BIOMIMETIC MOLECULARLY IMPRINTED POLYMERS:
A NEW QUORUM SENSING CAPTURING AGENT TO PREVENT BACTERIAL
BIOFILM FORMATION

by

Luyao Ma

B.Sc., Zhejiang University, 2014

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Food Science)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

September 2016

© Luyao Ma, 2016
Abstract

Biofilm is a bacterial community that is responsible for most clinical infections and shows increased resistance to the conventional antimicrobials. Biofilm formation is mediated by quorum sensing (QS), by which bacteria produce and recognize autoinducers (AIs) and thereby coordinate their behaviors in a cell-density dependent manner. The purpose of this thesis project was to design and apply molecularly imprinted polymers (MIPs) to capture AIs, interrupt QS, and subsequently inhibit the formation of bacterial biofilms.

*Pseudomonas aeruginosa* and *N*-((3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-AHL) were selected as the bacterial model and target AI molecule, respectively. Photo-initiated bulk polymerization method was conducted to synthesize MIPs using 3-oxo-C12-AHL as the template, itaconic acid (IA) or 2-hydroxyethyl methacrylate (HEMA) as the functional monomer, ethylene glycol dimethacrylate as the crosslinker, 2,2’-azobis(2-methylpropionitrile) as the initiator and *N,N*-dimethylformamide as the porogen. Different functional monomers and different molar ratios of template: functional monomer: crosslinker were examined to optimize the adsorption capacity and affinity of the synthesized MIPs.

Equilibrium rebinding study was conducted to evaluate the adsorption performance of MIPs. MIPs captured 55.2%-61.2% of 3-oxo-C12-AHL in 20% acetonitrile. However, none of them showed good adsorption affinity due to the dominant non-specific binding. In 50% acetonitrile, IA-based MIPs (*i.e.*, 1:6:25 and 1:8:25) demonstrated good adsorption affinity with imprinting factor >1.
In biofilm inhibitory studies, *P. aeruginosa* biofilm was incubated with or without the presence of MIPs for 24 h. Biofilm biomass and sessile cell viability were determined by crystal violet assay and 2,3,5-triphenyl-tetrazolium chloride assay, respectively. Selective HEMA-based polymers (i.e., 1:8:25, 1:6:48 and 1:8:48) significantly (*P* < 0.05) inhibited the formation of *P. aeruginosa* biofilms, while all IA-based polymers had no impact on biofilm development. The viability of sessile cells was significantly (*P* < 0.05) reduced by selective HEMA-based polymers (i.e., 1:6:48 and 1:8:48), but was increased by some IA-based polymers (*P* < 0.05). Moreover, some HEMA-based polymers (e.g., 1:6:48) showed antimicrobial effect against *P. aeruginosa* planktonic cells.

The current study investigated the inhibitory effect of MIPs against *P. aeruginosa* biofilm. However, more studies need to be conducted to optimize the capturing performance of MIPs towards 3-oxo-C$_{12}$-AHL, thereby increase the anti-biofilm effect.
Preface

The author, Luyao Ma, under the guidance of Dr. Xiaonan Lu, designed the research study, performed the experimental work, analyzed the data and wrote the thesis.

The work is original and has not been previously published.
# Table of contents

Abstract ............................................................................................................................................. ii

Preface .............................................................................................................................................. iv

Table of contents ............................................................................................................................... v

List of tables ....................................................................................................................................... viii

List of figures ..................................................................................................................................... ix

List of abbreviations ......................................................................................................................... xiii

Acknowledgements ........................................................................................................................... xv

Dedication .......................................................................................................................................... xvii

Chapter 1: Introduction ..................................................................................................................... 1

Chapter 2: Hypotheses and objectives ............................................................................................... 4

2.1 Hypotheses ................................................................................................................................. 4

2.2 Objectives .................................................................................................................................... 4

Chapter 3: Literature review ............................................................................................................. 6

3.1 Quorum sensing .......................................................................................................................... 6

3.1.1 Mechanism of quorum sensing ............................................................................................. 6

3.1.2 Quorum sensing autoinducers .............................................................................................. 7

3.2 Bacterial biofilm .......................................................................................................................... 13

3.2.1 Prevalence and hazards of biofilms in the food industry ....................................................... 13

3.2.2 Process of biofilm formation ................................................................................................. 15

3.2.3 *Pseudomonas aeruginosa*: an example of the relationship between QS and biofilm formation .................................................................................................................... 17

3.2.4 Antimicrobial resistance ........................................................................................................ 22
3.3 Current strategies to control biofilm: QS interruption ........................................... 24
  3.3.1 Three major QS interruption strategies ........................................................... 25
  3.3.2 Limitation ......................................................................................................... 26
3.4 A novel biofilm inhibition strategy: application of MIPs ......................................... 26
  3.4.1 Principle and categories .................................................................................... 27
  3.4.2 Important factors in polymerization .................................................................. 30
  3.4.3 Previous applications of imprinted polymers in biofilm inhibition ......................... 33

Chapter 4: Materials and methods .................................................................................. 37
  4.1 Chemicals ............................................................................................................ 37
  4.2 Synthesis of MIPs ............................................................................................... 37
    4.2.1 Polymerization condition ............................................................................... 38
    4.2.2 Ratio of AHL: IA: EGDMA ........................................................................... 39
    4.2.3 Ratio of AHL: HEMA: EGDMA .................................................................... 40
  4.3 Effect of MIPs on capturing 3-oxo-C_{12}-AHL ....................................................... 41
    4.3.1 Equilibrium rebinding study .......................................................................... 41
    4.3.2 High performance liquid chromatographic condition ...................................... 42
  4.4 Effect of MIPs on *P. aeruginosa* biofilm formation ............................................... 42
    4.4.1 Media, bacterial strain and culture preparation ................................................. 42
    4.4.2 Anti-biofilm activity test ................................................................................. 43
      4.4.2.1 Immobilization and sterilization of MIPs ...................................................... 43
      4.4.2.2 Biofilm cultivation with MIPs .................................................................... 43
      4.4.2.3 Biofilm quantification .............................................................................. 44
    4.4.3 Antimicrobial activity test .............................................................................. 46
4.5 Statistical analysis .................................................................................................................. 46

Chapter 5: Results and discussion .................................................................................................. 47

5.1 Effect of MIPs on capturing 3-oxo-C12-AHL ........................................................................... 47

5.1.1 Effect of polymerization condition on 3-oxo-C12-AHL stability ........................................... 47

5.1.2 Synthesis and characterization of IA-based polymers ............................................................. 50

5.1.3 Synthesis and characterization of HEMA-based polymers ..................................................... 56

5.1.4 Summary ................................................................................................................................. 62

5.2 Effect of MIPs on P. aeruginosa biofilm formation ...................................................................... 62

5.2.1 Anti-biofilm activity ............................................................................................................... 62

5.2.1.1 Crystal violet assay ............................................................................................................ 63

5.2.1.2 TTC assay .......................................................................................................................... 67

5.2.2 Antimicrobial activity ............................................................................................................. 73

5.2.3 Summary ................................................................................................................................. 75

Chapter 6: Conclusion ...................................................................................................................... 77

6.1 Study outcomes ......................................................................................................................... 77

6.2 Future directions ......................................................................................................................... 78

Bibliography .................................................................................................................................... 81
List of tables

Table 3.1 Chemical structures of the representative N-acyl-homoserine lactones (AHLs) in Gram-negative bacteria ................................................................. 8

Table 4.1 Synthetic formulations of IA-based polymers .......................................................... 40

Table 4.2 Synthetic formulations of HEMA-based polymers .................................................. 40

Table 5.1 Adsorption affinity of itaconic acid (IA)-based molecularly imprinted polymers (MIPs) towards 3-oxo-C_{12}-AHL in 50% acetonitrile (n=3). ............................................. 55

Table 5.2 Adsorption affinity of 2-hydroxyethyl methacrylate (HEMA)-based molecularly imprinted polymers (MIPs) towards 3-oxo-C_{12}-AHL in 50% acetonitrile (n=3). ..... 60

Table 5.3 P-values between each pair of molecularly imprinted polymers (MIPs) and the corresponding non-imprinted polymers (NIPs) determined using crystal violet assay. ........................................................................................................ 64

Table 5.4 Toxicity test of 0.1% TTC solution on P. aeruginosa planktonic cells after 6 h incubation (n=3). ........................................................................................................ 69

Table 5.5 P-values between each pair of molecularly imprinted polymers (MIPs) and the corresponding non-imprinted polymers (NIPs) using TTC assay. .............................. 70
List of figures

Figure 3.1 Chemical structure of an N-acyl-homoserine lactone. R1 can be assigned to H, OH, or O. R2 is a carbon chain. .................................................................................................................................................. 9

Figure 3.2 Schematic illustration of LuxI/LuxR-type quorum sensing system. AHL denotes N-acyl-homoserine lactones. Image is adapted from a previous work by Defoirdt and coauthors (Defoirdt, Boon, & Bossier, 2010). ......................................................................................... 10

Figure 3.3 Chemical structures of representative autoinducing peptides in Gram-positive bacteria (Guo, Gamby, Zheng, & Sintim, 2013; Schauder & Bassler, 2001). ................................. 11

Figure 3.4 Chemical structure of AI-2. DPD: 4,5-dihydroxy-2,3-pentanedione; DHMF: methyl dihydroxy tetrahydrofuran; THMF: methyl tetrahydroxy tetrahydrofuran. Image is referenced from the works by Guo and colleagues (Guo et al., 2013). ...................... 12

Figure 3.5 Schematic illustrations of the stages of biofilm formation: (1) initial attachment, (2) irreversible attachment, (3) microcolony formation, (4) biofilm maturation, and (5) biofilm dispersion. Image is adapted from the work by Monroe (Monroe, 2007). ... 17

Figure 3.6 Chemical structures of three P. aeruginosa autoinducers: (1) C4-AHL denotes N-butyryl-L-homoserine lactone; (2) 3-oxo-C12-AHL denotes N-3-oxo-dodecanoyl-L-homoserine lactone; (3) PQS denotes Pseudomonas quinolone signal. ....................... 19

Figure 3.7 Regulation of three quorum sensing systems in the formation of P. aeruginosa biofilm. .................................................................................................................................................................................. 20

Figure 3.8 Principle of molecularly imprinted polymers. Image is referenced from the work by Chen and colleagues (Chen, Xu, & Li, 2011)........................................................................................................... 28

Figure 4.1 Schematic illustration of the experimental setup in the representative well of a 24-well microtiter plate. ................................................................................................................................................... 44
Figure 5.1 Degradation profile of 3-oxo-C12-AHL under different polymerization conditions when itaconic acid (IA) was used as the functional monomer. Thermo: thermal initiation (60°C). Photo: photo initiation (4°C, 365 nm). The range of pH was determined based upon the potential concentrations of IA in N,N-dimethylformamide (DMF). Error bars indicate standard deviation (n=3).

Figure 5.2 Chemical structures of reagents used for the synthesis of IA-based polymers. 3-oxo-C12-AHL: N-(3-oxododecanoyl)-L-homoserine lactone; IA: itaconic acid; EGDMA: ethylene glycol dimethacrylate; DMF: N,N-dimethylformamide.

Figure 5.3 Adsorption capacity of itaconic acid (IA)-based molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) towards 3-oxo-C12-AHL in 20% acetonitrile. Molar ratios of template: functional monomer: crosslinker vary from 1:6:25 to 1:12:148. M: MIPs; N: NIPs. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Games Howell’s test (unequal variance) was conducted with a statistical confidence coefficient of 0.95.

Figure 5.4 Chemical structure of 2-hydroxyethyl methacrylate (HEMA).

Figure 5.5 Adsorption capacity of 2-hydroxyethyl methacrylate (HEMA)-based molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) towards 3-oxo-C12-AHL in 20% acetonitrile. Molar ratios of template: functional monomer: crosslinker vary from 1:6:25 to 1:8:48. M: MIPs; N: NIPs. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Tukey’s test (equal variance) was performed. Polymers with different letters are statistically different (P < 0.05).

Figure 5.6 Comparative figures on the adsorption capacities (Q) of itaconic acid (IA)-based polymers and 2-hydroxyethyl methacrylate (HEMA)-based polymers at different
synthetic formulations (template: functional monomer: crosslinker) in 20% acetonitrile: 1:6:25 (A); 1:8:25 (B); 1:6:48 (C); and 1:8:48 (D). M: MIPs; N: NIPs. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Tukey’s test or Games Howell’s test was performed. Within each panel, polymers with different letters are statistically different (P < 0.05). .................................................. 59

Figure 5.7 Comparative figures on the adsorption capacities (Q) of itaconic acid (IA)-based polymers and 2-hydroxyethyl methacrylate (HEMA)-based polymers at different synthetic formulations (template: functional monomer: crosslinker) in 50% acetonitrile: 1:6:25 (A); 1:8:25 (B); 1:6:48 (C); and 1:8:48 (D). M: MIPs; N: NIPs. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Tukey’s test or Games Howell’s test was performed. Within each panel, polymers with different letters are statistically different (P < 0.05). .................................................. 61

Figure 5.8 Relative biofilm formation of P. aeruginosa PAO1 with the presence of polymers. Crystal violet assay was employed for the quantification of biofilm biomass. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Games Howell’s test was performed to determine statistical significance between pairs of treatments (asteroid indicate significant difference of polymers against control, P < 0.05). IA: itaconic acid; HEMA: 2-hydroxyethyl methacrylate; M: molecularly imprinted polymers; N: non-imprinted polymers. .................................................. 65

Figure 5.9 Principle of TTC assay: 2,3,5-triphenyltetrazolium chloride (TTC) is enzymatically reduced to 1,3,5-triphenylformazan (TPF) by viable bacterial cells.......................... 68

Figure 5.10 Relative cell viability of P. aeruginosa PAO1 with the presence of polymers. TTC assay was employed to quantify the live sessile cells. Error bars indicate standard
deviation (n=3). One-way ANOVA followed by Games Howell’s test was performed to determine statistical significance of polymers against control (* indicates $P < 0.05$). IA: itaconic acid; HEMA: 2-hydroxyethyl methacrylate; M: molecularly imprinted polymers; N: non-imprinted polymers.

Figure 5.11 Effect of representative non-imprinted polymers (NIPs) on the viability of *P. aeruginosa* PAO1 planktonic cells after 24-h incubation (n=3). Dash-line represented the initial concentration of *P. aeruginosa* ($1.86 \times 10^6$ CFU/mL). Student’s two-tailed t-test was conducted for statistical analysis. Different letters indicated statistical significance between polymer group and control group ($P < 0.05$). Error bar indicated the standard deviation. IA: itaconic acid; HEMA: 2-hydroxyethyl methacrylate.
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-oxo-C&lt;sub&gt;12&lt;/sub&gt;-AHL</td>
<td>N-(3-oxododecanoyl)-L-homoserine lactone</td>
</tr>
<tr>
<td>3-oxo-C&lt;sub&gt;6&lt;/sub&gt;-AHL</td>
<td>N-3-oxo-hexanoyl-L-homoserine lactone</td>
</tr>
<tr>
<td>ABCN</td>
<td>1,1’-azobis(cyclohexanecarbonitrile)</td>
</tr>
<tr>
<td>ABVN</td>
<td>2,2’-azobis(2,4-dimethyl)valeronitrile</td>
</tr>
<tr>
<td>AHLs</td>
<td>acyl homoserine lactones</td>
</tr>
<tr>
<td>AI-2</td>
<td>autoinducer 2</td>
</tr>
<tr>
<td>AIBN</td>
<td>2,2’-azobis(2-methylpropionitrile)</td>
</tr>
<tr>
<td>AIs</td>
<td>autoinducers</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>C&lt;sub&gt;4&lt;/sub&gt;-AHL</td>
<td>N-butyryl-L-homoserine lactone</td>
</tr>
<tr>
<td>C&lt;sub&gt;6&lt;/sub&gt;-AHL</td>
<td>N-hexanoyl-homoserine lactone</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detector</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DPD</td>
<td>4,5-dihydroxy-2,3-pentanedione</td>
</tr>
<tr>
<td>eDNA</td>
<td>extracellular DNA</td>
</tr>
<tr>
<td>EGDMA</td>
<td>ethylene glycol dimethacrylate</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substances</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>H-bond</td>
<td>hydrogen bond</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IA</td>
<td>itaconic acid</td>
</tr>
<tr>
<td>IF</td>
<td>imprinting factor</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MAA</td>
<td>methacrylic acid</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>MIPs</td>
<td>molecularly imprinted polymers</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MMA</td>
<td>methyl methacrylate</td>
</tr>
<tr>
<td>NaOCl</td>
<td>sodium hypochlorite</td>
</tr>
<tr>
<td>NIPs</td>
<td>non-imprinted polymers</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>optical density at 600 nm</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEGDA</td>
<td>poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PQS</td>
<td>Pseudomonas quinolone signal</td>
</tr>
<tr>
<td>PVC</td>
<td>poly(vinyl chloride)</td>
</tr>
<tr>
<td>Q</td>
<td>adsorption capacity</td>
</tr>
<tr>
<td>QS</td>
<td>quorum sensing</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescence protein</td>
</tr>
<tr>
<td>TPF</td>
<td>1,3,5-triphenylformazan</td>
</tr>
<tr>
<td>TRIM</td>
<td>trimethylolpropane trimethacrylate</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5-triphenyltetrazolium chloride</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescence protein</td>
</tr>
</tbody>
</table>
Acknowledgements

Foremost, I would like to thank my supervisor and mentor Dr. Xiaonan Lu for giving me this unique opportunity to study in his laboratory, for providing me with freedom to explore my ideas, for answering my questions and leading me to overcome problems, for always encouraging me when I was stressed out, and for opening my eyes on this fantastic scientific world.

I have always felt fortunate to be supported by so many excellent scientists. First, I want to give my sincere gratitude to my committee members, Dr. Christine H. Scaman and Dr. Bruce A. Vallance, who provided me with invaluable suggestions on experimental design and thesis writing. Second, I want to thank Benny Chan and Peter Hoffman for their technical support during my research study. I also want to express my heartfelt appreciation to Lufiani Lina Madilao, who showed great willingness to share her analytical knowledge and took care of me like a mom.

I am so glad to work with all my wonderful Lu lab colleagues: Dr. Lina Ma, Dr. Yiwei Tang, Jinsong Feng, Shaolong Feng, Mohammed Hakeem, Yaxi Hu, Gracia Windiasti, Bowen Zhao, Jiaqi Li and Jingyi Feng. Thanks for teaching me basic experimental skills, brainstorming about the science and enriching my life. I also want to thank UBC safewalk for their kind volunteering and driving me home safe.

My special thanks are owed to my best friends: Sijia Ju, Danfeng Wang, Ruosu Fang, Yutong Wu, and Wen Liao. Thanks for always being my side at my every achievement and struggle.
Nothing I have achieved or experienced will become possible without my beloved parents, Jun Ma and Ling Liu. They gave me life and then have devoted their lives to love me, educate me and unconditionally support me with any decisions I made. Lastly, I would thank my boyfriend and best friend, Junyu Zhou, for his continuous company, encouragement and belief in me.

Thanks for this unique graduate study journey, which makes me more skillful and confident to deal with potential problems in my future study. Challenges? Accepted!
Dedication

To all

Who dedicate to explore the unknown and never give up
Chapter 1: Introduction

Bacterial biofilms include sessile bacterial cells and extracellular polymeric substances (EPS), and have been considered as the dominant form of bacterial communities in nature compared to the free-floating counterparts (Costerton, Stewart, & Greenberg, 1999; Poulsen, 1999). Biofilms pose a significant threat to both public health and the food industry. According to the report by National Institute of Health in 2002, over 80% of all microbial infections are associated with bacterial biofilms (National Institutes of Health, 2002). In the United States, biofilms are responsible for ~1.3 million hospitalization cases annually, which costs about 6 billion US dollars for medical treatment (Dongari-Bagtzoglou, 2008). In the food industry, biofilms are not only associated with the contamination of fresh produce and processed food products, but also lead to the damage of the food processing equipment (Shi & Zhu, 2009).

Conventional strategy to control bacterial infections involves the use of antimicrobial agents, such as antibiotics (Shi & Zhu, 2009). This method has been mainly used to control planktonic bacterial cells, but it is validated to be less effective against bacterial biofilms (Hoyle, Alcantara, & Costerton, 1992; Mah & O’Toole, 2001). Although many studies have been conducted to explore new antimicrobial agents that can antagonize bacterial biofilms, rapid development of bacterial antimicrobial resistance is always a major concern.

One of the most promising strategies to inhibit bacterial biofilm formation is to interrupt bacterial quorum sensing (QS). QS is a cell-to-cell signaling communication system, by which bacteria can regulate the expression of certain genes in a cell-density-dependent manner (Bassler,
Bacterial biofilm formation is mediated by QS (Bassler, 1999). In a QS system, small diffusible molecules called “autoinducers (AIs)” are produced by specific synthetases and transported into the extracellular environment for intra- and inter-species bacterial communication. The concentration of AIs is related to bacterial density. When the concentration of AIs in the environment reaches to a critical threshold level, specific receptors can recognize and bind to AIs. Consequently, these receptors are fully activated to bind to the promoter of the target genes and induce different patterns of gene expression (Reading & Sperandio, 2006). QS includes different types of AIs and is widely used by both Gram-negative and Gram-positive bacteria. One of the most common types of AIs is acyl homoserine lactones (AHLs) utilized by Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Salmonella enterica* (Watson, Minogue, Val, Von Bodman, & Churchill, 2002).

A variety of strategies to attenuate bacterial QS have been evaluated. For example, some structural analogues of AIs have been validated to inhibit the expression of AIs synthases (Hentzer & Givskov, 2003; Parsek, Val, Hanzelka, Cronan, & Greenberg, 1999). In addition, enzymatic degradation and antibody sequestration can be applied to reduce the accumulation of AIs in the extracellular environment (Hong, Koh, Sam, Yin, & Chan, 2012). The structural analogues of AIs also demonstrate interfering capability of specific recognition and binding between AIs and their corresponding receptors (Smith, Bu, & Suga, 2003). However, either the structural analogues of AIs or antibodies are too difficult and/or expensive to be produced in a large scale. Further, some of these candidates are not stable in the extreme environmental conditions (*e.g.*, high or low temperature, low pH) that limit their further applications.
The alternative and novel QS attenuating strategy evaluated in the current thesis project is to specifically capture AIs by molecularly imprinted polymers (MIPs), which consequently disrupt QS and inhibit QS phenotypes (e.g., biofilm formation). MIPs are highly cross-linked polymers that contain specific binding sites with the memory of shape, size, and functional groups of the template molecule (Mosbach & Ramström, 1996). MIPs are stable in the environment and easy to be generated in a large scale (Whitcombe, Alexander, & Vulfson, 1997). In the current study, MIPs were synthesized by bulk polymerization. Bulk polymerization is an easy-to-operate method with fewer variable factors and less template molecules required than other polymerization methods, such as precipitation polymerization and suspension polymerization (Vasapollo et al., 2011). Thus, this method is suitable for exploring or optimizing the formulation of MIPs synthesis.

MIPs have exhibited high specific adsorption capacity towards many chemicals, such as pesticides (e.g., bentazone and atrazine), antibiotics (e.g., penicillin G and ampicillin), and food components (e.g., tocopherol, quercetin, and lycopene) (Baggiani, Trotta, Giraudi, Giovannoli, & Vanni, 1999; Feng et al., 2013; Matsui, Miyoshi, Dobhlhoff-Dier, & Takeuchi, 1995; Molinelli, Weiss, & Mizaikoff, 2002; Urraca, Hall, Moreno-Bondi, & Sellergren, 2006). However, only three research studies were conducted to evaluate the capturing effect of polymers on quorum sensing molecules and all of these studies were performed by a single research group (Cavaleiro et al., 2015; Piletska et al., 2010, 2011). Therefore, we aim to comprehensively evaluate the potential inhibitory effect of MIPs against QS-mediated bacterial biofilm formation. *P. aeruginosa* was used as a model bacterium because it has been extensively studied for quorum sensing and biofilm formation (Klausen et al., 2003; Sharma et al., 2014).
Chapter 2: Hypotheses and objectives

In this thesis project, *Pseudomonas aeruginosa* was selected as the model bacterium. *N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂-AHL)*, one of the major autoinducers produced by *P. aeruginosa*, served as the template molecule for the synthesis of MIPs.

2.1 Hypotheses

**Hypothesis 1**: An optimal initiation method, functional monomer, and the ratio of template: functional monomer: crosslinker can be identified and developed for the synthesis of MIPs.

**Hypothesis 2**: MIPs can obtain high adsorption capacity and affinity towards 3-oxo-C₁₂-AHL in the aqueous condition.

**Hypothesis 3**: MIPs can efficiently inhibit the formation of *P. aeruginosa* biofilm by capturing 3-oxo-C₁₂-AHL from the environment.

2.2 Objectives

**Objective 1**: To develop and optimize the synthesis of MIPs considering three major parameters, namely 1) initiation method, 2) nature of functional monomer, and 3) ratio of template: functional monomer: crosslinker.

**Objective 2**: To evaluate the adsorption capacity and affinity of MIPs towards 3-oxo-C₁₂-AHL in aqueous media.
Objective 3: To investigate the inhibitory effect of MIPs against *P. aeruginosa* biofilm formation.
Chapter 3: Literature review

This chapter includes a detailed scientific background for the understanding of the entire thesis. Firstly, the mechanism and major types of quorum sensing (QS) systems are described. Secondly, QS-mediated biofilm formation is introduced, including its prevalence, hazard, formation process, and antimicrobial resistance. *Pseudomonas aeruginosa* is introduced as the bacterial model for the discussion of the relationship between QS and biofilm formation in details. Thirdly, QS inhibition strategies are discussed as the novel approach to inhibit bacterial biofilm. Lastly, the principle and synthesis of molecularly imprinted polymers (MIPs) is reported due to its potential ability to interrupt QS and inhibit bacterial biofilm formation.

3.1 Quorum sensing

Bacterial cells mediate collective physiological behaviors through cell-to-cell communication system in order to respond to the environmental stresses (Atkinson & Williams, 2009). This cell-to-cell communication system is referred to as quorum sensing (QS).

3.1.1 Mechanism of quorum sensing

In the QS system, bacteria can regulate the expression of certain genes in a cell density-dependent manner (Reading & Sperandio, 2006). The communication among bacteria is mediated by low-molecule-weight signaling molecules, called “autoinducers (AIs)” (Gobbetti, De Angelis, Di Cagno, Minervini, & Limitone, 2007). In general, bacteria can produce AIs at low levels inside of cells and release these molecules into the extracellular environment. These AIs can be specifically detected and bonded by the cognate receptors. At low cell density,
bacteria produce few AIs and the diffusion of AIs into the environment further reduces their concentration. Therefore, few AIs can bind to the receptors. When bacteria population increases, the concentration of AIs increases accordingly and finally reaches to a critical threshold level. At this point, the complex of AIs-receptors are formed and fully activated to bind to the promoter of target genes, resulting in the expression of certain genes (Miller & Bassler, 2001; Ng & Bassler, 2009). A variety of physiological behaviors of bacteria are mediated by QS, such as biofilm formation, bioluminescence, secretion of virulence factors, and surface motility (Bassler & Losick, 2006).

3.1.2 Quorum sensing autoinducers

QS is widely employed by both Gram-negative and Gram-positive bacteria within and among species (Miller & Bassler, 2001). There are three major classes of AIs in different types of QS systems (Reading & Sperandio, 2006). Gram-negative and Gram-positive bacteria primarily use N-acyl-homoserine lactones (AHLs) and autoinducing peptides for intra- and inter-species communication, respectively (Lazazzera & Grossman, 1998). A furanosyl borate diester, also known as autoinducer 2 (AI-2), is commonly available in both Gram-negative and Gram-positive bacteria for intra- and inter-species communication (Lowery, Park, Kaufmann, & Janda, 2008).

In Gram-negative bacteria, LuxI/LuxR-type QS system is the primary intraspecies communication system. This is also the QS system that we selected to study in this thesis project. Over 50 Gram-negative bacterial species have been validated to use AHLs as AIs in the LuxI/LuxR-type QS system (Bassler, 2002). The examples of bacteria species and their corresponding AHLs are listed in Table 3.1.
Table 3.1 Chemical structures of the representative $N$-acyl-homoserine lactones (AHLs) in Gram-negative bacteria.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>AHL(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td><img src="attachment" alt="Structure" /> N-3-oxo-octanoyl-L-homoserine lactone</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td><img src="attachment" alt="Structure" /> N-octanoyl-L-homoserine lactone</td>
</tr>
<tr>
<td><em>Erwinia carotovora</em></td>
<td><img src="attachment" alt="Structure" /> N-3-oxo-hexanoyl-L-homoserine lactone</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td><img src="attachment" alt="Structure" /> N-butyryl-L-homoserine lactone N-3-oxo-dodecanoyl-L-homoserine lactone</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td><img src="attachment" alt="Structure" /> N-3-oxo-dodecanoyl-L-homoserine lactone</td>
</tr>
<tr>
<td><em>Ralstonia solanacearum</em></td>
<td><img src="attachment" alt="Structure" /> N-hexanoyl-L-homoserine lactone</td>
</tr>
</tbody>
</table>
**Vibrio fischeri**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Vibrio fischeri structure" /></td>
<td>N-3-oxo-hexanoyl-L-homoserine lactone</td>
</tr>
</tbody>
</table>

**Vibrio harveyi**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image2.png" alt="Vibrio harveyi structure" /></td>
<td>N-3-hydroxybutyryl-L-homoserine lactone</td>
</tr>
</tbody>
</table>

N-(3-oxohexanoyl)-L-homoserine lactone used by *V. fischeri* was the first AHL identified as an autoinducer (Nealson & Hastings, 1979). Over 20 AHLS have been identified in different bacterial species, such as N-3-oxo-dodecanoyl-L-homoserine lactone in *P. aeruginosa*, N-octanoyl-L-homoserine lactone in *B. cepacia*, and N-hexanoyl-L-homoserine lactone in *R. solanacearum* (Gobbetti et al., 2007). All of these AHLS contain a homoserine lactone ring, but vary in the length of acyl chain (from C₄ to C₁₈), oxidation at the C₃ position, and saturation (contain oxo-/hydroxyl- group or no substitution) (Neumann, Patzelt, Wagner-Döbler, & Schulz, 2013; Watson et al., 2002). The general structure of an AHL is shown in Figure 3.1.

![Figure 3.1](image3.png)

**Figure 3.1** Chemical structure of an N-acyl-homoserine lactone. R1 can be assigned to H, OH, or O. R2 is a carbon chain.
The general process of LuxI/LuxR-type QS system is shown in Figure 3.2. Briefly, LuxI protein or its homolog is responsible for the synthesis of AHLs. After the synthesis of AHLs, the short chain AHLs (C₄-C₈) can passively diffuse into and out of the cells. In contrast, the transportation of the long chain AHLs (C₈-C₁₈) follows the active transportation mechanism (Fuqua, Parsek, & Greenberg, 2001). Localized in the cytoplasm, LuxR protein or its homolog serves as AHLs receptor that detects and binds to AHLs specifically. Once the concentration of AHLs increases to a threshold level, the formation of AHLs-receptor complex can induce the expression of certain genes (Galloway, Hodgkinson, Bowden, Welch, & Spring, 2011).

Figure 3.2 Schematic illustration of LuxI/LuxR-type quorum sensing system. AHL denotes N-acyl-homoserine lactones. Image is adapted from a previous work by Defoirdt and coauthors (Defoirdt, Boon, & Bossier, 2010).
Similar to Gram-negative LuxI/LuxR QS system, Gram-positive bacteria produce and recognize specific AIs (*i.e.*, autoinducing peptides) for intraspecies communication. Autoinducing peptide is an oligopeptide. The representative examples of autoinducing peptides are listed in Figure 3.3 (Schauder & Bassler, 2001). In general, autoinducing peptides are transported into the environment through ATP-binding cassette transporter. The extracellular autoinducing peptides can be recognized by two-component regulatory system that consists of histidine kinase receptor on the membrane and cognate response regulator in the cytoplasm (Kleerebezem, Quadri, & Oscar, 1997; Ng & Bassler, 2009). After serial steps of phosphorylation on the histidine kinase receptor and cognate response regulator, phosphorylated response regulators are activated to control gene expression.

Autoinducing peptides in *Staphylococcus aureus*

Autoinducing peptide in *Streptococcus pneumoniae*

Figure 3.3 Chemical structures of representative autoinducing peptides in Gram-positive bacteria (Guo, Gamby, Zheng, & Sintim, 2013; Schauder & Bassler, 2001).
In many species (~70) of Gram-negative and Gram-positive bacteria, AI-2/LuxS-type QS system is commonly used for multicellular communication (Guo, Gamby, Zheng, & Sintim, 2013; Xavier & Bassler, 2003). Since AI-2 is universal to different bacterial species, it can be used for both intra- and inter-species communication. LuxS protein synthesizes 4,5-dihydroxy-2,3-pentanedione (DPD), which can be further spontaneously converted to various chemically distinctive forms (Figure 3.4). The availability and concentration of different forms are related to the environmental conditions. For instance, S-DHMF and R-DHMF are generated after the cyclization of DPD. S-THMF, R-THMF and hydrated DPD exist in water condition. S-THMF-borate is generated in the presence of borate (Galloway et al., 2011). The diversity of AI-2 mixture leads to the challenges in the identification of AI-2 receptors (Pereira, Regt, Brito, Miller, & Xavier, 2009). Limited number of receptors has been identified so far, such as LuxP in Vibrio and LsrB in Salmonella enterica serovar Typhimurium (Guo et al., 2013).

![Chemical structure of AI-2](image)

**Figure 3.4** Chemical structure of AI-2. DPD: 4,5-dihydroxy-2,3-pentanedione; DHMF: methyl dihydroxy tetrahydrofuran; THMF: methyl tetrahydroxy tetrahydrofuran. Image is referenced from the works by Guo and colleagues (Guo et al., 2013).
Taken together, different QS systems employ different AIs. The specificity of QS system is extremely high due to the specific binding between AIs and their corresponding receptors.

3.2 **Bacterial biofilm**

Most microorganisms in nature are not in a free-floating form. Instead, ~90% of microorganisms form communities by irreversibly attaching to each other and/or to substrate and interface (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995; Yawata et al., 2010). These communities, called “biofilms”, are comprised of adherent cells and their self-produced matrix of extracellular polymeric substances (EPS) (Poulsen, 1999). In general, up to 97% of water exists in a bacterial biofilm, with the presence of 2-5% of microbial cells and 3-6% of EPS and ions (Meliani & Bensoltane, 2015). The major components of EPS include polysaccharides, lipids, proteins and extracellular DNA (eDNA) (Flemming & Wingender, 2010). The component and quantity of EPS vary among different types of microorganisms, different ages of biofilms, and different environmental conditions (Vu, Chen, Crawford, & Ivanova, 2009). EPS builds up a three-dimensional structure that allows the transportation of nutrients, waste, and water into and out of the biofilm (Vu et al., 2009). It can also enclose and protect sessile cells from the environmental stresses, such as shear stress, nutrient deprivation, extremely high or low temperature, and from antimicrobial agents (Jayaraman & Wood, 2008).

3.2.1 **Prevalence and hazards of biofilms in the food industry**

Both pathogenic and spoilage bacteria can attach to food products and food contact surfaces and subsequently form biofilms (Myszka & Czaczyk, 2011). Many studies have been conducted to
investigate bacterial attachment and biofilm formation under simulated conditions. For example, the surface attachment and biofilm formation of *Campylobacter jejuni* were both enhanced in the presence of chicken juice (Brown et al., 2014). *Listeria monocytogenes* irreversibly attached to food processing-related materials, such as stainless steel, plastics and rubber (Beresford, Andrew, & Shama, 2001). *Pseudomonas fluorescens* formed strongly adherent biofilms on a polystyrene substrate (Aswathanarayan & Vittal, 2014).

During the past two decades, the microbial composition of biofilms from the surfaces of food processing facilities have been identified (Shi & Zhu, 2009; Srey, Jahid, & Ha, 2013). For example, a group of researchers placed stainless steel coupons (i.e., removable biofilm growth surfaces) near food contact surfaces in shrimp plants (Guðbjörnsdóttir, Einarsson, & Thorkelsson, 2005). After three months, two biofilm-formers *P. fluorescens* and *P. putida* were identified from the stainless steel coupons. In another study, *Acinetobacter*, *Neisseriaceae* and *Pseudomonas* were identified as the major microflora adhering to the processing equipment in smoked salmon plants (Bagge-Ravn et al., 2003). In the dairy industry, the spoilage microorganism *Bacillus cereus* was found to form biofilms on the piping system (Kumari & Sarkar, 2016).

The prevalence of pathogenic and spoilage bacterial biofilms increases the food safety risk and economic loss. Biofilm is difficult to be eradicated from the food contact surfaces by conventional cleaning steps, such as the application of disinfections and combination of disinfections and physical forces (e.g., water turbulence and scrubbing) (Chmielewski & Frank, 2003; Kumar & Anand, 1998). The presence of bacterial biofilms increases the opportunity for
the cross-contamination of food products, leading to an increased probability of foodborne outbreaks and food spoilage. It is estimated that over 80% of microbial infections are due to biofilms (Jahid & Ha, 2014; National Institute of Health, 2002). In addition, biofilms can reduce the heat transfer efficiency and life-time of equipment used in the food industry (Winkelstroter, 2015).

3.2.2 Process of biofilm formation

QS system plays a key role in the formation of bacterial biofilms (Cvitkovitch, Li, & Ellen, 2003; García-Aljaro, Melado-Rovira, Milton, & Blanch, 2012; Kong, Vuong, & Otto, 2006; Shrout et al., 2006; Barrios, Zuo, Hashimoto, Yang, Bentley, & Wood, 2006). Biofilm formation follows a dynamic and stepwise process (Annous, Fratamico, & Smith, 2009). As shown in Figure 3.5, the process of biofilm formation is characterized by five steps (Srey et al., 2013).

(1) Initial attachment: Bacteria attach to the substrate either in a passive or active manner (Chmielewski & Frank, 2003). Passive attachment is dependent upon the gravity and fluid shear force. Active attachment is driven by cell motility (e.g., flagella and pili) and surface charges. At this stage, the interaction between bacteria and substrate is weak, involving van der Waals’s force, electrostatic force, and hydrophobic interaction (Srey et al., 2013). The attachment is reversible and bacterial cells can be easily removed by mild shear force.

(2) Irreversible attachment: After the initial attachment, bacteria cells anchor their appendages (e.g., flagella and pili) onto the substrate. In the meanwhile, bacteria secrete EPS to irreversibly facilitate the adhesion between cells and surfaces. The interaction between bacteria and substrate
involves hydrogen bond, hydrophobic interaction, and ionic interaction (Chmielewski & Frank, 2003).

(3) Microcolony formation: The growth and aggregation of bacteria take place simultaneously, resulting in the formation of microcolonies. The microcolonies enlarge and coalesce to form a cellular layer covering onto the substrate. In the meanwhile, these bacterial communities keep secreting EPS through QS and are gradually enclosed by EPS.

(4) Biofilm maturation: As cells proliferate and EPS accumulates, biofilm develops into a dynamic and mature three-dimensional structure, which can resist environmental stresses.

(5) Biofilm dispersion: When the environmental condition is unfavorable to the bacterial community (e.g., limited nutrients and space), some sessile cells detach and disperse from the biofilm and transit to planktonic cells. These planktonic cells can relocate to another surface and start a new cycle of biofilm formation. The detachment can be induced by many factors, such as the production of EPS-degradation enzymes and release of specific EPS component (e.g., rhamnolipid in *P. aeruginosa*) (Miller & Bassler, 2001).
3.2.3 *Pseudomonas aeruginosa*: an example of the relationship between QS and biofilm formation

To understand the relationship between QS and biofilm formation, *P. aeruginosa* is widely used as the bacterial model for study (Sharma et al., 2014). *P. aeruginosa* is a Gram-negative, rod-shaped, aerobic bacterium. The size of a single cell varies from 0.5-0.8 μm by 1.5-3.0 μm. This microorganism can grow at temperatures between 10°C and 43°C (Gacesa & Rusell, 1990).

*P. aeruginosa* can form biofilms and survive in almost any moist environment, from soil and water to animals and human. In the food industry, *P. aeruginosa* is commonly isolated from floors, drains, walls, and pipes (Meliani & Bensoltane, 2015). In the clinical settings, *P. aeruginosa* can easily develop a strong biofilm in different hospital reservoirs, such as respiratory equipment and catheters (Lyczak, Cannon, & Pier, 2000). As an opportunistic human
pathogen, *P. aeruginosa* can colonize the tissues of immunocompromised people through biofilm formation, such as the lungs of cystic fibrosis patients and the wounds of burn patients.

QS plays a key role in the formation of *P. aeruginosa* biofilm. Two LuxI/LuxR-type QS systems (*i.e.*, LasI/LasR and RhlI/RhlR) are identified in *P. aeruginosa* (Sharma et al., 2014). In the LasI/LasR QS system, LasI protein synthesizes N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C$_{12}$-AHL) and LasR protein senses and binds to 3-oxo-C$_{12}$-AHL. In the RhlI/RhlR QS system, RhlI protein synthesizes N-butyryl-L-homoserine lactone (C$_{4}$-AHL), which is specifically detected by transcriptional regulator RhlR. 3-oxo-C$_{12}$-AHL can interfere with the binding between C$_{4}$-AHL and RhlR protein. When 3-oxo-C$_{12}$-AHL binds to its receptor LasR, the complex can induce the transcription of *rhlR*. In this way, LasI/LasR QS system is established prior to RhlI/RhlR QS system (Miller & Bassler, 2001). A third QS system, which involves *Pseudomonas* quinolone signal (2-heptyl-3-hydroxy-4-quinolone, also known as PQS), interacts with two LuxI/LuxR-type QS systems in an intricate way (Rasamiravaka, Labtani, Duez, & El Jaziri, 2015). The structure of three AIs is shown in Figure 3.6.
Figure 3.6 Chemical structures of three *P. aeruginosa* autoinducers: (1) C$_4$-AHL denotes N-butyryl-L-homoserine lactone; (2) 3-oxo-C$_{12}$-AHL denotes N-3-oxo-dodecanoyl-L-homoserine lactone; (3) PQS denotes *Pseudomonas* quinolone signal.

Davies and colleagues firstly studied the role of LasI/LasR QS system in biofilm formation (Davies, 1998). In that study, the depth and structure of biofilms formed by *P. aeruginosa* PAO1 wild type and Δ*lasI* mutant were evaluated. The biofilm of *lasI* mutant was 20% thinner than that of wild type. The biofilm formed by wild type exhibited a loose structure with considerable intervening space among bacterial cells, while the biofilm formed by *lasI* mutant showed a flat and compressed structure with a uniform cell distribution.

Other research works continuously unraveled the controlling mechanism of QS in the formation of *P. aeruginosa* biofilm, including the production of EPS (*i.e.*, polysaccharides Psl and Pel, eDNA, and rhamnolipid) and administration of bacterial motility (*i.e.*, swarming and twitching) (Figure 3.7) (Rasamiravaka et al., 2015). Below is the detailed illustration of each mechanism.
Figure 3.7 Regulation of three quorum sensing systems in the formation of *P. aeruginosa* biofilm.

(1) Polysaccharide Psl: Psl is one of the major polysaccharides identified in *P. aeruginosa* biofilm. Rich in galactose and mannose, Psl plays an important role in bacterial initial attachment and biofilm maturation (Wei & Ma, 2013). Gilbert and coauthors identified that LasR protein can bind to the promoter of *psl* operon, indicating that LasI/LasR-type QS system can regulate the production of Psl (Gilbert, Kim, Gupta, Greenberg, & Schuster, 2009).

(2) Polysaccharide Pel: Pel is another major polysaccharide identified in *P. aeruginosa* biofilm. Pel is a glucose-rich exopolysaccharide. It is essential for the formation of pellicles (*i.e.*, biofilms forming at the air-liquid interface) as well as the initial attachment on solid surfaces (Vasseur, Vallet-Gely, Soscia, Genin, & Filloux, 2005; Wei & Ma, 2013). Sakuragi and colleagues
knocked out pelA (encoding oligogalacturonide lyase) or lasI in P. aeruginosa PA14. Compared to the wild-type strain, pelA or lasI mutant could not form wrinkly colonies and had the similar reduction level of β-galactosidase activity. After the addition of 3-oxo-C_{12}-AHL, these mutants formed wrinkly colonies and restored their β-galactosidase activity to a comparable level to that of the wild-type strain. Thus, LasI/LasR QS system can activate the transcription of pel genes (Sakuragi & Kolter, 2007).

(3) eDNA: eDNA functions as the cell-cell interacting component within a biofilm. The release of eDNA has been validated to be controlled by LasI/LasR, RhlII/RhlR, and PQS QS systems (Allesen-Holm et al., 2006). Allesen-Holm and coauthors compared the release of eDNA between P. aeruginosa PAO1 wild type and its lasIrhlII and pqsA mutants and reported that the biofilms of lasIrhlII and pqsA mutants formed less eDNA compared to the wild-type biofilm.

(4) Rhamnolipid: Rhamnolipid is a type of glycolipid biosurfactant in biofilm. Rhamnolipid is essential in forming microcolonies, maintaining open channels within the biofilm architecture, and facilitating biofilm dispersion (Boles, Thoendel, & Singh, 2005; Rasamiravaka et al., 2015). RhlII/RhlR QS system mediates the formation of rhamnolipid in P. aeruginosa (Dusane et al., 2010).

(5) Motility: Flagellum-mediated swarming and type IV pili-mediated twitching are essential to the initial attachment of P. aeruginosa cells on solid surfaces (Deziel, Comeau, & Villemur, 2001; Kearns, 2010). Kievit and coauthors studied the effect of lasI and rhlII genes on P. aeruginosa motility by using M9 minimal medium plates (Kievit, Gillis, Marx, & Brown, 2001).
Good twitching motility was observed in *P. aeruginosa* PAO1, but all of the mutant strains (*i.e.*, Δ*lasI*, Δ*rhlI*, and Δ*lasIrhl*) did not exhibit any twitching motility. Flagellum-mediated swarming was decreased in all mutants strains compared to that of the wild type strain.

### 3.2.4 Antimicrobial resistance

Sessile bacterial cells residing in the formed biofilms have a higher resistance to the conventional antimicrobial agents compared to the planktonic counterparts (Mah & O’Toole, 2001). For example, Anderl and colleagues studied the inhibitory effect of ampicillin and ciprofloxacin against *Klebsiella pneumonia* (Anderl, Franklin, & Stewart, 2000). After 4-h treatment at 10× minimal inhibitory concentration (MIC), ampicillin and ciprofloxacin caused 4.43 ± 0.33 log- and 4.14 ± 0.33 log-reduction on *K. pneumonia* planktonic cells, respectively. In comparison, these two antibiotics only resulted in -0.06 ± 0.06 log- and 1.02 ± 0.04 log-reduction on *K. pneumonia* biofilms. In another study, the resistance of *Campylobacter jejuni* biofilms to ciprofloxacin treatment was investigated by calculating the frequency of ciprofloxacin-resistant cells (Bae & Jeon, 2013). After 24-h treatment at 1× MIC, the frequency of planktonic cells was ~3.2-fold lower than that of sessile cells in the biofilm.

Compared to the monospecies biofilm as aforementioned, multispecies biofilms are dominant in the natural environment and show a higher resistance to the antimicrobial treatment (Burmolle, Ren, Bjarnsholt, & Sorensen, 2014; Yang et al., 2011). For example, Kart and coauthors studied the inhibitory effect of two disinfectants (NaOCl and H₂O₂) against monospecies biofilms of *S. aureus, Candida albicans*, and *P. aeruginosa*, as well as their corresponding multispecies biofilms (Kart, Tavernier, Van Acker, Nelis, & Coenye, 2014). After treated with 0.05% NaOCl
for 5 min, a significantly higher ($P < 0.05$) amount of $P. \text{aeruginosa}$ and $C. \text{albicans}$ cells survived in the multispecies biofilms than in the monospecies biofilms. After 0.2% (v/v) $H_2O_2$ treatment for 15 min, more $C. \text{albicans}$ cells survived in the multispecies biofilms than that in its monospecies biofilm.

The mechanism of antimicrobial resistance in biofilms has not been in fully agreement yet because it is related to multiple factors (Fuente-Nunez, Reffuveille, Fernandez, & Hancock, 2013; Drenkard, 2003; Mah & O’Toole, 2001; Taylor, Yeung, & Hancock, 2014). Specifically, the penetration of antimicrobial agents may be delayed by EPS (Ishida et al., 1998). Further, nutrient and oxygen depletion in a biofilm can result in a lower growth rate of the encased bacterial cells, leading to a less susceptibility to the growth-dependent antimicrobial agents (e.g., tetracycline) than the planktonic cells (Mah & O’Toole, 2001). In addition, general stress responses are triggered in the biofilms due to the environmental stresses, such as limited nutrients. For example, during the stationary phase, the sigma factor $rpoS$ (a general stress response regulator) is expressed at a higher level in $P. \text{aeruginosa}$ biofilm than in the planktonic cultures (Xu, Franklin, Park, McFeters, & Stewart, 2001). QS system may also participate in the antimicrobial resistance. For example, $P. \text{aeruginosa}$ wild-type biofilm has a higher antimicrobial resistance to the kanamycin treatment compared to its $lasI$ and $rhlI$ mutant counterparts (Shih & Huang, 2002). Finally, multidrug efflux pumps may play a role in biofilm resistance as well. Multidrug efflux pumps can extrude antimicrobial agents from the bacterial cells (Drenkard, 2003). In a previous study, the expression of multidrug efflux pump-related genes was up-regulated in $E. \text{coli}$ biofilm (Ma, Alberti, Lynch, Nikaido, & Hearst, 1996).
3.3 Current strategies to control biofilm: QS interruption

Due to the high antimicrobial resistance of biofilms, other efficient control strategies against biofilms have been explored. The control strategies are mainly based upon (1) inhibiting the attachment of planktonic cells to the surfaces; (2) disrupting biofilm matrix (i.e., EPS); and (3) interrupting QS circuits (Francolini & Donelli, 2010). To inhibit the adhesion of bacterial cells, antimicrobial agents (especially antibiotics) are commonly coated on the contact surfaces (Tran, Hamood, & Reid, 2014). This method shows great reduction on bacterial attachment. However, long-term usage of antimicrobials increases the risks of bacterial resistance. Besides, there is a possibility that antimicrobials can release from the surfaces and contaminate food products (Simões, Simões, & Vieira, 2010). Several attempts have been made to disperse biofilm by using EPS-degrading enzymes (i.e., combination of proteases, polysaccharide-degrading enzymes and DNases) (Thallinger, Prasetyo, Nyanhongo, & Guebitz, 2013). Nevertheless, the high price and environmental limitation of EPS-degrading enzymes pose great challenges on their application.

Alternatively, interrupting the QS system is gaining more and more attention for biofilm control. In theory, QS interruption does not induce any resistance since no ‘life-or-death’ selection is imposed (Dong, Wang, & Zhang, 2007). Further, biofilm formation can be controlled from its early stage. Three major QS interruption strategies are discussed in the next section. Since we select the LuxI/LuxR-type QS system as the representative QS system in this thesis project, we will only discuss the interruption strategies on the LuxI/LuxR-type QS system in this thesis.
3.3.1 Three major QS interruption strategies

According to the process of QS circuit, three major interruption strategies have been studied: (1) prevention of AIs production; (2) degradation or sequestration of AIs in the extracellular environment; and (3) blocking of AIs detection (Hentzer & Givskov, 2003).

(1) Prevention of AIs production

Inhibition of AIs synthesis can reduce the accumulation of AIs in the extracellular environment, which is an effective method to control QS. Some AI analogues have been validated to inhibit the expression of AIs synthases. For example, various structural analogues of AHL precursors (i.e., S-adenosyl-methionine), such as S-adenosylhomocysteine and S-adenosylcysteine, have been confirmed to have the inhibitory effect against the synthesis of AHL in *P. aeruginosa* (Parsek et al., 1999).

(2) Degradation or sequestration of AIs in the extracellular environment

After AIs are produced and released into the extracellular environment, it is critical to prevent the accumulation of AIs by degradation and/or sequestration methods. Enzymatic inactivation methods are the most commonly used ones. For example, two types of AHLs degradation enzymes (i.e., AHL-lactonase and AHL-acylase) have been identified to inhibit QS in *P. aeruginosa* and *V. paradoxus*. AHL-lactonase hydrolyzes the ester bond of the lactone ring to form acyl homoserine. AHL-acylase cleaves the amide bond of AHLs, forming a fatty acid and homoserine lactone. The degradation of AHLs inhibits the specific binding between AHLs and receptors, thus inhibiting QS (Hong et al., 2012).
Another approach is to apply antibody to sequestrate AIs. For example, Kaufmann and coauthors indicated that an antibody RS2-1G9, which is generated against 3-oxo-C\textsubscript{12}-AHL analogue hapten RS2, could protect mammalian cells from the cytotoxic effect caused by 3-oxo-C\textsubscript{12}-AHL (Kaufmann, Park, Mee, Ulevitch, & Janda, 2008).

(3) Blocking of AIs detection

The blocking of AIs detection is another approach to inhibit QS. Due to the highly specific binding between AIs and the corresponding receptors, AIs analogues can interfere the detection of AIs from the corresponding receptors. For example, Smith and others screened a library of AHLs analogues and identified that these analogues could block the interaction between AHLs and LasR receptor, thus inhibited the formation of \textit{P. aeruginosa} biofilms (Smith et al., 2003).

3.3.2 Limitation

Although the aforementioned QS interruption methods are effective in attenuating QS process, they are either too expensive or difficult to apply in a large scale. Further, some compounds (\textit{e.g.}, antibodies) are not tolerant to high or low temperature, pH, and/or salinity, limiting their application in the food industry. Thus, a novel and alternative QS inhibition strategy is in demand.

3.4 A novel biofilm inhibition strategy: application of MIPs

Molecularly imprinted polymer (MIPs), known as “artificial antibodies”, are highly cross-linked, synthetic molecular recognition materials that can mimic recognition entities (\textit{e.g.}, antibodies and enzymes) (Vasapollo et al., 2011). MIPs have been validated to be useful in recognizing both
biological and chemical molecules, such as amino acids (Scorrano, Mergola, del Sole, & Vasapollo, 2011), proteins (Xu et al., 2016), and food chemical hazards (e.g., pesticide and herbicide) (Murray & Örmeci, 2012). MIPs have been recently proposed as alternative AIs capturing agent in the extracellular environment, thus attenuating QS in bacteria (Piletska et al., 2011). Compared to other recognition entities, MIPs obtain more advantages, including high chemical and physical stability, simple preparation and low cost. Taken together, MIPs are promising tools to interrupt QS and biofilm formation by capturing AIs.

### 3.4.1 Principle and categories

The specific recognition of MIPs to target compounds is based upon the formation of specific binding sites and shape in a highly cross-linked polymer matrix (Haupt, 2003; Vasapollo et al., 2011). The procedure for MIPs synthesis is shown in Figure 3.8 (Chen, Xu, & Li, 2011). MIPs are produced from a mixture of template molecules, functional monomers, crosslinkers, initiators and porogen solvents by polymerization. Target compounds or their structural analogues serve as templates. Functional monomers interact with the templates by covalent or non-covalent bonds, such as hydrogen bond, ionic interaction, and van der Waal force. Free radical polymerization is the most commonly used method to convert monomers into polymers, which starts from the decomposition of initiators upon heating or irradiation (Cormack & Elorza, 2004). After initiation, free radicals produced by initiators can react with crosslinkers and start the generation of polymer chains. The vinyl groups on functional monomers can also react with the vinyl groups on crosslinkers, by which the template-functional monomer complex is fixed in a cross-linked three-dimensional matrix. After the removal of templates from the polymers, complementary space and binding sites to the templates are permanently left in the polymers.
Consequently, the obtained polymers can specifically recognize and bind to the target compounds.

Figure 3.8 Principle of molecularly imprinted polymers. Image is referenced from the work by Chen and colleagues (Chen, Xu, & Li, 2011).

Depending upon the type of bonds between templates and functional monomers, molecular imprinting strategies can be categorized into two groups: covalent and non-covalent imprinting (Spivak, 2005). Covalent imprinting involves the formation of reversible covalent bonds between templates and functional monomers. For example, the functional monomer with a boronic acid group can form a boronic ester bond with the diol-containing template (Wulff, 2002). The covalent bonds can be cleaved for the removal of templates and reformed for the adsorption of target compounds. Due to the high stability of template-functional monomer complex, MIPs have rather homogenous binding sites and high adsorption affinity. However, this method is restricted to the templates and functional monomers with appropriate reversible covalent
functionalities. Besides, covalent imprinting requires an extra step to synthesize the template-functional monomer complex prior to polymerization (Chen et al., 2011). In contrast, non-covalent imprinting does not have such limitations. Non-covalent imprinting involves the self-assembly of templates and functional monomers by various non-covalent bonds. A wide variety of commercially available functional monomers can interact with almost all types of templates by non-covalent bonds (Wei & Mizaikoff, 2007). The synthetic step of non-covalent imprinting is relatively simple, since only the mixing of templates and functional monomers is required. Therefore, non-covalent imprinting is the most commonly used approach for the synthesis of MIPs. However, MIPs generated by non-covalent imprinting suffer from the relatively low adsorption affinity (Yan & Row, 2006). In the non-covalent imprinting, the formation of template-functional monomer complex is in an equilibrium process. In order to push the equilibrium towards the template-functional monomer complex, excessive functional monomers are added, resulting in more non-specific binding sites (Vasapollo et al., 2011).

MIPs can also be categorized by polymerization methods, mainly including bulk polymerization, suspension polymerization, and precipitation polymerization (Lok & Son, 2009). Each polymerization method has its advantages and disadvantages. Bulk polymerization generates rigid monoliths of MIPs, followed by grinding and sieving to obtain fine particles. The operation of bulk polymerization is straightforward. However, grinding may destroy the binding sites and cause MIPs with irregular sizes and shapes (Chen et al., 2011). Suspension polymerization can be used to generate spherical particles with high reproducibility. However, surfactants are required, which are expensive and may introduce interferences into the MIPs (Mayes & Mosbach, 1996). Precipitation polymerization is free from surfactants and can generate spherical
particles in relatively homogenous sizes, but it requires large amount of template and porogen solvents (Lok & Son, 2009).

3.4.2 Important factors in polymerization

The molecular recognition performance of MIPs is affected by various factors, which make the optimization of polymerization to be complicated. These factors include the nature and levels of templates, functional monomers, crosslinkers and porogens, as well as initiation methods (Yan & Row, 2006).

(1) Templates

Template is the most critical element during polymerization because it directs the organization of functional monomers and thus determines the distribution of functional groups. Template should be soluble in the porogen and not have any functional groups to participate in the radical reaction. In addition, the template should remain stable during polymerization (Cormack & Elorza, 2004).

In this thesis project, 3-oxo-C12-AHL was used as the template due to its key role in the QS system of P. aeruginosa. This compound is chemically inert in the radical reaction. However, some studies have demonstrated that AHLs degrade in a pH- and temperature-dependent manner (Yates et al., 2002). Although no data is available about the stability of 3-oxo-C12-AHL, some other AHLs have been studied. For example, N-butanoyl-homoserine lactone (C4-AHL) and N-hexanoyl-homoserine lactone (C6-AHL) undergo hydrolysis when the pH is above 5, which falls in the common pH range of imprinting solution. Further, more extended hydrolysis occurs with
the increase in temperature (e.g., 22°C and 37°C). Therefore, it is critical to study the stability of 3-oxo-C₁₂-AHL in different initiation conditions (i.e., thermal and photochemical initiation) before the synthesis of MIPs. Three major questions should be addressed: 1) Does 3-oxo-C₁₂-AHL degrades under the thermal initiation condition (usually 60°C-80°C)? 2) If the answer is “yes”, would photo initiation at a relatively low temperature (e.g., 22°C or below) be an alternative initiation method? 3) If both of the methods lead to the degradation of 3-oxo-C₁₂-AHL, can we find any structural analogues for the synthesis of MIPs?

(2) Functional monomers

Functional monomers are responsible for offering binding sites to the templates. It is critical to match the functional groups of the functional monomers and the functional groups of templates in a complementary manner. In this thesis project, we used non-covalent imprinting to develop MIPs towards 3-oxo-C₁₂-AHL. Hence, we will introduce some strategies in selecting functional monomers in terms of non-covalent bonds. Based upon the property of functional groups, functional monomers can be divided into three groups, namely acidic, basic, and neutral monomers (Vasapollo et al., 2011). For example, carboxylic groups on acidic monomers, amino groups on basic monomers, or carbonyl groups on neutral monomers can form hydrogen bonds with the templates that bear H-bond acceptors or H-bond donors.

For the non-covalent imprinting, the ratio of template to functional monomers is critical. The association between templates and functional monomers proceeds in equilibrium (Chen et al., 2011). In general, the excessive functional monomers are added. Relatively high ratios can induce more template-functional monomer complexes, resulting in more binding sites. However,
extensively high ratios can also lead to high amount of free functional monomers fixed in the MIPs matrix and subsequently generate more non-specific binding sites. According to the previous studies, the common molar ratio between templates and functional monomers varies from 1:4 to 1:12 (Gao et al., 2014; Piletska et al., 2011; Vasapollo et al., 2011).

(3) Crosslinkers

Crosslinkers have three major functions in MIPs, namely controlling the morphology of polymer matrix, stabilizing the binding sites and imparting the mechanical stability of MIPs. Generally, a high amount of crosslinkers is preferred to obtain the mechanical stability and permanent cavities for the adsorption of the target compounds (Cormack & Elorza, 2004). Crosslinkers commonly comprise over 80% of the MIPs (Yan & Row, 2006). In fact, the ratio of crosslinker to functional monomer also should be considered to get high adsorption affinity. For example, Yoshimatsu and colleagues synthesized propranolol-imprinted polymers by precipitation polymerization method (Yoshimatsu, Yamazaki, Chronakis, & Ye, 2012). Methacrylic acid (MAA) and trimethylolpropane trimethacrylate (TRIM) were used as functional monomer and crosslinker, respectively. The molar ratios of MAA: TRIM were 3:2 and 2:5. Adsorption affinity was determined by comparing the adsorption capacity of MIPs to its control polymer (non-imprinted polymer synthesized with the absence of propranolol). MIPs at the ratio of 3:2 showed ~2.5-time higher adsorption affinity than the ones at the ratio of 2:5.

(4) Porogens

A porogen is the solvent that dissolves the template, functional monomer, crosslinker and initiator. Porogen plays an important role in the morphology of macroporous MIPs (Vasapollo et
al., 2011). With appropriate amount of porogen, large pores can be produced to achieve good flow-through properties of MIPs. During prepolymerization, porogen may influence the interactions between templates and functional monomers. For instance, protic solvents (e.g., water and methanol) may interfere the hydrogen binding between templates and functional monomers. In this case, aprotic organic solvents (e.g., acetonitrile) are selected (Pichon & Chapuis-Hugon, 2008).

(5) Initiation methods

For free radical polymerization, azoinitiators are commonly used as the radical source, such as 2,2’-azobis(2-methylpropionitrile) (AIBN) and 2,2’-Azobis(2,4-dimethyl)valeronitrile (ABVN) (Yan & Row, 2006). Thermal and photo initiations are two common initiation methods to trigger free radical polymerization. Polymers produced at a lower temperature obtain a higher adsorption affinity than the polymers produced at an elevated temperature. For example, Sreenivasan and coauthors compared the adsorption affinity of MIPs obtained from thermal initiation (70°C) and photo initiation (366 nm, 26°C). Photo-initiated polymers had a higher adsorption affinity towards the target compounds than thermo-initiated polymers (Sreenivasan, 1999). The relatively high temperature might have an adverse impact on the stability of template-functional monomer complex.

3.4.3 Previous applications of imprinted polymers in biofilm inhibition

To the best of our knowledge, only one study was conducted using MIP to inhibit bacterial biofilm formation (Piletska et al., 2011). In that study, 3-oxo-C12-AHL was selected as the template, along with itaconic acid (IA) as the functional monomer, ethylene glycol
dimethacrylate (EGDMA) as the crosslinker, 1,1’-azobis(cyclohexanecarbonitrile) (ABCN) as the initiator, and N,N-dimethylformamide (DMF) as the porogen. The ratio of template: functional monomer: crosslinker was 1:12:148. Bulk polymerization was performed at 80°C for 12 h. Non-imprinted polymers (NIPs) was synthesized without the addition of 3-oxo-C_{12}-AHL. The adsorption capacities of MIPs and NIPs towards 3-oxo-C_{12}-AHL were 6.69 mg/g and 4.19 mg/g in 20% acetonitrile, respectively. Compared to NIPs, the higher adsorption capacity of MIPs indicated the higher adsorption affinity towards the target compound (i.e., 3-oxo-C_{12}-AHL). Adsorption selectivity was determined by comparing the adsorption capacities of MIPs towards different compounds [i.e., 3-oxo-C_{12}-AHL and its structural analogue N-butyryl-L-homoserine lactone (C_{4}-AHL)]. MIPs showed no adsorption capacity towards C_{4}-AHL, while NIPs could capture 0.05 mg/g of C_{4}-AHL. In this case, high selectivity was preferred so that polymers could have better performance to capture target compound and interrupt QS. *P. aeruginosa* biofilm was cultivated with or without the presence of polymers for 24 h. Crystal violet assay was conducted to quantify the formation level of biofilm. Approximately an 80% reduction in the *P. aeruginosa* biofilm was achieved by the MIPs (20 mg/mL), while a 40% reduction was achieved with the presence of NIPs. In that study, MIP showed sufficient capacity and good affinity to capture 3-oxo-C_{12}-AHL from the environment, thus interrupting QS and inhibiting biofilm formation. However, no other studies have been conducted to evaluate the performance of 3-oxo-C_{12}-AHL-imprinting polymers. Therefore, there is not sufficient evidence to confirm the biofilm inhibitory effect by using this approach.

Other types of polymer-based AIs capturing agents were developed in two studies. Piletska and colleagues evaluated the effect of NIPs against *Vibrio fischeri* biofilm (Piletska et al., 2010). To
synthesize NIPs, IA was used as the functional monomer, along with EGDMA as the crosslinker, ABCN as the initiator, and DMF as the porogen. No template was added. The ratio of functional monomer and crosslinker was 1:12. In V. fischeri biofilm, N-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C\_6-AHL) mediates the QS system. Hence, 3-oxo-C\_6-AHL was used as the target compound for adsorption test. The adsorption capacity of the polymer was 46.3 ± 5.7 mg/g in nutrient broth containing 2% NaCl. In biofilm inhibition test, the effect of the polymer against V. fischeri biofilm formation was studied with or without exogenous 3-oxo-C\_6-AHL. Crystal violet assay was conducted to quantify the entire biomass of biofilm. Without the presence of the polymer, V. fischeri biofilm formation was increased by 40% with the exogenous 3-oxo-C\_6-AHL. By adding the polymer (20 mg/mL), V. fischeri biofilm was significantly (P < 0.05) reduced compared to both control groups (i.e., V. fischeri +/- exogenous 3-oxo-C\_6-AHL), indicating that the polymer could effectively capture 3-oxo-C\_6-AHL and subsequently inhibit biofilm formation.

In another study, the researchers developed two linear polymers to inhibit the biofilm formation in Aeromonas hydrophila, a human opportunistic pathogen (Cavaleiro et al., 2015). The polymers were synthesized by copolymerizing methyl methacrylate (MMA) with IA or methacrylic acid (MAA), without the addition of any templates. Since MMA, IA and MAA have one vinyl group, water-soluble and linear polymers were generated. Two AIs in A. hydrophila biofilm, N-butyryl-L-homoserine lactone (C\_4-AHL) and N-hexanoyl-L-homoserine lactone (C\_6-AHL) were selected as the adsorption target molecules. IA-MMA copolymer could capture 0.24 ± 0.12 mg/g C\_4-AHL and 0.13 ± 0.03 mg/g C\_6-AHL in acetonitrile, respectively. MAA-MMA copolymer could capture 0.66 ± 0.11 mg/g C\_4-AHL and 0.71 ± 0.11 mg/g C\_6-AHL in acetonitrile,
respectively. Both of the copolymers significantly ($P < 0.05$) reduced the formation level of *A. hydrophila* biofilm.

Taken together, the results from these two studies indicated that polymers (*i.e.*, NIPs and linear polymers) without specific imprinting process could be used as AIs capturing agents to inhibit bacterial biofilm formation. However, these two types of polymers usually showed a relatively low adsorption selectivity towards the target compounds in a complex medium, limiting their real world application (Curcio et al., 2010). Therefore, applying MIPs is still a pre-mature approach to be considered for the inhibition of bacterial biofilm formation. Due to these aforementioned reasons, we aim to develop MIPs for capturing 3-oxo-C$_{12}$-AHL and evaluate its inhibitory effect against *P. aeruginosa* biofilm formation.
Chapter 4: Materials and methods

4.1 Chemicals

\(N\)-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C\(_{12}\)-AHL; \(\geq 98\%\) purity), itaconic acid (IA; \(\geq 99\%\) purity), 2-hydroxyethyl methacrylate (HEMA; \(\geq 99\%\) purity), ethylene glycol dimethacrylate (EGDMA; 98\% purity), 2,2'-azobis(2-methylpropionitrile) (AIBN; 98\% purity), \(N,N\)-dimethylformamide (DMF; 99.8\% purity), acetonitrile (HPLC grade, \(\geq 99.9\%\) purity;), formic acid (reagent grade, \(\geq 95\%\) purity), crystal violet (\(\geq 90\%\) purity, certified by the Biological Stain Commission), and 2,3,5-triphenyltetrazolium chloride (TTC; \(\geq 95\%\) purity) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Phosphate buffered saline (1× PBS, pH 7.4) was obtained from VWR International (Mississauga, ON, Canada). Methanol, ethanol, acetone, and acetic acid were obtained from Thermo Fisher Scientific (Toronto, ON, Canada). Deionized water (Millipore, Darmstadt, Germany, 18.2 M\(\Omega\)/cm) was prepared in the laboratory.

For the synthesis of MIPs, stock solution of 3-oxo-C\(_{12}\)-AHL was prepared in DMF to achieve the concentration of \(10^5\) mg/L. For equilibrium rebinding study, another stock solution of 3-oxo-C\(_{12}\)-AHL was prepared in acetonitrile to achieve the concentration of \(10^3\) mg/L. Stock solutions were stored at -20°C for further usage.

4.2 Synthesis of MIPs

The synthesis of MIPs was conducted by bulk polymerization method. Three major factors were taken into consideration to optimize the adsorption affinity of MIPs towards 3-oxo-C\(_{12}\)-AHL.
These factors include polymerization condition (section 4.2.1), nature of functional monomer (section 4.2.2), and the ratio of template: functional monomer: crosslinker (section 4.2.3).

### 4.2.1 Polymerization condition

The stability of template-functional monomer complex developed during polymerization influences the final imprinting effect of MIPs. Therefore, degradation of template should be avoided. In the present study, 3-oxo-C_{12}-AHL was used as the template. AHL molecules were reported to degrade in a pH- and temperature-dependent manner, which are two major parameters in polymerization condition (Yates et al., 2002).

Regarding the effect of temperature and pH on the stability of 3-oxo-C_{12}-AHL, the degradation profiles of 3-oxo-C_{12}-AHL under thermal initiation and photo initiation were compared. The range of pH was determined based upon the potential concentrations of functional monomer (i.e., IA) in porogen (i.e., DMF). Briefly, 3-oxo-C_{12}-AHL (stock solution: 10^5 mg/L) was 10-fold serially diluted in DMF in a 1.5-mL glass vial, making the final concentration to be 100 mg/L. IA was added into the solution to adjust the pH value from 3 to 5. Vials were sealed and incubated at 60°C in water bath or 4°C under UV light exposure (365 nm, 6 watt; VWR, Radnor, PA, USA) for different time points (0, 3, 6, 12, 18, 24 h). The residual 3-oxo-C_{12}-AHL was quantified using high performance liquid chromatography coupled with diode array detector (HPLC-DAD; Agilent Technologies, Santa Clara, CA, USA). Degradation percentage was calculated following Equation 4.1. Experiment was conducted in triplicate.
\[
\text{Degradation} \% = \frac{C(i) - C(f)}{C(i)} \times 100 \quad (4.1)
\]

where \( C(i) \) is the initial concentration of 3-oxo-C\(_{12}\)-AHL (mg/L) and \( C(f) \) is the final concentration of 3-oxo-C\(_{12}\)-AHL at a given time (mg/L).

### 4.2.2 Ratio of AHL: IA: EGDMA

After confirming the appropriate initiation method, MIPs were generated by using IA as the functional monomer, EGDMA as the crosslinker, AIBN as the initiator, and DMF as the porogen. The composition of reagents is listed in Table 4.1.

Briefly, defined amounts of 3-oxo-C\(_{12}\)-AHL and IA were added into DMF. The mixture was stirred at 100 rpm for 2 h to achieve the equilibrium of interaction between 3-oxo-C\(_{12}\)-AHL and IA. Then, EGDMA and AIBN were added into the solution. To remove oxygen, nitrogen was purged for 5 min. Afterwards the solution was sealed and polymerized under UV light (365 nm, 6 watt, 10 cm-distance between the central point of solution and UV light source) for 12 h. All procedures were conducted at 4°C in a cold room. The monolithic product was ground and sieved through a steel sieve (standard 200-mesh, 75 micron; Thermo Fisher Scientific, Waltham, MA, USA). Soxhlet extraction was applied to remove the template using methanol/acetic acid mixture (7:3, v/v) for 24 h, followed by pure methanol for another 48 h. Then, MIPs were dried in vacuum drying oven at 70°C overnight. The non-imprinted polymers (NIPs) were synthesized following the same procedure without the addition of the template.
Table 4.1 Synthetic formulations of IA-based polymers

<table>
<thead>
<tr>
<th>T: FM: X</th>
<th>3-oxo-C_{12}-AHL (μL)</th>
<th>IA (mg)</th>
<th>EGDMA (μL)</th>
<th>AIBN (mg)</th>
<th>DMF (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:12:148</td>
<td>48</td>
<td>24.7</td>
<td>447</td>
<td>5.0</td>
<td>500</td>
</tr>
<tr>
<td>1:6:48</td>
<td>143</td>
<td>37.5</td>
<td>435</td>
<td>5.0</td>
<td>750</td>
</tr>
<tr>
<td>1:8:48</td>
<td>143</td>
<td>50.0</td>
<td>435</td>
<td>5.2</td>
<td>750</td>
</tr>
<tr>
<td>1:6:25</td>
<td>190</td>
<td>50.0</td>
<td>302</td>
<td>3.8</td>
<td>500</td>
</tr>
<tr>
<td>1:8:25</td>
<td>190</td>
<td>66.6</td>
<td>302</td>
<td>4.1</td>
<td>500</td>
</tr>
</tbody>
</table>

^1 T: FM: X- the molar ratio of template: functional monomer: crosslinker

4.2.3 Ratio of AHL: HEMA: EGDMA

HEMA was used to increase the hydrophilicity of MIPs in the previous studies (Cirillo et al., 2011; Puoci et al., 2007). Since MIPs would be applied in aqueous condition where bacteria normally grow, HEMA-based MIPs were synthesized following the same procedure in section 4.2.2, but replacing IA with HEMA. The composition of reagents is listed in Table 4.2.

Table 4.2 Synthetic formulations of HEMA-based polymers

<table>
<thead>
<tr>
<th>T: FM: X</th>
<th>3-oxo-C_{12}-AHL (μL)</th>
<th>HEMA (μL)</th>
<th>EGDMA (μL)</th>
<th>AIBN (mg)</th>
<th>DMF (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:6:48</td>
<td>143</td>
<td>35</td>
<td>435</td>
<td>5.0</td>
<td>750</td>
</tr>
<tr>
<td>1:8:48</td>
<td>143</td>
<td>47</td>
<td>435</td>
<td>5.2</td>
<td>750</td>
</tr>
<tr>
<td>1:6:25</td>
<td>190</td>
<td>47</td>
<td>302</td>
<td>3.8</td>
<td>500</td>
</tr>
<tr>
<td>1:8:25</td>
<td>190</td>
<td>63</td>
<td>302</td>
<td>4.1</td>
<td>500</td>
</tr>
</tbody>
</table>

^1 T: FM: X- the molar ratio of template: functional monomer: crosslinker
4.3 Effect of MIPs on capturing 3-oxo-C\textsubscript{12}-AHL

The capacity and affinity of adsorption are the two major properties to be characterized for MIPs.

To test these two properties, equilibrium rebinding study was carried out as follows.

4.3.1 Equilibrium rebinding study

Equilibrium rebinding study was conducted following the procedure by Feng and others with some modifications (Feng et al., 2013). The working solution of 3-oxo-C\textsubscript{12}-AHL was prepared by 10-time serial dilution from the stock solution (1000 mg/L in acetonitrile). Five milligram of MIPs or NIPs was suspended in 500 μL of 3-oxo-C\textsubscript{12}-AHL working solutions (100 mg/L in 20% or 50% acetonitrile). The mixtures were shaken for 6 h. After centrifugation at 15,000 × g for 5 min, the supernatant was filtrated through a 0.45-μm nylon syringe filter (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of free 3-oxo-C\textsubscript{12}-AHL was determined using HPLC-DAD. Experiment was conducted in triplicate.

Briefly, adsorption capacity (Q) can be calculated according to the Equation 4.2 (Qian, Fang, He, Pan, & Wang, 2010),

\[
Q = \frac{[C(i) - C(\text{free})] \times V}{m} \quad (4.2)
\]

where Q represents the adsorption capacity of polymers (mg/g), C(i) is the initial concentration of 3-oxo-C\textsubscript{12}-AHL (mg/L), C(\text{free}) is the concentration of free 3-oxo- C\textsubscript{12}-AHL (mg/L), V is the volume of 3-oxo- C\textsubscript{12}-AHL solution (mL), and m is the mass of polymers (mg).
Imprinting factor (IF), which reveals the adsorption affinity of MIPs, can be calculated according to the Equation 4.3 (Nezhadali, Feizy, & Beheshti, 2014). If there is a significant difference in the adsorption capacity between MIPs and NIPs, IF should be higher than 1 and therefore higher IF is equivalent to higher affinity.

\[
IF = \frac{Q_{MIP}}{Q_{NIP}} \quad (4.3)
\]

where \(Q_{MIP}\) and \(Q_{NIP}\) are the adsorption capacities of MIPs (mg/g) and NIPs (mg/g) to 3-oxo-C\(_{12}\)-AHL, respectively.

### 4.3.2 High performance liquid chromatographic condition

The quantification of 3-oxo-C\(_{12}\)-AHL using HPLC-DAD was optimized from a previous protocol by Piletska and colleagues with minor modification (Piletska et al., 2011). HPLC separation was conducted using Agilent 1260 series HPLC system with a binary pump. A 5 μL aliquot was injected into a C\(_8\) reverse-phase column (100 × 3.0 mm, 2.6-μm particle size; Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) 40% water with 0.5% formic acid and (B) 60% acetonitrile. Isocratic elution at a constant flow rate of 0.5 mL/min was conducted for 5.5 min. The temperature in sampler and column were 4°C and 22°C, respectively. The retention time of 3-oxo-C\(_{12}\)-AHL was 1.9 min and 3-oxo-C\(_{12}\)-AHL was detected at 195 nm.

### 4.4 Effect of MIPs on \(P.\) aeruginosa biofilm formation

#### 4.4.1 Media, bacterial strain and culture preparation

Luria-Bertani (LB; Becton, Dicknson and Company, Franklin Lakes, NJ, USA) was used as the non-selective media for the cultivation of \(P.\) aeruginosa PAO1 as the microbial model. \(P.\)
*P. aeruginosa* PAO1 was preserved in glycerol supplemented LB broth (2:8, v/v) at -20°C. Ten microliter of stock culture was cultivated on LB agar at 37°C for 24 h. One single colony was selected and inoculated in 5 mL of LB broth. The solution was incubated in incubation shaker (150 rpm, 37°C) for 16-18 h.

### 4.4.2 Anti-biofilm activity test

#### 4.4.2.1 Immobilization and sterilization of MIPs

Three hundred milliliters of 70% ethanol was added into each well of 24-well microtiter plate (Non-treated plate, polystyrene, flat; Corning, Corning, NY, USA). Fifteen milligram of polymers were gently dispensed in the solution and evenly settled on the surface of plate well. The solution was allowed to evaporate for 3 h at room temperature (Chianella et al., 2013). Before biofilm study was performed, the package (*i.e.*, 24-well microtiter plate containing dried polymers) was sterilized using UV radiation for 15 min.

#### 4.4.2.2 Biofilm cultivation with MIPs

A conventional biofilm cultivation method was conducted following the work by Piletska and coauthors with modifications (Piletska et al., 2011). Briefly, overnight bacterial culture was adjusted to OD$_{600}$= 0.003 ($10^6$ CFU/mL) by 10-fold serial dilutions. A five hundred milliliter aliquot was added into each well of 24-well microtiter plate containing polymers. A piece of polystyrene coupon (VWR, Mississauga, ON, Canada; 10 mm ×10 mm) was immersed into bacterial culture, which could stay on the top of polymer layer (Figure 4.1). Biofilm was incubated under static condition at 37°C for 24 h. Biofilm cultivated without the presence of
polymers was used as the negative control. Bacteria-free LB broth with or without the addition of polymers was applied as the blank control.

![Figure 4.1 Schematic illustration of the experimental setup in the representative well of a 24-well microtiter plate.](image)

**4.4.2.3 Biofilm quantification**

After incubation, planktonic cells and media were taken out from each well. Then, polystyrene coupon was gently removed from the plate and washed with PBS three times, and then placed in another 24-well microtiter plate to air dry for 15 min. All procedures were performed in aseptic condition. Subsequently, biofilms formed on the polystyrene coupons were quantified by two independent approaches, namely crystal violet assay and 2,3,5-triphenyl-tetrazolium chloride (TTC) assay.

**4.4.2.3.1 Crystal violet assay**

Crystal violet assay was carried out following the protocol by Cady and colleagues with minor modifications (Cady et al., 2012). Briefly, biofilm was stained using 0.05% crystal violet solution (500 μL/well) for 10 min at room temperature. Then, the crystal violet solution was removed from each well and the polystyrene coupon was washed with PBS three times to remove the extra crystal violet solution. Biofilm developed on the polystyrene coupon was air
dried for 15 min, followed by immersing in 1 mL of 95% ethanol for another 15 min. The solution was collected and centrifuged at 15000 \( \times g \) for 3 min. A 100 \( \mu L \) aliquot of the supernatant was transferred into another 96-well microtiter plate (sterile clear flat-bottom plate with low evaporation lid; Corning, Corning, NY, USA). The absorbance was determined at 595 nm using a microtiter plate reader (SpectraMax M2; Molecular Devices LLC, Sunnyvale, CA, USA).

### 4.4.2.3.2 TTC assay

To quantify the viable cell number in a biofilm, TTC assay was applied following the procedure described by Sabaeifard and colleagues with minor modification (Sabaeifard, Abdi-Ali, Soudi, & Dinarvand, 2014). In brief, 0.5% TTC stock solution was prepared by dissolving TTC in PBS and sterilized by passing through a 0.45-\( \mu m \) cellulose acetate filter (Sigma-Aldrich, Oakville, ON, Canada). To prepare the TTC working solution, one part of 0.5% TTC stock solution was mixed with four parts of LB broth (final concentration: 0.1%). Biofilm on the polystyrene coupon was incubated at 150 rpm with the fresh TTC working solution (500 \( \mu L \)) in dark at 37°C for 6 h. Then, the supernatant was withdrawn and ethanol/acetone mixture (8:2, \( v/v \); 1 mL/well) was added into each well. The aliquot was transferred and centrifuged at 15000 \( \times g \) for 3 min, followed by the measurement of absorbance at the wavelength of 490 nm using a microtiter plate reader.

For both crystal violet assay and TTC assay, relative biofilm formation was calculated based upon the Equation 4.4.
\[
\text{relative biofilm formation} = \frac{\text{Ab}_{\text{polymer}} - \text{Ab}_{\text{blank1}}}{\text{Ab}_{\text{control}} - \text{Ab}_{\text{blank2}}} (4.4)
\]

where \( \text{Ab}_{\text{polymer}} \) represents the absorbance of polymer group (\textit{i.e.}, polymers and \textit{P. aeruginosa} biofilm), \( \text{Ab}_{\text{control}} \) represents the absorbance of control group (\textit{i.e.}, only \textit{P. aeruginosa} biofilm), \( \text{Ab}_{\text{blank1}} \) is the absorbance of blank group (\textit{i.e.}, without \textit{P. aeruginosa} biofilm) with the addition of polymers, and \( \text{Ab}_{\text{blank2}} \) is the absorbance of blank group without the addition of polymers.

### 4.4.3 Antimicrobial activity test

The antimicrobial activity of polymers against planktonic bacterial cells was tested following the protocols by Dave and coauthors with modifications (Dave, Joshi, & Venugopalan, 2011). First, overnight bacterial culture was diluted to \( \text{OD}_{600} = 0.003 \) (\( 10^6 \text{ CFU/mL} \)). Then, 2 mL of inoculum was added into a 10-mL glass tube containing 0 mg or 60 mg of sterilized polymers. The mixture was incubated in a shaking manner (150 rpm) for 24 h at 37°C. After incubation, bacterial cell counts were quantified using the conventional plating count assay (Cady et al., 2012). Experiment was conducted at least in triplicate.

### 4.5 Statistical analysis

The results are presented in terms of the mean ± standard deviation. Data analysis was conducted using SPSS Statistics v17.0 software (IBM, USA). In the stability study (section 4.1.2), three-way ANOVA and two-way ANOVA were conducted. In other studies, Student’s t-test or one-way ANOVA followed by appropriate post-hoc test was employed to compare the means of different treatment data. Significant difference was determined when \( P < 0.05 \).
Chapter 5: Results and discussion

5.1 Effect of MIPs on capturing 3-oxo-C\textsubscript{12}-AHL

5.1.1 Effect of polymerization condition on 3-oxo-C\textsubscript{12}-AHL stability

In the current study, MIPs for 3-oxo-C\textsubscript{12}-AHL were developed by the free radical bulk polymerization method, followed by grinding and sieving. Bulk polymerization is the most conventional and popular method compared to other polymerization methods, such as precipitation polymerization and suspension polymerization (Turiel & Martin-Esteban, 2010; Wei & Mizaikoff, 2007). The major advantages of bulk polymerization include simple preparation, no involvement of expensive or sophisticated facilities, and no addition of surfactants. In the previous studies, Piletska and colleagues prepared polymers to capture 3-oxo-C\textsubscript{12}-AHL and \textit{N}-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C\textsubscript{6}-AHL) by bulk polymerization (Piletska et al., 2010, 2011).

To synthesize MIPs, free radical bulk polymerization was conducted with AIBN as the initiator in the current study. The polymerization follows three steps: initiation, propagation, and termination. During the initiation stage, AIBN is decomposed and forms free radicals under thermal or photo initiation. Then, the free radicals are transferred from AIBN to the vinyl monomers (\textit{i.e.}, functional monomer and crosslinker), followed by rapid sequential propagation. The growth of chains ceases when no active centers that carry free radicals are available (Colombani, 1997; Tobita & Hamielec, 1989). Generally, a temperature range between 50\degree C and 80\degree C is employed for thermal initiation of AIBN (Guan, Combes, Menceloglu, & DeSimone, 1993; Piletsky et al., 2005; Yin, Yang, & Chen, 2005). In comparison, ultraviolet light at 365 nm
is usually applied for photo initiation with the temperature at or below room temperature (González, Hernando, & Alegría, 2006).

Although both thermal and photo initiation methods are theoretically appropriate for molecularly imprinting process, the stability of template (i.e., 3-oxo-C₁₂-AHL) during polymerization needs to be secured. According to the study by Yates and colleagues, the degradation of acyl homoserine lactones [e.g., 3-oxo-C₆-AHL, N-butanoyl-homoserine lactone (C₄-AHL), and N-octanoyl-AHL (C₈-AHL)] increased as a function of increase in temperature (22-37°C) and pH (5.8-7.4) (Yates et al., 2002). No data is available about the stability of 3-oxo-C₁₂-AHL under different polymerization conditions yet. Therefore, degradation test of 3-oxo-C₁₂-AHL was conducted in the current study.

Figure 5.1 illustrates the degradation profile of 3-oxo-C₁₂-AHL under thermal and photo initiations. To mimic the real polymerization condition, itaconic acid (IA) was used as the representative functional monomer (Piletska et al., 2011). The pH range from 3.4 to 4.5 was prepared based upon the potential concentrations of IA. There was a significant three-way interaction among time, pH and initiation method with F(4, 40) = 6.141, P = 0.001. Therefore, we conducted multiple two-way ANOVA tests at the fixed level of time, pH, or initiation method. In the thermal initiation, there was a significant interaction between the effect of pH and time on the degradation of 3-oxo-C₁₂-AHL with F(4, 20) = 7.652, P = 0.001. 3-oxo-C₁₂-AHL gradually degraded with the increase in time and pH (P < 0.05). At a higher pH, thermal initiation caused 30.38 ± 1.42% and 47.93 ± 3.06% of degradation after 12 h and 24 h, respectively. In the photo initiation, time period significantly (P < 0.05) influenced the stability of 3-oxo-C₁₂-AHL, while
pH was not a statistically significant factor ($P > 0.05$). Less than 5% of 3-oxo-C$_{12}$-AHL degraded after 24 h. Compared to thermal initiation at the same time point or pH, photo initiation resulted in significantly lower ($P < 0.05$) degradation of 3-oxo-C$_{12}$-AHL. Taken together, photo initiation was validated to be the better method for imprinting 3-oxo-C$_{12}$-AHL-based MIPs.

![Degradation profile of 3-oxo-C$_{12}$-AHL under different polymerization conditions when itaconic acid (IA) was used as the functional monomer. Thermo: thermal initiation (60°C). Photo: photo initiation (4°C, 365 nm). The range of pH was determined based upon the potential concentrations of IA in N,N-dimethylformamide (DMF). Error bars indicate standard deviation (n=3).](image)

Moreover, relatively low temperature in photo initiation is preferable for the stability of template-functional monomer complex compared to that developed by thermal initiation (Sreenivasan, 1999; Urraca et al., 2008; Zsebi, Horvath, Safrany, & Horvai, 2008). Stable self-assembly between template and functional monomer during polymerization is critical to obtain
specific shape, size, and functional groups within the MIPs network. Since the temperature in photo initiation can be adjusted freely, a lower temperature can be set up, resulting in a lower kinetic energy of template-functional monomer complex and a higher adsorption capacity and specificity of MIPs (Sreenivasan, 1999).

5.1.2 Synthesis and characterization of IA-based polymers

As mentioned in section 3.4.3, only one study has been conducted to synthesize MIPs towards 3-oxo-C_{12}-AHL (Piletska et al., 2011). In that study, 3-oxo-C_{12}-AHL was selected as the template, along with IA as the functional monomer, EGDMA as the crosslinker, ABCN as the initiator and DMF as the porogen. The molar ratio of template: functional monomer: crosslinker was 1:12:148. Bulk polymerization was performed at 80°C for 12 h. The adsorption capacities of MIPs and NIPs were 6.69 mg/g and 4.19 mg/g in 20% acetonitrile, respectively. However, no standard deviation and initial concentration were shown in that paper. In our preliminary study, MIPs and NIPs were synthesized and tested following the same procedure, but no adsorption affinity could be identified in the MIPs (Q_{MIP} = 7.76 ± 0.05 mg/g, Q_{NIP} = 7.88 ± 0.10 mg/g, initial concentration=100 mg/L). There might be two reasons. Firstly, free radical polymerization is a probability-based process where continuous initiation, propagation, and termination take place at every instant, resulting in an uncontrolled molecular structure (Zhang, 2013). Piletska and colleagues did not indicate the performance of MIPs from different batches, thus the reproducibility of their synthesized MIPs was unknown. It is highly possible that we may not be able to generate the desired MIPs even following the same experimental procedure. Secondly, free radical polymerization is an exothermic process. Temperature as high as 180°C in the reactant system can be reached during polymerization when the initial temperature is 80°C.
(Piletsky et al., 2002, 2005). Hence, the absence of specific binding might be due to the degradation of 3-oxo-C12-AHL as well as unstable interaction between 3-oxo-C12-AHL and IA at an extremely high temperature.

To improve the adsorption affinity, photo initiation was applied in the current study. 3-oxo-C12-AHL was used as the template, along with IA as the functional monomer, EGDMA as the crosslinker, AIBN as the initiator, and DMF as the porogen (Figure 5.2). Compared to ABCN, AIBN is more commonly used in photo-initiated polymerization, has shorter half-life, and requires less reaction time. Polymerization was carried out under UV exposure (λ = 365 nm) at 4°C. Polymerization times varied in different previous studies, normally from 2 h to 24 h (Gadzala-Kopciuch, Ricanyova, & Buszewski, 2009; Pan, Wang, Fang, Tang, & Wang, 2010; Sellergren & Shea, 1993). Longer polymerization time causes a higher degree of cross-linking and produces a more rigid polymer, which may affect the adsorption capacity of the polymer (Piletsky et al., 2005). Since MIPs and NIPs follow the same polymerization principle, the effect of polymerization time on the adsorption capacity is expected to be identical. Therefore, preliminary tests were conducted to compare the adsorption capacity of NIPs that were generated at 2 h, 4 h, 8 h, 12 h and 24 h. Different NIPs were not significantly different (P > 0.05) from each other, indicating that polymerization time might not influence the adsorption capacity of polymers in our case. Therefore, we selected 12 h as the polymerization time in this study, as it is the most widely used one.
The ratio of template: functional monomer: crosslinker is of essential importance to obtain chemical and spatial specificity in MIPs as well. The nature and number of functional groups determine the complementary effect on the target compound (i.e., 3-oxo-C$_{12}$-AHL). Crosslinker is used to stabilize the cavity and functional groups during polymerization (Vasapollo et al., 2011). Appropriate degree of crosslinking is required to obtain good mechanical stability and accessibility. The molar ratio between templates and functional monomers commonly varies from 1:4 to 1:12 (Gao et al., 2014; Piletska et al., 2011). The molar ratio between functional monomers and crosslinkers commonly varies from 1:3 to 1:8 (Feng et al., 2013; Matsui et al., 1995). Tedious trails are usually required to optimize the synthetic formulation. In this work, four extra molar ratios (i.e., 1: 6: 25, 1: 8: 25, 1: 6: 48, 1: 8: 48) were used besides of the original ratio (i.e., 1: 12: 148).
To simulate the real bacterial growth condition, equilibrium rebinding study was performed in an aqueous media. Due to the low solubility in water, 3-oxo-C\textsubscript{12}-AHL working solution was prepared in 20% acetonitrile instead of pure water. The concentration of 100 mg/L was selected as the working concentration since this is the concentration that can be commonly detected in \textit{P. aeruginosa} biofilm (Charlton et al., 2000). Adsorption capacity (Q) and imprinting factor (IF) were calculated to evaluate the adsorption performance of MIPs. NIPs were used as the control.

The adsorption capacities of IA-based MIPs and NIPs from different formulations are shown in Figure 5.3. The adsorption capacity of IA-based polymers was higher than 5.63 ± 0.17 mg/g. In other words, over 56.3\% of 3-oxo-C\textsubscript{12}-AHL could be captured from 20\% acetonitrile when the initial concentration was 100 mg/L. Since no initial concentration was specified when Piletska and colleagues presented their adsorption results, we could not compare the adsorption efficacy between these two studies (Piletska et al., 2011).

One-way ANOVA followed by Games Howell’s test (unequal variance) was conducted to test the significant difference between each pair of MIPs and NIPs. There were no significant difference ($P > 0.05$) in the adsorption capacity between each pair of MIPs and NIPs regardless the ratio of template: functional monomer: crosslinker, indicating that there was no specific adsorption existing in MIPs in 20\% acetonitrile aqueous solution.
Figure 5.3 Adsorption capacity of itaconic acid (IA)-based molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) towards 3-oxo-C₁₂-AHL in 20% acetonitrile. Molar ratios of template: functional monomer: crosslinker vary from 1:6:25 to 1:12:148. M: MIPs; N: NIPs. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Games Howell’s test (unequal variance) was conducted with a statistical confidence coefficient of 0.95.

The possible explanation might be as follows. During polymerization, specific interactions exist between 3-oxo-C₁₂-AHL and IA due to the hydrogen bonds. As a polar protic solvent in the equilibrium rebinding study, water can easily interrupt these hydrogen bonds (Curcio et al., 2010). In addition, non-specific binding (i.e., hydrophobic interaction) dominates both MIPs and NIPs towards 3-oxo-C₁₂-AHL since water tends to repel 3-oxo-C₁₂-AHL out of its dynamic
hydrogen network (Gao et al., 2014; Ma, Pan, Zhang, Guo, & Zhang, 2013; Manesiotis, Borrelli, Aureliano, Svensson, & Sellergren, 2009). Therefore, it is reasonable that no significant difference in the adsorption capacity can be identified between MIPs and NIPs in the present study.

However, when water proportion was decreased to 50%, some MIPs showed specific binding compared to their corresponding NIPs due to the decrease in hydrophobic interaction. When the ratios of template: functional monomer: crosslinker were 1:6:25 and 1:8:25, MIPs exhibited significantly higher ($P < 0.05$) adsorption capacity than that of NIPs (Table 5.1). Their imprinting factors (IFs) were $1.68 \pm 0.24$ and $1.43 \pm 0.17$, respectively. Polymers at the ratio of 1:6:48 and 1:8:48 also showed high IF values ($i.e., 1.74 \pm 0.46$ and $1.34 \pm 0.26$) even though no significant difference ($P > 0.05$) can be identified between MIPs and NIPs.

Table 5.1 Adsorption affinity of itaconic acid (IA)-based molecularly imprinted polymers (MIPs) towards 3-oxo-C$_{12}$-AHL in 50% acetonitrile (n=3).

<table>
<thead>
<tr>
<th>Ratio of T: FM: X$^1$</th>
<th>Imprinting factor$^2$</th>
<th>$P$-value$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:12:148</td>
<td>0.93 ± 0.28</td>
<td>0.76</td>
</tr>
<tr>
<td>1:6:48</td>
<td>1.74 ± 0.46</td>
<td>0.07</td>
</tr>
<tr>
<td>1:8:48</td>
<td>1.34 ± 0.26</td>
<td>0.17</td>
</tr>
<tr>
<td>1:6:25</td>
<td>1.68 ± 0.24</td>
<td>0.01</td>
</tr>
<tr>
<td>1:8:25</td>
<td>1.43 ± 0.17</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Imprinting factor = \( \frac{Q_{\text{MIP}}}{Q_{\text{NIP}}} \). Results are presented as the mean of triplicates ± standard deviation.

P-value: P-value between each pair of MIPs and NIPs generated by the same formulation. Student’s t-test (two-tailed, \( \alpha=0.05 \)) was applied to determine the statistically significant difference.

The high IF values of some IA-based MIPs (\( i.e., \) ratio of template: functional monomer: crosslinker = 1:6:25, 1:8:25, 1:6:48 and 1:8:48) showed great potential adsorption affinity in the aqueous media. Therefore, further modification on the synthesis of MIPs was performed based upon these selective ratios.

5.1.3 Synthesis and characterization of HEMA-based polymers

To increase the adsorption affinity of MIPs, hydrophobic interaction should be eliminated when the proportion of water is high (\( e.g., \) 20\% acetonitrile). One alternative approach is to introduce hydrophilic monomers for molecularly imprinting. HEMA, a hydrophilic and biocompatible material, has been used to achieve high adsorption affinity of MIPs in the aqueous condition (Cirillo et al., 2011; Puoci et al., 2007; Sreenivasan, 1997). For example, Cirillo and colleagues evaluated the adsorption affinity of HEMA-based MIPs towards glycyrrhizic acid in the aqueous media (Cirillo et al., 2011). The IF value of HEMA-based MIPs was 5.00 after 24 h incubation in an ethanol-water mixture (6:4, v/v). Puoci and coauthors developed a cholesterol adsorbent (\( i.e., \) HEMA-based MIPs) to extract cholesterol from food matrices (Puoci et al., 2007). In a mixture of acetonitrile and water (7:3, v/v), 21\%-28\% of cholesterol was rebounded by MIPs while only 11\%-14\% of cholesterol was captured by NIPs.
Considering the good performance of HEMA-based MIPs in the aqueous condition, HEMA (Figure 5.4) was selected as the alternative functional monomer in the current study. Four formulations (i.e., the ratio of template: functional monomer: crosslinker = 1:6:25, 1:8:25, 1:6:48, 1:8:48) were used in the synthesis of HEMA-based MIPs because they already showed a great potential of high adsorption affinity in IA-based MIPs as aforementioned in section 5.1.2.

\[
\text{Figure 5.4 Chemical structure of 2-hydroxyethyl methacrylate (HEMA).}
\]

Equilibrium rebinding study was conducted in 20% acetonitrile to evaluate the adsorption affinity of the polymers. The adsorption capacities of all HEMA-based polymers are shown in Figure 5.5. One-way ANOVA followed by Tukey’s test was conducted to check the statistical significance in adsorption capacity between MIPs and the corresponding NIPs. All pairs of MIPs and NIPs showed no significant difference \((P > 0.05)\) between each other, indicating that the adsorption affinity of HEMA-based MIPs was not modified compared to that of IA-based MIPs.
Figure 5.5 Adsorption capacity of 2-hydroxyethyl methacrylate (HEMA)-based molecularly imprinted polymers (MIPs) and non-imprinted polymers (NIPs) towards 3-oxo-C12-AHL in 20% acetonitrile. Molar ratios of template: functional monomer: crosslinker vary from 1:6:25 to 1:8:48. M: MIPs; N: NIPs. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Tukey’s test (equal variance) was performed. Polymers with different letters are statistically different (P < 0.05).

The adsorption performance of IA-based and HEMA-based polymers was compared as well (Figure 5.6). When the formulation was 1:6:48 HEMA (Figure 5.6, panel C) or 1:8:48 HEMA (Figure 5.6, panel D), polymers showed significantly higher (P < 0.05) adsorption capacities than the corresponding IA-based polymers. This might be due to the increase in the number of hydrogen bonds between HEMA-based polymers and 3-oxo-C12-AHL. When the amount of
crosslinker was decreased (i.e., 1:6:25 and 1:8:25), no increase in the adsorption capacities could be observed for HEMA-based polymers (Figure 5.6, panel A and B). A previous study showed that higher amount of crosslinker could lead to more porous morphology, which was related to the accessibility of the target compound to the binding sites (Nicolescu, Sarbu, Ghiurea, & Donescu, 2011). Therefore, 3-oxo-C₁₂-AHL might have low accessibility to get into 1:6:25 and 1:8:25 HEMA-based polymers and form less hydrogen bonds.

![Comparative figures on the adsorption capacities (Q) of itaconic acid (IA)-based polymers and 2-hydroxyethyl methacrylate (HEMA)-based polymers at different synthetic formulations (template: functional monomer: crosslinker) in 20% acetonitrile: 1:6:25 (A); 1:8:25 (B); 1:6:48 (C); and 1:8:48 (D). M: MIPs; N:]
NIPs. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Tukey’s test or Games Howell’s test was performed. Within each panel, polymers with different letters are statistically different (P < 0.05).

There was no significant difference (P > 0.05) between MIPs and NIPs in the adsorption capacity towards 3-oxo-C\textsubscript{12}-AHL in 50% acetonitrile (Table 5.2). IF values were relatively low (i.e., 0.79-1.27). Hence, HEMA-based MIPs had poor adsorption affinity even though the proportion of water was low. This might be due to the increase in hydrogen binding on both MIPs and NIPs towards 3-oxo-C\textsubscript{12}-AHL, leading to no difference in adsorption (Figure 5.7). The adsorption capacities of IA-based polymers were in the range of 0.23-0.52 mg/g. The adsorption capacities of HEMA-based polymers were in the range of 0.41-1.14 mg/g.

Table 5.2 Adsorption affinity of 2-hydroxyethyl methacrylate (HEMA)-based molecularly imprinted polymers (MIPs) towards 3-oxo-C\textsubscript{12}-AHL in 50% acetonitrile (n=3).

<table>
<thead>
<tr>
<th>Ratio of T: FM: X\textsuperscript{1}</th>
<th>Imprinting factor\textsuperscript{2}</th>
<th>P-value\textsuperscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:6:48</td>
<td>1.27 ± 0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>1:8:48</td>
<td>1.16 ± 0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>1:6:25</td>
<td>0.79 ± 0.17</td>
<td>0.21</td>
</tr>
<tr>
<td>1:8:25</td>
<td>0.80 ± 0.27</td>
<td>0.34</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Ratio of T: FM: X- molar ratio of template: functional monomer: crosslinker.

\textsuperscript{2}Imprinting factor = Q\textsubscript{MIP}/Q\textsubscript{NIP}. Results are presented as the mean of triplicates ± standard deviation.
$^3$P-value: P-value between each pair of MIPs and NIPs generated by the same formulation. Student’s t-test (two-tailed, $\alpha=0.05$) was applied to determine the statistically significant difference.

Figure 5.7 Comparative figures on the adsorption capacities (Q) of itaconic acid (IA)-based polymers and 2-hydroxyethyl methacrylate (HEMA)-based polymers at different synthetic formulations (template: functional monomer: crosslinker) in 50% acetonitrile: 1:6:25 (A); 1:8:25 (B); 1:6:48 (C); and 1:8:48 (D). M: MIPs; N: NIPs. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Tukey’s test or Games Howell’s test was performed. Within each panel, polymers with different letters are statistically different ($P < 0.05$).
5.1.4 Summary

In conclusion, both MIPs and NIPs generated in the current study had good adsorption capacity towards 3-oxo-C₁₂-AHL in the aqueous condition. The adsorption capacities varied from 5.52 ± 0.02 mg/g to 6.12 ± 0.02 mg/g in 20% acetonitrile when the initial concentration of 3-oxo-C₁₂-AHL was 100 mg/L. In other words, ~55.2% to 61.2% of 3-oxo-C₁₂-AHL could be captured from the aqueous environment. However, MIPs did not show specific binding in 20% acetonitrile solution even when favorable initiation method (i.e., photo initiation), different functional monomers (i.e., IA and HEMA), and different ratios of template: functional monomer: crosslinker were applied. Non-specific interaction (i.e., hydrophobic interaction) predominantly existed between 3-oxo-C₁₂-AHL and polymers due to the high proportion of water. Nevertheless, IA-based polymers (i.e., 1:6:25, 1:8:25) showed good adsorption affinity when 50% acetonitrile was used as the rebinding solvent.

Therefore, our first two objectives were partially achieved. The synthesized MIPs could efficiently capture 3-oxo-C₁₂-AHL from the aqueous environment, but the synthetic formulation still need to be optimized to obtain good adsorption affinity.

5.2 Effect of MIPs on P. aeruginosa biofilm formation

5.2.1 Anti-biofilm activity

P. aeruginosa biofilm formation is regulated by quorum sensing system via the production of AIs (Mangwani, Kumari, & Das, 2016; Solano, Echeverz, & Lasa, 2014). Conversely, quorum quenching can lead to interference in biofilm development (Abraham, 2016). Since MIPs and NIPs (i.e., IA-based and HEMA-based polymers) were validated to have a good efficacy on
capturing 3-oxo-C_{12}-AHL in section 5.1, it was logical to further study their effect on the formation of *P. aeruginosa* biofilm.

### 5.2.1.1 Crystal violet assay

Anti-biofilm study was performed in 24-well polystyrene plates. *P. aeruginosa* biofilm was cultivated on the surface of polystyrene coupon with or without the presence of MIPs and NIPs. The incubation period was set as 24 h because this time period is sufficient for the maturation of *P. aeruginosa* biofilm (Taylor et al., 2014) and frequently selected to test the anti-biofilm effect (Rasamiravaka et al., 2015). After 24-h incubation, polystyrene coupons with the formed biofilm were rinsed and transformed to a clean 24-well plate. Otherwise, polymers in the former 24-well plate could bind to crystal violet dye and contribute strong background signals. Crystal violet staining assay was conducted to quantify the whole biomass of *P. aeruginosa* biofilm. A relative biofilm formation value was calculated by dividing the absorbance value of polymer group by control group (*i.e.*, biofilm cultivated without the presence of polymers). If the relative biofilm formation value is higher than 1, polymers can be validated to have a good biofilm inhibitory efficacy.

One-way ANOVA followed by Games Howell’s test were performed due to the unequal variances among groups. Comparing each pair of MIPs and NIPs, no significant difference could be identified on *P. aeruginosa* biofilm formation (Table 5.3). These results were consistent with the equilibrium rebinding study in 20% acetonitrile (Figure 5.3 and 5.5), indicating that MIPs did not exhibit specific binding towards 3-oxo-C_{12}-AHL in microbial growth media.

63
Table 5.3 $P$-values between each pair of molecularly imprinted polymers (MIPs) and the corresponding non-imprinted polymers (NIPs) determined using crystal violet assay.

<table>
<thead>
<tr>
<th>Ratio of T: FM: X</th>
<th>$P$-value $^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IA-based polymers $^2$</td>
</tr>
<tr>
<td>1:6:25</td>
<td>1.00</td>
</tr>
<tr>
<td>1:8:25</td>
<td>1.00</td>
</tr>
<tr>
<td>1:6:48</td>
<td>1.00</td>
</tr>
<tr>
<td>1:8:48</td>
<td>1.00</td>
</tr>
<tr>
<td>1:12:148</td>
<td>1.00</td>
</tr>
</tbody>
</table>


$^2$ IA-based polymers: polymers synthesized with itaconic acid (IA) as the functional monomer.

$^3$ HEMA-based polymers: polymers synthesized with 2-hydroxyethyl methacrylate (HEMA) as the functional monomer.

$^4$ One-way ANOVA followed by Games Howell’s test was applied for statistical significant analysis ($\alpha = 0.05$, n=3).

The anti-biofilm performance was dependent upon functional monomers (Figure 5.8). Surprisingly, IA-based polymers did not show any significant reduction ($P > 0.05$) in the formation of *P. aeruginosa* biofilm. However, 1:6:48 and 1:8:48 HEMA-based polymers significantly suppressed biofilm ($P < 0.05$), with the reduction of biofilm ranging from 43.79% to 62.48%. Thus, HEMA-based polymers had a higher adsorption capacity towards 3-oxo-C$_{12}$-AHL than IA-based polymers in microbial growth media. This result was generally consistent
with the rebinding equilibrium study (Figure 5.5 and 5.6), where 1:6:48 and 1:8:48 HEMA-based polymers showed significant higher adsorption capacity \( (P < 0.05) \) than others.

![Figure 5.8 Relative biofilm formation of P. aeruginosa PAO1 with the presence of polymers. Crystal violet assay was employed for the quantification of biofilm biomass. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Games Howell's test was performed to determine statistical significance between pairs of treatments (asteroid indicate significant difference of polymers against control, \( P < 0.05 \)). IA: itaconic acid; HEMA: 2-hydroxyethyl methacrylate; M: molecularly imprinted polymers; N: non-imprinted polymers.]

The inefficient biofilm inhibition by IA-based polymers and some HEMA-based polymers might be due to the insufficient adsorption towards 3-oxo-C\(_{12}\)-AHL. Although MIPs have been widely developed as the selective absorbents for the treatment of a complex matrix (e.g., a food), sample pretreatment is still required to remove the interferences, such as proteins, ions, and fats (Gao et al., 2014; Ge, Wu, Wang, Liang, & Sun, 2015). Therefore, adsorption capacity towards the target compounds may be compromised by a complex matrix. Insufficient adsorption in the current
study might be caused by the interferences from the complex media and bacterial metabolites. In addition, all MIPs showed no adsorption affinity towards 3-oxo-C12-AHL, indicating that more non-specific binding of interference molecules likely take place. However, other factors should also account for low biofilm inhibitory efficacy because over half of the 3-oxo-C12-AHL could be captured from the aqueous condition in the equilibrium rebinding study (section 5.1). Inconsistently, 1:8:25 HEMA-NIPs significantly \((P < 0.05)\) inhibited \(P. \ aeruginosa\) biofilm formation, but its adsorption capacity was even lower than that of 1:8:25 IA-based polymers in 20% acetonitrile (Figure 5.6, panel B). Therefore, other factors may impact biofilm formation as well.

We observed that some polymers settled onto the surface of polystyrene coupon when the polystyrene coupon was immersed into the cell culture. The presence of polymers may change the roughness of the polystyrene surface (Sikiti, Msagati, Mamba, & Mishra, 2014), which is a critical factor for biofilm development (Myszka & Czaczyk, 2011). Jones and coauthors validated the positive correlation between surface roughness and cell attachment and biofilm formation (Jones, Adams, Zhdan, & Chamberlain, 1999). More microorganisms can colonize onto the rougher surfaces due to greater surface areas and more favorable sites \((i.e.,\) depressions) (Myszka & Czaczyk, 2011). Therefore, the increase in cell attachment and decrease in adsorption capacity towards 3-oxo-C12-AHL may lead to an inefficient biofilm inhibition in the current study. This assumption can also explain the contrary results compared with the previous studies (Cavaleiro et al., 2015; Piletska et al., 2011). Cavaleiro and colleagues generated water-soluble linear polymers by copolymerization of IA with methyl methacrylate (MMA), which has only one vinyl group. This IA-MAA copolymer \((0.1 \text{ mg/mL})\) caused a \(\sim31\%\) reduction of the
formation of Aeromonas hydrophila biofilms. Since the copolymers were solubilized in media, the roughness of the attached surface was not enhanced (Cavaleiro et al., 2015). In another study, Piletska and colleagues inhibited the growth of P. aeruginosa biofilm on a glass slide with the presence of IA-based polymers (1:12:148), which was placed vertically into the cell culture (Piletska et al., 2011). In this case, surface roughness of the glass slide may not be changed as well. Actually, preliminary test in this thesis project was conducted by using this method, but only a small amount of biofilm was formed in the air/liquid/solid three-phases without statistical significance.

In addition, the smaller the particle size, the easier these particles tend to be refloated and thus may increase the roughness of the substrate surface. Polymers generated by bulk polymerization obtain a dispersed size distribution due to grinding (Balamurugan, Gokulakrishnan, & Prakasam, 2012; Yan & Row, 2006), which may be the reason that different polymers showed different trends in biofilm formation and were not fully consistent to the results of equilibrium rebinding study.

5.2.1.2 TTC assay

It is necessary to assess the effect of polymers on P. aeruginosa sessile cells, which are one of the major compositions of biofilms (Donlan & Costerton, 2002; Mangwani et al., 2016). Three factors may affect cell growth with the presence of polymers. First, polymers might inhibit bacterial growth (Cavaleiro et al., 2015). Second, capturing 3-oxo-C12-AHL by polymers may lead to the decrease in cell growth (Flickinger et al., 2011). Third, we assume that the initial
attachment of bacterial cells may be enhanced (section 5.2.1.1). In the current study, biofilm formation (i.e., sessile cells) of *P. aeruginosa* was evaluated by using TTC assay.

TTC is one of the commonly used tetrazolium salts to evaluate the metabolic activity of viable cells (Moussa, Tayel, Al-Hassan, & Farouk, 2013; Peeters, Nelis, & Coenye, 2008). The principle of TTC assay is described as follows. Water-soluble, colorless TTC is enzymatically reduced to water-insoluble, red 1,3,5-triphenylformazan (TPF) by succinate dehydrogenase (Figure 5.9). The absorbance of TPF in organic solvent (e.g., acetone and ethanol) is proportional to the amount of live cells (Peeters et al., 2008). TTC assay offers more information on cell viability, while crystal violet assay stains all negatively charged components without specificity (e.g., bacterial surface molecules and polysaccharides in EPS).

![Figure 5.9 Principle of TTC assay: 2,3,5-triphenyltetrazolium chloride (TTC) is enzymatically reduced to 1,3,5-triphenylformazan (TPF) by viable bacterial cells.](image)

*P. aeruginosa* biofilm was cultivated on polystyrene coupons with or without the presence of polymers. After 24-h incubation, biofilm was harvested and rinsed by PBS to remove unattached cells. LB broth supplemented with 0.1% TTC was prepared for TTC assay (Sabaeifard et al.,
Biofilm was incubated with 0.1% TTC working solution for 6 h. The absorbance value of TPF produced by the biofilm was obtained using a microtiter plate reader at 490 nm.

A previous study reported that TTC in high concentration may be toxic to bacterial cells (Weinberg, 1953). Therefore, toxicity of 0.1% TTC on P. aeruginosa was first evaluated before applying the TTC assay on biofilm/polymer samples. Overnight culture of P. aeruginosa was adjusted to 10⁶ CFU/mL. Then, the inoculum was incubated with or without 0.1% TTC solution at 37°C for 6 h. Cell counts were quantified by conventional plate counting assay (Cady et al., 2012). No toxicity was observed after incubation for 6 h (Table 5.4).

Table 5.4 Toxicity test of 0.1% TTC solution on P. aeruginosa planktonic cells after 6 h incubation (n=3).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cell count (CFU/mL)¹</th>
<th>0.1% TTC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>(1.41 ± 0.64) × 10⁶A</td>
<td>(1.48 ± 0.14) × 10⁶A</td>
</tr>
<tr>
<td>6</td>
<td>(1.67 ± 0.14) × 10⁸a</td>
<td>(1.22 ± 0.78) × 10⁸a</td>
</tr>
</tbody>
</table>

¹ Data are shown in terms of mean ± standard deviation. Statistical significance between control and 0.1% TTC group was determined by Student’s t-test (two-tailed, α = 0.05). The same upper case or lower case letter indicates no significant difference between control and 0.1% TTC group (P > 0.05).

Table 5.5 demonstrates the P-values of cell viability between each pair of polymers by TTC assay. Comparing MIPs with the corresponding NIPs, no significant difference (P > 0.05) in cell
viability could be observed, which was consistent to the result by crystal violet assay (Table 5.3). Therefore, MIPs had no specific binding towards 3-oxo-C_{12}-AHL in the aqueous media.

Table 5.5 $P$-values between each pair of molecularly imprinted polymers (MIPs) and the corresponding non-imprinted polymers (NIPs) using TTC assay.

<table>
<thead>
<tr>
<th>Ratio of T: FM: X</th>
<th>IA-based polymers$^2$</th>
<th>HEMA-based polymers$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:6:25</td>
<td>0.91</td>
<td>1.00</td>
</tr>
<tr>
<td>1:8:25</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1:6:48</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1:8:48</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1:12:148</td>
<td>1.00</td>
<td>-</td>
</tr>
</tbody>
</table>


2 IA-based polymers: polymers synthesized with itaconic acid (IA) as the functional monomer.

3 HEMA-based polymers: polymers synthesized with 2-hydroxyethyl methacrylate (HEMA) as the functional monomer.

4 One-way ANOVA followed by Games Howell’s test was applied for statistical significant analysis ($\alpha = 0.05$, n=3).

Compared to the control group, IA-based polymers either promoted or had no influence on cell viability (Figure 5.10). The random promotion effect indirectly confirmed our assumption that bacterial attachment might be increased due to the increase in substrate roughness (section 5.2.1.1). Since bulk polymers have large particle size distribution (Yan & Row, 2006), various
amounts of polymers might refloat and settle onto the substrate, resulting in a variable impact on the surface roughness. Consequently, different promotion effects may occur.

HEMA-based polymers with the ratios of 1:6:48 and 1:8:48 significantly ($P < 0.05$) inhibit $P. aeruginosa$ cell viability (Figure 5.10). In addition, the inhibitory effect of polymers on cell viability was slightly lower than its effect on biofilm biomass. In brief, 37.93%-47.04% of viable cells were reduced (Figure 5.10), while 43.78%-62.48% of biofilm biomass production was prevented (Figure 5.8). The higher reduction on biomass might be due to the reduction in EPS (Lade, Paul, & Kweon, 2014; Packiavathy, Priya, Pandian, & Ravi, 2014).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5_10.png}
\caption{Relative cell viability of $P. aeruginosa$ PAO1 with the presence of polymers. TTC assay was employed to quantify the live sessile cells. Error bars indicate standard deviation (n=3). One-way ANOVA followed by Games Howell’s test was performed to determine statistical significance of polymers against control (* indicates $P < 0.05$). IA: itaconic acid; HEMA: 2-hydroxyethyl methacrylate; M: molecularly imprinted polymers; N: non-imprinted polymers.}
\end{figure}
As aforementioned, two possible mechanisms may contribute to the reduction of cell viability with the presence of polymers: 1) the reduction of extracellular 3-oxo-C\textsubscript{12}-AHL (Flickinger et al., 2011) and 2) cell inactivation effect by polymers (Cavaleiro et al., 2015). Regarding the first mechanism, Flickinger and coauthors validated the relationship between the amount of 3-oxo-C\textsubscript{12}-AHL and \textit{P. aeruginosa} biofilm formation (Flickinger et al., 2011). In that study, wild type \textit{P. aeruginosa} PAO1 biofilm was incubated in the center of 81-well poly(ethylene glycol) diacrylate (PEGDA) hydrogel plate. Since 3-oxo-C\textsubscript{12}-AHL is highly diffusible through PEGDA, chambers in different distances to the center could contain different amounts of AHL. To determine the amount of 3-oxo-C\textsubscript{12}-AHL, AHL reporter strain (\textit{i.e.}, \textit{P. aeruginosa} PAO-MW1 pUM15), which does not synthesize 3-oxo-C\textsubscript{12}-AHL but can produce yellow fluorescence protein (YFP) in the presence of exogenous 3-oxo-C\textsubscript{12}-AHL, was cultivated in different chambers around the center. To determine the cell density with the presence of different amounts of 3-oxo-C\textsubscript{12}-AHL, \textit{P. aeruginosa} PAO-MW1 p67T1 was employed, which cannot produce 3-oxo-C\textsubscript{12}-AHL but can express red fluorescence protein (RFP). The correlation between the amount of AHL (proportional to YFP fluorescence) and the growth of sessile cells (proportional to RFP fluorescence) was established. Generally, 3-oxo-C\textsubscript{12}-AHL enhanced the cell density of sessile cells, and the increase was dependent upon the amount of AHL. The growth of sessile cells was significantly enhanced (\(P < 0.01\)) when the concentration of 3-oxo-C\textsubscript{12}-AHL increased from 0.1 \(\mu\text{M}\) to 1 \(\mu\text{M}\). However, no significant difference (\(P > 0.05\)) was observed when the concentration of 3-oxo-C\textsubscript{12}-AHL was between 0.4 \(\mu\text{M}\) and 1 \(\mu\text{M}\). This observation shed a light on the complex mechanism of biofilm inhibition by polymers in the current study. Even though all polymers could capture 3-oxo-C\textsubscript{12}-AHL from the environment (section 5.1), different extent of capturing
leads to different impacts on the proliferation of sessile cells (Figure 5.10). Only when the concentration of 3-oxo-C12-AHL was lower than a certain level, the inhibitory effect against biofilms could be eventually observed (i.e., 1:6:48 HEMA- and 1:8:48 HEMA-based polymers). Even though there was a decrease in cell viability, the effect might also be compensated by the increase in bacterial attachment (e.g., IA-based polymers).

Regarding the second mechanism, there were controversial conclusions from the previous studies. In one study, the effect of linear polymers on *V. fischeri* and *Aeromonas hydrophila* was investigated (Cavaleiro et al., 2015). IA-MMA copolymer significantly inhibited the growth of *A. hydrophila* at 25 h. This polymer also inhibited the growth of *V. fischeri* between 12-30 h. However, MAA-MMA copolymer increased the growth of *A. hydrophila* at 7 h, but it inhibited the growth of *A. hydrophila* at 9 h. No explanation was offered in that study. In another study, Piletska and colleagues validated that IA-based polymers had no antimicrobial effect against *P. aeruginosa* (Piletska et al., 2011). Since no agreement was reached in the previous studies, it is necessary to evaluate the antimicrobial activity of polymers in the current study.

### 5.2.2 Antimicrobial activity

As mentioned in section 5.2.1.2, polymers may cause reduction in the number of bacterial cells. Hence, the antimicrobial activity of polymers was evaluated. Since MIPs and their corresponding NIPs showed equal inhibitory effect against *P. aeruginosa* biofilm formation (i.e., crystal violet assay and TTC assay), only NIPs were selected as the representatives for testing. Furthermore, 1:8:25 and 1:6:48 IA-based and HEMA-based NIPs were selected as the representative polymers due to their different anti-biofilm performances.
Briefly, *P. aeruginosa* planktonic cell (initial concentration: $10^6$ CFU/mL) were incubated with 0 mg or 60 mg of polymers in a shaking incubator for 24 h (37°C, 150 rpm). The growth of *P. aeruginosa* was evaluated by determining cell counts. To quantify the cell number, conventional plate counting assay was applied instead of the measurement of absorbance at OD$_{600nm}$ (Cady et al., 2012; Chen et al., 2011; Liong, France, Bradley, & Zink, 2009). Although the latter method is simpler and faster, the presence of insoluble polymers could interfere with the results.

The antimicrobial effects of the representative NIPs on the viability of *P. aeruginosa* planktonic cells are shown in Figure 5.11. After 24-h incubation, 1:8:25 IA-, 1:6:48 IA-, and 1:8:25 HEMA-based NIPs did not inhibit bacterial growth compared to the control group. However, 1:6:48 HEMA-based polymers showed significant reduction in cell viability ($P < 0.05$).

![Figure 5.11](image-url)
indicated statistical significance between polymer group and control group \((P < 0.05)\). Error bar indicated the standard deviation. IA: itaconic acid; HEMA: 2-hydroxyethyl methacrylate.

In fact, HEMA-based polymers \((e.g.,\) HEMA-hydrogel\) have been widely used in antimicrobial and anti-biofilm studies. However, all of these studies employed HEMA-based polymers as an antimicrobial agent carrier, which can release the antimicrobial agents and thus inactive bacteria \((\text{Jones, McCoy, Andrews, McCrory, } \& \text{ Gorman, 2015; Norris et al., 2005; Rad, Khameneh, Sabeti, Mohajeri, } \& \text{ Bazzaz, 2016})\). No study has yet been conducted to test the antimicrobial or anti-biofilm effect of HEMA-based polymers solely. Therefore, we could not compare our results to any previous works.

5.2.3 Summary

In conclusion, MIPs and the corresponding NIPs had no significant difference \((P > 0.05)\) in anti-biofilm effect, which was consistent to the equilibrium rebinding study. The anti-biofilm effect was related to the nature of functional monomers. HEMA-based polymers showed a higher anti-biofilm effect against \(P. \text{ aeruginosa}\) compared to IA-based polymers. Biofilm formation was significantly \((P < 0.05)\) inhibited by 1:6:48 and 1:8:48 HEMA-based MIPs and NIPs, as well as 1:8:25 HEMA-based NIPs after incubation for 24 h. The reduction could reach to as high as 62.48\%. Nevertheless, other polymers failed to reduce biofilm development. The inefficient anti-biofilm effect might be due to the increase in bacterial attachment and decrease in adsorption capacity towards 3-oxo-C\(_{12}\)-AHL.
The effect of polymers against *P. aeruginosa* sessile cells was further evaluated. IA-based polymers randomly increased the cell viability within *P. aeruginosa* biofilm. We assume that water-insoluble polymers might increase the surface roughness of substrate and thus accelerate the bacterial attachment. Since the same polymers showed no significant inhibitory effect on biofilm formation, there may be a complemented effect between the decrease in biofilm biomass and the increase in bacterial attachment. However, 1:6:48 and 1:8:48 HEMA-based polymers significantly reduced the cell viability (*P* < 0.05). The decrease in cell growth may be caused by the sufficient capture of 3-oxo-C\(_{12}\)-AHL (Flickinger et al., 2011). The reduction of cell viability was relatively lower than the reduction of biofilm biomass, indicating that other biofilm components (e.g., EPS) may also be reduced (Lade et al., 2014).

To investigate whether the anti-biofilm effect was also related to the antimicrobial effect of polymers, four representative NIPs were selected and incubated with *P. aeruginosa* cell culture. The results showed that 1:6:48 HEMA-based NIPs had potential antimicrobial effect, while other NIPs did not affect the growth of planktonic cells.
Chapter 6: Conclusion

6.1 Study outcomes

In this thesis project, MIPs towards 3-oxo-C\textsubscript{12}-AHL, a representative bacterial quorum sensing signaling molecule, has been synthesized by considering initiation condition, nature of functional monomers, and ratio of template: functional monomer: crosslinker. Photo-initiation (365 nm, 4°C) is an appropriate initiation condition to prevent template molecule (i.e., 3-oxo-C\textsubscript{12}-AHL) from degradation (< 5%). IA-based polymers were synthesized with different formulation ratios (i.e., 1:6:25, 1:8:25, 1:6:48, 1:8:48 and 1:12:148). Using IA-based polymers in 20% acetonitrile aqueous solution, over 50% of 3-oxo-C\textsubscript{12}-AHL was efficiently captured. However, MIPs showed no significant difference ($P > 0.05$) in the adsorption capacity compared to the corresponding NIPs because the non-specific interaction (i.e., hydrophobic interaction) dominated the rebinding.

When the water proportion was decreased to 50%, some MIPs (i.e., 1:6:25, 1:8:25, 1:6:48 and 1:8:48) showed potential adsorption affinity with imprinting factor >1. To increase the hydrophilicity of MIPs, HEMA was used as the alternative functional monomer. The adsorption capacity of HEMA-based polymers was increased up to 62% in 20% acetonitrile. Nevertheless, HEMA-based MIPs still did not show any adsorption affinity in both 20% and 50% acetonitrile. Further studies need to be conducted to optimize the adsorption affinity of MIPs.

Consistent to the equilibrium rebinding study, each MIPs showed the similar performance against the development of \textit{P. aeruginosa} biofilm to the corresponding NIPs. Compared to IA-based polymers, HEMA-based polymers showed a higher inhibitory effect against \textit{P. aeruginosa} biofilm formation. The 1:6:48 and 1:8:48 HEMA-polymers significantly ($P < 0.05$) inhibited the
biofilm formation after 24-h incubation. In contrast, all IA-based polymers showed no impact on biofilm development. In addition, IA-based polymers randomly increased the cell viability, but HEMA-based polymers (i.e., 1:6:48 and 1:8:48) significantly reduced the cell viability ($P < 0.05$). Besides, one representative HEMA-based polymer (i.e., 1:6:48) showed antimicrobial effect against *P. aeruginosa* planktonic cells. Taken together, the effect of polymers on the *P. aeruginosa* biofilm formation might be determined by a complex mechanism, including the increase of bacterial attachment, the capturing of 3-oxo-C$_{12}$-AHL from the environment, and/or antimicrobial effect.

### 6.2 Future directions

In the current study, HEMA-based MIPs (i.e., 1:6:48 and 1:8:48) showed high adsorption capacity towards 3-oxo-C$_{12}$-AHL, as well as good inhibitory effect against *P. aeruginosa* biofilm. However, the adsorption affinity of these MIPs was relatively low and needs to be further optimized. To optimize the adsorption performance of HEMA-based MIPs, the amounts of HEMA and EGDMA are two major factors that can be adjusted. Besides, different polymerization methods can be conducted. In the current study, MIPs was synthesized by bulk polymerization method, which might result in irregular sizes and shapes and also destroyed the binding sites due to grinding process. In contrast, precipitation polymerization can generate spherical particles in a relatively homogenous size and high surface area. According to some previous studies, precipitation polymerization method showed a better performance of adsorption affinity and selectivity compared to bulk polymerization method (Ho, Yeh, Tung, & Liao, 2005; Mohajeri, Karimi, Aghamohammadian, & Khansari, 2011). Thus, precipitation polymerization method can be used as an alternative polymerization method in the future study.
Due to time constraint, it was challenging to conduct more equilibrium rebinding studies in other adsorption media (e.g., water waste from the dairy industry, where *P. aeruginosa* is commonly identified) or with 3-oxo-C_{12}-AHL structural analogues (e.g., C_4-AHL). It would be beneficial to conduct such studies and collect more information about the adsorption performance of MIPs with the presence of inference compounds in the real environment (e.g., food processing lines).

In addition, the capturing mechanism of MIPs towards 3-oxo-C_{12}-AHL is rarely uncovered. Future studies can be continuously conducted by investigating the functional groups and types of bonds in MIPs. For example, Fourier transform infrared spectroscopy can be applied to determine the interaction mechanism by comparing the spectra that are collected from the template, functional monomer, MIPs alone, and template-carrying MIPs (Yusof, Rahman, Hussein, & Ibrahim, 2013). Computational modeling is also an available method to simulate the interaction between functional monomer and template molecule (Liu et al., 2014; Yanez, Chianella, Piletsky, Concheiro, & Alvarez-Lorenzo, 2010).

Considering the real application in agri-food systems, MIPs can be further synthesized into different formats. The most promising application is to coat MIPs onto the food processing facilities, where biofilms are commonly discovered. For example, MIPs can be synthesized with the presence of poly(vinyl chloride) (PVC, the composition of conveyor belts) to generate a functionalized MIP-coating film (Gao, Grant, & Lu, 2015; Jones et al., 2015).
In terms of biofilm inhibitory studies, three more research directions could be approached. Firstly, more works could be conducted to characterize the profiles of biofilms with the treatment of MIPs. For example, it is worthwhile of testing the effect of MIPs on biofilm structure and the production of polysaccharides because both of them are mediated by 3-oxo-C_{12}-AHL-based QS system in *P. aeruginosa* (section 3.2.3). Secondly, the effect of MIPs on the development of multispecies biofilms is of significant importance to be evaluated. Most natural biofilms are formed by multiple species of microorganisms and have a higher antimicrobial resistance than the monospecies biofilms (section 3.2.4). Besides, *P. aeruginosa* has been reported to promote the biofilm formation of other bacteria, such as *E. coli* and *C. jejuni* (Culotti & Packman, 2014; Ica et al., 2012). In the current study, some MIPs (*i.e.*, HEMA-based MIPs, 1:6:48 and 1:8:48) showed significant inhibitory effect against the formation of *P. aeruginosa* monospecies biofilm. Therefore, it is practical to investigate the effect of such MIPs on the formation of multispecies biofilm including *P. aeruginosa*. Thirdly, the effect of MIPs on *P. aeruginosa* biofilm can also be evaluated in a flow condition, which is the most common condition for biofilm formation in the food industry, such as in the pipelines. To achieve this goal, MIPs can be incorporated into a microfluidic “lab-on-a-chip” system, where bacterial biofilms can be cultivated in a micron-scale chamber and under a defined rate of fluid flow (Benoit, Conant, Ionescu-Zanetti, Schwartz, & Matin, 2010).
Bibliography


Gadzala-Kopciuch, R., Ricanyova, J., & Buszewski, B. (2009). Isolation and detection of...


Piletska, E. V., Stavroulakis, G., Karim, K., Whitcombe, M. J., Chianella, I., Sharma, A.,


Xavier, K. B., & Bassler, B. L. (2003). LuxS quorum sensing: more than just a numbers game.


Pseudomonas aeruginosa. Infection and Immunity, 70(10), 5635–5646.


