend with the increase in Ca^{2+} concentration in the supplied medium (i.e. $5 \times 10^5\text{ um}^3$ when calcium absent, and $4 \times 10^6\text{ um}^3$ when calcium reached 0.68 mM) (**Figure 6.3B**). Moreover, surface coverage and roughness (termed as the standard deviation of biofilm thickness) (Heydorn et al. 2000) of biofilm were maintained at similar levels with the increase of Ca^{2+} in the medium, with coverages less than 20% and roughness larger than 0.4. However, at positions where Ca^{2+} reached a level higher than 0.5 mM , biofilms coverage on the surface increased dramatically, reaching almost 100% while the roughness decreased to 0.1 (**Figure 6.3C**). The quantitative analysis of biofilm development under defined ion gradients showed positive influence of ion in substrate to biofilm development, and indicated a presence of key concentration for biofilm attachment and formation.

Figure 6.3 A. Representative CLSM images of MR1-gfp biofilms at different x positions (y position was kept at y = 1.4mm) taken on day 3, day 5 and day 7. CaCl₂ concentration for each x position is predicted according to simulation results. B. Biofilm biovolume at day 7 were calculated based on tile images by Imaris, and were plotted along x-axis together with predicted calcium concentrations. C. Roughness and surface coverages at day 7 were calculated of image area, and were plotted along calcium concentrations. Surface coverage was present in percentage, and roughness was determined by the standard deviation of average thickness of the biofilms.

6.3.3 Biofilm comparison of different strains under same nitrate-gradients

To investigate the responses of different strains to same substrate-gradients, we developed *C. testosteroni* WDL7 and I2-rfp single-species biofilms under a defined gradient of potassium nitrate, from 0 to 4 mM, in the chamber. The biofilm development was monitored by CLSM tile imaging at early maturation state at $y = 3.5\text{mm}$ along x direction respectively. Bacterial cells were stained with SYTO9/PI and representative images under various nitrate concentrations were shown in **Figure 6.4A**. Quantitative analysis was carried out based on the tile images, that representative biovolumes corresponding to four x positions were plotted together with estimated nitrate concentration (**Figure 6.4B**).

As shown by the quantitative results, biovolumes of both WDL7 and I2 showed positive relation with the nitrate supply in the medium. The biofilm development of WDL7 at positions where nitrate supply was higher than 3 mM was significantly higher than that of I2, reaching almost 6- to 8- folds of difference. The quantitative biovolume of I2 was less affected by nitrate supply once it reached 1 mM in the substrate, whereas minimal biofilm attachment and formation was observed for WDL7, where nitrate supply was as low as 1 mM. Meanwhile, the appearance of the microcolonies observed within each biofilm was different from the single colonies, which was present only in I2. These observational and quantitative comparisons revealed different responses to the same substrate-condition by different organisms. More specifically, these results showed significant variation in biofilm developmental capability and in nitrate metabolism of WDL7 and I2.

Figure 6.4 A. CLSM images of WDL7 and I2 monoculture biofilms under different nitrate concentrations in medium. Images were taken at early maturation stage (day 4) by bacterial live/dead staining along x-axis where $y = 1.4\text{mm}$. B. Quantitative analysis of CLSM tile images for WDL7 and I2 monoculture biofilms at selected positions were plotted along x-axis together with predicted nitrate concentration.

6.3.4 Development of dual-species biofilms under nitrate-gradients

To study the substrate-gradients on the development of biofilm communities, we developed *C. testosteroni* WDL7-wt and I2-rfp dual organism biofilms in the gradient generator flow chamber in which nitrate concentrations were generated from 0 to 4 mM. The biofilm was monitored by CLSM imaging at $y = 3.5\text{mm}$ along x direction at early maturation by staining both strains with SYTO9 (Ex/Em at 495/535 nm). As shown in the

representative images (**Figure 6.5A**), the dual organism culture was capable of developing into aggregative biofilm structures with separate microcolonies throughout the chamber regardless of nitrate supply. The I2-rfp showed a tendency to be present in the central position of the biofilms, accounting for a relatively less portion of the overall biofilm biomass. Moreover, quantitative analysis of biofilm biovolume was carried out to elucidate the development of each organism (**Figure 6.5B**). The dual organism biofilms showed a positive relation between total biovolume and nitrate supply, though there was still significant biovolume developed, where no nitrate was present in the substrate. Also, WDL7-wt appeared to be dominant in the overall biovolume comparison with I2-rfp, which showed less relevance with nitrate supply in the cocultures. The development of dual organism under defined nitrate gradients gave a hint on the structural distribution, metabolic interactions and cooperation among different organisms in the biofilm communities.

Figure 6.5 A. CLSM images of WDL7 and I2-rfp dual-organism biofilms under nitrate concentration gradients in medium (all images were kept at $y = 1.4\text{mm}$); all cells were stained by SYTO9 and were observed at 495/535 nm (in green); I2-rfp cells were observed directly at 561/593 nm (in red). All scale bars are 50.0 μm . B. Biofilm biovolume of WDL7 and I2-rfp in biofilms were calculated based on the CLSM images and were plotted along x-axis together with predicted nitrate concentration.

6.4 Discussion

Biofilm development and biofilm-based functions are sensitive to environmental chemical conditions, such as substrate nutrients, oxygen and signal molecules. The study of biofilm responses to chemical environments, which is always dynamic, requires fine spatial control of local chemical concentrations and chemical distributions during biofilm development.

Generation of chemical gradients to mimic natural stimuli for biological studies has been successfully investigated for tissue cell migration, differentiation and chemotaxis, and for

microbial gene expressions, antibiotic resistance and chemotaxis motility (Hong et al. 2012; Kim et al. 2010). Several different approaches have been developed to achieve controllable chemical gradients in microenvironments. Traditionally, chemical gradients were generated in hydrogel matrix either by molecular diffusion or micropipette injection, which gives low reproducibility and spatial control (Velve-Casquillas et al. 2010). Other approaches that generated gradients in chamber and on membrane by molecular diffusions are also limited by poor stability (Lin and Levchenko 2015).

More recently, the emerging technology of microfluidic design and fabrication enables the creation of complex and stable chemical gradients (Hol and Dekker 2014). The most basic design is “T” mixer, which contains two inlet channels for two different chemical concentrations and one output channel, where two inlet flows merge together and diffusion of molecules occurs across the interface (Lin and Levchenko 2015; Wu et al. 2012). Based on the modified “T” mixer design, microfluidic device has generated glucose gradients for biofilm development study, although width of the gradient generated was limited in the chamber (Song et al. 2014). The chemical mixing of the two streams are mainly driven by chemical diffusion, which is highly time and length dependent according to the advection-diffusion equation (**Eq.3**) for most chemical particles in fluid based “T” mixer (Walker et al. 2005). For instance, the chemical particles can only travel 2 mm max according to the advection-diffusion estimation of two streams (flow rate between 1 mL/h to 10 mL/h) in a “T” chamber of 30 mm in length. The gradient pattern generated by simple “T” mixer is not ideal for biofilm development study as biofilm formation usually occurs in a relatively larger surface area in engineering and environmental applications. Upgraded version of the flow-based gradient generator includes pre-mixer and dilution networks (microfluidic mixer), which have already been utilized to study bacterial chemotaxis responses to effectors (Englert et al. 2010; Jeon et

al. 2000; Ricart et al. 2011). Also, the dilution network, which is able to generate stable and reproducible chemical gradients has been investigated for the application of scanning bacterial / initially attached biofilm susceptibility to antibiotics (Kim et al. 2010).

Being inspired by the microfluidic design for chemotaxis study (Englert et al. 2010; Kim et al. 2012), we developed a microfluidic gradient generator-flow chamber for biofilm development, combining a flow-based microfluidic gradient mixer and a biofilm cultivation chamber. Dimensions of the whole device are designed to maintain laminar flow for biofilm experiments, to reduce the effects from physical dynamic environments. The Reynolds number in our centre chamber with rectangular cross section was estimated smaller than 10 when the flow rate was lower than 10 mL/h for biofilm development, which was minimal compared to the critical Reynolds number at 1,000 for the turbulence (Ismagilov et al. 2000; Stroock et al. 2002). Thereafter, chemical gradient in the substrate supply was considered as the sole environmental variable for biofilm development within this gradient generator-flow chamber system.

The two-inlets-gradient generator utilizes a cascaded-mixing to overcome the disadvantages present in “T” mixers and fluid-filled reservoirs that rely on advection-diffusion and / or diffusion over the short distance. In our device, a series dilution of the initial source was achieved by a cascade of mixings, where the concentration of mixed stream in each output of the i^{th} level can be calculated by **Eq.4**. Meanwhile, the diffusion of particles also took place between each mixed stream in each level and in the centre chamber, as modeled by the advection-diffusion equation (**Eq.3**). In the centre chamber, advection-diffusion is responsible for the downstream chemical mixing. Advection occurs only along y -axis and diffusion is minimal for consideration except along x -axis, though

the effects are estimated as minimal in our preset experimental conditions (Walker et al. 2005).

The experimental validation results of both BSA and calcium had goodness of fit above 0.9 to simulation, whereas the goodness of fit was only 0.76 for potassium nitrate to the simulation. This is supposed to be a consequence of molecular adsorption onto the material surface of the chamber, and the limitation of the quantification process.

Single-species biofilm development of *S. oneidensis* MR-1-gfp under calcium gradients was designed and carried out to elucidate the application of this device in biofilm formation study. According to the observation (**Figure 6.3**), calcium was proposed to be a critical impact factor to enhance biofilm attachment and development which is consistent with the previously reported phenomenon (Theunissen et al. 2010). Moreover, a critical calcium concentration around 0.5 mM was observed from where both bottom coverages and roughness dramatically changed (**Figure 6.3B, C**). Limited surface coverages with significant roughness of biofilms where calcium concentration was lower than the critical concentration suggested a strong limitation of biofilm attachment and development in the lack of calcium. The dramatic changes of all these parameters indicated a minimal calcium supply for MR-1-gfp biofilm formation such that MR-1-gfp developed into relatively uniform 3D structures covering the whole bottom surface instead of separated microcolonies.

Calcium supplemented in growth medium has been revealed to have positive impacts on biofilm formation, by affecting cell attachment, motility, and biofilm structures (Cruz et al. 2012). Calcium regulates extracellular polymeric substance production during biofilm formation and development in many species (Patrauchan et al. 2005; Sarkisova et al. 2005), and calcium-binding proteins play important role in bacterial surface adhesion

(Porsch et al. 2013). Specifically, in *S. oneidensis* MR-1, calcium-binding proteins have been reported to be involved in initial attachment, and calcium stimulates auto-aggregation of MR-1 cells significantly (Satchell 2011; Sivakumar et al. 2015; Theunissen et al. 2010). Hence, the positive relation between biofilm formation and calcium supply in substratum was as expected, indicating the practicability of this gradient generator flow chamber.

Furthermore, this gradient generator flow chamber can be utilized to analyze and compare biofilm development of different organisms under the same chemical gradients, since it is able generate defined and reproducible chemical environment. The positive impact of nitrate supply for single-species biofilm development was observed in the gradient generator flow chamber for both *C. testosteroni* WDL7 and I2, whereas different structures and minimal requirements of nitrate supply were found. It has been reported that denitrification, which is a sequential reduction of nitrate, contributes to anaerobic growth of biofilms, and that most *C. testosteroni* has denitrification metabolic pathways (Borriello et al. 2004; Wu et al. 2015).

According to the genomic information of WDL7 and I2, both organisms were capable of nitrate reduction, while only I2 was revealed to be able to utilize nitrate as energy source during denitrification (Wu et al. 2015). Our observation of I2-rfp biofilm was consistent with the prediction from genomic information that nitrate promotes biofilm attachment and development especially when oxygen was limited in the heterogeneous matrix. And that the effect from nitrate supply is not significantly changed for I2-rfp biofilms when concentrations of nitrate were above 3 mM, suggesting a saturation of nitrate utilization of nitrate-based biofilm induction. While the genomic information suggested that WDL7 was less likely to utilize nitrate as energy source for biofilm development, Nap system in

WDL7 of nitrate metabolic pathway may play a role by balancing the intracellular redox state (Dietrich et al. 2013). It has been reported in *P. aeruginosa* that nitrate sensor and response system, especially the NarX, affects the mobility and virulence significantly and thereafter affects the biofilm attachment and formation as well (Van Alst et al. 2007). The phenomenon gave a hint of positive enhancement on biofilm development of WDL7-wt in the presence of nitrate, especially a concentration above 3 mM was able to promote the biofilm development significantly. Hence, we hypothesize that nitrate is a factor other than nitrogen or energy source that promotes the biofilm formation.

Therefore, the single species biofilm formation within the gradient generator flow chamber showed the application of this device for fast and convenient testing of biofilm development under a defined gradient of target substrate. It can also be used easily to determine the threshold and working range of the target chemicals for biofilm formation or removal. Meanwhile, there is a possibility to utilize this gradient generator flow chamber to compare the biofilm development of different organisms under same chemical gradients.

Dual organism biofilms of *C. testosteroni* WDL7-wt and I2-rfp were studied as a model within the gradient generator flow chamber to further investigate the interactions and colocalizations of multispecies biofilms. Though the two organisms were inoculated at an equal ratio of cell numbers, WDL7-wt was responsible for almost 80% of the overall biomass and for the positive enhancement by the presence of nitrate. On the contrary, I2-rfp was observed to be only located within the central and bottom of the biofilms 3D structures, and showed no apparent relation with the nitrate induction. We hypothesize that the competition between WDL7 and I2, besides their respective growth rate, leads to this incomparable development in the dual-organism biofilms; and that I2 is more

favorable in oxygen-limited conditions. The mechanisms of collaboration and competition between these two organisms are not clear and require further investigation.

The dual-organism biofilms development in the gradient-generator flow chamber systems support the hypothesis that this device is applicable for multispecies biofilm studies, especially the investigation on interactions and inter-species relations.

6.5 Conclusions

In summary, by integrating microfluidic gradient generator with flow chamber, we developed a novel biofilm reactor that generates controllable and well-defined chemical gradients for biofilm development. We demonstrated that the gradient pattern is predictable by fluid simulation, and it is reproducible and applicable for a wide range of chemicals.

Using *S. oneidensis* and *C. testosteroni* as model organisms, we studied biofilm formation of single and dual organisms under the generated chemical gradients, and we compared the biofilm formation of different organisms under the same chemical environment. We showed that the gradient-generating flow chamber is a promising tool to understand the impacts of chemical environments on biofilms with single batch of experiment. Using the gradient generator flow chamber, it is possible to resolve the dynamics of biofilm development in response to environmental cues and signals. Further, it becomes convenient to optimize the bacterial responses to the dynamic environmental conditions.

CHAPTER 7: Conclusions and Future Recommendations

7.1 Summary and conclusions

This study focused on engineering reproducible biofilm with predictable and controllable biofilm-based bioprocesses through the approach of biofilm mimics. To better control and manipulate biofilm for industrial application, hydrogel is applied as the artificial matrix for bacterial encapsulation.

The hydrogel matrix has been proven to be a promising platform for enzyme and whole cell encapsulation in industry, enhancing the catalytic stability, recyclability, retentions, etc. Our work (Chapter 3) successfully introduced the idea of biofilm mimics in artificial matrix and clearly showed the possible application of hydrogel matrix in biofilm study. Our finding showed that the growth rate and surface properties of encapsulated bacterial populations were comparable to those in flow-cell reactors. Proteomic profiling of hydrogel immobilized cultures were studied and compared with those in flow cell biofilms, improving the understanding of impacts from alginate polymers and encapsulation processes.

To better understand and evaluate the bioprocesses and activities of biofilm mimics, transcriptomic analysis was proposed to be a more powerful tool providing thorough information, when compared with proteomic analysis. Our study (Chapter 4) compared the most frequently utilized RNA extraction methods and set up our own standardized protocol for high quality RNA extraction for downstream transcriptomic analysis.

To better regulate biofilm mimics-based bioprocesses, two approaches were proposed in our work. The first is to regulate the signaling systems and the second is to control the environmental conditions, because the biofilm metabolic activities are tightly associated with these regulatory networks and environmental cues. We utilized *P. aeruginosa* as

model organism to study the biofilm mimics in responding to synthetic QS signals introduced in the artificial matrix (Chapter 5). And we profiled and compared the QS target genes with those reported in other biofilms studies, providing a better understanding of QS regulation in the artificial matrix. Furthermore, to better control the chemical environmental conditions, we developed the gradient-generator-flow cell system (Chapter 6), which was able to generate defined and reproducible chemical gradients within the chamber. Our finding of biofilm cultivation under different chemical gradients strongly suggests the application of this device in controlling the biofilm mimics, especially in generating the defined chemical gradients in the matrix.

Taken together, this study coherently shows the possibility of biofilm mimics in hydrogel matrix, the approaches to evaluate biofilm mimics-based bioprocesses, and the strategies to regulate biofilm mimics for industrial applications. This study provides an applicable platform towards controllable and reproducible biofilm mimics development and bioprocesses for future industrial application.

7.2 Future Recommendations

The findings in this thesis have demonstrated that hydrogel supported biofilm mimics is a promising approach toward the controllable and reproducible biofilm and biofilm-based bioprocesses. Basic understanding of the hydrogel supported biofilm mimics and the regulation through environmental signals has been illustrated, whereas several limitations of this study need further investigation in the future. Future recommendations may involve several aspects:

- Matrix characterization and modification;
- Regulation of biofilm mimics-based bioprocesses through single and mixed species of signaling molecules;

- Exploration of inducible biofilm mimics-based bioprocesses;
- Modification of artificial matrix with defined chemical gradients by coupling matrix functionalization and the gradient generator flow cell;
- Investigation of multispecies biofilm mimics in the hydrogel matrix;

Here we would like to discuss two possible future plans in detail as follows:

7.2.1 Inducible metabolic activities in biofilm mimics

Our finding in chapter 5 has indicated the possibility to introduce external signals to affect and regulate biofilm mimics-based activities. The alternation of biofilm-based processes on the sensing of external signals is generally regulated through secondary messenger signaling system. Hence, manipulation of c-di-GMP, one major secondary messenger in a broad range of Gram negative bacteria, by environmental chemicals is proposed to control target metabolic activities in biofilm mimics.

The intracellular signaling system of c-di-GMP involves in a wild range of gene expressions, including those responsible for motility, virulence, and extracellular polymers secretion. Meanwhile, the c-di-GMP is also involved in the regulation of important environmental processes, such as aromatic compounds degradation. Our preliminary results of biofilm mimics using engineered *C. testosteroni* showed the positive relationship between c-di-GMP level and 3-chloroaniline (3CA) degradation. Briefly, the engineered cells with different c-di-GMP levels were encapsulated in hydrogel matrix at the same level of cell numbers, and their 3CA removal capability was measured simultaneously. The improved 3CA degradation by the engineered strain with upregulated c-di-GMP levels indicated the possibility to induce biofilm mimics-based bioprocess through mediating intracellular c-di-GMP levels.

However, the enhanced c-di-GMP level not only advances biofilm-based remediation but also promotes the biofilm formation. The overproduction of biofilm formation related factors is a challenge that promotes growth rate of biofilm mimics, leading to the loss of biomass by active dispersion and the burst of hydrogel matrix. Hence, inducible c-di-GMP levels are required in biofilm mimics, in which biofilm development and bioremediation processes can be balanced and regulated.

The inducible c-di-GMP levels in biofilm mimics is proposed to be achieved by genetic programming, where the expression of c-di-GMP regulating genes is activated by the introduction of external chemical molecules into the hydrogel matrix or in the substrate. Therefore, engineered *Comamonas testosteroni* WDL7 with inducible intracellular c-di-GMP levels is designed as the model organism for biofilm mimics, to achieve a better control on overproduction of biomass and efficiency of 3CA degradation. The c-di-GMP synthesis gene *yedQ* and degradation gene *yhjH* from *E.coli* are proposed to be transcribed into WDL7 under the control of IPTG inducible promoters (**Figure 7.1**). Thereafter, biofilm mimics of engineered WDL7 strains in hydrogel matrix can be developed and used for 3CA remediation. The 3CA degradation efficacy is proposed to be inducible by the treatment of IPTG molecules introduced in the medium. Evaluation of degradation efficiency and the recyclability of the biofilm mimics are required be carried out for further assessment.

Figure 7.1 The design of the inducible *yedQ* / *yhjH* expression. The fragment consists of both *lacI* and *yedQ* / *yhjH*.

7.2.2 Exploring the application of biofilm mimics in fluid reactors

The biofilm mimics in hydrogel matrix has been demonstrated for its application towards construction of reproducible biofilms; meanwhile, regulation of biofilm mimics-based bioprocesses has been investigated through different approaches, either through introduction of external signals to mediate bacterial regulatory networks or construction of engineered strains expressing specific functional genes. Another approach proposed is to regulate biofilm mimics-based bioprocess through functionalizing the matrix with chemical gradients / patterns. Bioactive molecules are able to be coupled into the hydrogel matrix as shown in previous studies to mediate gene expressions. However, generation of defined and reproducible chemical gradients / pattern in the matrix is a challenge, due to the limitation of chemical diffusions by different molecules in different phases.

Figure 7.2 Examples of chemical gradients generated by the gradient-generator flow cell system. **A**, Gradient of single chemical molecule; **B**, Gradient of double chemical molecules with reversed gradient direction; **C**, Gradient of double chemical molecules with same gradient direction; **D**, Gradient of multiple chemical molecules with either reverse or same gradient direction.

The gradient-generator flow cell that we have designed (Chapter 6) has shown its promising application in biofilm studies by generating defined and reproducible chemical gradients. The chemical gradients are consistent over a wide range of molecules as well as various inlet flow rates with good reproducibility. Moreover, different chemical compounds can be coupled together to generate more complex gradient patterns by the 2-input gradient mixer (**Figure 7.2**).

By coupling the gradient-generator flow cell with hydrogel preparation, the artificial matrix is able to be functionalized with gradients of bioactive molecules, facilitating its application in study of biofilm mimics under dynamic chemical conditions. The defined gradients of bioactive molecules present in the hydrogel matrix will advance our understanding on biofilm mimics-based activities in response to the environmental dynamics. Meanwhile, the integrated device provides a platform for convenient and high throughput scanning of the development, survivability and other metabolic activities of biofilm mimics.

Moreover, modified design based on the microfluidic-gradient generator (Chapter 6) was proposed with separate channels supporting independent chemical concentrations (**Figure 7.3**). This modified device replaces the center chamber of previous version with 9 independent channels, which are able to be disassembled independently. This device is proposed to facilitate sample collection and analysis in high throughput fashion by generating reproducible chemical patterns for biofilm and biofilm mimics studies.

Figure 7.3 Illustration of modified microfluidic gradient generator flow cell system. This design includes two major components—microfluidic gradient mixer with two inlets and 9 outlets; 9 independent chambers with each chamber connecting with one outlet of the gradient mixer. All scales in milli meter.

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