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Attenuation of quorum sensing using computationally designed polymers

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Abstract

It is generally accepted that the majority of Gram-negative and Gram-positive bacteria communicate via production and sensing of small signal molecules, autoinducers. The ability of bacteria to sense their population density is termed quorum sensing (QS). Quorum sensing controls certain phenotypic traits, particularly virulence factors and biofilm formation. In this project a new solution for the attenuation of quorum sensing which involves selective sequestering of the signal molecules using rationally designed synthetic polymers was explored.

In order to prove the concept, the Gram-negative, marine bacterium *Vibrio fischeri* was used. The bioluminescence of *V. fischeri* is controlled by QS (signal molecule- *N*-(β-ketocapryloyl)-L-homoserine lactone, 3-oxo-C_6-AHL) and is considered a sensitive marker for quorum sensing bacterial communication. Six polymers with affinity towards 3-oxo-C_6-AHL were computationally designed and tested. The best polymer, which was based on 5% of itaconic acid, demonstrated the highest capacity towards 3-oxo-C_6-AHL (45.5±1.5 mg per g of polymer) and was shown to inhibit completely the bioluminescence of *V. fischeri* for almost 5 h. This polymer could be considered as a first synthetic polymer which was able to stop the communication between the bacteria and inhibit the development of the QS-controlled traits.

In order to apply the knowledge gained to the practical problems of the modern health care, the specific polymers were designed for the signal molecules which are used by more problematic microorganisms that also possess QS-regulated virulence determinants, such as *Pseudomonas aeruginosa* (signal molecules- C_4-AHL, 3-oxo-C_{12}-AHL) and *Burkholderia cepacia* (signal molecules- C_6-AHL and C_8-AHL). Several signal molecules- sequestering polymers were prepared for each autoinducer. The physical properties of the polymers were

characterised and their binding capacity towards corresponding targets was measured and considered suitable for the practical application.

The molecularly imprinted polymer (MIP) for a key signal molecule of *P. aeruginosa* (3-oxo-C_{12}-AHL) was produced and exhibited higher affinity for the template (K_d=145±2 μM) as compared to the corresponding Blank polymer (K_d=312±3 μM). The *in vitro* testing showed that the MIP almost completely stopped the biofilm formation by *P. aeruginosa* (80% inhibition in comparison with control samples).

In order to develop different formats of signal molecule-sequestering polymers, the modification of commercial hydrophilic polypropylene microfiltration membranes with polymers was conducted. It was found that a MIP- modified membrane specific for 3-oxo-C_{12}-AHL demonstrated higher binding of the template in comparison with reference polymeric membrane (14.9±0.1 versus 5.8±0.1 mg AHL per g of grafted polymer).

Custom-made signal molecule-sequestering polymers could be considered as a new tool for the control of life-threatening infections in a wide variety of applications (e.g. catheter-associated infections) whilst minimising the risk of inducing bacterial resistance.

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ABBREVIATIONS

AA	acrylamide
ACN:	acetonitrile
ACP:	acyl carrier protein
agr:	accessory gene regulator
AHL:	*N*-acyl-homoserine lactone
AiiA:	AHL-inactivating enzyme
AiiD:	AHL-inactivating enzyme
AIP:	autoinducing peptide
AMPSA:	2-acrylamido-2-methyl-propanesulfonic acid
ATCC:	American Type Culture Collection
ATR:	attenuated total reflection
BP:	benzophenone
C:	Celcius
CBMA:	carboxybetaine methacrylate
CDC:	Center for Disease Control and Prevention
CF:	cystic fibrosis
CFU:	colony forming units
CV:	Chromobacterium violaceum
D:	dimension
DDT:	dichloro-diphenyl-trichloroethane
DEAEM:	*N*,*N*-diethylaminoethyl methacrylate
DEEDA:	*N*,*N*-diethylethylenediamine
DG:	degree of grafting

DMAEM: *N*,*N*-dimethylaminoethyl methacrylate

DMAP: 4-dimethylaminopyridine

DMF: *N*,*N*-dimethyloformamide

DNA: deoxyribonucleic acid

DTC: diethyldithiocarbamate

EGDMA: ethylene glycol dimethacrylate

EGMP: ethylene glycol methacrylate phosphate

ELISA: enzyme-linked immunosorbent assay

EPS: extracellular polymeric substances

EtOH: ethanol

FT-IR Fourier Transfer-Infra Red

g: gram

GFP: green fluorescent protein

GST: glutathione-S-transferase

H: hydrogen

h: hour

HEM: hydroxyethyl methacrylate

HPLC-MS: high performance liquid chromatography with mass-spectrometry detection

IA: itaconic acid

IPN: interpenetrating polymer network

K: Kelvin

kcal: kilocalories, energy unit

K_d: dissociation constant

kg: kilogram

L: litre

LB: Luria-Bertani broth

LSCM: Laser Scanning Confocal Microscopy

LuxI: synthase protein of AHL

LuxR: receptor protein of AHL

M: molarity

m: mass

MAA: methacrylic acid

mAb: monoclonal antibody

MBAA: *N*,*N*'-methylene bisacrylamide

mg: milligram

MIC: minimum inhibitory concentration

MIM: molecularly imprinted membrane

min: minute

MIP: molecularly imprinted polymer

mL: millilitre

MM: molecular mechanics

mM: millimolar

mol: mole, mass unit

MPC: 2-methacryloyloxyethyl phosphorylcholine

MRSA: meticillin-resistant *Staphylococcus aureus*

MS: mass spectrometry

m/z: mass-per-charge value

NaCl: sodium chloride

NB: nutrient broth

ng: nanogram

NIP: non-imprinted polymer, blank polymer

nM: nanomolar

nm: nanometre

4-NPO: 4-nitropyridine-N-oxide

OD: optical density

OPA: o-phthalic dialdehyde

OTA: ochratoxin A

P: polymer

PBS: phosphate buffered saline

PE: polyethylene

PEG: poly (ethylene glycol)

PEO: poly (ethylene oxide)

PET: poly (ethylene terephtalate)

pH: acidity measurement unit

PI: phase inversion

PMN: polymorphonuclear leukocytes

PP: polypropylene

PTFE: poly (tetrafluoro ethylene)

PUR: polyurethane

QS: quorum sensing

QSI: quorum sensing inhibitor

RAP: RNAIII-activating polypeptide

Rh: rhodamine

RIP: RNAIII-inhibiting peptide

RNA: ribonucleic acid

rpm:	rounds per minute
SA:	salicylic acid
SAM:	S-adenosyl methionine
SDS:	sodium dodecyl sulphate
SE:	standard error
SEM:	scanning electron microscopy
SPE:	solid phase extraction
SSP:	signal-sequestering polymer
TEPA:	tetraethylenepentamine
TFMAA:	2-(trifluoromethyl)acrylic acid
TRAP:	target protein of RAP
TRIM:	trimethylopropane trimethacrylate
TSST-1:	toxic shock syndrome toxin-1
UAEE:	urocanic acid ethyl ester
UV:	ultraviolet
V:	Volt
WGA:	Wheat germ agglutinin
μg:	microgram
μM:	micromolar
μL:	microlitre

CHAPTER 1

INTRODUCTION – AIMS AND OBJECTIVES

The current project is a continuation of the feasibility study, which was conducted by our group in collaboration with Dr Gary Robinson (University of Kent). It was aimed to find the answer if rationally-designed polymers could affect or control the communication between cells of the Gram-negative, marine bacterium, *Vibrio fischeri*. The aim of the project was to continue this study and to design synthetic polymers which are able to specifically sequester the signal molecules, thus attenuating QS-controlled phenotypes, in particular bioluminescence (*V. fischeri*) and biofilm formation (*Pseudomonas aeruginosa* and *Burkholderia cepacia*). Considering the importance of the new ideas which were aimed at attenuation of the bacterial infections, the project was sponsored by Pump-priming fund (Professor Anant Sharma, Bedford Hospital, NHS Trust) and by the British Council in Germany which supported the collaboration between Cranfield University (Prof. S. Piletsky) and University of Essen (Prof. Mathias Ulbricht) on the development of membranes which are resistant to biofouling. 3-oxo-C_6-AHL (signal molecule used by *V. fischeri*), C_4-AHL and 3-oxo-C_{12}-AHL (used by *P. aeruginosa*), C_6-AHL and C_8-AHL (used by *B. cepacia*) were selected as the target molecules for the polymer synthesis. Computer modelling was used in the present work for the design of a synthetic receptor for AHLs. The selected polymers were characterised in terms of their affinity, selectivity and capacity towards the corresponding signal molecules using solid phase extraction (SPE) and high performance liquid chromatography equipped with MS detector (HPLC-MS). Subsequently, the leads selected on the basis of their superior selectivity and binding properties were tested in the biological system where their role in the attenuation of QS of corresponding bacteria was estimated.

Finally, in the frame of the collaboration project with University of Essen sponsored by the British Council in Germany a method for photo-grafting on

hydrophilic polypropylene (PP) membranes was developed and optimised in order to combine the advantages of molecular imprinting and membrane technology. Membranes were modified with computationally designed polymers and tested for the adsorption of the corresponding signal molecules and for inhibition of biofilm formation.

The objectives of this study were to develop specific signal-molecule-sequestering polymers which could attenuate the QS of *V. fischeri*, and opportunistic pathogens *P. aeruginosa* and *B. cepacia.*

It was envisaged that produced materials could find their application as either adsorbents in the wound dressings or by being integrated as a polymer layer on the surface of medical tools for prevention of biofilm formation (e.g. catheter sleeves, contact lenses). Part of the work was dedicated to the development of polymeric membranes resistant to biofilm formation.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The discovery of the first antibiotic penicillin by Alexander Fleming in 1928 could be considered as one of the greatest accomplishments of modern medicine. Since then many more antibiotics have been discovered and introduced into clinical practice. It was found that the mechanism of antibiotic action consists of growth-inhibitory effects on a broad range of target microbial organisms. Thus, a marked decrease in the number of deaths from bacterial infections was achieved. Unfortunately, it was soon realised that the use of antibiotics led to an increased frequency in the bacterial mutation resulted in a significantly increased antibiotic resistance. More recently, many infections became increasingly difficult to treat because therapeutic options have been severely limited or even non-existent due to the prevalence of resistant bacteria. In the UK, the Public Health Laboratory Service has reported that the meticillin-resistant *Staphylococcus aureus* (MRSA), a multiple-resistant organism that causes septicemia, has risen from 2% in 1990 to greater than 40% in 2000 (Department of Health, London, 2002). Today, we are entering a post-antibiotic era with a reduced ability to combat microbes and, hence, the development of novel antimicrobials that can target bacteria without succumbing to bacterial resistance constitutes an important pharmaceutical target (Cámara *et al*., 2002).

Historically, the main strategy for the drug development has been aimed at introducing agents that prevent bacterial growth, e.g. antiseptic agents. Another therapeutic approach consisted of the attenuation of bacterial virulence. The idea behind this approach was that if the microorganism does not establish a successful infection it would be healed easily by the host immune system. Compounds with such abilities were termed anti-pathogenic drugs (i.e., halogenated furanones and fungal compounds) as opposed to antibacterial drugs (i.e., most conventional antibiotics)

(Hentzer and Givskov, 2003). Unfortunately these anti-pathogenic compounds are usually pathogenic themselves and not suitable for the medical application due to their toxicity and mutagenic properties (Rasmussen and Givskov, 2006).

The discovery of the communication systems which regulate bacterial virulence has offered a novel opportunity to control infectious bacteria without affecting their growth. In order to overcome the host defences, bacteria synthesise either cell-associated or extracellular virulence determinants, which serve as communication signals. These virulence determinants are not strictly essential for the viability of organism and exist only in bacteria while the determinants are absent in the host. Therefore they represent an attractive therapeutic target and also provide a degree of selectivity which is a pre-requisite for antimicrobial therapies (Cámara *et al*., 2002). One of the alternative antibacterial approaches was introduced by Hentzer (Hentzer *et al*., 2003). The authors envisaged that compounds that can override communication signals could be used for the control of infectious bacteria without affecting the growth and inducing the bacterial mutagenesis.

2.2 Quorum sensing (QS)

It is well-known that both Gram-negative and Gram-positive bacteria do not exist individually but coordinate community behavior through cell-to-cell communication. This cell-to-cell communication is mediated by small, diffusible signal molecules, also termed “pheromones” or “autoinducers” (Xavier and Bassler., 2003).

The term “quorum sensing” (QS) has been adopted to describe the situation when the accumulation of the signal molecules has reached a threshold, which ultimately causes a population-wide alteration in gene expression and subsequent

production of virulence factors and biofilm formation (Cámara *et al*., 2002). This phenomenon is prevalent among both symbiotic and pathogenic bacteria associated with plants and animals (Hentzer and Givskov, 2003).

2.3 The chemical "languages" of Gram-negative bacteria

In Gram-negative bacteria the most common type of signal molecules belongs to the *N*-acyl-homoserine lactone (AHL) family (Figure 2.1). The chemical structure of AHLs consists of a homoserine lactone ring attached, via an amide bond, to an acyl side chain of varying length. Up to now, naturally occurring AHLs with 4-14 carbons in length have been revealed. They could be saturated or unsaturated and with or without a hydroxyl-substituent, oxy-substituent or no substituent at the 3 position of the N-linked acyl chain (Withers *et al*., 2001).

AHLs are synthesised by the family of LuxI homologue proteins. They employ the appropriate charged acyl carrier protein (ACP) as the major acyl chain donor and S-adenosyl methionine (SAM), which provides the homoserine lactone ring (Parsek *et al*., 1999; Rasmussen and Givskov, 2006).

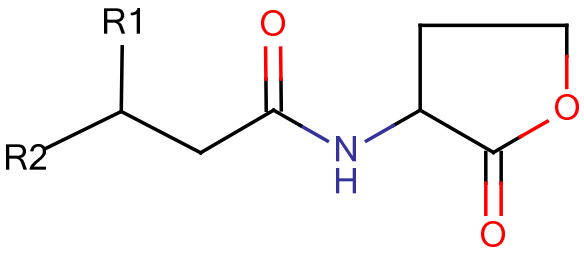


Figure 2.1: Basic structure of the AHL signal molecule where R1=H, OH, or O and R2=C_1–C_{18} (modified version of structure available in Rasmussen and Givskov, 2006).

The AHL-mediated QS system consists, briefly, of a four-component circuit: an AHL signal molecule, a LuxI-type signal synthase, a LuxR-type signal receptor and the target genes (Figure 2.2). The AHL synthase produces the AHL signal (or autoinducer) at a low basal level. The latter usually, depending upon chain length and lipophilicity, diffuses out of the bacteria and into the surrounding environment. An increase in the population of bacteria leads to an increase in extracellular AHL concentration, and, when threshold is reached, this signal binds to and activates a transcriptional regulator protein (receptor) to form a complex that is responsible for activating or repressing the target genes. Often, QS is subject to autoinduction, because the gene encoding the signal synthase is one of the target genes. Hence, a positive feedback regulatory loop is generated (Hentzer and Givskov, 2003).

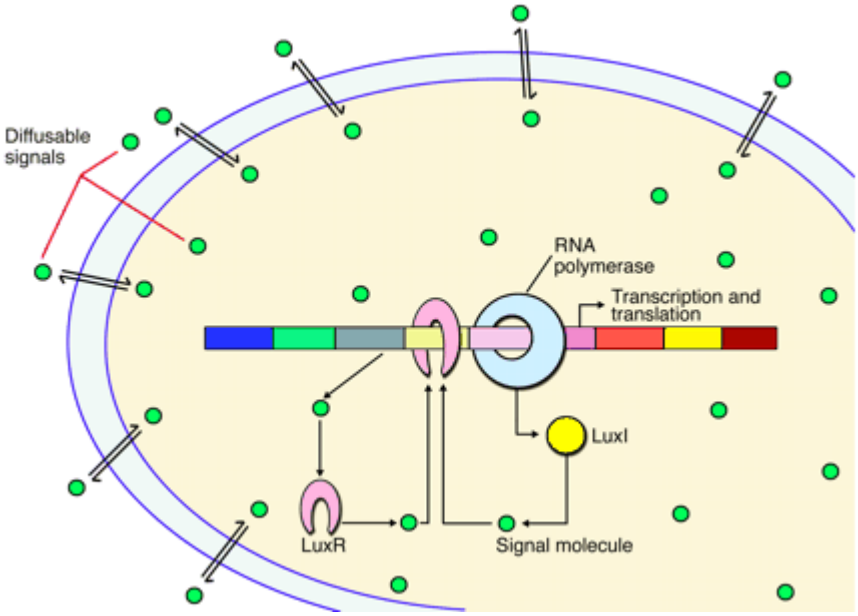


Figure 2.2: The archetypical Lux quorum sensor. The AHL signal (green circles) is synthesised by the *luxI* gene product LuxI (the synthase protein). At a certain threshold concentration, the AHL signal interacts with the receptor protein LuxR (encoded by *luxR*), which binds to the promoter sequence of the target genes (the *lux* operon) and in conjunction with the RNA polymerase promotes transcription (Hentzer and Givskov, 2003).

2.4 QS in Gram-positive bacteria: its role in virulence and its potential as a therapeutic target for novel antimicrobials

It is known that Gram-positive bacteria also have QS ability, but the nature of the signal molecules is different from those in Gram-negative bacteria. Gram-positive bacteria employ small peptides (Figure 2.3) as QS-signalling molecules instead of AHLs, and signal transduction is basically consisted of a two- component system phosphorylation cascade which eventually brings about the required change in the gene expression. Peptide-based autoinduction is used by several pathogenic bacteria to regulate a variety of cellular processes. For example, *Bacillus subtilis* and *Streptococcus pneumoniae* use peptides as signal molecules to induce bacterial competence. Furthermore, in staphylococcal species, peptide-regulated QS correlates with virulence gene expression (Novick and Muir, 1999).

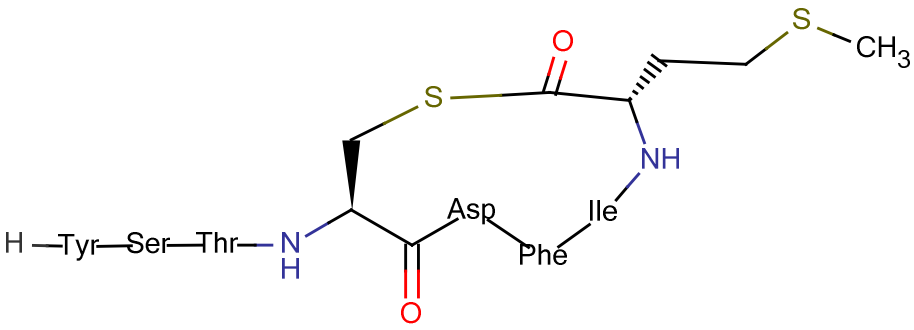


Figure 2.3: The QS peptide produced by the Gram-positive bacterium *S. aureus* (modified version of structure available in Cámara *et al*., 2002).

Staphylococcus aureus is, for instance, a major pathogen causing a variety of suppurative infections and toxinoses to human beings and animals. Due to antibiotic resistance, *S. aureus* constitutes a primary health threat, especially in hospital environments (Crossley and Archer, 1997). The less toxigenic *Staphylococcus epidermidis* and other staphylococci are associated with chronic infections,

particularly those related to indwelling medical devices (Otto, 2001). Both *S. aureus* and *S. epidermidis* secrete several surface proteins and exoproteins, the expression of which is controlled via QS through the accessory gene regulator system (*agr*) (Figure 2.4; Novick and Muir, 1999; Otto, 2001). In *S. aureus*, cell surface proteins such as adhesins, coagulase and protein A, a major surface antigen, are produced in the early stages of growth while, at a later time, their production is suppressed. Most exoproteins, including toxins, hemolysins, and tissue-degrading enzymes, are produced at the onset of the stationary phase of growth. The production of all of these virulence factors is regulated primarily by *agr* in a cell-density-dependent manner (Novick and Muir, 1999). The crucial role of the *agr* locus in virulence has been underlined in several experimental infection models (Booth *et al*., 1995; Novick and Muir, 1999; Mayville *et al*., 1999).

In staphylococci, the *agr* locus (Figure 2.4) consists of two divergent transcriptional units, which use two main operons, P2 and P3. The P2 transcript comprises four genes which are called *agrA, B, C,* and *D*. Genes *agrA* and *C* encode the signal regulator and the signal receptor respectively. The rest of the genes, *agrB* and *D*, are involved in the synthesis of the autoinducer, a small peptide (Figure 2.3) that binds to and activates the membrane protein AgrC. AgrB is also responsible for secretion of the autoinducer. The peptide interacts with the AgrAC two-component signal transduction pathway, which is subsequently activated by phosphorylation (see Figure 2.4). P3 triggers the transcription of a DNA fragment encoding an RNA species termed RNAIII, which is responsible for the regulation of the *agr* target genes (Novick and Muir, 1999).

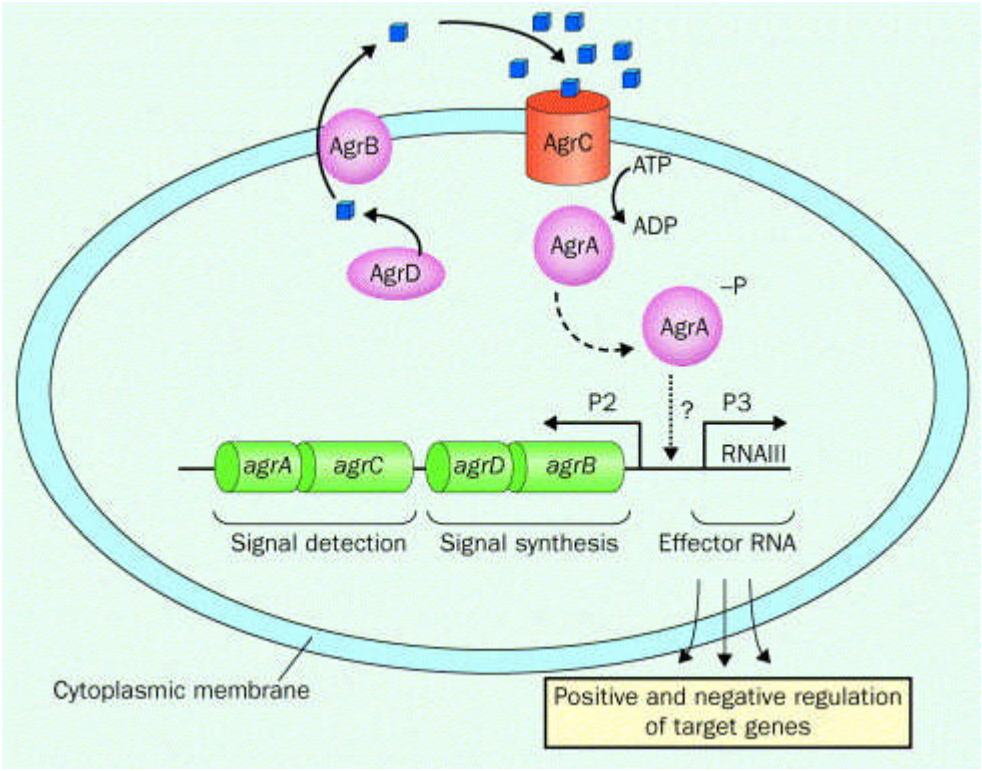


Figure 2.4: QS in *S aureus*. The QS molecule is generally a small peptide and signal transduction involves a two-component system comprising a sensor protein and a response- regulator protein. The *agr* locus consists of two divergent transcriptional units. As shown, the leftward operon is expressed from the P2 promoter and consists of *agrABCD*, which encode the proteins that are responsible for generating the peptide; the rightward operon, transcribed from the P3 promoter, encodes RNAIII. The QS peptide is derived from AgrD while AgrB is responsible for the export of the peptide out of the cell. As the bacterial population increases, the peptide accumulates in the extracellular environment. Once a critical concentration is reached, the peptide interacts with the AgrC membrane-associated sensor protein causing to the reaction of autophosphorylation. The phosphate is then transferred to the response regulator and subsequently AgrA activates expression of RNAIII, the effector molecule, which mediates the changes in expression of multiple target genes resulting in the agr response. Since the genes responsible for peptide production (*agrBD*) are also activated, this results in the production of more peptide signal and the generation of an autoinduction feedback loop (Cámara *et al*., 2002).

S. aureus strains can be divided into four different specificity groups (I-IV), a group which was defined as a number of strains that can mutually cross-activate the

agr response. In all cases, members of one group suppressed *agr* expression, and thus virulence, by members of the other groups. Other staphylococci strains generally inhibited the response in *S aureus* strains of all four groups. This finding represents a novel form of bacterial interference, since it involves the inhibition of the virulence rather than the inhibition of growth, which is the common manifestation of bacterial interference (Ji *et al*., 1997). For example, a reduction in the production of some virulence factors, such as toxic shock syndrome toxin-1 (TSST-1), enterotoxin C3, and lipase was achieved by adding a group III peptide pheromone to the culture of a group I strain (McDowell *et al*., 2001). In *in vivo* testing, co-inoculation of a group I strain with the group II peptide attenuated virulence of the group I strain dramatically in a mouse subcutaneous abscess model (Mayville *et al*., 1999).

In the case of *S. epidermidis* (Otto *et al*., 1998 and 1999) and *Staphylococcus ludgunensis* (Ji *et al*., 1997) only one peptide pheromone group has been detected so far. The *agr* autoinducing peptides (AIPs) comprise 7-9 residues and although the amino acid sequences differ substantially among the various peptide groups, each of the peptides contains a thiolactone bond between the central cysteine residue and the carboxy-terminal carboxyl (Figure 2.3) (Mayville *et al*., 1999; McDowell *et al*., 2001; Otto *et al*., 1998; Otto, 2004). Meanwhile, several synthetic pheromones and pheromone analogues have been produced (Mayville *et al*., 1999; Otto *et al*., 1998; Otto *et al*., 1999). Synthetic thiolactone-containing peptides of both *S. aureus* and *S. epidermidis* were proved to have biological, i.e. autoinducing activity. In contrast, in these two strains, lactam and lactone derivatives retained this activity (Mayville *et al*., 1999; Otto *et al*., 1999). These results suggested that the thiolactone moiety is essential for activation of the *agr* response (Mayville *et al*., 1999; Otto *et al*., 1998;

Otto, 2004). Based on these experiments, a pheromone derivative was produced by rational design which significantly inhibited the *agr* response in each one of the four *S. aureus* peptide groups but showed only weak inhibition against *S. epidermidis*. This substance was originated from the *S. aureus* group II pheromone and contained only the thiolactone moiety without the tail region of the peptide (Lyon *et al*., 2000). Moreover, while all *S. aureus* subgroups except for subgroup 4 were sensitive towards the *S. epidermidis* pheromone, only the subgroup 4 was able to inhibit the *S. epidermidis agr* response (Otto *et al*., 2001).

It can be assumed that staphylococci have acquired an innate mechanism for fighting off competitors and, also taking into consideration the information above, suggest that the peptide thiolactones constitutes an outstanding starting point for the rational design of potential anti-staphylococcal therapeutics. However, the existence of different *agr* groups is very important when considering the design of the novel inhibitors of the *agr* response because an inhibitor of one group can be a potent activator of another (Cámara *et al*., 2002). A promising global inhibitor of the virulence response in *S. aureus* may be the truncated pheromone produced by Lyon *et al*. (2000).

The fact that targeting the *agr* system is a key for inhibiting virulence in *S. aureus* has also been underlined in a report by Balaban *et al*. (1998). In this study, inhibition of the *agr* response and suppression of *S. aureus* infection in mice were achieved by an RNAIII-inhibiting peptide (RIP) and antiserum against an RNAIII-activating polypeptide (RAP). RAP is the autoinducer of another QS mechanism that has been described in *S. aureus*. When RAP reaches a threshold concentration, it elicits the phosphorylation of TRAP, its target protein, which results in upregulation of cell-surface adhesion and activation of the *agr* response, encoding AIP and AgrC.

Subsequently, AIP down-regulates cell adhesion by suppressing TRAP phosphorylation and stimulates the phosphorylation of AgrC, its cognate receptor (Balaban *et al*., 1998 and 2001). This triggers the production of the RNA molecule RNAIII, which ultimately stimulates toxin production (Novick *et al*., 1993). By suppressing TRAP phosphorylation, RIP reduces cell adhesion and at the same time abolishes *agr*-regulated toxin production. It was believed that *agr* up-regulated toxin secretion and down-regulated cell adhesion, and that any peptide which suppresses *agr* response would elicit an upsurge in adhesion. It was then speculated that TRAP plays a crucial role in both *agr* response and cell adhesion. Consequently, a peptide, such as RIP, which suppresses TRAP phosphorylation, would lead to reduced biofilm formation (Balaban *et al*., 2001 and 2003a). Giacometti *et al*. (2003) tested RIP for its ability to inhibit *S. aureus* biofilm formation in a rat Dacron graft model. Pre-treatment of grafts with a solution containing RIP resulted in a 1,000-fold reduction in the bacterial load of any strain tested. Significantly, RIP in conjunction with antibiotics, which have been commonly used for the treatment of *S. aureus* infection, was able to achieve 100% inhibition. In other *in vivo* experiments, the results for RIP-based eradication of *S. epidermidis* biofilm formation were similar to those described previously (Giacometti *et al*., 2003), considering the possibility that RIP is a global inhibitor of staphylococcal strains (Balaban *et al*., 2003b). In another study by Yang *et al*. (2003), RAP was used to screen a peptide phage library to search for RAP-binding peptides that would affect its activity and, hence, suppress RNAIII production. Several peptides were found to bind RAP. One of them, which had the strongest inhibitory activity, was synthesised and expressed in *E. coli* as a GST-fusion. Importantly, the GST-fusion peptide was shown to eradicate infection in a murine cutaneous *S. aureus* infection model (Yang *et al*., 2003).

Consequently, it was suggested to use RIP in indwelling medical devices as a coating agent to inhibit biofilm formation by *S. aureus* and *S. epidermidis* (Otto, 2004). Nevertheless, a series of other studies showed that inhibition of the *agr* system leads to increased rather than decreased colonisation (Vuong *et al*., 2000; 2003; 2004), so the biofilm-supressing activity of RIP is not in accordance with its RNAIII-inhibitory activity (Otto, 2004). What is more, the RIP concentration used in the above studies (~10-50 μg mL^{-1}) is in the range, in which peptides with a certain degree of amphipathy can inhibit biofilm formation mainly by their detergent-like properties, rather than by a regulatory effect that involves QS (Vuong *et al*., 2000 and 2003). Taken together, the use of RIP to coat implants does not have an advantage over other suggested coating agents and the question whether QS control in staphylococci has potential as a therapeutic target for novel antibacterial agents has yet to be answered (Otto, 2004; Kong *et al*., 2006).

However, other results from a recent study were very promising. An anti-autoinducer monoclonal antibody, AP4-24H11 was elicited against a rationally designed hapten, and efficiently inhibited QS *in vitro* by sequestering the autoinducing-peptide (AIP)-4 (Group IV) produced by *S. aureus* RN4850. Significantly, AP4-24H11- mitigated *S. aureus* pathogenicity was employed *in vivo* using an abscess- formation mouse model. It was found that this vaccine provided a total protection against a lethal *S. aureus* dose. Based on these findings, antibodies generated against bacterial autoinducers constitute an alternative strategy which used immuno-pharmacotherapy for the treatment of bacterial infections in which the expression of virulence factors was controlled by QS (Park *et al*., 2007).

2.5 Biofilm formation

The success of human pathogens, particularly *P. aeruginosa* in causing a chronic infection is mainly attributed to its ability to form biofilms and adapt to almost any environmental niche. Often, the only cure is the surgical removal of the infected organ (Bjarnsholt and Givskov, 2007). A biofilm is defined as a microbial sessile community which is irreversibly attached to a substratum and embedded in a matrix of extracellular polymeric substances (EPS) formed by the bacteria (Costerton *et al*., 1995). Initially, it was believed that biofilms are homogeneous monolayers of attached bacterial cells. Later, however, it was found that biofilms consisted of a heterogeneous structure in which individual microcolonies are located in mushroom-like structures surrounded by EPS (Lewandowski, 2000). It is known that there is a relationship between QS and biofilm development in *P. aeruginosa* (Bjarnsholt and Givskov, 2007).

2.6 Strategies to interfere with QS

Several strategies aiming at the interruption of bacterial QS are possible. There are basically three different targets in Gram-negative QS systems: the signal generator, the signal molecule and the signal receptor. Obviously, one strategy is to screen for compounds that prevent the synthesis of the signal molecules, e.g. AHL by the *luxI*-encoded AHL synthase. If no AHL is produced, the bacteria cannot sense when a quorum is reached, hence, QS-controlled genes will not be activated. Alternatively, the signals themselves can be subjected to degradation, which may be metabolic, chemical or enzymatic. Finally, one of the most studied targets is the signal receptor. By preventing the signal molecule from binding to the receptor protein, the latter can no longer act as a transcriptional regulator (Rasmussen and Givskov, 2006).

2.6.1 Drug targets: the signal generator

The first QS-disrupting technique focuses on inhibiting the signal molecule's biosynthesis (Figure 2.5). The majority of bacteria that produce AHLs encode one or more genes homologous to *luxI* of *Vibrio fischeri* (see Figure 2.2). Expression of these genes in heterologous host backgrounds has shown that the LuxI-type protein is required for production of AHL signals. The catalysis of AHL synthesis has been studied in vitro for three LuxI family members. AHL signals are produced by a sequentially ordered reaction mechanism that uses acyl carrier proteins (ACP) for the acyl chain and S-adenosyl methionine (SAM) for the homoserine lactone moiety as substrates (Parsek *et al*., 1999).

In attempts to block biosynthesis of AHLs Parsek *et al*. (1999) found that various analogs of SAM, such as S-adenosylhomocysteine, S-adenosylcysteine and sinefungin, could slow down the activity of the *Pseudomonas aeruginosa* LuxI homologue RhlI by up to 97%. It should be noted that these tests were performed only *in vitro*.

AHL synthases differ from most other SAM-utilising enzymes in that the SAM acts as an amino donor rather than a methyl donor. The reaction chemistry of AHL synthase with SAM seems to be unique, although SAM is a common intermediate in many prokaryotic and eukaryotic pathways. This raises the hope that SAM analogs could be used as specific inhibitors of autoinducer synthases, without affecting eukaryotic enzymes that use SAM as a substrate (Parsek *et al*., 1999).

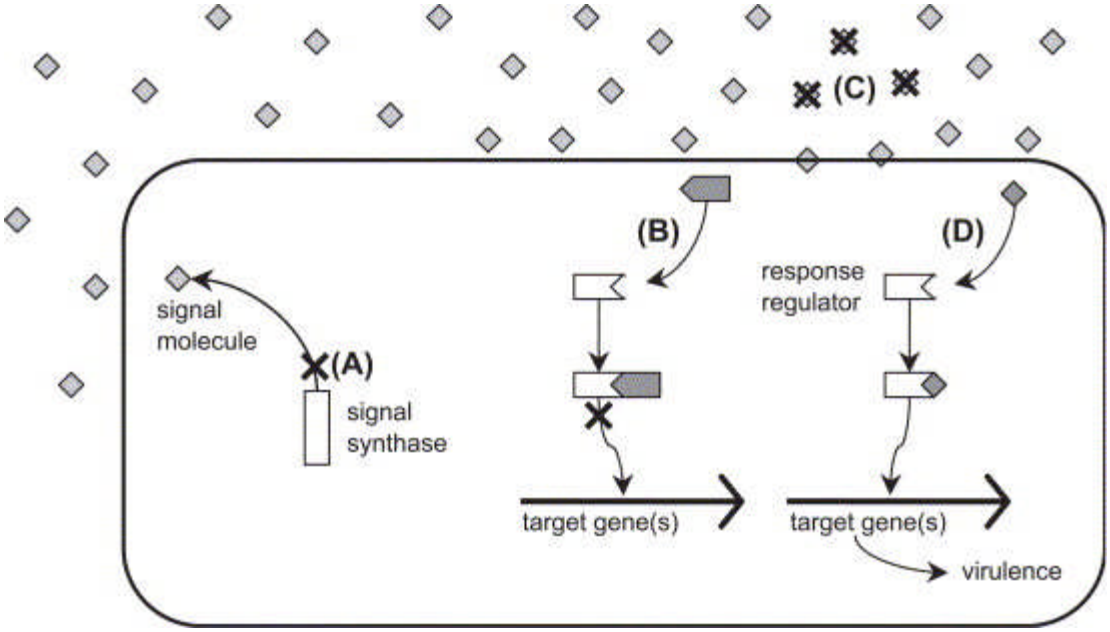


Figure 2.5: Schematic overview of different strategies that have been developed to inhibit bacterial QS. (a) Inhibition of signal molecule biosynthesis by the application of substrate analogues. (b) Blocking signal transduction by the application of QS antagonists. (c) Chemical inactivation and biodegradation of signal molecules. (d) Application of QS agonists to evoke virulence factor expression at low population density (Defoirdt *et al*., 2004).

Some recent reports have shown that certain macrolide antibiotics have the ability to repress *P. aeruginosa* AHL synthesis when they were applied in sub-minimal growth-inhibitory concentrations. Azithromycin has been reported to inhibit the QS circuitry of *P. aeruginosa*. Addition of synthetic autoinducers partially restored the expression of the transcriptional activator LasR but not that of the autoinducer synthase LasI (Tateda *et al*., 2001). It was found that erythromycin, could suppress the production of *P. aeruginosa* hemagglutinins (including lectins), protease, hemolysin and homoserine lactone autoinducers (Sofer *et al*., 1999). Macrolides are basically recognised as inhibitors of protein synthesis at the ribosomes. However, it remains unclear how these antibiotics interfere with QS circuits.

2.6.2 Drug targets: the signal molecule

Bacterial cell-to-cell communication can also be disrupted by decreasing the concentration of the signal molecules (Figure 2.5c). Decay of AHLs might be a result of a non-enzymatic reaction. Indeed, AHLs are prone to alkaline hydrolysis at high pH values. Due to the nature of the homoserine lactone ring moiety, the AHLs are also unstable under exposure to alkaline pH. The molecule of AHL is subjected to lactonolysis-ring opening-and subsequent loss of the biological activity (Yates *et al*., 2002). Yates *et al.* (2002) have shown that when either *Yersinia pseudotuberculosis* or *P. aeruginosa* are grown in a complex nutrient media, AHLs are accumulated during the exponential growth phase, while they are largely decomposed during the stationary phase. This is due to the pH-dependent opening of the ring, such that in the more alkaline pH achieved during the stationary phase of the culture, the structures with opened ring are accumulated. The kinetics of the ring opening relies on the length of the acyl side chain as well as on the temperature (the higher the temperature the higher the rate of ring opening). Also, molecules with extended side chains undergo lactonolysis slower than their short-chain counterparts. Hence, to be functional under physiological conditions in the mammalian tissue fluids, AHL signal molecules must have a side chain length of at least 4 carbon atoms (Yates *et al*., 2002).

Such chemical instability of AHLs could be exploited for the development of the novel therapies in order to reduce the AHL concentration and to reduce or eliminate the production of the virulence factors. Enzymes that are able to inactivate AHLs have been discovered in some Gram-negative bacteria species (Dong *et al*., 2000; Dong *et al*., 2001; Xu *et al*., 2003; Molina *et al*., 2003; Huang *et al*., 2003; Lin *et al*., 2003). These bacteria might block the QS systems of their competitors to obtain

a selective advantage over them. The inactivation of the signal compound can be accomplished by two types of enzymes: AHL lactonases and AHL acylases (Figure 2.6). Furthermore, eukaryotic acylases might be able to act as AHLs inactivators as well (Xu *et al*., 2003).

In order to discover AHL lactonases, Dong *et al*. (2000) screened more than 400 field bacterial isolates and about 100 strains of the laboratory bacteria culture collection for AHL inactivation activity. Of these, 24 isolates showed different levels of enzymatic activity in depleting AHLs. The enzyme responsible for the AHL-inactivating activity (AiiA) was isolated from the strain that showed the highest level of activity, *Bacillus* sp. strain 240B1. The purified protein, at a concentration of 50 mg/L, decreased the concentration of *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C_6-AHL) from 20 µM to about 5 µM in 10 min. Electrospray ionisation-mass spectrometry of the hydrolysis product revealed that the AiiA enzyme catalyses the lactonolysis reaction to produce *N*-(3-oxohexanoyl)-L-homoserine and hence lowers the amount of bio-active AHL (Dong *et al*., 2001).

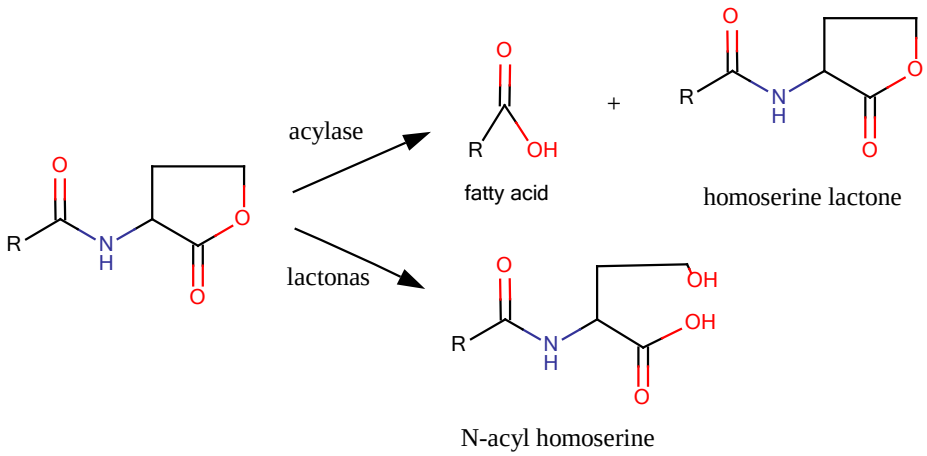


Figure 2.6: Enzymatic inactivation of AHLs. Cleavage of the amide bond by an AHL acylase enzyme yields a fatty acid and homoserine lactone. Cleavage of the lactone ring by an AHL lactonase enzyme yields the corresponding acylated homoserine. R is an alkyl group comprising between 3 and 13 carbons, which can have an oxo or hydroxyl substitution at the second carbon (Defoirdt *et al*., 2004).

The first evidence which indicated that enzymatic depletion of AHLs could be used as a biocontrol strategy was shown in the study of Dong *et al*. (2000). In this study, expression of the *aiiA* gene in the plant pathogen *Erwinia carotovora* resulted in the reduced release of AHL signals, decreased extracellular pectolytic activity, and attenuated soft rot disease symptoms in all tested plants. Moreover, transgenic plants expressing AiiA enzyme have been shown to be considerably less prone to infection by *E. carotovora* (Dong *et al*., 2001). In another *in vivo* study, Molina *et al*. (2003) tested the possibility to use a wild-type soil bacterium, such as the *Bacillus* sp. strain A24. This strain was able to diminish potato tuber soft rot caused by *E. carotovora* to about 15% and crown gall in tomato caused by *Agrobacterium tumefaciens* to about 10%. Biocontrol activity of this AHL-degrading strain was greater than that observed with a *Pseudomonas chlororaphis* strain, which relies on antibiotic production for disease suppression.

Furthermore, degradation of AHLs could be considered both as a preventive and a curative biocontrol activity. Specifically, it was found that even when the pathogen was given a 2-day head start to establish disease symptoms, subsequent application of an AHL-degrading strain reduced further disease development. This is one of the rare examples of curative disease control and it may have significant practical implications for bacterial diseases that usually have an incubation period before any outward symptom reveal themselves (Molina *et al*., 2003).

Some other researchers cloned the *aiiA* gene into a particular expression vector and the correspondent AiiA protein was overexpressed in *E. coli* strain. They found that not only could the lysate of this strain suppress the pigment production of the QS system in *C. violaceum* ATCC 12472, but it also reduced the bioluminescence intensity of *V. harveyi* VIB391 by 85% (Bai *et al*., 2008).

On the other hand, since the lactonolysis which AiiA-type enzymes deploy to inactivate AHLs is a pH-dependent reversible chemical reaction, there is a possibility that inactivated ring-opened forms could revert to the active ring-closed structure. More to the point, such enzymes are unlikely to be useful for systemic administration due to problems associated with the delivery of large proteinaceous therapeutic agents to the site of infection (Cámara *et al*., 2002).

Looking for AHL-acylases, Lin *et al*. (2003) isolated an AHL signal-inactivating bacterium, identified as *Ralstonia* sp. strain XJ12B, from a mixed-species biofilm. The protein responsible for the AHL-inactivating activity (AiiD) was purified and, subsequently, incubated with 3-oxo-C_{12}-AHL. Electrospray ionisation-mass spectrometry analyses demonstrated that AiiD hydrolysed the amide bond of AHLs, releasing homoserine lactone and the corresponding fatty acid. Expression of the AiiD enzyme in transformed *P. aeruginosa* PAO1 strain inhibited QS by this bacterium, by means of decreasing swarming of the pathogen, elastase and pyocyanin production, and led to reduced mortality of the nematode *Caenorhabditis elegans* by 15% as compared to wild type PAO1 (Lin *et al*., 2003).

Another study by Huang *et al*. (2003) showed that some substrate specificity of AHL-inactivating enzymes can exist. A novel soil pseudomonad, strain PAI-A, was isolated and found to be able to inactivate AHLs via an AHL acylase enzyme. In contrast to *Ralstonia* sp. strain XJ12B (Lin *et al*., 2003), the pseudomonad was able to use AHLs for the growth if the acyl side chain was longer than eight carbons. Since the small-subunit rRNA gene from strain PAI-A was almost identical to that of the pathogen *P. aeruginosa* strain PAO1, the ability of the latter to utilise AHLs for growth was investigated as well. In accordance with the results obtained from the strain PAI-A, the pathogen was able to grow on long-acyl but not short-acyl AHLs. It

was indicated that both strains (PAI-A and PAO1) were able to degrade long chain AHLs using an AHL acylase and a homoserine-generating HSL lactonase. A *P. aeruginosa* gene, *pvdQ*, has previously been identified as being the closest homologue of the *Ralstonia* acylase gene (Lin *et al*., 2003). When *pvdQ* was expressed in *Escherichia coli*, the culture was able to catalyse the rapid inactivation of exogenously supplied AHLs. Moreover, when expressed in *P. aeruginosa*, the culture was unable to accumulate AHLs. Interestingly, *pvdQ* knockout mutants of *P. aeruginosa* were still able to grow by utilising 3-oxo-C_{12}-AHL as sole carbon, energy and nitrogen source indicating that *P. aeruginosa* might contain several AHL-degradation systems (Huang *et al*., 2003).

Xu *et al*. (2003) investigated the ability of a eukaryotic counterpart of bacterial enzymes for AHL-degrading activity. Indeed, the porcine kidney acylase I enzyme was shown to inactivate two different kinds of AHLs. Moreover, the acylase could reduce a model biofilm, mainly consisting of a *Pseudomonas* and a *Microbacterium* species, indicating that the enzyme might have the ability to degrade AHLs at environmentally relevant concentrations. Nevertheless, the above results should be followed by systematic investigation so that more insight into the mechanism of this reaction could be obtained and further research is needed to investigate whether such enzymes have any significance in *in vivo* disruption of QS-mediated gene expression (Xu *et al*., 2003; Defoirdt *et al*., 2004).

As far as chemical inactivation of QS signal molecules is concerned, Borchardt *et al*. (2001) investigated the potential for oxidised halogens to react with acylated homoserine lactone-based signal molecules. These antimicrobials, at a concentration of about 0.14 mM, were found to reduce the concentration of 3-oxo-substituted AHLs to approximately one-fourth after 1 min incubation, but had no

effect on unsubstituted AHLs. Experiments with the marine alga *Laminaria digitata* demonstrated that natural haloperoxidase systems are able to deactivate 3-oxo-AHL molecules. This alga secretes oxidised halogen compounds, like hypochlorous and hypobromous acids. These compounds have been used extensively for eradication of microbes in both natural and industrial environments. Furthermore it was noted that the reaction between AHLs and oxidised halogens can occur even under conditions in which biofilm components - mostly exopolysaccharides - are present at much greater concentrations than the AHLs levels. Since QS is found to be correlated with biofilm development (Smith and Iglewski, 2003), secretion of compounds that degrade the AHLs and hence inhibit the QS, it might be advantageous to the alga in its competition with other species (Borchardt *et al*., 2001; Rasmussen and Givskov, 2006).

Using HPLC-MS Michels *et al*. (2000) unravelled the degradation pathway of AHL signal molecules by oxidised halogen antimicrobials. The reaction kinetics was largely influenced by the pH of the reaction mixture. In particular, at pH 6 a molecule of 3-oxo-AHL reacts with two molecules of hypochlorous or hypobromous acid, yielding a 2,2-dihalo-3-oxo-AHL molecule. Subsequently, hydrolation of the acyl chain yields a fatty acid and 2,2-dihalo-*N*-ethanoyl-L-homoserine lactone (Figure 2.7). At pH 3, only the mono- and di-halogenated products are detected, whereas at pH 8, the lactone ring of 2,2-dihalo-*N*-ethanoyl-L-homoserine lactone is hydrolised, yielding 2,2-dihalo-*N*-ethanoyl-L-homoserine (Michels *et al*., 2000; Defoirdt *et al*., 2004). Such pH-dependent degradation of the β-keto-amides may be considered as one of the potential mechanism for control of industrial biofilms (Michels *et al*., 2000).

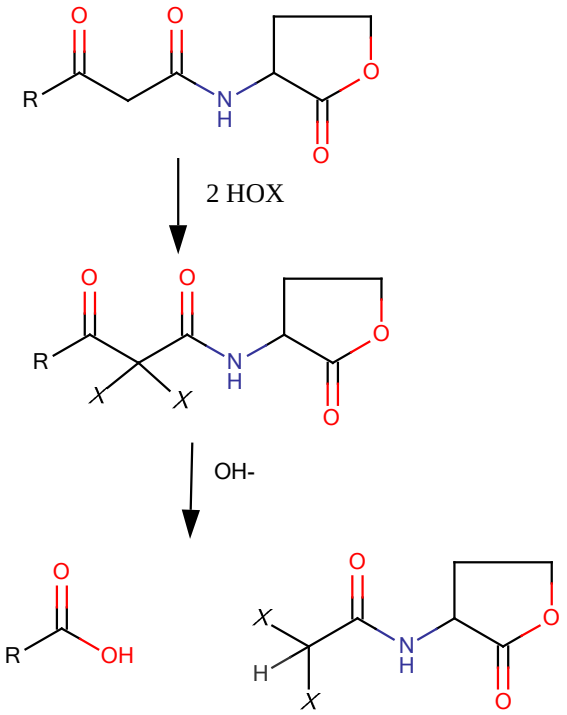


Figure 2.7: Reaction between 3-oxo-AHL and halogen antimicrobials (HOX; hypobromous or hypochlorous acid) at pH 6. First, two α-halogenation reactions take place, yielding 2,2-dihalo-3-oxo-AHL. Ultimately, the acyl chain is subjected to hydrolysis, yielding a fatty acid and 2,2-dihalo-*N*-ethanoyl-L-homoserine lactone. R is an alkyl group comprising between 3 and 13 carbons, which can have an oxo or hydroxyl substitution at the second carbon (Defoirdt *et al*., 2004).

Another effective non-conventional strategy which was employed to inhibit the AHL-mediated QS includes the sequestering of AHLs by antibodies. The first use of antibodies was recently reported by Marin *et al* (2007). The investigators screened a number of antibodies that were previously obtained via immunisation of mice with a reactive hapten based on a squaric monoester monoamide motif (Figure 2.8) for their ability to catalyse the hydrolysis of 3-oxo-C_{12}-AHL to its ring-opened product (see Figure 2.4). From 17 monoclonal antibodies, which were tested, XYD-11G2 was found to catalyse the hydrolysis of 3-oxo-C_{12}-AHL with moderate catalytic efficiency. Furthermore, 10 μM concentration of mAb XYD-11G2 was shown to reduce

pyocyanin production to about 40% in wild-type *P. aeruginosa* after 12-h incubation. The above results suggest that antibody catalysis could provide a new way of disrupting QS in bacteria (Marin *et al*., 2007).

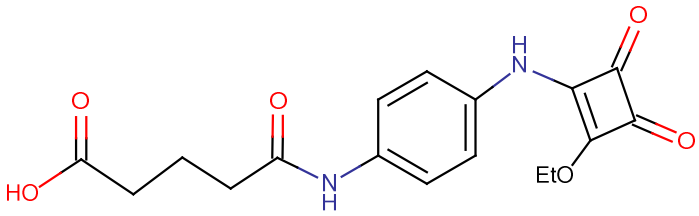


Figure 2.8: Squaric monoester monoamide hapten (Marin *et al*., 2007).

2.6.3 Drug target: the signal receptor

One of the most effective approaches of interference with QS is to use small molecules to compete with the cognate AHLs for binding to the LuxR transcriptional regulator proteins. It would allow attenuating the pathogen by switching off virulence gene expression. QS-mediated processes often exist in the interactions between bacteria and plant or animal hosts. Therefore, it is not surprising that these higher organisms have acquired mechanisms to inhibit QS. One of these mechanisms is the production of QS antagonists: molecules that can bind to and occupy the AHL-binding cite but fail to activate the LuxR-type receptor protein (see Figure 2.2 and Figure 2.5b) (Defoirdt *et al*., 2004).

The Australian macroalga *Delisea pulchra* has developed a mechanism to protect itself from extensive bacterial colonisation (Givskov *et al*., 1996). The alga produces secondary metabolites, in particular halogenated furanones, as antagonists for AHL-mediated QS. Givskov *et al*. (1996) showed that swarming of the pathogen *Serratia liquefaciens* could be inhibited completely in the presence of 100 mg L^{-1} of (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone (see Figure 2.9b, where

R_1=Br and R_2=H). It was found that the reduction of motility of the swarm cells was not correlated by factors which influence flagellar synthesis, cell differentiation or growth rate. Essentially, it was shown that the same quantity of this furanone could also inhibit bioluminescence in growing cultures of *S. liquefaciens* containing a reporter plasmid, without affecting the growth rate of the bacterium. Rasmussen *et al*. (2000) proposed that the furanone interferes with the putative protein receptor and thereby displaces the AHL molecule. The structural similarity of the furanone to AHLs (see Figure 2.9b and Figure 2.1) together with the findings of Manefield *et al*. (1999) which indicate a displacement of radiolabeled AHL molecules from a strain overproducing LuxR by the furanone, suggested that halogenated furanones most probably bind to LuxR type proteins without activating them. Manefield *et al*. (2000) found that the furanone inhibited the toxin production in the prawn pathogen *Vibrio harveyi*. Additionally, a significant reduction in the toxicity of the diluted cell supernatant extracts from *V. harveyi* cultures grown in the presence of the signal antagonist, as measured in *in vivo* assays in shrimps and mice, was observed.

Moreover, a further study by Manefield *et al*. (2001) indicated that halogenated furanones of *D. pulchra* inhibited QS-controlled virulence of *E. carotovora* favoured this hypothesis. However, more recent evidence suggested that the reported inhibition of AHL-mediated gene expression is the outcome of the rapid degradation of the LuxR protein by the furanones which resulted in the reduced concentrations of the protein available to interact with AHL and act as transcriptional regulator (Manefield *et al*., 2002).

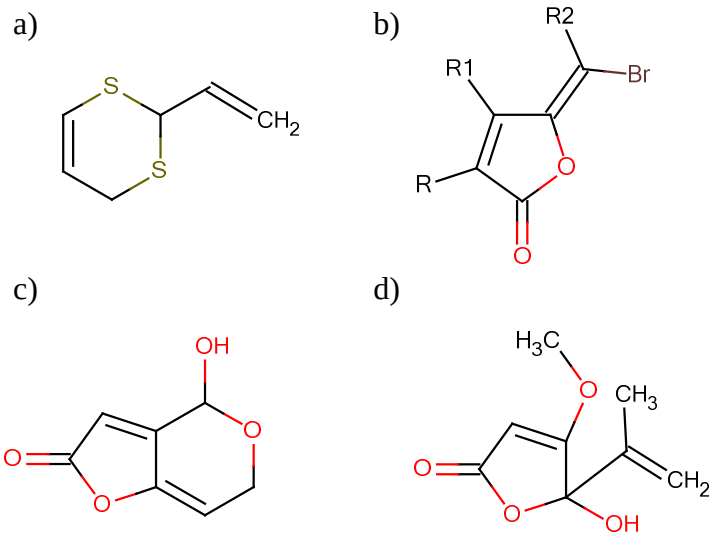


Figure 2.9: QS inhibitors from natural sources. (a) Cyclic sulphur-containing compounds isolated from garlic (Persson *et al.*, 2005). (b) Basic structure of halogenated furanone from *Delisea pulchra*, R1 and R2 can be either H or Br (Givskov *et al.*, 1996). (c) Patulin and (d) penicillic acid produced by fungi (Rasmussen *et al.*, 2005b).

More to the point, plants such as bean sprout, chamomile, carrot, habanero, water lily, and yellow pepper produce compounds that interfere with bacterial QS. In addition propolis, which is produced by bees, also contains QS inhibitor (QSI) activity (Rasmussen *et al.*, 2005a). Garlic extracts contain at least three different QSI compounds, one of which is a hetero-cyclic ring containing four carbon and two sulphur atoms (Figure 2.9a) (Persson *et al.*, 2005). This compound is a strong inhibitor of LuxR-based QS. DNA microarray analysis revealed that 34% of the QS regulon in *P. aeruginosa* was down-regulated by garlic treatment. RhlR-controlled genes (34% of total RhlR-controlled genes) are more frequently targeted by garlic than LasR-controlled genes (8% of total LasR-controlled genes), indicating that garlic preferably targets RhlR. Furthermore, *P. aeruginosa* biofilms treated with garlic extract were highly susceptible to tobramycin, and garlic extract significantly reduced the mortality of the worms in a *Caenorhabditis elegans* nematode model (Rasmussen

et al., 2005a). Garlic extract could also attenuate pathogenic bacteria in vivo. In an experimental setup conducted by Bjarnsholt *et al*. (2005), two different groups of mice were treated with either garlic extract or placebo. The treatments were given as single subcutaneous injections daily for 7 days. The dosage of garlic extract was calculated as to be 1.5% of the total mass of the mouse. After two days of prophylactic treatment, the mice were intra-tracheally challenged with alginate beads containing *P. aeruginosa*, at 10^7 CFU/lung. On average, there was a three order of magnitude difference in CFU/lung in each of the days up to day 5 where all bacteria were cleared from 9/10 mice in the garlic-treated group whereas the placebo group still contained *P. aeruginosa* at 10^5 CFU/lung (Bjarnsholt *et al*., 2005). Interestingly, garlic extract, apart from the efficient eradication of pulmonary infections in the described above animal model, also enabled a more appropriate PMN (polymorphonuclear leukocytes) response in the presence of *P. aeruginosa* biofilms. However, in order to receive the dose of garlic extract used in this animal model, an 80 kg person would have to ingest 50 bulbs of garlic each day.

QSI compounds have been recognised by random screening of pure chemical compound libraries. Using the QSI selector, Rasmussen *et al*. (2005a) identified various compounds which were able to down-regulate both LuxR- and LasR-based genes. The most effective QSI of this group of compounds was found to be 4-nitro-pyridine-*N*-oxide (4-NPO) (Figure 2.10a). Using DNA array-based analysis, it was shown that 4-NPO down-regulated a total of 37% of the QS regulon in *P. aeruginosa*. Affected genes were preferably genes regulated either by RhlR alone or by RhlR and LasR in conjuction. This indicates that 4-NPO, similar to garlic compounds, probably targets the RhlR receptor. Moreover, in the *C. elegans* infection model, 4-NPO

significantly decreased the mortality of the worms from 100% in an untreated culture to 5% in a 4-NPO-treated culture (Rasmussen *et al*., 2005a).

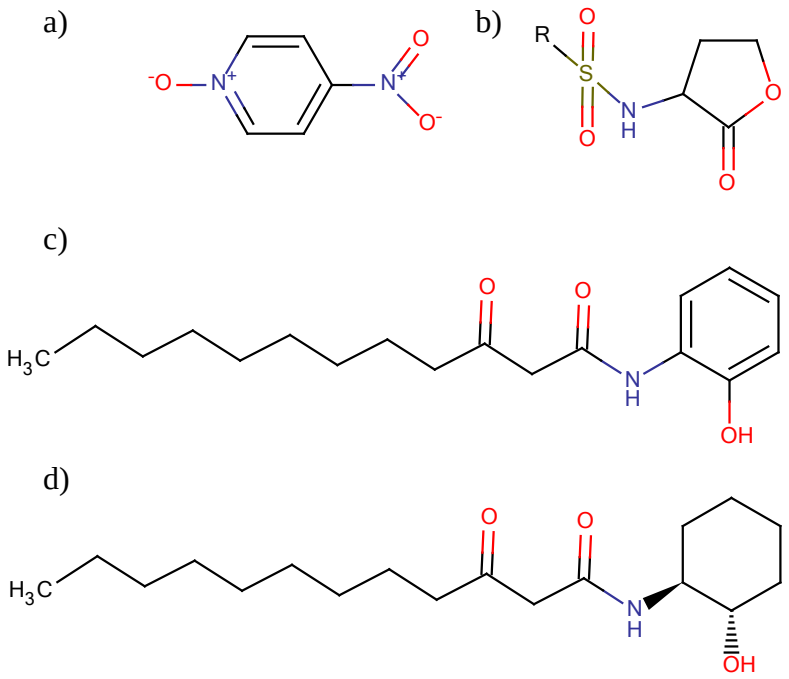


Figure 2.10: QSIs – the small molecules: (a) 4-NPO (Rasmussen *et al*., 2005a). (b) AHL analogue where the carbonyl group was replaced with a sulfonyl group (Castang *et al*., 2004). (c) 3-oxo-C_{12}-(2-aminophenol) is a QS antagonist in *P. aeruginosa*, while (d) 3-oxo-C_{12}-(2-aminocyclohexanol) is an agonist (Smith *et al*., 2003b).

A much explored source of anti-microbial compounds are fungi. The QSI selector (Rasmussen *et al*., 2005a) was used once more for screening of *Penicillium* species. Rasmussen *et al*. (2005b) found several strains which produced QSI compounds. Two of these compounds were identified as patulin (Figure 2.9c) and penicillic acid (Figure 2.9d). Their effect on QS-dependent gene expression in *P. aeruginosa* was verified by DNA microarray transcriptomics. Patulin and penicillic acid were found to down-regulate 45 and 60 %, respectively, of the QS-regulated genes. *P. aeruginosa* biofilms were easily eradicated by tobramycin when they were pre-treated with patulin or penicillic acid. Furthermore, the wild-type of *P. aeruginosa*

biofilms, which was formed in the presence of either of these two specific QSI compounds, led to oxidative burst in PMNs that settled on top of the biofilm. By monitoring the bacterial load in the lungs over several days, it was shown that mice which were treated with patulin cleared the infecting *P. aeruginosa* more rapidly than mice treated with a placebo (Rasmussen *et al*., 2005b). However, due to the fact that the two compounds mentioned above are well- known mycotoxins, their pharmaceutical relevance is limited (Rasmussen and Givskov, 2006).

Except for furanones from *D. pulchra* and other QSI compounds, which were obtained from natural sources, several reports have described the *in vitro* application of naturally occurring AHL analogs used to disrupt the QS circuit in various bacteria (Schaefer *et al*., 1996; McClean *et al*., 1997; Zhu *et al*., 1998; Swift *et al*., 1997; Swift *et al*., 1999). Schaefer *et al*. (1996) found analogues of 3-oxo-C_6-AHL, with substitutions both in the acyl side chain and in the ring, which could displace ^{3}H-labelled 3-oxo-C_6-AHL (the cognate AHL) from LuxR. Most of the compounds, which were bound to LuxR, were not only able to displace the AHL but were also able to activate LuxR. Such compounds are competitive agonists. However, some compounds with a side chain length of at least 10 carbons and others with a conservative substitution of a thiolactone ring for the homoserine lactone ring inhibited 3-oxo-C_6-AHL activity by 80% or more in a concentration of 200 nM. These compounds constitute true inhibitors of QS. Furthermore, a decrease in the chain rotation by introducing an unsaturated bond close to the amide bond almost completely abolished the binding to the receptor protein. Compliant with this finding, no naturally occurring AHL signal has ever been found to contain a 2,3 unsaturated bond (Schaefer *et al*., 1996). In another piece of research by McClean *et al*. (1997), it was indicated that natural AHL molecules could either induce or inhibit QS-regulated

pigment production in the Gram-negative bacterium *Chromobacterium violaceum*. The stimulatory or disruptive effect was dependent on the length of the acyl side chain of the molecules: AHLs with an acyl side chain of up to eight carbons were stimulatory and acted as QS agonists, whereas, AHLs with an acyl side chain of ten carbons or more were inhibitory and acted as QS antagonists (McClean *et al*., 1997). Zhu *et al*. (1998) examined the ability of two strains of *Agrobacterium tumefaciens* to distinguish between its cognate autoinducer (3-oxo-C_8-AHL) and a related compound library. The wild-type strain was much effectively stimulated by its cognate autoinducer than by any other tested compound and was almost non-responsive to almost all of these compounds. In contrast, a congenic strain that over-expressed TraR protein, was far more sensitive to these compounds, since the majority of them were stimulatory. Moreover, in the former strain most compounds acted as inhibitors, while in the latter strain the compounds acted as agonists (Zhu *et al*., 1998). Interestingly, two reports by Swift *et al*. (1997 and 1999) indicated that heterologous AHLs can reduce virulence factor induction by the Gram-negative pathogens *Aeromonas hydrophila* and *Aeromonas salmonicida*. In the first report, Swift pointed out that the application of 10 μM 3-oxo-C_{12}-AHL reduced the final concentration of the serine protease in *A. salmonicida* by approximately 50% and delayed the appearance of protease in the culture. The second report showed that in *A. hydrophila*, a significant reduction in exoprotease production regulated by C_4-AHL (its cognate AHL) was inhibited by AHLs analogs with much longer acyl side chains *in vitro*. In particular, it was demonstrated that the inclusion of 3-oxo-C_{12}-AHL or 3-oxo-C_{14}-AHL, both at a concentration of 10 μM, abolished QS-regulated exoprotease production by the aquatic pathogen *A. hydrophila* as well (Swift *et al*., 1999).

According to the findings mentioned above, many researchers started to study the possibility of using different synthetic AHL and furanone analogues for controlling QS (Castang *et al*., 2004; Persson *et al*., 2005; Smith *et al*., 2003a; Smith *et al*., 2003b; Reverchon *et al*., 2002; Manefield *et al*., 2002; Hentzer *et al*., 2002; Hentzer *et al*., 2003; Wu *et al*., 2004). Castang *et al*. (2004) synthesised AHL analogues in which the carboxamide group was replaced by a sulphonamide one (Figure 2.10b). These compounds were assessed for their ability to competitively inhibit the activity of 3-oxo-C_6-AHL, the cognate molecule of the QS transcriptional regulator LuxR, which in turn induces bioluminescence in *V. fischeri*. A non-active LuxR protein was obtained by using either a phenyl group at the end of the side chain in carboxamide analogues or sulfonamide moiety in sulfonamide analogues. Interestingly, there was no synergic effect in the compounds bearing two structural modifications (Castang *et al*., 2004). In a similar way, Persson *et al*. (2005) synthesised AHL analogues with a sulphur atom in the alkyl chain. This strategy generated potent inhibitors of the LuxR and LasR/RhlR QS systems. The strongest inhibitors were those that resembled C_{10} and C_{12} AHL molecules (Persson *et al*., 2005).

Substituents attached to the end of the acyl chain yielding alkyl, cycloalkyl or aryl AHLs showed strikingly different effects. Most of the analogues bearing either acyclic or cyclic alkyl sustituents acted as agonists in a LuxR dependent QS system. On the contrary, the aryl compounds displayed significant antagonist activity. Compounds with tertiary alkyl derivatives as well as larger alkyl or aryl moieties were deprived of any activity, indicating that these groups were too bulky to allow the molecule to interact with the receptor protein (Reverchon *et al*., 2002).

Replacing the lactone ring of the AHL with another ring structure is an alternative way to create compounds, which would be able to bind and inhibit the AHL receptor protein. In the two signal molecules from *P. aeruginosa*, C_4-AHL and 3-oxo-C_{12}-AHL, the side chains were retained and the HSL moiety was altered to various amines cycloalcohols or cycloketones containing either 5 or 6 atoms in the ring. Interestingly, the compound harbouring an amino cyclohexanol ring was the strongest agonist of the *las* system whereas in contrast the ketone derivatives were the strongest activators of the *rhl* system. Thus, based on the above findings, it can be proposed that the two *P. aeruginosa* AHL receptor proteins differ significantly in their AHL-binding sites (Smith *et al*., 2003a). Smith *et al*. (2003a) succeeded in generating a compound, 3-oxo-C_{12}-(2-aminocyclo-hexanone) which was able to inhibit the QS cascade of a p*lasI- gfp* fusion and reduce the expression of important virulence factors and biofilm in *P. aeruginosa*. Yet, the concentration required in order to achieve this inhibition (100 μM) was too high to be applied in medical practice.

In a further research by Smith *et al*. (2003b), the compound library described above was enlarged to include phenolic compounds. It was shown that a common structural motif for antagonists is an aniline ring together with an appropriate H bond acceptor at the either ortho- or meta- position. Although the only difference between the agonist 3-oxo-C_{12}-(2-aminocyclohexanol) (Figure 2.10d) and 3-oxo-C_{12}-(2-aminophenol) is an aromatic ring, the latter turned out to be a very strong inhibitor of LasR-based QS in *P. aeruginosa*. Significantly, this molecule displayed a potent inhibitory activity in the reporter gene assays, reduced elastase activity and interfered with normal biofilm formation (Smith *et al*., 2003b). Similarly, a compound containing the side chain of 3-oxo-C_6-AHL attached to a cyclohexane ring was found

to be an agonist, whereas, if the side chain was attached to a benzyl ring the compound was a potent inhibitor. These findings point out that the nature of the ring moiety of the AHL analogue is crucial for its function as agonist or antagonist (Reverchon *et al*., 2002).

The fact that natural QSIs produced by *D. pulchra* proved to be ineffective against *P. aeruginosa,* pushed researchers to synthesise halogenated furanone analogues able to inhibit QS in this bacterium. Compounds without side chains or with an electro-negative C1 substituent were found to be capable to inhibit the QS cascade in *P. aeruginosa* (Manefield *et al*., 2002; Hentzer *et al*., 2002; Hentzer *et al*., 2003; Hjelmgaard *et al*., 2003; Wu *et al*., 2004). DNA array technology was applied to demonstrate that the presence of the synthetic furanone compounds result in repression of QS-controlled genes in *P. aeruginosa*. Microarray analysis of wild-type *P. aeruginosa* PAO1 showed that a synthetic furanone compound, which does not contain an acyl side chain (Figure 2.11b), influenced the expression of 93 genes (1.7% of the *P. aeruginosa* genome). The next step was to elucidate whether the furanone-repressed genes were in fact controlled by QS. Parallel mapping of the QS regulon was conducted in a *lasI rhlI* mutant grown with or without AHL signals. Remarkably, the comparative analysis demonstrated that 80% of these genes were indeed regulated by QS indicating a high specificity of this furanone compound for QS-controlled genes. One-third of the furanone-affected genes has previously been reported as QS-controlled and produced the virulence factors of *P. aeruginosa*. Moreover, this furanone compound and one of its close analogues (Figure 2.11a), in 10 μM concentration, could almost completely suppress the production of some QS-controlled extracellular virulence factors, namely protease, chitinase and pyoverdin in *P. aeruginosa* cultures. Interestingly, those furanones were equally active on biofilm

bacteria compared to planktonic cells, making them more susceptible for tobramycin and more prone to dispersal by the detergent sodium dodecyl sulphate (SDS) (Hentzer *et al*., 2002 and 2003). The use of the GFP-based single-cell technology in conjuction with SCLM revealed that the furanones could penetrate the microcolonies, interfere with cell signalling and block QS in the majority of the biofilm-forming cells. In addition, furanone treatment led to the formation of flat, less differentiated biofilms, which eventually were detached (Hentzer *et al*., 2002). When classical antibiotics were used to treat *P. aeruginosa* infections instead of furanone treatment, the concentrations, which were required in order to eradicate the biofilm bacteria, were 100- to 1000-fold higher than those to eradicate the planktonic counterparts (Anwar and Costerton, 1990). Hentzer *et al*. (2003) monitored bacterial load in mouse lungs for several days and showed that mice, which were treated with halogenated furanones, cleared the infection by *P. aeruginosa* faster than mice treated with a placebo. *P. aeruginosa* strain which contained a fluorescent AHL reporter plasmid was used to cause infection. After subcutaneous injection of the furanone, a significant reduction in the fluorescence signal from the *P. aeruginosa* strain was observed. Yet, after 8 h, the signal reappeared, meaning that the furanone was cleared from the mouse blood circulation. In another experiment, 88% of the mice in the placebo group passed away from the lethal lung infection whereas, in the furanone-treated group the mortality of the mice was only 55% (Wu *et al*., 2004).

a) b)

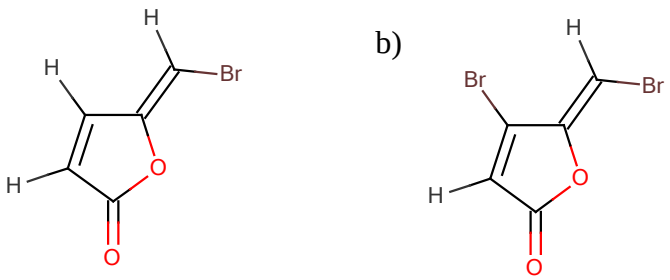
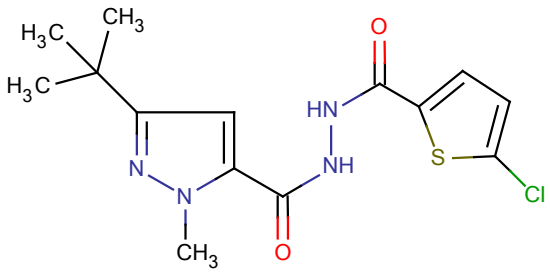


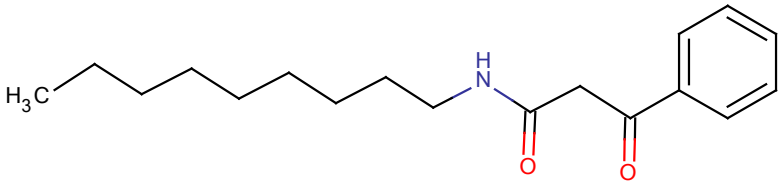
Figure 2.11: Synthetic furanones with QSI activity (Hentzer *et al*., 2002 and 2003).

Recently, some studies have been conducted towards the rational design of new QSI substances which were totally unrelated to AHL signal molecules or furanone compounds that were previously shown to interfere with QS (Riedel *et al*., 2006; Müh *et al*., 2006). Riedel *et al*. (2006) screened a database of commercially available compounds *in silico*. After the screening of over 1 million substances, a hydrazide derivative compound (Figure 2.12a) was found to efficiently inhibit the QS system of *Burkholderia cepacia*. Subsequently, it was tested whether this compound had the ability to interfere with the expression of pathogenic traits in *B. cepacia* and, therefore, to attenuate its pathogenicity. It was found that extracellular proteolytic activity, swarming motility, expression of the virulence factor AidA, biofilm formation and mortality of worms in a *C. elegans* nematode model were markedly reduced in the presence of the hydrazide derivative compound (Riedel *et al*., 2006). In another report, Müh *et al*. (2006) developed an ultra-high-throughput cell-based assay and screened a library of approximately 200,000 compounds for inhibitors of QS-regulated gene expression in *P. aeruginosa*. Interestingly, the two best inhibitors that were identified out of this screening resembled the cognate signal molecule 3-oxo-C_{12}-AHL, despite the big variety of the chemical structures of the library. Each inhibitor had a 12-carbon alkyl tail and either a benzyl head or a tetrazole (Figure 2.12b and 2.12c, respectively). Microarray analysis revealed that both compounds down-regulated most of the QS-controlled genes. Both compounds also inhibited a secretion of two QS-dependent extracellular virulence factors by wild-type *P. aeruginosa*, elastase and pyocyanin. The above findings provide evidence that these classes of molecules constitute general inhibitors of QS and can be applied in therapeutic applications (Müh *et al*., 2006).

a)



b)



c)

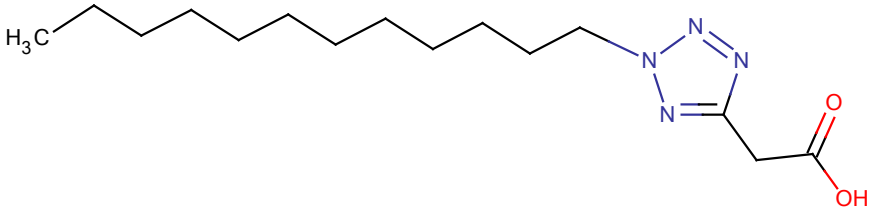


Figure 2.12: New classes of molecules with enhanced QSI activity (Riedel *et al*., 2006; Müh *et al*., 2006).

Recently, Vattem *et al*. (2007) investigated the effect of sub-lethal concentrations of bioactive dietary fruit-, herb- and spice- extracts on modulating QS in model bioassay test systems. Disruption of QS was evaluated in violacein pigment production in *C. violaceum O26* (CVO26) and CV 31532 system, known to be mediated by AHL. Additionally, the effect of sub-lethal concentrations of the extracts on swarming motility of *E. coli* O157:H7 and *P. aeruginosa* PAO1 was assessed. The testing showed that all extracts substantially inhibited QS by either interfering with AHL activity or modulating AHL synthesis. The phytochemical extracts also inhibited swarming of the above pathogenic bacteria, known to be regulated by QS. These observations open up a new strategy for antimicrobial chemotherapy and can

lead to the discovery of alternative drugs with reduced toxicity and minimal risk of the development of bacterial resistance (Vattem *et al*., 2007).

2.6.4 Application of QS agonists

All techniques discussed so far were aimed at inhibition of QS-dependent virulence factor expression. Mäe *et al*. (2001) applied the opposite approach which included the triggering of the QS-controlled virulence factor expression by means of QS agonists. The idea behind this technique was that by applying the active pheromone (or signal molecule) of the pathogen, virulence factor expression would be evoked at low population density (Figure 2.5d). Subsequently, host's defence system would be capable to fight the infection when the bacteria population is not sufficient to suppress this response. It was expected that the infection could be easier eradicated. Mäe *et al*. (2001) showed that the disease in tobacco plants caused by *E. carotovora* was decreased to 10% by using 5 mM-concentration of the pathogen's own signal molecule. Moreover, the ability of *E. carotovora* to elicit disease after local inoculation with 2×10^6 pathogens per plant was reduced to about half in transgenic tobacco plants, which produced the pathogen's own signal molecule as compared with control lines. It was observed that if the infection was already initiated or if the inoculum size was increased by a factor of four or more, the pheromone production appeared to be less effective because there was no significant difference in bacterial growth between transgenic and control plants. Apparently, the bacteria's load was sufficient enough to overwhelm the plant defence responses in these cases. All in all, the results of this study are in compliance with the hypothesis that QS was used by pathogenic bacteria to avoid premature virulence factor expression and premature activation of the plant defence system (Mäe *et al*., 2001).

2.6.5 The advantages and disadvantages of QS inhibitor therapy

It was found that several distinct signal molecules are produced by bacterial pathogens and there is no doubt that many more have still to be identified. However, any therapeutic agent able of disrupting QS will probably have a narrow range of activity as compared with conventional antibiotics, due to the lack of a universal bacterial language. On the other hand, QSIs, unlike conventional antibiotics, will not necessarily inhibit bacterial growth but, by switching off virulence, will promote clearance of the pathogen by the host's own defence system. As a consequence, disruption of QS is unlikely to pose the selective pressure for the emergence of resistance. Yet, since the mode of action of QSIs relies on host defences, it is far less likely that they will be capable of treating the immune-compromised patients than conventional antibacterial compounds do. Their general use would be probably prophylactic rather than therapeutic. What is more, the development of the new diagnostic systems to justify their effectiveness will also be indispensable, since the use of conventional *in vitro* sensitivity assays will not be applicable for QSI compounds (Cámara *et al*., 2002). Of the several compounds capable of disrupting QS, a few have been tested in animal models quite successfully but, unfortunately not one of them is suitable for human use. The halogenated furanones are unstable and toxic while the fungal compounds are well-known mycotoxins (Rasmussen and Givskov, 2006). In the case of garlic, the active components exist in very low concentrations (Bjarnsholt *et al*., 2005). Regarding the development of vaccines, a serious problem could be that once the patients are immunised against bacterial QS, re-vaccination could bring unpredictable immune reaction.

The results obtained using various techniques and assays that inhibit QS systems of pathogenic bacteria suggest that it is a promising alternative for antibiotics

in fighting the bacterial diseases. Not only are many virulence factors are down-regulated by QSI compounds but also biofilm bacteria become more vulnerable to conventional antibiotics. This indicates that the activity of QSIs is synergistic with antibiotics activity and that a combination of both may make the antibiotics treatment more effective. Thus, the development of QSI drugs will facilitate the antibiotic therapy and drug development (Pan and Ren, 2009). Much work has still to be done to fully understand the intricacies of QS-controlled gene expression. It is important to understand that the QSI substances have a future as anti-pathogenic drugs and that successful bacterial eradication relies on the synergistic activity of multiple bioactive components.

2.7 Molecularly Imprinted Polymers

The new approach behind this project was to see if it would be possible to stop the bacterial communication and therefore the expression of the virulence factors by removing the signal molecules from the system. The aim of this project was to develop the rationally designed synthetic polymers for selected AHLs, which are signal molecules in pathogenic Gram-negative bacteria *Pseudomonas aeruginosa (P. aeruginosa)* and *Burkholderia cepacia (B. cepacia*). If the signal molecule gets adsorbed by polymers and removed from the system, it will lead to disruption of the communication between the bacteria and in such way to inhibition of the QS and expression of virulence. Unlike conventional antibodies these polymers can be synthesised in bulk using easily available substrates and technologies.

Molecular imprinting was selected as a popular tool for the design of the specific resins with recognition properties, which are sometimes called as “plastic antibodies” and characterised by high selectivity and robustness in comparison with

natural receptors and antibodies. The main principle of the molecular imprinting is a generation of the molecular complex between the selected analyte (template) and a number of complementary functional monomers which possess functional groups and polymerisable double bond. The polymerisation in presence of the high amount of cross-linker serves as a stabiliser of the complex. When the template molecule is removed from the complex, the binding pocket is complementary to the analyte of interest and resembles the binding pocket of the natural receptors. Over the past few decades many research groups have attempted to generate specific molecularly imprinted polymers for the variety of analytes. The concept of using a specific imprint molecule to orchestrate the assembly of synthetic monomers around a target molecule has been discussed for quite a long time (Dickey, 1949; Dickey, 1955; Wulff and Sarham, 1972; Wulff *et al*., 1977).

Up to the present time, many advances were achieved in molecular imprinting technology and advanced techniques and processes have been developed and optimized with aiming to using molecularly imprinted polymers:

(1) as tailor-made separation materials,
(2) as antibody and receptor binding site mimics in recognition and assay system,
(3) for catalytic applications as enzyme mimics,
(4) as recognition elements in biosensors.(Mosbach, 1994; Wullf, 1995; Andersson *et al*., 1996).

A molecularly imprinted polymer (MIP) is a polymer that is formed in the presence of a template. Afterwards the template is extracted, thus creating the selective recognition sites.

The imprinting procedure involves 3 steps.

In the first step the functional monomers form the molecular complex around the template molecule by interactions between functional groups on both the template and monomers. These interactions can be either non-covalent or reversible covalent. Other components of the polymer include cross-linker, the polymerisation initiator and a porogenic solvent. Since a very high degree of cross-linking (70-90%) is required for maintaining the specificity, the choice of cross-linker is very important. The most popular cross-linkers include ethylene glycol dimethacrylate (EGDMA), divinylbenzene and trimethylopropane trimethacrylate (TRIM). Among them, the ethylene glycol dimethacrylate is the cheapest, easy to purify and the most suitable cross-linker for the imprinting process (Wulff, 1995).

The second step involves co-polymerisation of the template-monomer(s) complex in the presence of cross-linker. The radical polymerisation can be either thermal or photochemical (UV irradiation), thus generating free radicals through the cleavage of the initiator. The radicals then initiate the polymerisation of the functional monomers and the cross-linker resulting in the formation of a cross-linked polymer network, where the template molecules are “trapped” inside the network.

In the final step, after polymerisation, the bulk polymer is ground and sieved. Before the MIP could be used for rebinding studies, the template (preferentially, completely) is removed from the matrix by washing with solvent or a combination of chemical treatment and washing. For the non-covalent imprinting approach, a polar solvent, often containing an acidic or basic component, is used to interrupt ionic or

hydrogen bonds and effectively extract the template molecules from their binding sites and non-reacted monomers. After extraction of the template a polymer with complementary cavity in size and shape of the template is produced (Figure 2.13) (Ramström and Yan, 2005).

Currently, two basic approaches to molecular imprinting may be distinguished: (1) the pre-organised approach, pioneered by Wulff and co-workers, where the interactions between template and monomers in solution prior to polymerisation are covalent, and (2) the self-assembly approach, developed by Mosbach and co-workers, where the pre-arrangement between the target molecule and the functional monomers is maintained by non-covalent interactions (Andersson *et al.*, 1996).

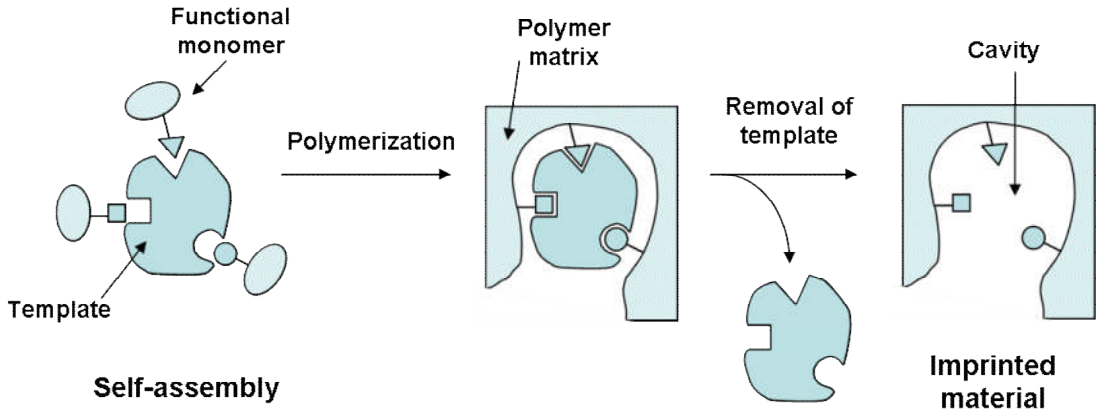


Figure 2.13: Schematic representation of the molecular imprinting.

The types of linkages involved in the covalent approach are mainly the esters of carboxylic/boronic acids, ketal or imines. After the construction of the polymer, the template is removed using some chemical cleavage, usually acid hydrolysis. Since it depends on the molecular structure of the template, it limits the application of this approach.

The non-covalent approach is based on non-covalent interactions between the molecules such as ionic interactions, hydrogen bonds, π-π interactions and hydrophobic interactions which form the molecular complexes between the template and monomers in solution. After polymer formation the imprint molecule can be almost quantitatively extracted from the matrix by mild extraction (Mosbach and Ramstrom, 1996). Today the non-covalent cross-linking is a predominant method used for molecular imprinting, because it offers much more versatility in terms of the functionalities of the functional monomers and of the imprinted analyte (template). Additionally, it requires from the researcher a lot less chemistry skills than the pre-arrangement of covalent adducts (Mayes and Whitcombe, 2005).

The simplest imprinting approach includes a non-covalent imprinting with single functional monomer. This approach is the most commonly used in the imprinting literature. Some functional monomers have been used very often in non-covalent imprinting, while others have been used rarely. Typical examples of the functional monomers are summarised below, where they are classified according to whether they are acid, bases or neutral molecules in nature (Mayes and Whitcombe, 2005).

In particular, methacrylic acid has been very successful monomer for imprinting amino acid derivatives (Sellergren *et al*., 1988), peptides (Ramström *et al*., 1994; Kempe and Mosbach, 1995; Titirici and Sellergren, 2004), theophyline (Vlatakis *et al*., 1993), propanolol (Andersson, 1996), morphine (Andersson *et al*., 1995) and monosaccharide derivatives (Mayes *et al*., 1994). This monomer benefits from the presence of the methyl group, which probably limits rotation and conformational changes and also provides additional van der Waals interactions (as compared to acrylic acid). The imprinting of β-blockers (*S*-(–)-timolol) (Fischer *et al*.,

1991) and (–)-norephedrine (Suedee *et al*., 1999) was accomplished by the use of itaconic acid, the dimeric form of acrylic acid.

Among the basic monomers, the vinyl pyridines have been the most widely used for imprinting of *S*-naproxen (Kempe and Mosbach, 1994) and amino acid derivatives (Kempe *et al*., 1993; Kempe and Mosbach, 1995). The molecular complexes between these compounds and vinyl pyridines involve the electron-rich π-electron ring systems, which provide the strong interactions with electron-deficient aromatic forms, as well as acid-base interactions and H-bond acceptance. Other basic monomers include the tertiary amino monomers, like diethylaminoethyl methacrylate (DEAEM) and dimethylaminoethyl methacrylate (DMAEM). These have been effective in the imprinting of atrazine (Piletsky *et al*., 1995) and the imprinting of acidic templates (Simon and Spivak, 2004), respectively. Nevertheless, the results yielded by the use of the above monomers were less satisfactory than the ones for acidic monomers, probably because they are found as not sufficiently charged once polymerised (Piletska *et al*., 2008). Among the neutral monomers which generated effective imprints are acrylamide which was successfully used in the imprinting of amino acid derivatives (Yu *et al*., 1997) and *N*,*N*-methylene bisacrylamide (MBAA).

MIPs offer several advantages over other synthetic receptors:

i. High affinity and selectivity, sometimes similar to those of natural receptors or even higher.
ii. Much high stability, superior to that of natural biomolecules.
iii. Low cost, simplicity of their preparation and the ease of adaptation to different applications.

There have been several attempts to develop generic procedure for MIP design however, the one that has been in prime focus recently is a computational design.

2.8 Molecular modeling and computer simulation: tools for the design of MIPs

In principle, the wide range of functional monomers, which are currently available, makes it possible to design specific MIP for any type of stable chemical compound. However, in practical terms, the selection of suitable functional monomers for polymer preparation is not trivial. The problem lies in the technical difficulty of carrying out detailed thermodynamic calculations on multi-component systems and the amount of time and resources required for the combinatorial screening of polymers. Considering even a simple two-component combination of 100 monomers, it would be a daunting task of producing and testing several thousand polymers. This task gets further complicated by the possibility that these monomers could be used in monomer mixtures in different ratios. The scientists working in the molecular imprinting field used to select the functional monomers depending mainly on their personal empirical experience and visual complementary of the molecules, e.g. used basic monomers if the template had some acidic functionality. Not surprising that this approach is possible only if the template has some obvious functional determinants like carboxylic- or amino- groups. It made methacrylic acid as one of the most popular and often used functional monomer in the history of molecular imprinting. It is much less obvious what monomers to select in case if the molecule of interest does not have distinctive acidic or basic functional groups (e.g. DDT, cholesterol or homoserine lactone). The need for the rational selection of the functional monomers inspired Cranfield to develop the technology which could help making a primary

selection of the most affinity or less affinity (depending on the task) monomers (Piletsky *et al*., 2001). The commercial software which was designed and used by pharmaceutical industry for the design of the peptide ligands was adopted for the screening and selection of the best affinity monomers towards compounds of interest such as AHLs (acyl homoserine lactones), which are the target compounds in this work.

The most known commercially available software used for the *de novo* design of receptors are Quanta/Charmn/MCSS/HOOK (MSI, San Diego, USA), Sybyl (Tripos, San Diego, USA), InsightII/Ludi (MSI, San Diego, USA) and Cerius 2 (MSI, San Diego, USA). In this work, Sybyl 7.0 (Tripos, San Diego, USA) was applied for the computational analysis.

Among the theoretical methods which allow predicting the molecules geometry, Molecular Mechanics (MM) (often called Force Field method) is one of the most commonly used. To facilitate calculations, MM considers molecules as a collection of points (atoms) connected by strings (bonds) with different elasticities (forces constant). The force holding the atoms together can be described by potential energy functions of structural features. The force field is a combination of these potential energy functions. MM always involves both structure and energy because of their intimate connection. To obtain the structure, it is necessary to evaluate the energy in order to find where energy minima occurs (Tripos). Tripos Force Field was used in this study.

In Tripos Force Field (Clark *et al*., 1989) the energy, E, of a molecule is given by the sum of the following energy contributions:

$$E_{tot} = \Sigma E_{str} + \Sigma E_{bend} + \Sigma E_{oop} + \Sigma E_{tors} + \Sigma E_{vdw}$$

where the sums extend over all bonds, bond angles, torsion angles and non-bonded interactions between atoms not bound to each other or to a common atom.

E_{str} energy of a bond stretched or compressed from its natural bond length

E_{bend} energy of bending bond angles from their natural values

E_{oop} energy of bending planar atoms out of the plane

E_{tors} torsional energy due to twisting about bonds

E_{vdw} energy due to van der Waals non-bonded interactions.

Energy calculations could be made with the MAXIMIN2 molecular mechanics proGram (Labanowski *et al*., 1986) using the Tripos force field. MAXIMIN2 is a function in Sybyl for optimising the geometry of a molecule. It uses a molecular mechanics approach. An atom by atom SIMPLEX technique is utilised to decrease the internal strain to acceptable limits. Then all the atoms are optimised simultaneously through the method of conjugate gradients. The method requires first derivatives and they are assessed analytically. Distance, angle and range constraints may be used.

LEAPFROG algorithm, a component of the Sybyl software package, is a second generation *de novo* drug discovery program that allows the evaluation of new, potentially active ligand molecules on the basis of their binding score. This is calculated with electrostatic/steric screening by applying repeatedly different ligands (one at a time) in different positions of the template and then either keeping or

discarding the results. Structures of ligands are saved in a Sybyl database and are referenced by a Molecular Spreadsheet containing the LEAPFROG binding energies.

At present, the molecular modelling of such complex systems such as molecularly imprinted polymers, is limited to modelling of the molecular complex between the template and functional monomers. The main paradigm of molecular imprinting can be described in statement that "the strength and type of interactions, existing between monomers and template in monomer mixture will determine the recognition properties of the synthesised polymer". The assumption is that the monomer-template complexes will survive the polymerisation stage and their structure will be preserved in the synthesised polymer. Importantly, the numerous testing performed in Cranfield (Piletsky *et al*., 2001; Karim *et al*., 2005) showed that some correlation exists between the strength of the molecular complex and the binding characteristics of the prepared polymers in the chromatographic and equilibrium conditions. Consequently, the computer modeling could be considered as a good starting point for any project.

The interactions between monomers and template can be evaluated and used for rational selection of monomers for polymer design. Although approximately 4000 different polymerisable compounds as potential functional monomers have been reported, in reality many of them have similar properties and structures. As an assumption, it might be sufficient to test possible interactions between a minimal library of functional monomers (20-30 compounds) and a target molecule. The design of a virtual library of functional monomers and its screening against target templates is relatively easy to perform. The library contains 22 functional monomers commonly used in molecular imprinting which possess polymerisable residues and residues able to interact with a template through ionic and hydrogen bonds, van der Waals' and

dipole–dipole interactions (Figure 2.14). Initial selection of the monomers is based upon the strength of the interactions with template, according to the equation referred earlier (page 49). The LEAPFROG algorithm was developed for such tasks and it is particularly useful for selection of monomers with high affinity to the template.

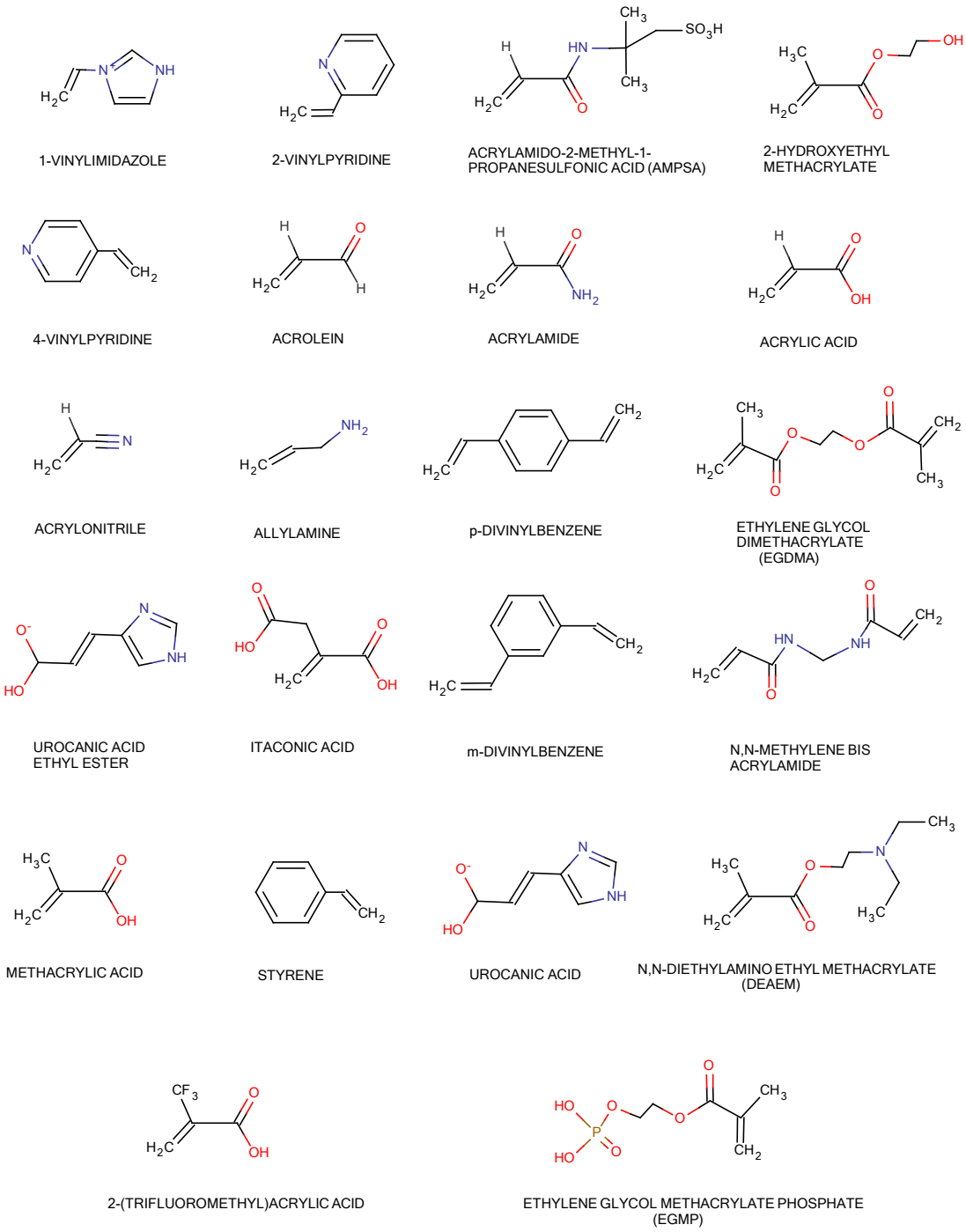


Figure 2.14: The functional monomers contained in the virtual library (Piletsky *et al.*, 2001)

One of the fundamental steps in the computer-aided rational design is the application of simulated annealing process, which could be used for the optimisation of MIP composition. Simulated annealing is a general purpose global optimisation technique for very large combinatorial problems which was pioneered by Kirkpatrick and co-workers (Kirkpatrick *et al.*, 1983). In their paper they report the following definition of simulated annealing applied at a generic system: *"The simulated annealing process consist of first 'melting' the system being optimised at a high effective temperature, then lowering the temperature by slow stages until the system freezes and no further changes occur. At each temperature, the simulation must proceed long enough for the system to reach a steady state."*

Simulated annealing which originates from statistical mechanics (Pannetier, 1990) has been used to a wide range of engineering optimisation problems in the areas like image processing, circuit placement, pollution control, even deployment of missile interceptors.

Practically, simulated annealing process is a type of molecular dynamics experiment which is used to obtain several different low energy conformations of a single molecule or a system of molecules, for example, a template surrounded by monomers.

In this work simulated annealing was applied to simulate the pre-arrangement of the functional monomers with the template in the monomer mixture prior to polymerisation, in order to determine the optimal monomers and ratio of the template and the monomers for MIP preparation.

In practice, computational simulation was performed in order to determine the best monomers that can form the molecular interactions with microcystin-LR (Chianella *et al*., 2002), creatinine (Subrahmanyam *et al*., 2001), ochratoxin A

(Turner *et al*., 2004), cocaine, deoxyephedrine, methadone and morphine (Piletska *et al*., 2005b), biotin (Piletska *et al*., 2004), abacavir (Chianella *et al*., 2006), tylosin (Piletsky *et al*., 2004), simazine (Piletska *et al*., 2005c), carbaryl (Barragán *et al*., 2007), triazines (Breton *et al*., 2007) and ephedrine (Piletsky *et al*., 2001).

In the case of the microcyctin-LR modelling (Chianella *et al*., 2002), it was concluded that the most stable complex was formed with one molecule of microcyctin-LR, one molecule of 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA) and six molecules of urocanic acid ethyl ester (UAEE) (Figure 2.15).

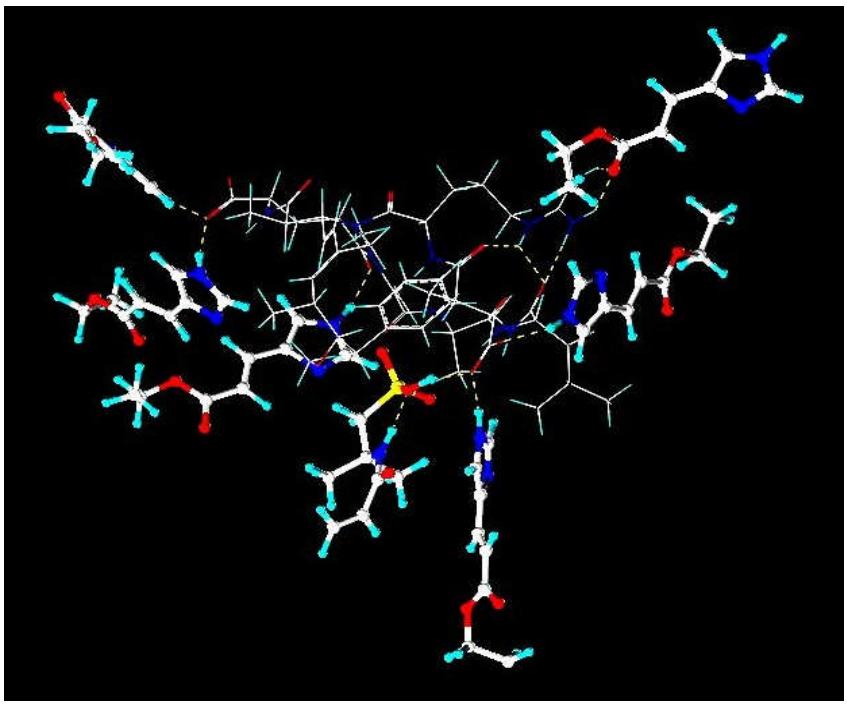


Figure 2.15: Interactions between microcystin-LR and monomers. Microcystin-LR, in balls and sticks in the center of the picture, interacts with six molecules of urocanic acid ethyl ester (UAEE) and 1 molecule of AMPSA (Chianella *et al*., 2002).

Two MIPs were then synthesised, one using the ratio obtained from the computer modelling, and the other using a "traditional" functional monomer methacrylic acid (MAA). Thereafter the synthesised polymers were tested for their

affinity for the target toxin by using ELISA (enzyme-linked immunosorbent assay). The affinity of the computationally MIP was found to be comparable to those of polyclonal antibodies, while superior chemical and thermal stabilities were obtained compared with those of biological antibodies. The computationally designed MIP also showed higher affinity than that of the MAA-MIP. Moreover, it was found that both MIPs had much lower cross-reactivity for microcystin-LR analogues than both polyclonal and monoclonal antibodies.

In another example of rational design using the protocol mentioned above (Piletsky *et al.*, 2001), our group demonstrated the proof of concept where the screening of the virtual library of monomers led to the optimised MIP composition specific for creatinine (Subrahmanyam *et al.*, 2001). Once again the computationally-designed polymer demonstrated superior selectivity in comparison with MIP which was prepared using a "traditional" functional monomer MAA.

Another polymer for the mycotoxin ochratoxin A (OTA), which is produced by several *Aspergillus* and *Penicillium* species, was designed using this protocol (Turner *et al.*, 2004). A polymer was synthesised using MAA and acrylamide functional monomers which were chosen computationally due to their high binding scores towards OTA molecule. The polymer has shown strong binding to OTA in aqueous conditions. It was suggested that the binding mechanism depends critically on the conformation of the polymeric binding pockets in combination with weak electrostatic interactions.

Efficient MIPs for drugs of abuse were also developed by Piletska and co-workers (2005b) using computational modelling (Piletsky *et al.*, 2001). The polymers for four drugs of abuse: cocaine, deoxyephedrine, methadone and morphine were designed and synthesised. The best candidates for molecular imprinting of cocaine

were: itaconic acid (IA), MAA and acrylamide; for imprinting of deoxyephedrine- IA, MAA, acrylamide and hydroxyethyl methacrylate (HEM); for methadone- IA, MAA and HEM; and for morphine- MAA, IA and HEM. The monomer: template ratio was determined by molecular dynamics. Figure 2.16 shows molecular complexes for each template. The synthesised polymers demonstrated good recognition properties under similar conditions which make them suitable for application in a multisensor device where several different synthetic receptors could be integrated in the same unit and which is rarely possible for a set of different natural receptors.

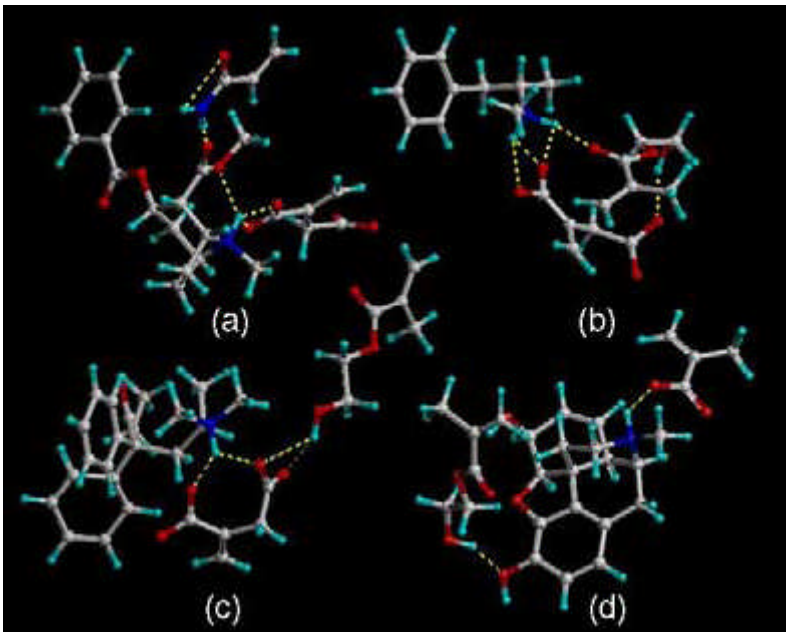


Figure 2.16: Molecular complex formed between: (a) cocaine, acrylamide and IA; (b) deoxyephedrine, IA and HEM; (c) methadone, IA and HEM; (d) morphine, MAA and HEM, as predicted from molecular modelling (Piletska *et al*., 2005b).

An important aspect which still hinders the practical application of MIPs is their poor performance in polar media. Although it is desirable to achieve affinity separation and sensing in water, MIPs usually do not work equally well in aqueous media due to the disruption of hydrogen bonds and competition process between

solvent and template molecules for their binding to the polymer functional groups. A loss of polymer affinity also originates from the difference in the structure of the polymer's binding sites in organic solvent (traditionally used for polymer preparation) and in water due to the differences in the polymer swelling. In an attempt to develop MIPs, which would be compatible with water, biotin-specific polymer was designed using the computational screening (Piletska *et al.*, 2004). In order to mimic aqueous conditions, the energy minimisation of monomers and template was performed using dielectric constant of water (ε = 80). The results of the modelling indicated three monomers MAA, 2-(trifluoromethyl)acrylic acid (TFMAA) and AMPSA which formed a strong complex with the template molecule in water via ionic and hydrogen bonds (Figure 2.17).

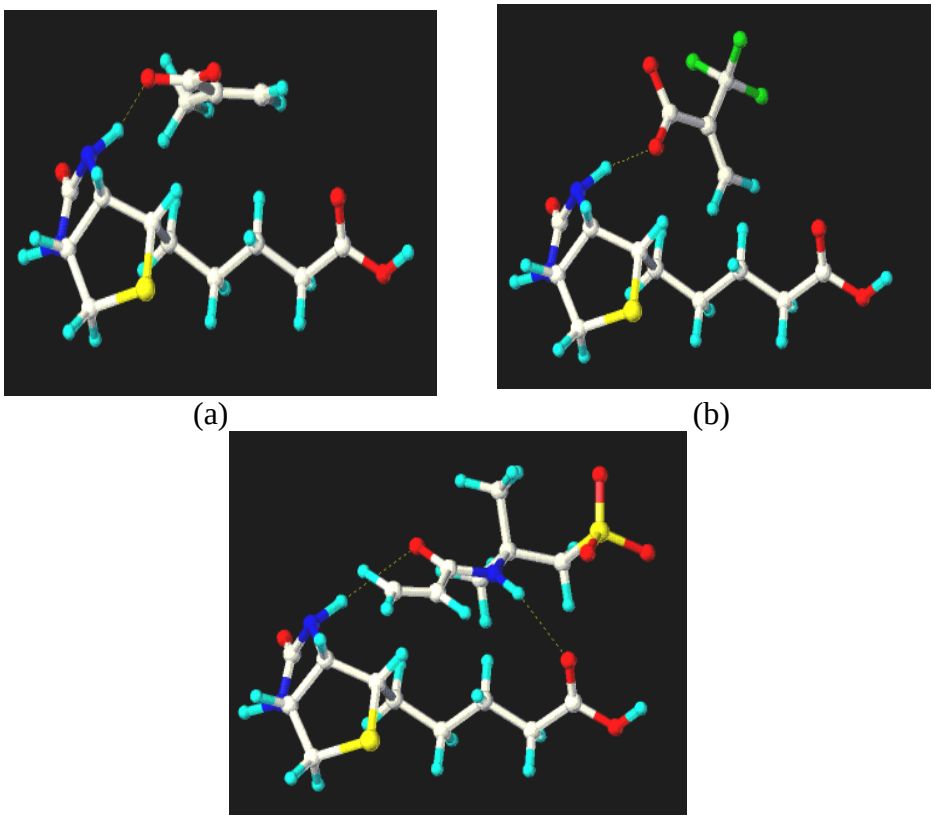


Figure 2.17: Computationally derived structures of biotin–monomer complexes: (a) biotin–MAA; (b) biotin–TFMAA; (c) biotin–AMPSA (Piletska *et al.*, 2004).

This was the first indication of the use of molecular modeling for rational selection of monomers capable of template recognition in water. The designed MIP was successfully grafted to the surface of polystyrene microspheres in aqueous environment using the benzophenone immobilisation method. The modified polymers demonstrated high affinity towards biotin in water.

Chianella *et al*. (2006) reported the development of MIP with high binding capacity for large scale solid phase extraction (SPE). The target analyte was abacavir, which is a HIV-1 transcriptase inhibitor. The MIP based on the functional monomer IA showed a surprisingly high binding capacity, up to 157 mg of drug/g of adsorbent in 50 mM Na-acetate buffer (pH 4.0).

The MIPs capable of controlled release of simazine in water for environmental control of algae were developed by Piletska (Piletska *et al*., 2005c). MAA and HEM were identified by LEAPFROG algorithm as two monomers which were showing the strongest binding to the template, which were used to produce polymers with different affinity and, correspondingly, different profile of the herbicide's release into environment. The speed of release of simazine was associated with the calculated binding characteristics. The high-affinity MAA-based polymer released ~2% and the low-affinity HEM-based polymer released ~27% of the template over 25 days.

Furthermore, our group succeeded in a design of a MIP for recognition of ephedrine enantiomers (Piletsky *et al*., 2001). The selected monomers giving the highest binding score in LEAPFROG were MAA, IA and HEM. The HEM-based MIP demonstrated a separation of ephedrine enantiomers with a separation factor of 1.42-2.09, bigger than those generally obtained with commercially available chiral phases.

In another study by Piletsky *et al*. (2004) a polymer specific for tylosin was synthesised. This MIP was examined for re-binding with the template and structural analogues such as tylactone, narbomycin and picromycin. HPLC analysis showed that the computationally designed polymer is specific and capable of separating the template from its related metabolites. The MIP was capable of recovering tylosin from crude broth extracts. This work demonstrated the ability of MIP "dialing" procedure – use of computational approach for the fast preparation of high-performance MIP for practical application. By using this approach it was possible to design MIP composition in 2-3 weeks, which is significantly quicker than to polymerise and perform a combinatorial screening of a multitude of polymers.

Other examples of the computationally-designed MIPs are polymers which were also prepared in Cranfield for photosynthesis-inhibiting herbicides: carbaryl (Barragán *et al*., 2007) and triazines (Breton *et al*., 2007). In the work by Breton *et al*. (2007) blank polymers were prepared using the monomers which were selected using computational modelling and tested in the SPE of herbicide from chloroform solutions. Interestingly, a good correlation was found between the binding score of the monomers and the affinities of corresponding polymers. This work also indicated that the computationally-designed blank polymers had a high affinity to the template which made them as potential substitutes for MIPs. In general, the screening of the functional monomers could be made by using the library of non-imprinted polymers (blanks). The results of such screening would allow selecting the most suitable functional monomer for the application under required experimental conditions. Since it did not require the template in the polymerisation mixture, this approach would be relatively easy and cheap to perform and the basic set of the polymers as a library

could be used for a screening of the functional monomers for a variety of templates and experimental conditions (Karim *et al*., 2005).

2.9 Design of the polymers for generation of the antimicrobial surfaces

It is well known that microorganisms can colonise artificial surfaces, such as heart valves, artificial joints, pacemakers, catheters, ventilator tubes and contact lenses that are used in a multitude of applications. Recent studies have shown that a wide range of medical device-related infections may be linked to the ability of infectious organisms to produce biofilms (Francolini *et al*., 2003).

The inherent resistance of biofilm-associated organisms to disinfectants and antibiotics hampers the eradication of biofilms using these agents (Costerton *et al*., 1999). Removal of biofilms is easier when they are directly accessible, as in the case of dental biofilms, which can be removed by mechanical cleaning in conjuction with the use of surface-active substances, like toothpastes. The strategies for controlling biofilm growth should be focused on preventing biofilm formation in the first place (Francolini *et al*., 2003).

As mentioned previously (section 2.7), the application of MIPs could provide a new tool in the attempt to eliminate biofilm growth on medical devices. In recent years, a series of different polymers and other antimicrobial chemicals have been tested in order to develop biomaterial surfaces which bacteria are unable to attach to, grow and colonise. The most important approaches are summarised as follow:

- Surfaces with non-covalently immobilised antimicrobial drugs
- Surfaces with covalently bound antimicrobial drugs

- Hydrophilic polymer coatings
- Negatively charged polymer coatings
- Antimicrobial polymers

Surfaces with non-covalently immobilised antimicrobial drugs

Immersion of a medical device into antimicrobial solution could be one of the simplest methods for loading antimicrobial drugs on its surface. The main problem associated with this technique is the limited affinity of biomaterials for such agents. Consequently, the limited concentration of the antibacterial agent that can be incorporated, it may result in a relatively short persistence of antibacterial action (Gorman and Jones, 2002). The additional coating of the medical devices with polymeric material could help adsorbing the antimicrobial agents on their surface using electrostatic interactions. For example, a coating of ciprofloxacin-containing liposomes sequestered in polyethylene glycol (PEG)-gelatin hydrogel seemed to inhibit completely *P. aeruginosa* adhesion to silicone catheter material (DiTizio *et al*., 1998).

Surfaces with covalently bound antimicrobial drugs

In order to achieve a permanent coating with long-term antibacterial activity, antimicrobial agents have been covalently attached to medical devices. The efficiency of this polymer-antibiotic system in terms of controlling biofilm growth depends on the device's ability to adsorb antibiotic molecules and to release them under clinically controlled conditions (Francolini *et al*., 2003). Importantly, prevention of the biofilm formation was achieved by attaching certain functional groups with antimicrobial activity, e.g. quaternary ammonium groups to the surface of the device. Fleming *et al*.

(1999) studied the influence of some specific functional groups on bacterial adhesion. Their data indicated that quaternary amino groups were the most active while the sulphonate and phosphonate groups were the less efficient in reducing bacterial attachment. The results of a following study (Grapski and Cooper, 2001) were consistent with this finding. In particular, experiments carried out on *S. aureus* showed that the quaternized polymers could kill up to 95% of adherent cells. The quaternary ammonium compounds have been shown to demonstrate a biocidal action through their ability to disrupt the bacterial cytoplasmic membranes but they appeared to be toxic to human cells (Nagamune *et al*., 2000).

Polymers able to absorb a high amount of the antibacterial agents on their surfaces have been also produced by Donelli *et al*. (2002). The authors showed that the introduction of the specific functional groups, which are able to interact with the drugs, to the side chain of urethane polymers allowed the production of biocidal polyurethane.

A secondary lichen metabolite possessing antimicrobial activity, (+)-usnic acid, was loaded into modified polyurethane and used against biofilm formation by either *S. aureus* or *P. aeruginosa*. (+)-Usnic acid-loaded polymers caused the inhibition of *S. aureus* biofilm by killing the attached cells, although they did not inhibit the initial bacterial attachment. Interestingly, the antibiotic-loaded polymers altered the morphology of the *P. aeruginosa* biofilm, suggesting that (+)-usnic acid interfered with QS pathways (Francolini *et al*., 2004). Recently, Wach *et al*. (2008) developed a natural product hybrid (Figure 2.18) composed of a siderophore and an antibiotic separated by a PEG linker that enabled the preparation of antimicrobial and cell-resistant surfaces.

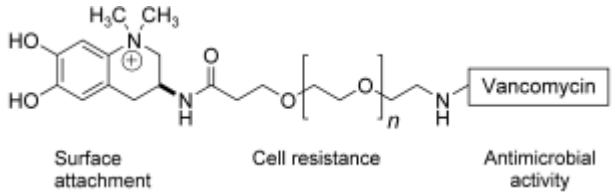


Figure 2.18: A natural product hybrid, which consists of an anachelin siderophore (that binds strongly to TiO_2 surfaces) and vancomycin (responsible for antibiotic activity) separated by a poly (ethylene glycol) (PEG) linker (which possesses antimicrobial and cell-resistant surface properties (Wach *et al*., 2008).

Hydrophilic polymer coatings

To prevent device-related infections increasing efforts have been made towards developing biomaterials with anti-adhesive properties. Chemical modifications of the polymer surfaces have improved either the anti-adhesive properties or biocompatibility of the existing biomaterials (Table 2.1).

Table 2.1: The polymers, which are employed in medical devices (Francolini *et al*., 2004a)

Polymers	**Patented names**
Poly (dimethyl siloxane)	Silastic (SR)
Polyethylene	Polyethylene, alkathene (PE)
Poly (ethylene terephtalate)	Mylar, melinex (PET)
Polypropylene	Propathene, prolene (PP)
Poly (tetrafluoro ethylene)	Teflon, fluon (PTFE)
Polyurethane	Texin, avcothane (PUR)

Based on experimental findings regarding the hydrophobic properties of bacteria (Van der Mei *et al*., 1988) hydrophilic polymer coatings were suggested as a

possible strategy for inhibiting bacterial adhesion. Studies performed either on hyaluronan (Bridgett *et al*., 1993) or poly-*N*-vinylpirrolidone (Morra and Cassinelli, 1999) coated polyurethane catheters showed a significant reduction in *S. epidermidis* adhesion.

Other experiments carried out by Baumgartner *et al*. (1997) demonstrated that the use of glycerophosphorylcholine as polymer chain extender in polyurethanes exhibited a strong effect on cell adhesion. In particular, hydrophilic surfaces were less colonised by cells than hydrophobic ones. This phenomenon is attributed to the increased tendency of the phosphonate polyurethane to absorb water.

In another study (Park *et al*., 2002) of *S. epidermidis* adhesion on poly (ethylene oxide)-based multiblock copolymer/ segmented polyurethane blends similar findings were obtained. The hydrated chains provided steric hindered barrier that minimised non-covalent interactions and reduced the number of adherent bacteria.

It is highly desirable for many applications to obtain polymer coatings that are capable of resisting long-term biofilm formation whilst maintaining non-fouling properties. In order to achieve this, a commonly used method is to graft the surfaces with PEG derivatives and, more recently, zwitterionic polymers. PEG-coated surfaces have been found to delay biofilm formation for 24 h (Johnston *et al*., 1997; Roosjen *et al*., 2004; Nejadnik *et al*., 2008; Wagner *et al*., 2004). It has been also shown that surfaces coated with 2-methacryloyloxyethyl phosphorylcholine (MPC) or its copolymers can decrease cell adhesion over 90% compared to untreated control surfaces over a 24 h period (Hirota *et al*., 2005; Patel *et al*., 2005; Fujii *et al*., 2008). Finally, results from a very recent study (Cheng *et al*., 2009) showed that glass surfaces grafted with zwitterionic poly (carboxybetaine methacrylate) (pCBMA)

suppressed *P. aeruginosa* and *P. putida* biofilm accumulation by more than 90%, with respect to uncoated glass cover slips.

Negatively charged polymer coatings

Preparing polymers with negative surface charge is an alternative approach for minimising bacterial adhesion (Jansen and Kohnen, 1995). Most bacteria carry a net negative surface charge at physiological conditions. Therefore, negatively charged biomaterials on the surface discourage adhesion, while positively charged ones facilitate it.

Antimicrobial polymers

It was shown that implantable, self-healing biomaterials that degrade into bioactive molecules could be a useful tool against bacterial colonisation. In particular, poly (anhydride-esters) composed of non-steroidal anti-inflammatory drugs that biodegrade to salicylic acid (SA) were found to possess anti-adhesive properties. Biofilm accumulation studies revealed a dramatic reduction in *P. aeruginosa* biofilm formation on the surfaces covered with salicylate-based polymer as compared to a control polymer that does not release SA upon biodegradation. What is more, by using a recombinant *P. aeruginosa* strain containing a fluorescent reporter gene, it was shown that salicylate-based polymer suppressed biofilm formation by inhibiting the *las* QS system (Bryers *et al*., 2006).

2.10 Molecularly Imprinted Membranes (MIMs)

One of the potential formats of the practical application of the specific polymers which are able to attenuate the QS is grafting them on the commercial

membranes. The introduction of the layer which would prevent the formation of the biofilms and biofouling of the membranes could be very important for the variety of practical applications. This part of the project is supported by prestigious collaboration grant funded by British Council in Germany in order to establish and support the collaboration between British and German scientists. Prof Piletsky group and Prof Ulbricht group (Universität Essen, Germany) are partners in this project.

In the frame of the project the task of the German group was to develop "tailored" MIM (molecularly imprinted membrane) adsorbents via surface-selective grafting onto base membranes with suitable pore structures. This goal was based on the promising results which have been achieved with respect to novel surface-selective grafting methods which enable the synthesis of highly specific MIPs from organic solvents (He and Ulbricht, 2006). Within the frame of the project our group worked on the development of polymeric membranes resistant to biofouling and biofilm formation, which at present represents a significant challenge for the application of membrane technology. Our task was based on the feasibility study performed by our group towards the development of polymers capable of the specific adsorption of the signal molecules used by bacteria for cell-cell communication (QS). The removal of the signal molecules from the system is leading to disruption of QS which prevents the expression of the virulence factors and the formation of biofilms. Our goal was to design materials with specificity for a number of different signaling molecules (for example: members of homoserine lactone family). In the frame of the collaboration with German partners the membranes based on computationally designed polymer compositions were prepared and tested for the adsorption of the corresponding signal molecules and disruption of QS.

A membrane is an interphase between two adjacent phases acting as a barrier, at the same time separating a system into compartments and regulating the exchange between these compartments. The driving force of the material transport is given by concentration, pressure, electrical or chemical gradient across the membrane. The main advantages of membrane technology lie in the unique separation principle, i.e. the transport selectivity of the membrane.

Synthetic separation membranes can be either porous or non-porous. The selectivity of porous membranes is based on size; examples of membrane separation are dialysis, ultra-filtration or micro-filtration. For non-porous membranes, permeability and selectivity depend on a solution-diffusion mechanism; examples of membrane separation in this case are gas separation, reverse osmosis, or pervaporation.

A molecular imprinted membrane (MIM) is a membrane either composed of MIP or containing MIP. A high membrane performance relies on high selectivity and permeability. The function of the membrane depends also on its well-defined morphology with respect to barrier pore size and layer topology, especially the thickness of the barrier layer.

In order to prepare MIMs three main strategies can be envisioned:

1. sequential approach - preparation of membranes from previously synthesised “conventional” MIPs (i.e. particles),
2. formation of “self-supported” MIMs,
3. grafting approach - preparation of MIPs on support membranes with suited shape (Ulbricht, 2004).

For strategy (1), only few attempts have been reported yet. A promising example is a “flat three-dimensional” arrangement of MIP nanoparticles as a filter cake between two microfiltration membranes (Lehmann *et al*., 2002).

For strategy (2), it was necessary to achieve macroscopic continuity – the “well-defined morphology” – and MIP formation in one step. Two main routes towards MIM preparation had been used, the “traditional” *in situ* radical polymerisation and the “alternative” polymer solution phase inversion, both in the presence of templates (Sergeyeva *et al*., 1999; Sergeyeva *et al*., 2007; Yoshikawa *et al*., 1999; Yoshikawa *et al*., 2001).

For strategy (3), the aim was to cover the support membrane externally or internally with a well-defined thin MIP film (Ulbricht, 2004).

Examples of MIM preparation as well as some membrane separations achieved using strategies (2) and (3) are outlined in the following paragraphs.

Preparation of self-supported MIMs by *in situ* radical polymerisation

In an early attempt, MIMs were prepared by a radical polymerisation of a mixture of acrylamide and acrylates including a photo-isomerisable functional acrylate (Marx-Tibbon and Willner, 1994). The authors observed that the MIM possessed a “poor mechanical stability”. Mathew-Krotz and Shea (1996) also prepared free-standing MIMs by thermally initiated radical copolymerisation of the one of the “standard” monomer mixtures (MAA/EDMA) for molecular imprinting. Scanning electron microscopy (SEM) revealed a regular porous structure, which was consisted of 50-100 nm diameter nodules. Apart from low mechanical stability, these membranes demonstrated extremely low fluxes, possibly, as consequences of high levels of cross-linking agent.

Sergeyeva *et al*. (1999) achieved a significant improvement of the mechanical stability of the self-supported MIMs by using oligourethane-acrylate macromonomer in *in situ* imprinted polymerisation mixtures with a thickness between 60 and 120 μm. MIMs were then tested as a recognition element of an atrazine-sensitive conductometric sensor. The conductivity response to atrazine was more than six times higher than for other triazine herbicides.

A step towards higher membrane permeability was to use polyester as a macromolecular pore former along with a radical copolymerisation of styrene monomers in a study by Kimaro *et al*. (2001). SEM and permeation analysis showed that "trans-membrane channels" were obtained, induced by the presence of the removable polyester from the MIM.

Recently, Sergeyeva and co-workers synthesised a new type of self-supported porous MIMs specific for atrazine using the principle of formation of semi-interpenetrating polymer networks (semi-IPNs) (Sergeyeva *et al*., 2007). Addition of linear polymers (polyethylene glycol and polyurethane) to the initial mixture of monomers before the polymerisation (Sergeyeva *et al*., 1999) resulted in high flux of the solvents through the MIMs. Again, the membranes imprinted with atrazine demonstrated substantially higher affinity to this herbicide as compared to structurally similar compounds.

Preparation of self-supported MIMs by polymer solution phase inversion (PI)

Polymer solution film casting and subsequent phase inversion can be applied in molecular imprinting. Instead of an *in situ* radical polymerisation, the solidification of a polymer is used. Yoshikawa and co-workers used specifically synthesised polystyrene resins with peptide recognition groups, in a blend with a matrix polymer,

for the MIM formation via a “dry PI” process, i.e. the polymer solidification was achieved by solvent evaporation (Yoshikawa *et al*., 1999; Yoshikawa *et al*., 2001). Importantly, the permeability was much higher for the MIM as compared with the blank membrane. Alternatively, Kobayashi and co-workers used functional acrylate copolymers for a “wet PI” process yielding asymmetric porous membranes (Kobayashi *et al*., 1995; Wang *et al*., 1996; Wang *et al*., 1997). In this case, polymer solidification was achieved by precipitation induced via contact with a non-solvent.

The formation of porous MIMs as a combination of a matrix polymer (for creating a permanent pore structure) and a functional polymer (for providing binding recognition sites) could provide even more alternatives (Ramamoorthy and Ulbricht, 2003). Moreover, polyethylene glycol was found to be compatible with the polymer blend casting solution and was subsequently used as pore forming agent, which increased the membrane permeability. MIMs which were prepared from cellulose acetate as the matrix polymer and sulfonated polysulfone as the functional polymer and using Rhodamine B (Rh B) as template and were tested as membrane adsorbers. MIMs possessed very high binding capacity, up to 72% of the applied Rh B (Ramamoorthy and Ulbricht, 2004).

It is remarkable that most MIMs prepared via phase inversion imprinting had acceptable binding performance in aqueous media. However, such MIMs were not stable in organic environment where swelling and chain rearrangement seemed to “erase” the imprinted information (Kondo and Yoshikawa, 2001). Simultaneous formation of the pore structure and imprinting sites in the phase inversion MIM becomes a significant limitation, since different optimal conditions for that are required (Ulbricht, 2004).

Preparation of composite MIMs

Composite membranes allow adjusting membrane pore structure and MIP recognition sequentially and by two different materials.

In the earliest attempts, MIP monomeric mixtures (e.g., MAA/EDMA) were polymerised in mm-thick glass filters to fill their pores. This approach was extended to other templates as well as to a comparison of covalent and non-covalent approach (Piletsky *et al*., 1990; Piletsky *et al*., 1998; Piletsky *et al*., 1999).

It was found that in order to maintain the membrane permeability, only thin layer of specific MIPs could be grafted to the membrane surface. In particular, Hong *et al*. (1998) performed *in situ* photoinitiated radical copolymerisation of a MAA/EDMA mixture on the surface of an alumina membrane with an asymmetric 20 nm pore size. Also, a commercial cellulose dialysis membrane was used as matrix for a two-step grafting procedure yielding a MIP by *in situ* copolymerisation into the pores of a symmetric micro-filtration membrane (Hattori *et al*., 2001).

Composite MIMs have been prepared by modifying commercially available polymeric membranes, substantially reducing the preparation time and enhancing the membranes permeability. Molecularly imprinted ultrafiltration membranes have been generated in this way from porous polypropylene (Piletsky *et al*., 2000) and poly(vinylidene fluoride) (Sergeyeva *et al*., 2001; Kochkodan *et al*., 2001; Kochkodan *et al*., 2002) for the removal of desmetryn and other herbicides from aqueous solutions. The monomers used for the preparation of the imprinted polymers for the triazine herbicides were AMPSA and MBAA. The same methodology was used in the synthesis of MIMs for the extraction of terbumeton, another member of the triazine herbicides, from water (Sergeyeva *et al*., 2001).

Piletsky *et al*. (2000) discovered that using a coated hydrogen abstraction photoinitiator very thin MIP-layers, which were covalently anchored and covered the entire surface of the base membrane (Figure 2.20b). This approach, which can be used to any membrane polymer with C-H bonds, was first explored with benzophenone as photo-initiator and a membrane made from polypropylene as support. Furthermore, it was found that a previously prepared thin hydrophilic layer on the support membrane can have two functions: (i) matrix for the radical polymerisation and forming an IPN, (ii) minimising non-specific binding during SPE (Sergeyeva *et al*., 2001).

Taking advantage of the intrinsic photo-reactivity of the membrane polymer (e.g. polysulfone), is an alternative for the initiation of a surface polymerisation without the addition of photo-initiator (Figure 2.20a) (Ulbricht *et al*., 2002). This approach is limited to a few polymers and the efficiency is quite low. Another notable discovery was that it is possible to create thin MIP films also via a controlled polymer deposition process, e.g. the very fast photo-initiation of a radical polymerisation by α-scission photoinitiator (Figure 2.20c) (Kochkodan *et al*., 2001). The latter approach can in principal be applied to any membrane material.

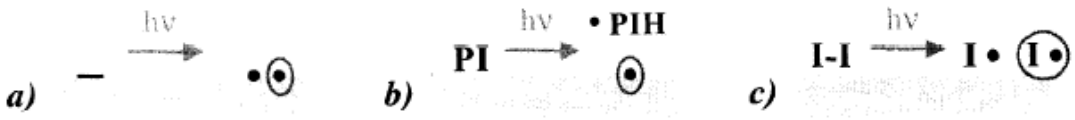


Figure 2.19: Different approaches for the photochemical initiation of a polymerisation yielding thin-layer MIPs on membrane surfaces: a) a direct UV excitation of the membrane polymer which produced starter radicals via chain scission; b) UV excitation of coated photoinitiator which produced starter radicals via hydrogen abstraction; c) UV excitation of a coated scission type photoinitiator which produced starter radicals close to the surface (Ulbricht *et al*., 2002).

CHAPTER 3

RATIONALLY DESIGNED POLYMERS FOR ATTENUATION OF *VIBRIO FISCHERI* QUORUM SENSING

3.1 Introduction

The process of quorum sensing (QS) (Section 2.2) was first discovered in the Gram-negative, bioluminescent bacterium, *Vibrio fischeri*. The expression of bioluminescence has been found to be highly dependent on cell density. This dependence is linked to a small molecule, namely *N*-(β-ketocapryloyl)-DL-homoserine lactone (3-oxo-C_6-AHL) (Eberhard *et al*., 1981). This chemical, commonly called the *V. fischeri* autoinducer, diffuses freely through the cell membrane (Kaplan and Greenberg, 1985). The bacteria harbour two proteins for regulation of the autoinducer: LuxI that directs the synthesis of the autoinducer and LuxR that controls transcription of the genes responsible for light production. Binding of the LuxR to the autoinducer results in three simultaneous phenomena: activation of the light-production gene, production of the enzyme luciferase and synthesis of LuxI (Kaplan and Greenberg, 1985; Kolibachuk and Greenberg, 1993). The bacterial luminescence reaction, which is catalysed by luciferase, involves the oxidation of the long-chain fatty aldehyde tetradecanal (RCHO) and luciferin ($FMNH_2$) with the liberation of excess free energy in the form of a blue-green light at 490 nm (Eberhard *et al*., 1981; Scheerer *et al*., 2006):

$FMNH_2 + RCHO + O_2$ ----> $FMN + RCOOH + H_2O$ + light (490nm)

When *V. fischeri* live freely in seawater, concentration of autoinducer is less than 10^2 cells/mL, and so it will diffuse out of the cells, resulting in no observable luminescence. However at the high cell densities found in the light organs of certain marine fishes and squids (10^{10} to 10^{11} cells/mL), autoinducer can accumulate, and luminescence is induced (Dunlap and Greenberg, 1991; Ruby and McFall-Ngai,

1992). The Hawaiian sepiolid squid *Euprymna scolopes* chose *V. fischeri* over all other species in the sea to be the specific bacterial symbiont to grow in its light organ (Ruby, 1996; McFall-Ngai, 1999). The symbiotic relationship between the squid and *V. fischeri* provides a remarkable example of specific cooperativity during the development and growth of both organisms. For example, once the newly hatched juvenile squid gets colonised by the bacteria, maturation of the nascent light organ begins leading to the development of the functional adult structure. Thus, there is a direct impact on the physical maturation of the squid's light organ as a result of its symbiotic relationship with *V. fischeri*. The luminescent bacteria are also advantageous to the squid by erasing the shadow that would normally be cast as the moon's rays struck the squid from above, thus protecting the squid from predators below. The squid, in turn, provides a stable source of nutrients for the bacteria (Montgomery and McFall-Ngai, 1994 and 1995).

V. fischeri was selected as the model system for this study. The aim was to produce rationally-designed polymers that recognise and specifically sequester the autoinducer of *V. fischeri*, 3-oxo-C_6-AHL. The bioluminescence characteristic for *V. fischeri* is a sensitive marker for the study of quorum sensing and bacterial communication (Kuttler and Hense, 2008).

3.2 Materials and Methods

3.2.1 Materials

The wild strain *of V. fischeri* ATCC 7744 was used throughout the study. *N*-(β-ketocapryloyl)-DL-homoserine lactone (3-oxo-C_6-AHL) and phosphate buffered saline (PBS), pH 7.4, were purchased from Sigma (Sigma-Aldrich, Gillingham, UK). Acrylamide (AA), *N*,*N*'-methylene bisacrylamide (MBAA), itaconic acid (IA),

methacrylic acid (MAA), *N*,*N*-diethylaminoethyl methacrylate (DEAEM), ethylene glycol methacrylate phosphate (EGMP), dimethylformamide (DMF), 1,1'-azobis (cyclohexanecarbonitrile), sodium chloride (NaCl) and polyacrylic acid were purchased from Aldrich (Sigma-Aldrich, Gillingham, UK). Methanol was obtained from Fisher, UK. Nutrient broth No.2 (NB) and Agar Bacteriological (Agar No.1) were purchased from Oxoid (Basingstoke, UK). Crystal Violet was obtained from Acros Organics (Fisher, Loughborough, UK).

3.2.2 Computational modelling

The molecular modelling was made using a workstation from Research Machines running the CentOS 5 GNU/Linux operating system. This system was used to execute the software packages SYBYL 7.0 (Tripos Inc., St. Louis, Missouri, USA). 3-oxo-C_6-AHL and each of the monomers were modelled by calculating the charges for each atom and the structures were refined using molecular mechanical (MM) methods applying an energy minimisation to a value of 0.01 kcal mol^{-1}.

The LEAPFROG algorithm was used to screen the library of functional monomers (Figure 2.14, Chapter 2) for their possible interactions with the template (3-oxo-C_6-AHL) resulting in a table, ranking the monomers with the highest binding score (kcal mol^{-1}) as the best candidates for polymer preparation.

3.2.3 Polymer preparation

Based on the computational modelling six functional monomers were selected for the preparation of SSPs: MBAA, DEAEM, IA, MAA, EGMP and AA. The composition of polymers is shown in Table 3.1.

Table 3.1: Polymer compositions (for abbreviations see Materials section).

Polymer	Masses of monomer, cross-linker, solvent and initiator (g, moles)			
	Functional monomer (g, moles)	**EGDMA (cross-linker)**	**DMF (solvent)**	**Initiator***
P1	20% MBAA (2, 0.013)	8, 0.04	10	0.1
P2	20% AA (2, 0.028)	8, 0.04	10	0.1
P3	20% DEAEM (2, 0.011)	8, 0.04	10	0.1
P4	20% EGMP (2, 0.01)	8, 0.04	10	0.1
P5	20% MAA (2, 0.023)	8, 0.04	10	0.1
P6	30% IA (3, 0.023)	7, 0.035	10	0.1
P7	5% IA (0.5, 0.004)	9.5, 0.048	10	0.1
P8	5%MAA (0.5, 0.006)	9.5, 0.048	10	0.1
P9	No functional monomer (0, 0)	10, 0.05	10	0.1

*1,1'-azobis (cyclohexanecarbonitrile)

Six polymers were prepared with high concentration (20% or 30%) of the functional monomers (P1 - P6), two polymers (P7 and P8) were prepared with reduced concentration (5%) of the functional monomers and one polymer (P9) was prepared without functional monomer. All polymers were prepared by thermal polymerisation at 80 ºC for 12 h. After synthesis polymers were ground, washed with methanol and sieved. The fraction with particles in the size range 38-125 μm was collected. Polymers were thoroughly washed in methanol using Soxhlet extraction, dried and used for SPE screening. The surface area of the polymers was characterised using Nova 1000e Series High Speed Gas Sorption Analyser (Quantachrome, Hartley Wintney, UK).

3.2.4 Screening of polymers against 3-oxo-C_6-AHL

100 mg of each polymer (P1 - P6) was packed in empty 1-mL capacity SPE cartridges between two polyethylene frits. In order to perform the screening of the polymers under physiological conditions 3-oxo-C_6-AHL was dissolved in PBS, pH 7.4. Cartridges were conditioned with 2 mL of water and loaded with 1 mL of 3-oxo-

C_6-AHL solution in PBS (1 mg mL^{-1}). The filtrate was collected and evaluated using a spectrophotometer by determining the absorbance at 201 nm.

3.2.5 Quantification of 3-oxo-C_6-AHL using HPLC-MS

In order to increase the sensitivity of the quantification of 3-oxo-C6-AHL the HPLC-MS method was optimised. HPLC separation was conducted using a Waters 2975 HPLC system equipped with Luna C18 (2) column (150 x 3 mm, 3 μm, Phenomenex). Mobile phase: water (A), methanol (B) in a binary system with 0.1% of formic acid as an additive. The gradient elution conditions were as follows: 0 - 10 min - 100% solution A (water/0.1% formic acid), then a linear gradient to from 0 to 70% B over 20 min, immediately switching to 100% A for a further 10 min in order to wash the column before the next injection (total analysis time - 40 min). The measurement of the sample was performed between the 25 to 30 minutes of the run, at all other times liquid was diverted to waste using the "solvent delay" option. The flow rate was 0.2 μL min^{-1}; the injection volume- 10 μL; the column temperature- +20 ºC. Fragment of AHL *m/z* 102 was detected by mass- spectrophotometer Micromass Quatro Micro (Waters, UK) equipped with an ESI interface in positive ion mode (Morin *et al*., 2003). The MS parameters were following: desolvation gas- 850 L h^{-1}, cone gas- 50 L h^{-1}, capillary- 4.5 kV, cone-25 V, CE- 20, source temperature- +120 ºC, desolvation temperature- +350 ºC, collision energy- 25 V, multiplier- 650 V. The analysis of the samples was performed by Dr Elena Piletska.

3.2.6 Determination of binding capacity of itaconic acid-based polymers

The polymers which contained 30% of IA (P6), 5% of IA (P7) and without IA (P9) correspondingly were tested for adsorption of 3-oxo-C_6-AHL from PBS, pH 7.4 and from nutrient broth containing 2% NaCl (NB/NaCl). 100 mg of each polymer was packed into 1-mL SPE cartridge. 1-mL aliquots of 3-oxo-C_6-AHL solution in PBS or in NB/NaCl (1.5 mg mL^{-1}) were filtered through the polymer. In order to determine the polymer capacity the concentration of 3-oxo-C_6-AHL in each fraction was quantified using a spectrophotometer at 204 nm. The volume from which more than 50% of 3-oxo-C_6-AHL was adsorbed was considered as a "breakthrough" volume and used to evaluate the capacity of the resin. The adsorbed AHL was then eluted with 2 x 1 mL of acetonitrile and quantified using HPLC-MS.

3.2.7 Effect of polymers on growth and bioluminescence of V. fischeri

A single colony of *V. fischeri* ATCC 7744 was inoculated into NB/NaCl and incubated overnight with orbital shaking (Yellowline OS 2 basic shaker, Fisher) at 200 rpm at 25 ^{o}C. A 0.25 mL aliquot of an overnight cell culture was added to 10 mL of NB/NaCl. Polymer (200 mg of the P7 or P8 polymer) was also added where required. All cultures were incubated at 25 ^{o}C in 15 mL Falcon tubes with constant inversion. The growth of bacteria and the effect of polymers on growth were determined during 10 hours by optical density measurements at 600 nm after tenfold dilution with NB/NaCl, using a spectrophotometer (UVPC 2100, Shimadzu, Japan).

The development of bioluminescence by the bacterial suspension was studied in the presence and absence of the P7 and P8 polymers. The bioluminescence was measured every 2.5 h using a HY-LiTE® 2 luminometer (Merck, Darmstadt,

Germany). For the measurement all samples incubated for 2.5 h were diluted tenfold with NB/NaCl and the bioluminescence was measured. The samples which were incubated for 5, 7.5 and 10 h were diluted one hundredfold with NB/NaCl.

In order to prove that it was specifically the 3-oxo-C_6-AHL which was adsorbed from the cell culture and therefore unavailable to up-regulate bacterial luminescence, 100 mg of the P7 polymer was recovered from the cell culture. The polymer was packed in an SPE tube, washed with 3 mL of distilled water and dried on a vacuum manifold. The elution was carried out with 1 mL of acetonitrile. The sample was filtered through a 0.22 µm nylon filter (Phenomenex, UK) and analysed using HPLC-MS.

3.2.8 Biofilm study

A single colony of *V. fischeri* ATCC 7744 was inoculated into NB/NaCl and was incubated for 24 h at 25 ºC with reciprocal shaking at 200 rpm. The inoculum was diluted 100 times with NB/NaCl and, where required, 100 µM of 3-oxo-C_6-AHL in PBS was added. Portions of the diluted cell suspension (200 µL) (either with or without 3-oxo-C_6-AHL) were added to each microtiter plate well, which contained, where required 4 ± 0.1 mg of sterile P7 polymer. The colorimetric assay of biofilms with Crystal Violet staining was performed as described by Burton *et al* (2007). After 22 h incubation without shaking, the medium was gently removed and the microtiter plate wells were washed three times with 200 µL of PBS buffer (0.1 M, pH 7.4) using a multichannel pipette and the plate was allowed to dry for 15 min. The biofilm on the microtiter plate wells was stained with 200 µL of a 0.4% solution of Crystal Violet for 15 min. Then unbound Crystal Violet stain was removed and the wells were washed gently three times with PBS buffer. The wells were air-dried for 15 min and the

Crystal Violet in each well was solubilised by adding of 200 µL of 95% ethanol. The plate was read at 600 nm using a microtiter plate reader from Dynex (Dynex Technologies, USA).

3.3 Results and Discussion

3.3.1 Analysis of the molecular structure of 3-oxo-C_6-AHL

The primary structural features that can be identified in the 3-oxo-C_6-AHL molecule include the lactone functionality, the secondary amide group and the β-dicarbonyl function (Figure 3.1).

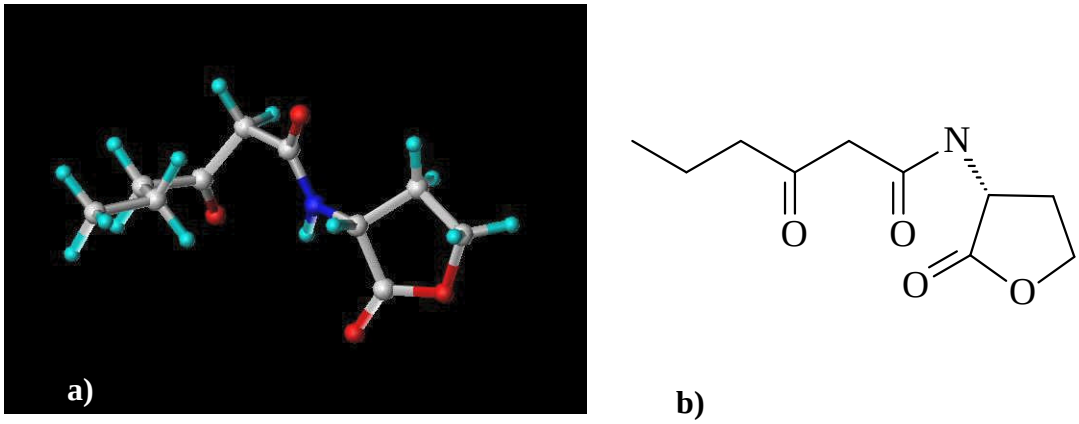


Figure 3.1: 3D molecular structure of 3-oxo-C_6-AHL which was computationally minimised in vacuum, b) 2D molecular structure of 3-oxo-C_6-AHL.

β-keto amide and (β-dicarbonyl) functional elements are rather rare in the literature describing the synthesis of affinity polymers, although isolated amide groups and to some extent keto-groups are common. The consensus is that acidic monomers (TFMAA or MAA) (Weiss *et al.*, 2003) are suitable for binding to keto-functional groups but the interactions tend to be weak. Both acidic monomers (MAA) (Lu *et al.*, 2004) and neutral amides (acrylamide) (Lepistö and Sellergren, 1989) were

mentioned in the literature for structures interacting with secondary amide groups, but two-centre bonds can easily be disrupted by conformational effects in the template. Acrylamide has been also used with lactone templates as the fixed geometry allows 2-centre H-bonding involving the -NH_2 function to be easily established (Zhu *et al*., 2006; Hu *et al*., 2005). Protonated acidic monomers, such as MAA and related compounds are also H-bond donors and can coordinate to either (or both) of the oxygen atoms of the lactone. Acrylamide is a common monomer for many of the structural features of 3-oxo-C_6-AHL; however safety issues may preclude its use in this application. Acidic monomers such as MAA may also prove useful, although conformational features of AHL will determine which monomer is most appropriate.

3.3.2 Computational screening

A molecular model of 3-oxo-C_6-AHL was designed and minimised to a value 0.001 kcal mol^{-1} (Figure 3.1). Then *in silico* screening of a library of functional monomers against 3-oxo-C_6-AHL was conducted. The monomers which demonstrated the highest binding scores represented the best candidates for polymer preparation, forming the strongest complexes with the target. The top six monomers with the highest binding energies were identified as acrylamide, MBAA, DEAEM, IA, EGMP and MAA (Table 2.2) and they were selected for polymer preparation.

Table 3.2: Results of the computational screening of the functional monomers and SPE screening of the polymers prepared with corresponding monomers (in the brackets are the names of the corresponding polymers).

Monomers	Binding energy, kcal mol^{-1}	Adsorption, %
MBAA (P1)	-28.23	87±4
AA (P2)	-27.32	88±1
DEAEM (P3)	-26.83	73±2
EGMP (P4)	-25.64	15±2
MAA (P5)	-15.33	80±5
IA (P6)	-25.44	79±1

It was necessary to highlight that AA, MBAA and IA monomers formed two-point interactions with 3-oxo-C_6-AHL and therefore were expected to demonstrate stronger interactions and higher affinity than DEAEM and EGMP which showed only single-point interaction during molecular modelling (Figures 3.2 and 3.3). The rational selection of the monomers was also influenced by the site of interaction with 3-oxo-C_6-AHL. In order to increase of the specificity of the binding for 3-oxo-C_6-AHL the preference was made for the monomers which could interact with functional groups close to the lactone ring. IA represented a good example of such a monomer (Figure 3.3) and was selected for further testing.

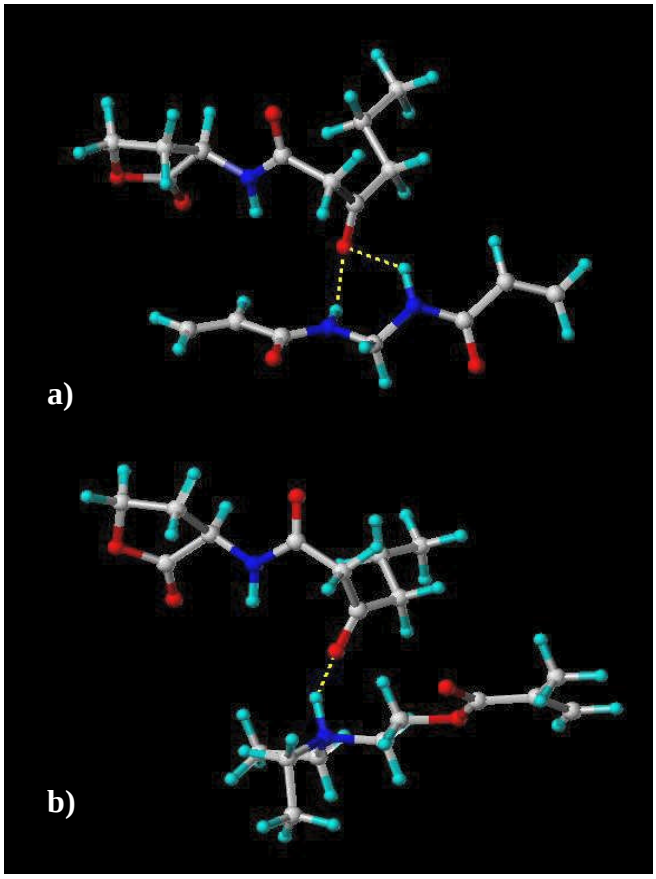


Figure 3.2: Complex of 3-oxo-C_6-AHL with MBAA (a) and DEAEM (b).

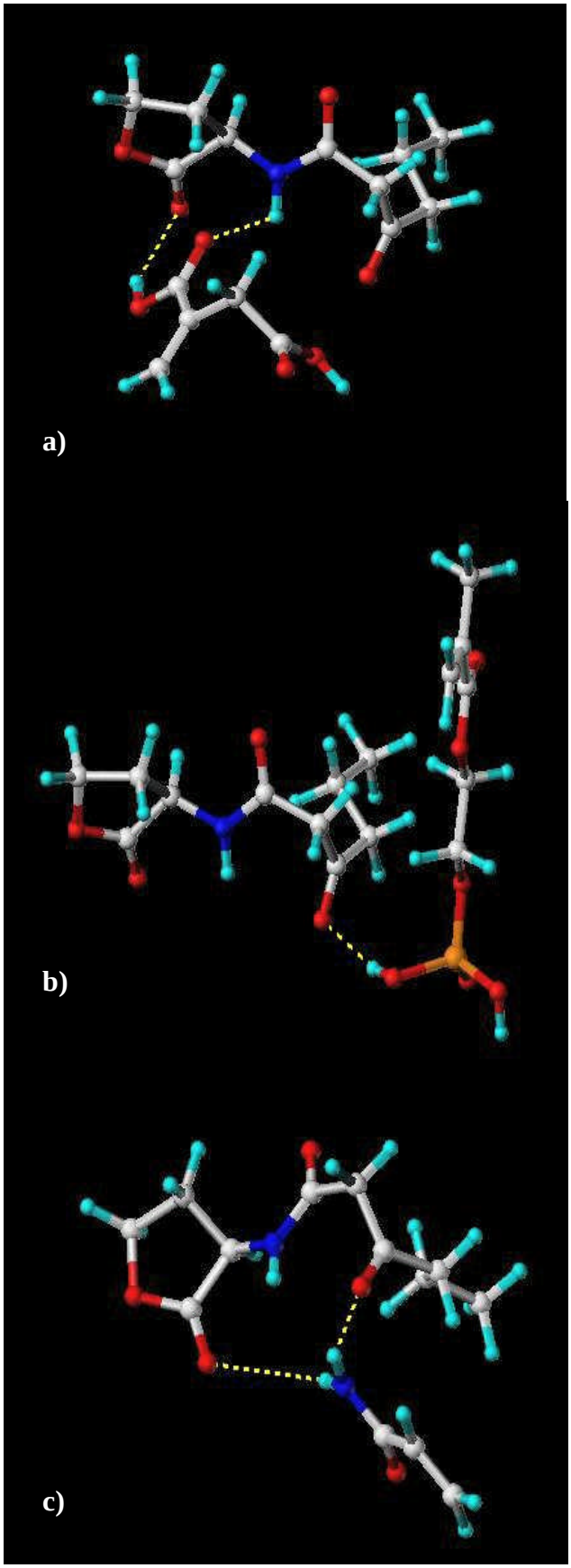


Figure 3.3: Complex of 3-oxo-C_6-AHL with IA (a), EGMP (b) and AA (c).

3.3.3 *In vitro* screening of polymers for adsorption of 3-oxo-C_6-AHL

The 3-oxo-C_6-AHL solution in PBS was filtered through the polymer cartridges packed with different polymers (P1 - P6) and the concentration of 3-oxo-C_6-AHL in the filtrate was measured. It was found that AA, MBAA, MAA and IA-based polymers demonstrated the highest retention of 3-oxo-C_6-AHL (88%, 87%, 80% and 79%, correspondingly, Table 2.2). Both screenings, *in silico* and by filtration, were in a good agreement with each other and with literature references discussed earlier. Due to the perceived high toxicity of acrylamide and of acrylamide derivatives, such as MBAA, which could preclude polymers containing these monomers from some potential applications, they were not considered for further development and IA-based polymers were therefore selected for the continuation of the testing.

3.3.4 Determination of binding capacity of itaconic acid-based polymers

Three polymers containing 30%, 5% IA and control polymer (P6, P7 and P9, respectively) were tested for adsorption of 3-oxo-C_6-AHL from PBS and NB/NaCl. NB/NaCl was selected for testing because it was used as the growth medium for *V. fischeri*. It was necessary to evaluate if such rich media as NB would hinder the binding of 3-oxo-C_6-AHL to the polymer. The polymer made without functional monomer (P9) was used as a reference polymer in order to estimate the specificity of binding.The capacity of the polymers was also evaluated accordingly to their surface area (Table 3.3).

Table 3.3: The binding capacity of the polymers towards 3-oxo-C_6-AHL in PBS and NB. Key: P6, P7 and P9 polymers based on 30% IA, 5% IA and 100% EGDMA.

Polymers	Surface area, $m^2\ g^{-1}$	Capacity in PBS		Capacity in NB	
		mg per g of polymer	µg per surface area, µg m^{-2}	mg per g of polymer	µg per surface area, µg m^{-2}
P6	215.7	30.5±4.5	141.4±20.9	26.5±3.5	122.8±16.3
P7	303.9	45.5±1.5	149.7±5.1	46.3±5.7	152.5±18.8
P9	353.8	48.5±1.5	137.1±4.4	37.3±2.7	105.4±7.6

The P7 polymer based on 5% IA demonstrated the highest binding capacity per surface area (~150 µg m^{-2}) in comparison with P6 polymer which contained 30% IA (binding capacity- 122 µg m^{-2}) and P9 polymer which was made without functional monomer (binding capacity- 105 µg m^{-2}). It was observed that the adsorption of 3-oxo-C_6-AHL from NB/NaCl was indeed reduced in comparison with adsorption from PBS. The decrease in polymer binding capacity towards 3-oxo-C_6-AHL could be explained by higher non-specific adsorption of other components of the nutrient broth by the polymer. The binding capacity of P9 polymer towards 3-oxo-C_6-AHL in NB/NaCl was reduced by 24% in comparison with adsorption from PBS. This polymer demonstrated very high non-specific adsorption of hydrophobic components of the nutrient broth which led to a reduction of the polymer capacity and prevention of the binding of 3-oxo-C_6-AHL. At the same time the P7 polymer (5% of IA) adsorbed as much 3-oxo-C_6-AHL in NB/NaCl as in PBS and it was selected for further testing.

3.3.5 Effect of polymers on growth and bioluminescence of *V. fischeri*

The effect of the P7 (5% IA) and P8 (5% MAA) polymers on the growth of *V. fischeri* was tested by measuring the optical density of the suspension over a 10-h

period. It was found that none of the polymers inhibited the growth of *V. fischeri* (Figure 3.4) indicating that the polymers were not toxic nor were they depleting nutrients from the media.

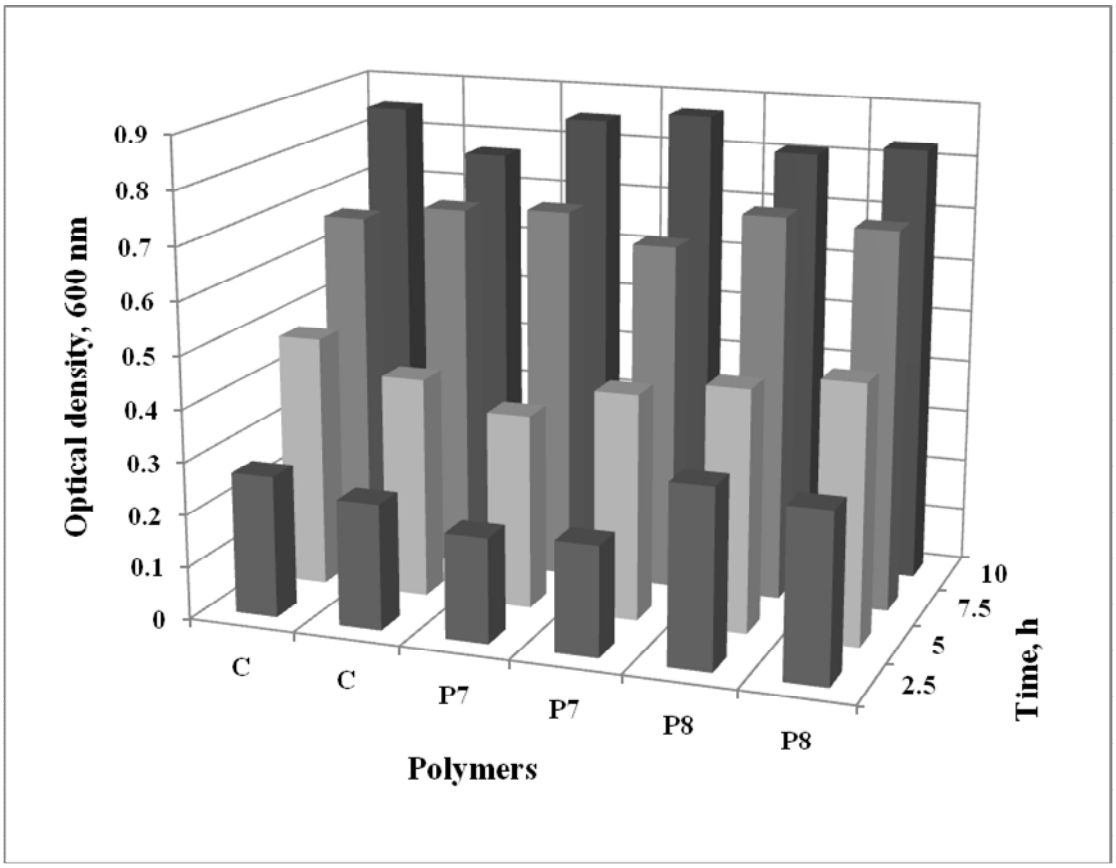


Figure 3.4: The effect of the IA- and MAA-based polymers on the growth of *V. fischeri*. P7-5% IA-based polymer, P8- 5% MAA-based polymer, C- control without polymers.

In parallel each sample was analysed on development of the bioluminescence, a phenotype of *V. fischeri* which is controlled by quorum sensing. The bioluminescence of the bacterial suspensions was monitored in the presence and absence of polymers. Figure 3.5 shows that the significant development of the bioluminescence in the control bacterial suspension which was growing without addition of the polymers took place between 5 h and 7.5 h. At the same time the bioluminescence of the samples which contained polymers was substantially reduced

(Figure 3.5). The *V. fischeri* culture which was incubated with either the P7 or P8 polymer for 10 h showed marked reductions in bioluminescence equal to 85 ± 8% and 84 ± 6% respectively. Since growth was not affected, this could be due to the sequestration of the signal molecule (3-oxo-C_6-AHL). Acrylic acid and activated charcoal (non-specific absorbent) were also used in this work. It was shown that the former did not have any effect on either growth or bioluminescence while the latter inhibited both.

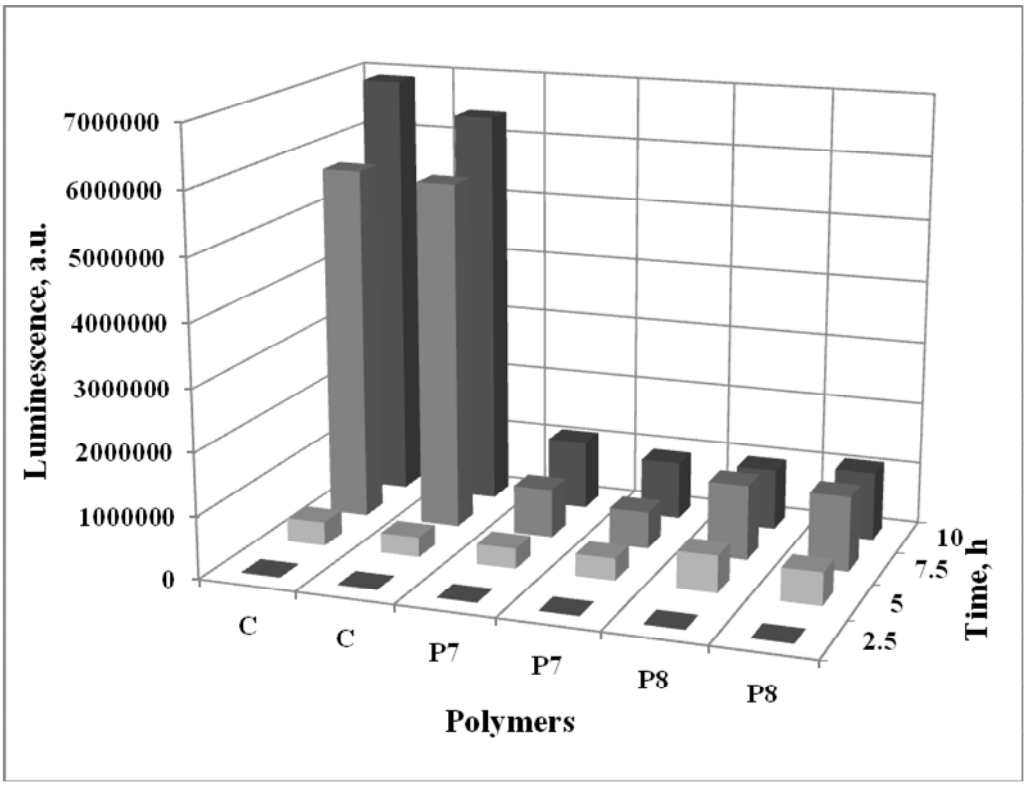


Figure 3.5: The effect of the IA- and MAA-based polymers on the bioluminescence of *V. fischeri*. P7-5% IA-based polymer, P8- 5% MAA-based polymer, C- control without polymers.

The P7 polymer which was incubated with the bacterial suspension was then extracted with acetonitrile and analysed using HPLC-MS to give the spectrum in Figure 3.6. It was found that the P7 polymer had adsorbed 0.27 ± 0.02 µg of 3-oxo-

C_6-AHL per g of polymer per 10 mL of bacterial culture (12.6 nM), explaining the quenching of bioluminescence which had been demonstrated in Figure 3.6. It correlates with a publication which reports that even as low as 10 nM of 3-oxo-C_6-AHL, which is equivalent to 1 or 2 molecules per cell, are sufficient for induction of bioluminescence in *V. fischeri* (Kaplan and Greenberg, 1985).

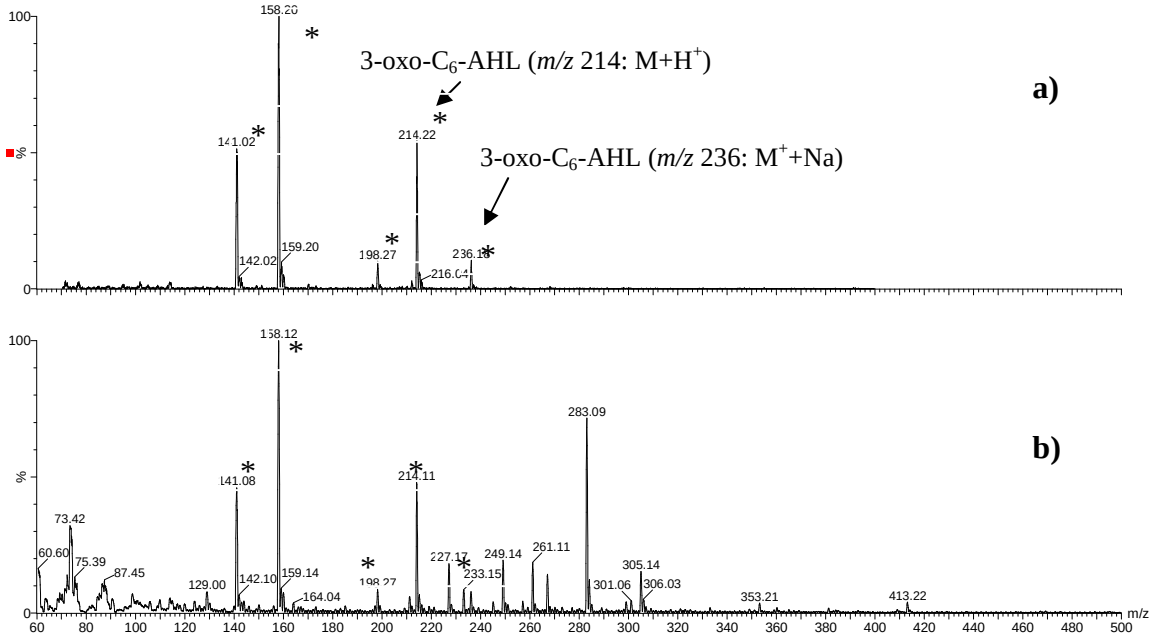


Figure 3.6: HPLC-MS spectrum of pure 3-oxo-C_6-AHL (a) and a spectrum of the compounds extracted from P7 polymer after its incubation with a bacterial suspension for 10 h (*-3-oxo-C_6-AHL-related fragments) (b).

3.3.6 Biofilm study

The effect of the polymers on biofilm formation by *V. fischeri* was studied. Since *V. fischeri* is a symbiotic bacterium which populates the light organs of squids, it is able to form biofilms, as has already been reported in the literature (Darnell *et al.*, 2008; Yildiz and Visick, 2009). Although the role of QS in biofilm formation by *V. fischeri* has not yet been thoroughly studied, it was interesting to find out whether this

process could be influenced by the addition of 3-oxo-C_6-AHL. It was shown that external addition of the cognate signal molecule increased the amount of biofilm by 40% (Figure 3.7) which suggests that 3-oxo-C_6-AHL might play some part in helping to trigger biofilm formation in this microorganism. The addition of P7 polymer to the suspension reduced biofilm production by *V. fischeri* by 56%, removing all effects of exogenous AHL (Figure 3.7). These results suggest that 5% IA- polymer was effective in the sequestration of the signal molecules which resulted in reduced biofilm formation by *V. fischeri*.

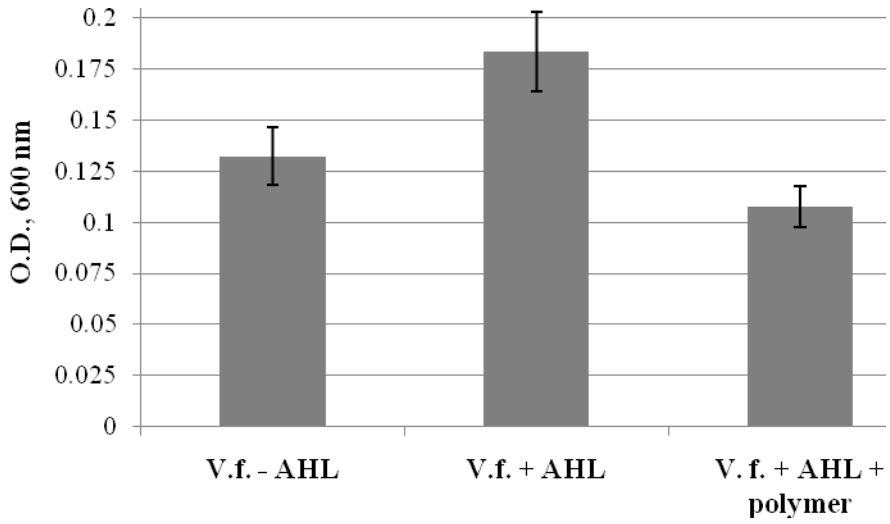


Figure 3.7: Effect of the P7 polymer ("polymer") on *V. fischeri* ("V. f.") biofilm formation, with and without (+ and -) exogenous 3-oxo-C_6-AHL ("AHL"). All standard deviations were based on six replicates.

3.4 Conclusions

The concept of QS attenuation was proven through *in vitro* tests which showed that it is possible to produce polymers with affinity to the signal molecule of the bacterium *V. fischeri*, *N*-(β-ketocapryloyl)-L-homoserine lactone. It was confirmed

that removal of the signal molecule disrupted the communication between the bacterial cells leading to inhibition of the expression of a post-quorate phenotype, i.e. to reduction of bioluminescence in *V. fischeri*. At the same time the polymer did not inhibit bacterial growth and in this aspect was superior to other non-specific commercial absorbents (such as activated charcoal) which inhibited bacterial growth through the non-specific absorption of nutrients. Although an influence of 3-oxo-C_6-AHL on *V. fischeri* biofilm formation was observed, a more detailed study of this phenomenon will be required.

The successful example of the attenuation of QS-controlled phenotype of *V. fischeri* by selective sequestering of the signal molecules suggests that it would also be possible to develop signal molecule-sequestering polymers for more problematic microorganisms that also possess QS-regulated virulence determinants, such as *Pseudomonas aeruginosa* (Chapter 4) or *Staphylococcus aureus*.

CHAPTER 4

RATIONALLY DESIGNED POLYMERS FOR ATTENUATION OF *PSEUDOMONAS AERUGINOSA* QUORUM SENSING

4.1 Introduction

Hospital-acquired (nosocomial) infections can be considered among the most serious problems in modern health care. These infections are caused by opportunistic pathogens of humans that easily infect any compromised tissues. Among the most dangerous opportunistic pathogens is the Gram- negative bacterium *Pseudomonas aeruginosa* which is responsible for urinary tract and respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections (Govan and Deretic, 1996; Wiblin, 1997; Dunn and Wunderink, 1995). *P. aeruginosa* is known to form antibiotic-resistant biofilms in the lungs of the patients affected by cystic fibrosis (CF), which is considered as one of the major causes of their mortality (Moreau-Marquis *et al*., 2008). This organism is uniquely problematic because of a combination of inherent resistance to many drug classes and its ability to acquire resistance to all relevant treatments. *P. aeruginosa* infections are usually associated with high mortality regardless of appropriate antimicrobial therapy. There are some data on the incidence of multidrug- resistant *P. aeruginosa* which is resistant to more than three agents (Rossolini and Mantengoli, 2005).

What should be done in order to control *P. aeruginosa* infections without generating the development of bacterial resistance? It is known that the virulence factor production and biofilm formation of *P. aeruginosa* are linked to the quorum-sensing (QS) system that uses diffusible *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-AHL) and *N*-butyryl-L-homoserine lactone (C_4-AHL) as intercellular messenger molecules. AHLs are used by bacteria to express certain phenotypic traits, particularly virulence factors, in a population density- dependent manner (Cámara *et al*., 2002). Both AHLs participate in a regulatory network that controls the production

of several virulence factors and biofilm formation, and therefore could be considered as potential therapeutic targets (Van Delden and Iglewski, 1998). It is known that biofilm-forming bacteria are surrounded by exopolysaccharide (EPS). The biofilm protects bacteria from antimicrobial agents (e.g. antibiotics and disinfectants) and make their elimination by disinfection even more difficult and unpredictable (Govan and Deretic, 1996; Costerton *et al*., 1999). By one estimate, provided by the Center for Disease Control and Prevention (CDC), 65% of human bacterial infections involve biofilms; therefore special efforts should be dedicated to the development of new materials and technologies for the reduction or elimination of biofilm formation.

The new concept which is presented here includes the application of synthetic polymeric materials for attenuation of bacterial quorum sensing of *P. aeruginosa* by sequestration of the signal molecules. This approach was already explored by our team using a model system based on the marine microorganism *Vibrio fischeri* (Chapter 3). In this study the aim was the design of signal molecule sequestering polymers for the control of *P. aeruginosa* infections and to progress further towards practical medical applications. 3-oxo-C_{12}-AHL was selected as a template for preparation of molecular imprinted polymer (MIP) because it is a key signal molecule of *P. aeruginosa* which regulates the expression of extracellular virulence factors (Muh *et al*., 2006; Smith and Iglewski, 2003), biofilm formation (Smith and Iglewski, 2003, Davies *et al*., 1998; De Kievit *et al*., 2001) and was found as an important factor in the development of *P. aeruginosa* infections in animal models (Tang *et al*., 1996; Teiber *et al*., 2008).

4.2 Materials and Methods

4.2.1 Materials

The wild strain of *P. aeruginosa* PAO1, *P. aeruginosa* PAO1 *lecA::lux* (University of Kent, Canterbury, UK) and the wild strain of *Escherichia coli* JM83 were used for testing. *N*-(3-oxododecanoyl)-DL-homoserine lactone (3-oxo-C_{12}-AHL) was purchased from GLSynthesis Inc. (Worcester, USA). *N*-butyryl-DL-homoserine lactone (C_4-AHL), *N*-hexanoyl-DL-homoserine lactone (C_6-AHL), *N*-(β-ketocaproyl)-DL-homoserine lactone (3-oxo-C_6-AHL), *N*-octanoyl-DL-homoserine lactone (C_8-AHL), methacrylic acid (MAA), itaconic acid (IA), *N*,*N*-methylene bisacrylamide (MBAA), acrylamide (AA), acrylamido-2-methyl-1-propanesulfonic acid (AMPSA), *N*,*N*-diethylaminoethyl methacrylate (DEAEM), ethylene glycol methacrylate phosphate (EGMP), ethylene glycol dimethacrylate (EGDMA), 1,1'-azobis (cyclohexanecarbonitrile), *N*,*N*-dimethyloformamide (DMF), Phosphate Buffered Saline (PBS, pH 7.4), acetonitrile and formic acid were purchased from Sigma and Alcian Blue from Fluka (Sigma-Aldrich, Gillingham, UK). Methanol, ethanol and Crystal Violet were obtained from Acros Organics (Fisher, UK). Wheat germ agglutinin-Alexa Fluor 488 conjugate (WGA) was purchased from Invitrogen (Carlsbad, CA, USA). Luria-Bertani broth (LB) and agar were from Merck (Poole, Dorset, UK). HPLC quality water which was purified using a Milli-Q system (Millipore, USA) was used in all experiments.

4.2.2 Computational modelling

Molecular modeling was done as described earlier (section 3.2.2). It included a refining step for optimisation of the polymer composition, which involved a molecular dynamics simulation of the pre-arrangement of the itaconic acid monomer

around the template prior to polymerisation (Piletska *et al*., 2005a). This was carried out by saturating the space around the template with monomers in a defined box which was heated to 600 K and cooled slowly to 300 K. Energy minimization was carried out to 0.05 kcal mol^{-1}. At the end of the simulation the number and the position of the functional monomers were examined. The type and quantity of the monomers participating in the complex with the template (first shell layer), or monomers which interact with the first shell layer of monomers, stabilising complex (second shell layer) indicate the type and ratio of the template (3-oxo-C_{12}-AHL) and monomers recommended for an optimised MIP composition.

4.2.3 Polymer preparation

Based on the computational modelling five functional monomers were selected as best candidates for polymer preparation: MAA, IA, MBAA, AA, AMPSA, DEAEM and EGMP. The composition of polymers is shown in Table 4.1.

Table 4.1: Polymer compositions (for abbreviations see 4.2.1. Materials section).

Polymer	**Masses of monomer, cross-linker, solvent and initiator (g, moles)**			
	Functional monomer (g, moles)	**EGDMA (cross-linker)**	**DMF (solvent)**	**Initiator***
P1	10% MAA (1, 0.012)	9, 0.045	10	0.1
P2	10% IA (1, 0.008)	9, 0.045	10	0.1
P3	10% MBAA (1, 0.006)	9, 0.045	10	0.1
P4	10% AA (1, 0.014)	9, 0.045	10	0.1
P5	10% AMPSA (1, 0.005)	9, 0.045	10	0.1
P6	10% DEAEM (1, 0.005)	9, 0.045	10	0.1
P7	10% EGMP (1, 0.005)	9, 0.045	10	0.1
P8	30% IA (3, 0.023)	7, 0.035	10	0.1
P9	5% IA (0.5, 0.004)	9.5, 0.048	10	0.1
P10	5% MAA (0.5, 0.006)	9.5, 0.048	10	0.1

*1,1'-azobis (cyclohexanecarbonitrile)

Primary screening was conducted using the polymers which were produced with 10% of corresponding functional monomer (selected as the best candidate for MIP preparation). All polymers were prepared as described in section 3.2.3. MIP composition consisted of 5 mg of 3-oxo-C_{12}-AHL as a template, 25 mg of functional monomer (IA), 0.47 g of cross-linker (EGDMA), 0.5 g of DMF and 5 mg of 1,1-azobis (cyclohexanecarbonitrile). The composition of the corresponding control (blank polymer, P9) was similar to MIP but without template. In order to ensure that no template remained in the MIP, the polymer was thoroughly washed in Soxhlet using methanol followed by regular changes of acidic methanol, acidic water, water and methanol. Washing continued for 3 days until the concentration of the template in the eluent measured using HPLC-MS became below quantification level (>2 ng mL^{-1}).

4.2.4 Screening of polymers against AHLs

100 mg of each polymer, prepared with 10% of functional groups (P1 – P7), was packed in empty 1 mL capacity SPE cartridges between two polyethylene frits. PBS was chosen as the principal media to perform the screening of the polymers under physiological conditions. Due to limited solubility of 3-oxo-C_{12}-AHL in water, it was dissolved in PBS containing 20% acetonitrile. Cartridges were conditioned with 5 mL of methanol, 5 mL of water and then loaded with 1 mL of AHL solution which contained 0.1 mg mL^{-1} of either C_4-AHL or 3-oxo-C_{12}-AHL. The eluent was collected and analysed using spectrophotometer at wavelength 204 nm to evaluate the concentration of the bound AHL.

4.2.5 Determination of binding capacity of polymers

In order to assess the binding capacity of the polymers towards C_4-AHL and 3-oxo-C_{12}-AHL two different methods were used:

a) Spectroscopy

100 mg of the IA-based polymers with 5% (P9), 10% (P2) and 30% concentration of monomer (P8) was packed in the 1 mL SPE cartridges. The conditioning was done with 5 mL of methanol and 5 mL of water. Sequential aliquots of C_4-AHL (0.1 mg mL^{-1} in PBS) or 3-oxo-C_{12}-AHL (0.2 mg mL^{-1} in PBS containing 20% acetonitrile) were loaded into the cartridges and the amount of AHL left in the filtrates was quantified by using a spectrophotometer at 204 nm. Thereafter, breakthrough curves were obtained and the binding capacity values were extrapolated from the 50% point of the breakthrough curve. The calibration curve based on several standards with concentration of 3-oxo-C_{12}-AHL (used in the concentration range between 12.5 μg mL^{-1} and 0.2 mg mL^{-1}) was built and used for quantification of the signal molecule.

b) HPLC-MS

100 mg of the polymers which contained 10% of MAA (P1) and 10% of IA (P2) was packed in the empty 1-mL SPE tubes. Cartridges were conditioned with 5 mL of methanol, 5 mL of water and then loaded with 1 mL of C_4-AHL or 3-oxo-C_{12}-AHL solution (10, 100 and 1000 ng mL^{-1}) in PBS or PBS containing 20% acetonitrile, respectively. The eluent was collected and evaluated in HPLC-MS. The samples with concentration 1000 ng mL^{-1} were diluted 10 times with water in order to fit into the calibration range optimal for the detection using mass-spectrometer.

In order to quantify the sequestration of 3-oxo-C_{12}-AHL and its analogues (C_4-, C_6-, 3-oxo-C_6- and C_8-AHLs) (Figure 4.1) the HPLC-MS methods for each homoserine

lactone was developed. HPLC separation was conducted using a Waters 2975 HPLC system equipped with Luna C18 (2) column (150x3 mm, 3 μm, Phenomenex). Mobile phase: water (A), methanol (B) in a binary system with 0.1% of formic acid as an additive. The elution gradient: linear gradient from 70% methanol/0.1% formic acid to 100% B from 0 to 10 min, then for 5 min solution B (100% methanol/0.1% formic acid) and for 5 min return to 70% methanol (total time- 20 min). The HPLC-MS parameters and the way of quantifying the analytes were as referred before (section 3.2.5). HPLC-MS analysis was carried out by Dr Elena Piletska.

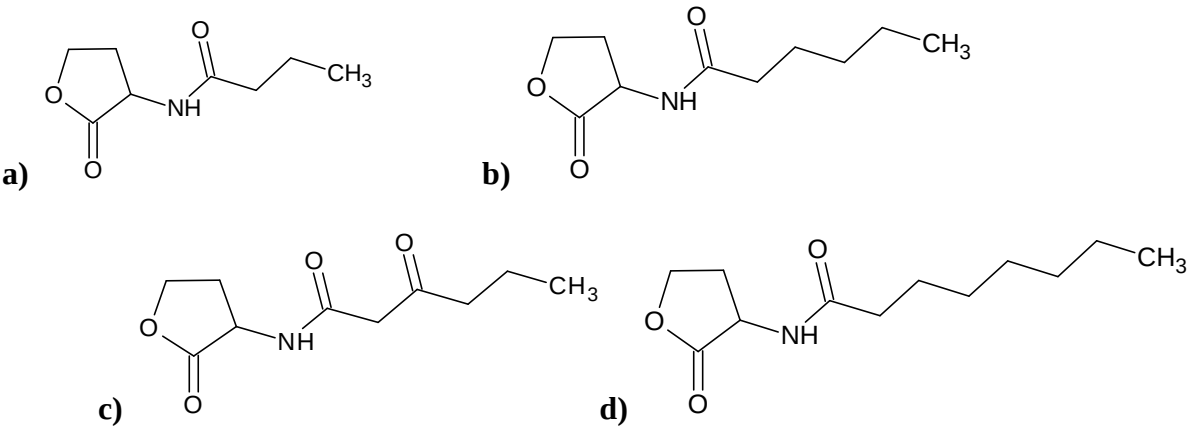


Figure 4.1: Molecular structures of tested analogues of 3-oxo-C_{12}-AHL: C_4-AHL (a), C_6-AHL (b), 3-oxo-C_6-AHL (c) and C_8-AHL (d).

4.2.6 Equilibrium binding studies of polymers designed for the signal molecules of *P. aeruginosa*

The evaluation of the binding capacity of the polymers towards 3-oxo-C_{12}-AHL was carried out as described by Lübke *et al*. (2000) with minor modifications. 10 mg of either MIP or corresponding blank polymer (P9) were suspended into 1 mL of 3-oxo-C_{12}-AHL solution (10 μg mL^{-1} in 20% acetonitrile). The vials were incubated overnight on an orbital shaker (Yellowline OS 2 basic shaker, Fisher) at

200 rpm at 20 ^{o}C. After incubation polymer suspensions were filtered through the syringe filters (Phenomenex, UK) with a pore size of 0.22 μm. The concentration of free AHL was assessed by HPLC-MS and the amount of bound 3-oxo-C_{12}-AHL was calculated by subtraction. The equilibrium binding of 10 μg mL^{-1} of 3-oxo-C_{12}-AHL by MIP and corresponding control polymer from LB broth was also determined as described above. As for C_4-AHL, 10 mg of polymers based on 10% MBAA (P3), 10% AA (P4), 10% EGMP (P7), 30% IA (P8), 5% IA (P9) and 5% MAA (P10) were added into 1 mL of C_4-AHL solution (100 μg mL^{-1} in 20% acetonitrile) and then the same procedure as before was followed.

4.2.7 Cross-reactivity study

The level of binding and cross-reactivity of MIP and corresponding control polymer (P9) for analogues of 3-oxo-C_{12}-AHL, particularly, C_4-AHL, C_6-AHL, 3-oxo-C_6-AHL and C_8-AHL was evaluated using equilibrium binding as described above (section 4.2.6).

4.2.8 Determination of polymer binding properties using Scatchard method

The binding of 3-oxo-C_{12}-AHL to either MIP or corresponding blank polymer (P9) was determined at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.5 25, 50, nd 100 μg mL^{-1} concentrations of AHL in polymer suspension in 20% acetonitrile/water, as described above. The binding characteristics were obtained by curve-fitting the binding isotherms for each section of the Scatchard plot using a single-site binding model (Grafit package, Erithacus Software).

4.2.9 Effect of polymers on growth of *P. aeruginosa* PAO1 and *Escherichia coli* JM83

A single colony of *P. aeruginosa* PAO1 was inoculated into LB and incubated overnight with reciprocal shaking at 200 rpm and 37 ºC. 0.5 mL of the overnight culture was diluted 1:20 in LB in 15 mL falcon tubes (Fisher, UK) in the presence, where required, of 200 mg of sterile P9 polymer. All cultures were incubated at 37 ºC with constant shaking. The bacterial growth and the effect of polymers on growth were determined by measuring the optical density at 600 nm over a period of 8 hours post-inoculation using a spectrophotometer (UVPC 2100, Shimadzu, Japan).

In order to investigate if polymers are toxic to other biological organism, *in vitro* testing was performed for *Escherichia coli* JM83. *E. coli* JM83 was grown overnight in LB at 37 ºC. 3 mL of the overnight culture was inoculated into 30 mL of LB in the presence or in the absence of 200 mg of 10% IA (P2), 10% AMPSA (P5), 10% DEAEM polymer (P6) and 10% EGMP (P7). Finally, the optical density at λ= 600 nm was measured with 2-h intervals for 8 h. Two replicates were used per treatment.

4.2.10 Static biofilm assay and laser scanning confocal microscopy (LSCM)

The biofilm study was carried out as described by Kievit *et al*. (2001) with some minor modifications. A single colony of *P. aeruginosa* PAO1 was inoculated into sterile LB broth and incubated overnight at 37 ºC with reciprocal shaking at 200 rpm. In order to prepare the microscope slides with biofilms, 1 mL of an overnight culture were diluted 1:20 in LB and placed in 4 mL top vials containing glass slides (25 mm × 7 mm) and, where required 5, 10 and 20 mg of sterile MIP or

corresponding blank polymer (P9). The vials were closed and incubated at 37 ºC for 24 hours under static conditions. Glass slides which contained biofilms were rinsed with sterile water and placed into vials containing 1 mL of 5 μg mL^{-1} wheat germ agglutinin-Alexa Fluor 488 conjugate (WGA), a fluorescent dye that stains cells green, in PBS. After incubation for 2 h at 4 ºC in the dark, the slides were removed from the solution and rinsed with sterile water. Biofilms, which were formed onto the glass slides in the presence of MIP and control polymer, as well as in the absence of polymer, were visualised using a Zeiss LSM 510 confocal microscope (Zeiss, Germany) equipped with an argon laser at excitation wavelength 488 nm (green fluorescence) and long pass emission filter 505 nm. All images were captured and processed using the Zeiss LSM software.

Alcian Blue staining was also used to assess the biofilm formation. Glass slides prepared as described in the previous paragraph were rinsed with sterile water and placed into vials containing 1 mL of 0.2 g L^{-1} Alcian Blue in water. The vials were incubated at 20 ºC for 30 min. Then solution of Alcian Blue was discarded and the slides were rinsed with sterile water, left to dry and photographed.

4.2.11 Quantification of biofilms of *P.aeruginosa* in the presence of signal molecule- sequestering polymers

The quantification of biofilm growth of *P.aeruginosa* was done using staining with Crystal Violet. The assay was performed as described by Schaber *et al*. (2004). Glass slides were carefully removed from each vial (static biofilm assay), rinsed with sterile water and placed into new vials containing 1 mL of 1 % Crystal Violet solution in water. After incubation at 20 ºC for 30 min, the slides were removed from the Crystal Violet solution, washed with sterile water, dried and placed into new vials

containing 1 mL of 95% ethanol. In order to quantify how much biofilm was formed the Crystal Violet- stained glass slides were incubated at 20 ºC for 1 h and solution was measured using a spectrophotometer (UVPC 2100, Shimazdu, Japan) at a wavelength of 600 nm.

4.2.12 Effect of polymers and external C_4-AHL on growth and bioluminescence of *P. aeruginosa* PAO1 *lecA::lux*

P. aeruginosa PAO1 *lecA::lux* was also used for *in vitro* testing of the selected polymers (P1-P4). The aim was to investigate whether polymers could sequester the signal molecule C_4-AHL, which regulates bioluminescence in *P. aeruginosa* PAO1 *lecA::lux* (Winzer *et al*., 2000). Cells were grown overnight in LB at 37 ºC. 0.25 mL of the overnight culture was inoculated into 10 mL of LB (1:40 dilution) containing either or both of the following:

(i) 50 mg of polymer

(ii) 100 µM C_4-AHL

Following this the turbidity at 600 nm and the luminescence were determined as a function of time.

4.3 Results and Discussion

4.3.1 Computational and SPE screening

a) 3-oxo-C_{12}-AHL modelling

The molecular structure of 3-oxo-C_{12}-AHL was drawn on the computer and minimised to a value 0.001 kcal mol^{-1}. The main structural features of 3-oxo-C_{12}-AHL include the lactone ring, the secondary amide group and the β-dicarbonyl function (Figure 4.2). In order to ensure the success of the molecular imprinting, preference

was made for the functional monomers which were able to form the interactions with functional groups close to lactone ring of 3-oxo-C_{12}-AHL.

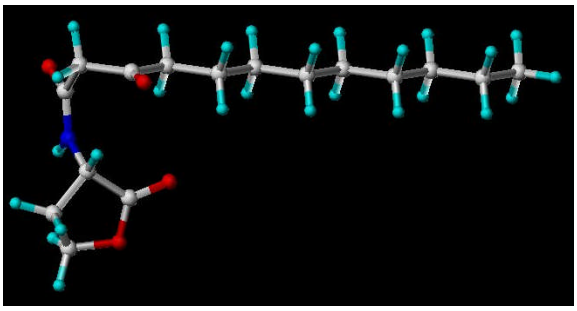
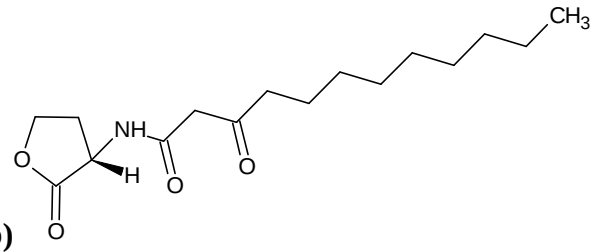
a)  b) 

Figure 4.2: 3D molecular structure of 3-oxo-C_{12}-AHL minimised in vacuum (oxygen atoms are shown in red, the nitrogen atoms in dark blue, the white atoms are carbon and the light blue atoms are hydrogens) (a), 2D molecular structure of 3-oxo-C_{12}-AHL (b).

The molecular library which contained 20 functional monomers was screened against 3-oxo-C_{12}-AHL. The screening generated a table ranking the monomers with the highest binding score (kcal mol^{-1}), as the best candidates for polymer preparation. The five monomers with the highest binding score were identified as AA, MAA, IA, MBAA and AMPSA (Table 4.2) (for abbreviations see 4.2.1. Materials section).

Table 4.2: Computational screening of the functional monomers (binding energy between 3-oxo-C_{12}-AHL and corresponding functional monomer, in kcal mol^{-1}) and SPE screening of the polymers prepared with corresponding monomers (binding from 100 µg mL^{-1} of 3-oxo-C_{12}-AHL solution in PBS containing 20% acetonitrile) (in the brackets are the names of the corresponding polymers).

Monomers	Binding energy, kcal mol^{-1}	Adsorption, %
MAA (P1)	-32.08	77±3
IA (P2)	-31.73	93±3
MBAA (P3)	-31.39	87±1
AA (P4)	-40.78	83±1
AMPSA (P5)	-25.62	42±5

It is important to report that all best monomers formed two-point interactions with the template and, therefore, were expected to exhibit high affinity towards 3-oxo-C_{12}-AHL. IA demonstrated three-point interactions with template, which made IA the most promising monomer for molecular imprinting. These expectations were supported by the results of primary SPE screening of five polymers which also confirmed that IA-based polymer had the highest binding properties towards 3-oxo-C_{12}-AHL (Table 4.2). The results of molecular dynamics showed that one molecule of 3-oxo-C_{12}-AHL could form the molecular complex with up to 5 molecules of IA (four IA molecules in direct contact with template and one IA molecule in the second shell which could be a stabiliser of the molecular complex (Figure 4.3). The molecular ratio which was suggested by molecular dynamics for MIP composition was 1:5 (3-oxo-C_{12}-AHL: IA) and was used for polymer preparation.

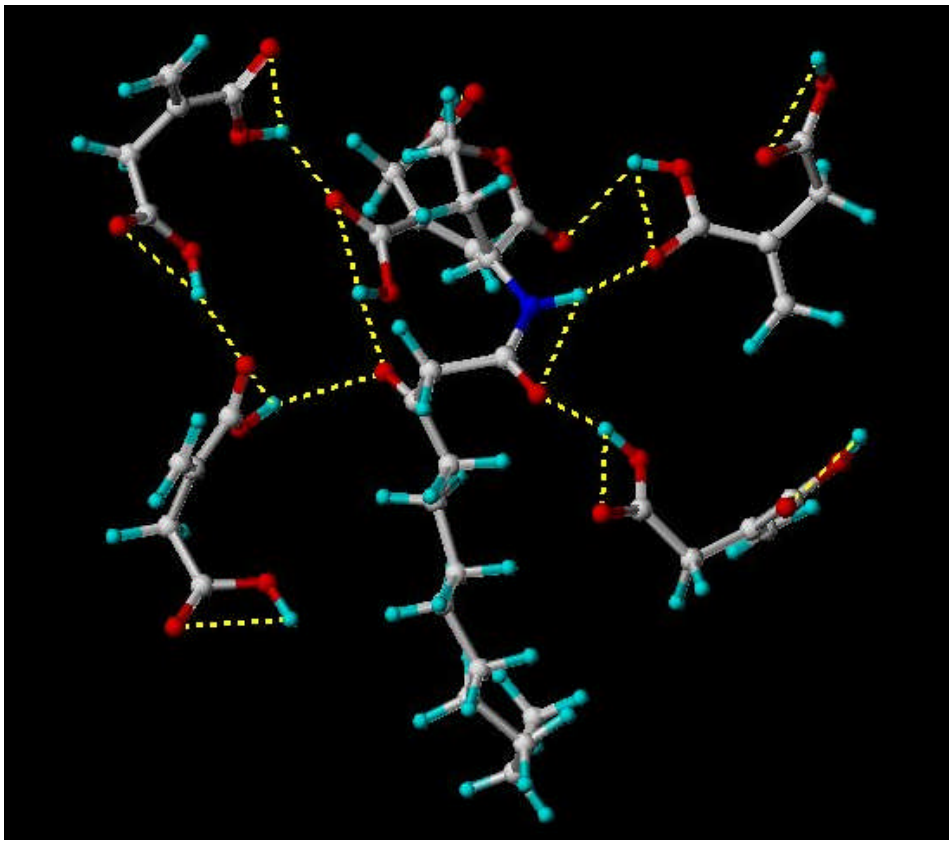


Figure 4.3: Molecular **c**omplex between 3-oxo-C_{12}-AHL and 5 molecules of IA.

b) C_4-AHL modelling

A minimised structure of C_4-AHL is shown in Figure 4.4.

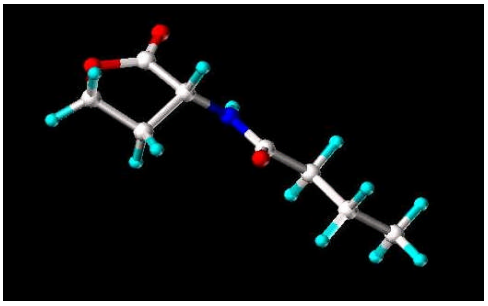
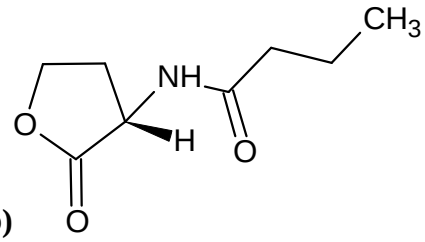
a)  **b)** 

Figure 4.4: 3D molecular structure of C_4-AHL minimised in vacuum (a), 2D molecular structure of C_4-AHL (b).

Screening of the template against the library of functional monomers resulted in a table ranking the monomers with the highest binding score (kcal mol^{-1}) as the best candidates for polymer preparation. The six best monomers were identified as MAA, IA, MBAA, AA, DEAEM and EGMP (Table 4.3) (for abbreviations see 4.2.1. Materials section).

Table 4.3: Computational screening of functional monomers (binding energy between C_4-AHL and corresponding functional monomer, in kcal mol^{-1}) and SPE screening of the polymers prepared with corresponding monomers (binding from 100 µg mL^{-1} of C_4-AHL solution in PBS) (in the brackets are the names of the corresponding polymers).

Monomers	Binding energy, kcal mol^{-1}	Adsorption, %
MAA (P1)	-28.57	81±3
IA (P2)	-28.20	86±2
MBAA (P3)	-26.47	79±3
AA (P4)	-26.84	68±3
DEAEM (P6)	-27.42	59±1
EGMP (P7)	-23.00	78±1

Of all monomers with the highest binding energy, only IA and MAA formed two-point interactions with the template. What is more, IA formed interactions with functional groups close to the lactone ring and, therefore, was expected to have the highest binding properties towards C_4-AHL (Figure 4.5). The results of primary SPE screening of six polymers confirmed that IA-based polymer exhibited the highest affinity towards C_4-AHL (Table 4.3).

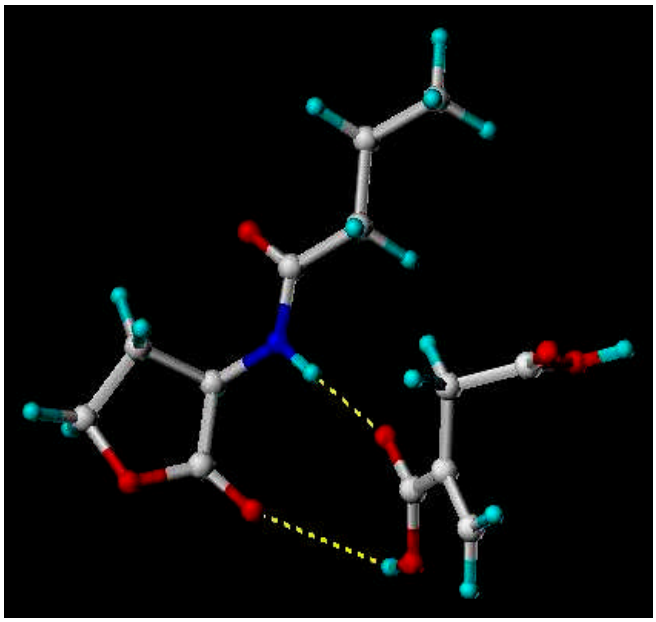


Figure 4.5: Complex of C_4-AHL with IA.

4.3.2 Determination of binding capacity of polymers towards C_4-AHL and 3-oxo-C_{12}-AHL

The IA-based polymers with 30% (P8), 10% (P2), and 5% (P9) concentration of corresponding functional monomer were tested for adsorption of C_4-AHL and 3-oxo-C_{12}-AHL from PBS and PBS containing 20% acetonitrile, respectively. In order to determine the capacity of the polymers, AHLs were filtered through the polymer in 1-mL aliquots and the concentration of filtrate in each fraction was quantified using a

spectrophotometer at 204 nm. It was found that the capacity values of polymers towards 3-oxo-C_{12}-AHL were much higher compared to the ones for C_4-AHL (Table 4.4). This could be explained by the "strong" hydrophobic properties of 3-oxo-C_{12}-AHL, which favours the binding to the hydrophobic polymer matrix. Furthermore, the P9 polymer with the lowest concentration of functional monomer (5% IA) demonstrated the highest binding capacity towards both AHLs tested and was selected for further testing.

Table 4.4: The binding capacity of the polymers towards C_4-AHL (0.1 mg mL^{-1}) in PBS and the binding capacity of the polymers towards 3-oxo-C_{12}-AHL (0.2 mg mL^{-1}) in PBS containing 20% acetonitrile.

Polymers	**Capacity (mg AHL per g of polymer)**	
	C_4-AHL	**3-oxo-C_{12}-AHL**
P8 (30% IA)	1.9±0.1	16.0±1.4
P2 (10% IA)	2.4±0.1	28.5±1.5
P9 (5% IA)	2.9±0.1	42.3±2.7

In order to quantify more precisely the AHL bound to the polymers, the following studies were conducted using HPLC-MS. Different solutions of C_4-AHL or 3-oxo-C_{12}-AHL were loaded through columns packed with polymers and the eluent was evaluated in HPLC-MS. The quantification of the samples showed that polymer based on MAA (P1) adsorbed 100 %, 100 % and 97.6 % of C_4-AHL from 10, 100 and 1000 ng mL^{-1} sample, respectively. The corresponding values for polymer based on IA (P2) were 100 %, 100 % and 99.2 % adsorption of C_4-AHL. Moreover, both polymers could completely sequester 3-oxo-C_{12}-AHL even from the most concentrated sample (1000 ng mL^{-1}).

4.3.3 Equilibrium binding studies of polymers designed for the signal molecules of *P. aeruginosa*

MIP and corresponding blank polymer were tested using equilibrium binding of 3-oxo-C_{12}-AHL from 20% acetonitrile and LB. Testing in suspension was chosen as the condition closest to the testing in the biological environment. The polymer suspension was incubated overnight in the corresponding solvent (20% acetonitrile or LB) which contained 10 μg mL^{-1} of 3-oxo-C_{12}-AHL, filtered through a syringe filter and binding of the signal molecules was assessed using HPLC-MS (Table 4.5).

Table 4.5: Binding of 3-oxo-C_{12}-AHL (10 μg mL^{-1}) and its structural analogues by 10 mg of MIP and Blank polymer from 20% acetonitile.*

Signal molecules	**Binding, %**		**Imprinting factor, $\alpha=B_{MIP}/B_{Blank}$**
	B_{MIP}	**B_{Blank}**	
3-oxo-C_{12}-AHL	86.8	78.3	1.1
C_4-AHL	0	4.2	0
C_6-AHL	29.8	29.9	0.99
3-oxo-C_6-AHL	44.7	17.1	2.6
C_8-AHL	63.7	75.9	0.83

*The standard deviations of these measurements were under 5%.

It was observed that the binding of 3-oxo-C_{12}-AHL to the MIP (87%) was higher when compared to that of the corresponding blank polymer (78%). In order to test the cross-reactivity of the MIP and blank polymer, binding studies were carried out using a number of structural analogues of 3-oxo-C_{12}-AHL, all of which belonged to the family of *N*-acyl-homoserine lactones (AHLs). All tested AHLs exhibited weaker binding to the MIP than 3-oxo-C_{12}-AHL. Binding of C_4-AHL to both polymers was very low with higher binding demonstrated by the blank polymer than

by the MIP, while C_6-AHL was bound to the same extent. Interestingly, 3-oxo-C_6-AHL, the closest structural analogue of the template, demonstrated the highest binding towards MIP and also the highest imprinted effect (α= 2.6). Having a shorter tail and functional groups identical to those of the template, 3-oxo-C_6-AHL likely fits ideally inside the cavity generated for 3-oxo-C_{12}-AHL (for molecular structures of both compounds see Figure 4.1c and Figure 4.2). The higher imprinting factor for 3-oxo-C_6-AHL than that for 3-oxo-C_{12}-AHL could be explained by low purity of 3-oxo-C_{12}-AHL, which was obtained by different company. The increased non-specific binding of C_4-, C_6- and C_8-AHL (Figure 4.1a, 4.2b and 4.2d respectively) to the blank polymer could be explained by relatively hydrophobic nature of these molecules and by higher hydrophobicity of the blank polymer in comparison with the more polar MIP.

LB was also selected for testing because it was used as the growth medium of *P. aeruginosa*. It was necessary to investigate whether such a rich medium as LB would prevent 3-oxo-C_{12}-AHL from binding to the polymer. It was shown that in the experimental conditions used LB did not affect binding to the polymer. Both MIP and corresponding control polymer demonstrated high binding of 3-oxo-C_{12}-AHL from LB quantitatively (100% and 99.7%, respectively) which was even higher than the binding observed in 20% acetonitrile (87% and 78%, respectively).

In order to measure the dissociation constant of the MIP and control polymer towards 3-oxo-C_{12}-AHL the equilibrium binding experiment was performed using a range of AHL concentrations ranging from 0.1 to 100 μg mL^{-1}. Scatchard graphs were constructed in order to assess the values of binding constants and population of binding sites. It was found that the MIP possessed high affinity with a dissociation constant calculated as K_d=145±2 μM and capacity calculated as 22.5 μmol g^{-1}.

Regarding the blank polymer, according to Scatchard plot its dissociation constant was characterised as K_d=312±3 μM and binding capacity 14.1 μmol g^{-1}.

Different polymers were also tested using equilibrium binding of C_4-AHL analysed in 20% acetonitrile. In this study AHL concentration was increased to 100 μg mL^{-1} (as opposed to 10 μg mL^{-1} for 3-oxo-C_{12}-AHL) to maximise the chances for the resins to sequester C_4-AHL. All polymers demonstrated moderate binding of the signal molecule (Table 4.6). This could be attributed to relative hydrophilic nature of C_4-AHL, which has higher affinity towards the polar solvent (20% acetonitrile) in comparison with the hydrophobic polymer.

Table 4.6: Binding of C_4-AHL (100 μg mL^{-1}) by 10 mg of polymer from 20% acetonitrile.

Polymers	Binding, %
P3 (10% MBAA)	15±3
P4 (10% AA)	17±2
P7 (10% EGMP)	23±3
P8 (30% IA)	17±3
P9 (5% IA)	19±1
P10 (5% MAA)	13±1

4.3.4 Effect of polymers on growth of *P. aeruginosa* PAO1 and *Escherichia coli* JM83

These studies were aimed to investigate whether polymers inhibit the growth of *P. aeruginosa* PAO1 and *E. coli* JM83. The effect of the P9 polymer (5% IA) on the growth of *P. aeruginosa* PAO1 as well as the effect of the P2 (10% IA), P5 (10% AMPSA), P6 (10% DEAEM) and P7 (10% EGMP) polymer on the growth of *E. coli* JM83 were determined by adding the corresponding resins into bacterial suspension

and measuring the optical density at 600 nm over a period of 8 hours after inoculation. It was found that none of the tested polymers affected bacterial growth of either *P. aeruginosa* PAO1 or *E. coli* JM83 at 20 mg L^{-1}. This was quite an important finding that bacteria are not affected by the presence of the polymeric materials, and therefore may not be prone to mutation and development of resistance.

4.3.5 *P. aeruginosa*: static biofilm assay

Since it is known that the 3-oxo-C_{12}-AHL signal molecule was responsible for biofilm formation in *P. aeruginosa* (Geske *et al*., 2005), whether the biofilm formation could be controlled by addition of MIP which was imprinted with 3-oxo-C_{12}-AHL was investigated. The corresponding blank polymer (P9) was also tested in order to evaluate the specificity of the binding.

Testing of the polymer effect on biofilm formation was conducted by studying the early stages of biofilm development. The static biofilm assay was considered a simple and reliable method to study the QS- controlled steps of biofilm formation (De Kievit *et al*., 2001). Cells were stained with fluorescent wheat germ agglutinin (WGA) conjugate and visualised using laser scanning confocal microscopy. WGA conjugate is known to bind specifically *N*-acetylglucosamine, a component of the EPS matrix of *P. aeruginosa* biofilms (Goldstein and Hayes, 1978; Wozniak *et al*., 2003). Furthermore, this lectin conjugate can also bind to the cell surface because of possible leakage or release from the periplasmic space to the cell surface of cell wall of peptidoglycan containing *N*-acetylglucosamine (Strathmann *et al*., 2002).

Biofilms were grown in the presence of different quantities of polymer ranging from 5 to 20 mg per mL of bacterial suspension. The addition of increasing quantities of either MIP or blank polymer resulted in a more sparse surface

distribution of cells and noticeable reduction of EPS matrix which was a signal that biofilm was not formed. 20 mg of MIP per 1 mL of bacterial suspension almost completely inhibited biofilm formation (Figure 4.6c). Blank polymer also suppressed biofilm formation (Figure 4.6b) with a marked difference to the control biofilm which was grown in the absence of polymer (Figure 4.6a). This could not be due to the depletion of nutrients since none of the polymers affected growth at this concentration (section 4.3.4). The thickness of the biofilms was observed using the z-stack capability of the LSCM and evaluating the thickness of the compiled image stack viewed edge-on. The thickness of non-treated biofilms was estimated as 20 μm; biofilm thickness in the presence of blank polymer was significantly less, with the presence of MIP appearing to show further improvement still.

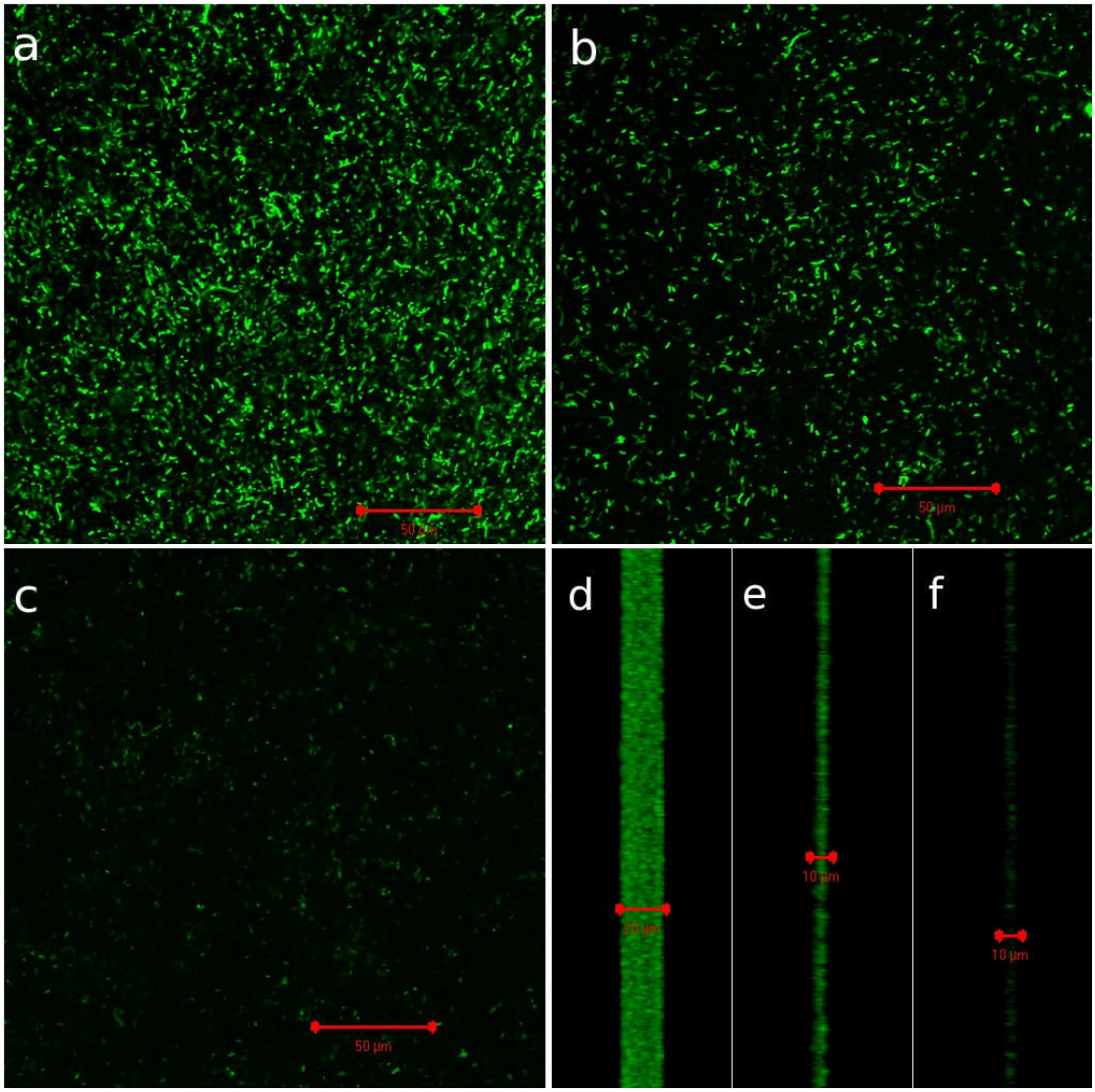
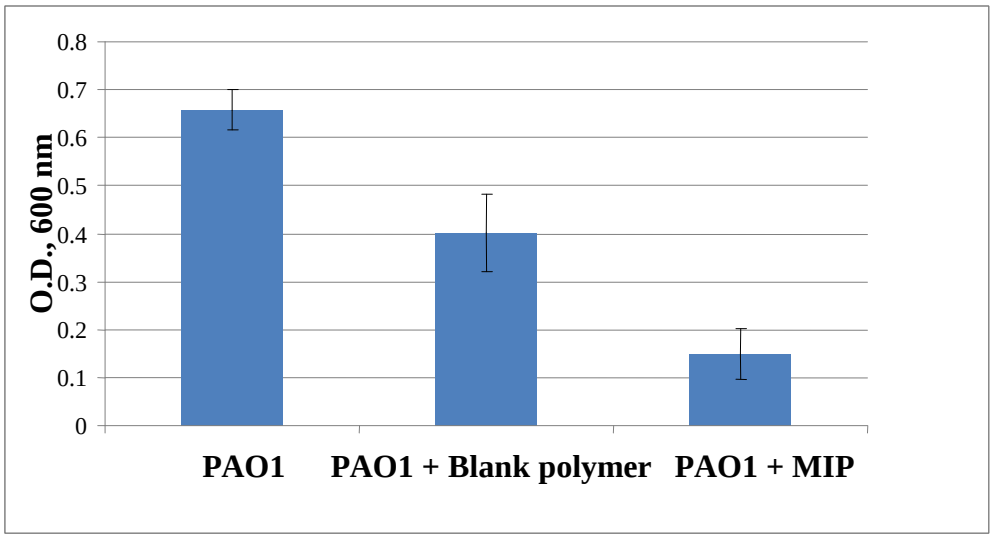


Figure 4.6: Effects of the polymer on *P. aeruginosa* biofilm formation grown on glass slides after 24 h incubation. Surface distribution of biofilm in control (a), in presence of 20 mg mL^{-1} of blank polymer (b) and in presence of 20 mg mL^{-1} of MIP (c). Scale bars are 50 µm. Corresponding biofilm thickness shown by z-stack in control (d), in presence of 20 mg mL^{-1} blank polymer (e) and in presence of 20 mg mL^{-1} of MIP (f). Scale bars are 20 µm, 10 µm and 10 µm respectively.

In order to confirm the above findings, the biofilms which were formed in presence of the MIP and blank polymers and without polymers were compared using two different dyes: Alcian Blue which binds specifically to acidic polysaccharides (Sauer *et al*., 2002), and Crystal Violet dye which stained all components of the biofilm including bacteria and is considered as a standard method of quantification of

biofilm growth (Schaber *et al.*, 2004). The polymer treated samples demonstrated inhibition of biofilm formation in both cases (Figures 4.7a and 4.7b). Addition of 20 mg mL^{-1} of MIP exhibited the most pronounced effect reducing the amount of biofilm by approximately 80% compared to control as opposed to 40% by the blank polymer (Figure 4.7b). These results clearly suggest that the MIP was effective in attenuating biofilm production of *P. aeruginosa* due to the specific sequestration of the "key autoinducer" 3-oxo-C_{12}-AHL.

a) **PAO1** **PAO1 + Blank polymer** **PAO1 + MIP**



b)

Figure 4.7: Alcian Blue staining of *P. aeruginosa* biofilms grown on glass slides after 24 h in the presence or absence of MIP or corresponding blank polymer (20 mg mL^{-1}) (a). Quantification of the Crystal Violet- stained biofilms from corresponding cultures (b). Error bars represent standard deviations between 6 replicates.

4.3.6 Effect of polymers and external C_4-AHL on growth and bioluminescence of *P. aeruginosa* PAO1 *lecA::lux*

The polymers were used for testing in the *P. aeruginosa* PAO1 *lecA::lux*, the QS-responsive bioluminescent variant of *P. aeruginosa*. The aim of this work was to investigate whether rationally designed polymers can function as synthetic receptors sequestering C_4-AHL, the signal molecule for the QS system regulating bioluminescence in *P. aeruginosa* PAO1 *lecA::lux* (Winzer *et al*., 2000). Monitoring of the bacterial growth in the presence and absence of polymers and/or external C_4-AHL revealed that none of the polymers inhibited growth, whether by toxicity or sequestration of nutrients, of *P. aeruginosa* PAO1 *lecA::lux* (Figure 4.8).

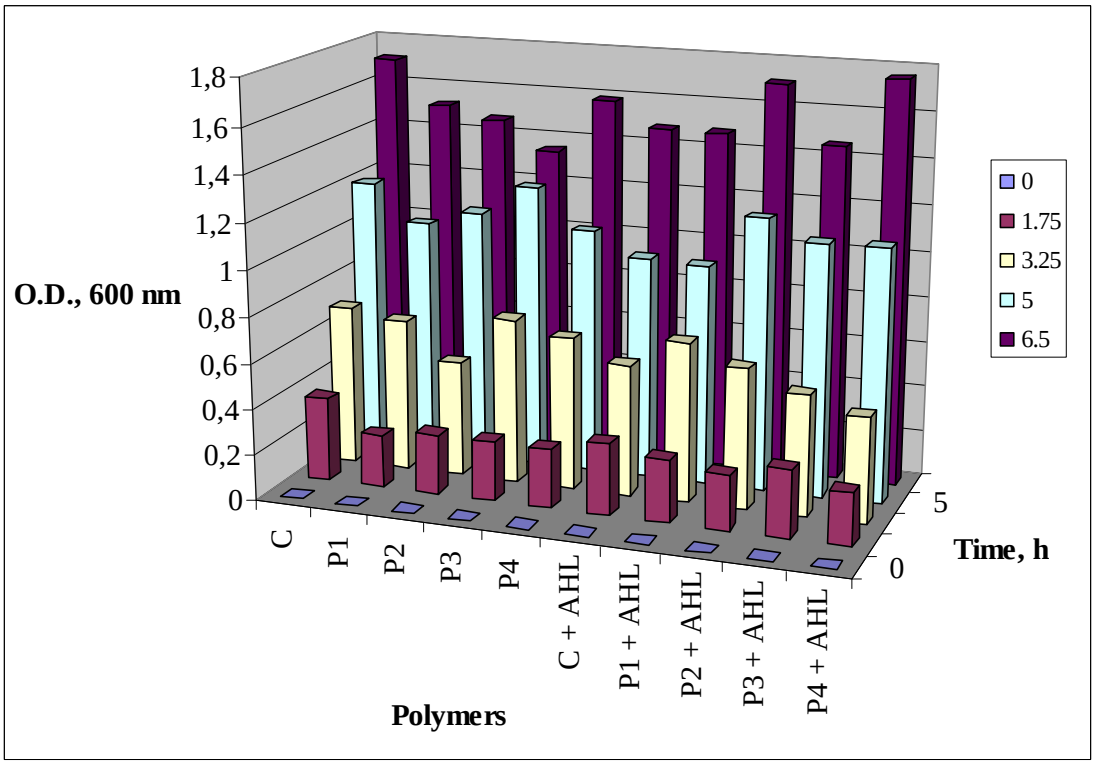


Figure 4.8: The effect of the polymers and external C_4-AHL ("AHL") on the growth of *P. aeruginosa* PAO1 *lecA::lux*. P1- 10% MAA-based polymer, P2- 10% IA-based polymer, P3- 10% MBAA-based polymer, P4- 10% AA-based polymer, C- control without polymers.

In parallel, bioluminescence of the bacterial suspension was monitored with and without polymer and in the presence or in the absence of external C_4-AHL (Figure 4.9). It was observed that the addition of external AHL enhanced bioluminescence, which was C_4-AHL-dependent. In addition, all polymers had capability to adsorb and remove C_4-AHL from the system. The retention of exogenous C_4-AHL by the MBAA-based polymer (P4) was approximately 100% at 3.25 h, 70% at 5 h and 60% at 6.5 h. It is necessary to highlight that external signal molecules could be used for the communication between the bacteria and activate the virulence determinants and biofilm formation.

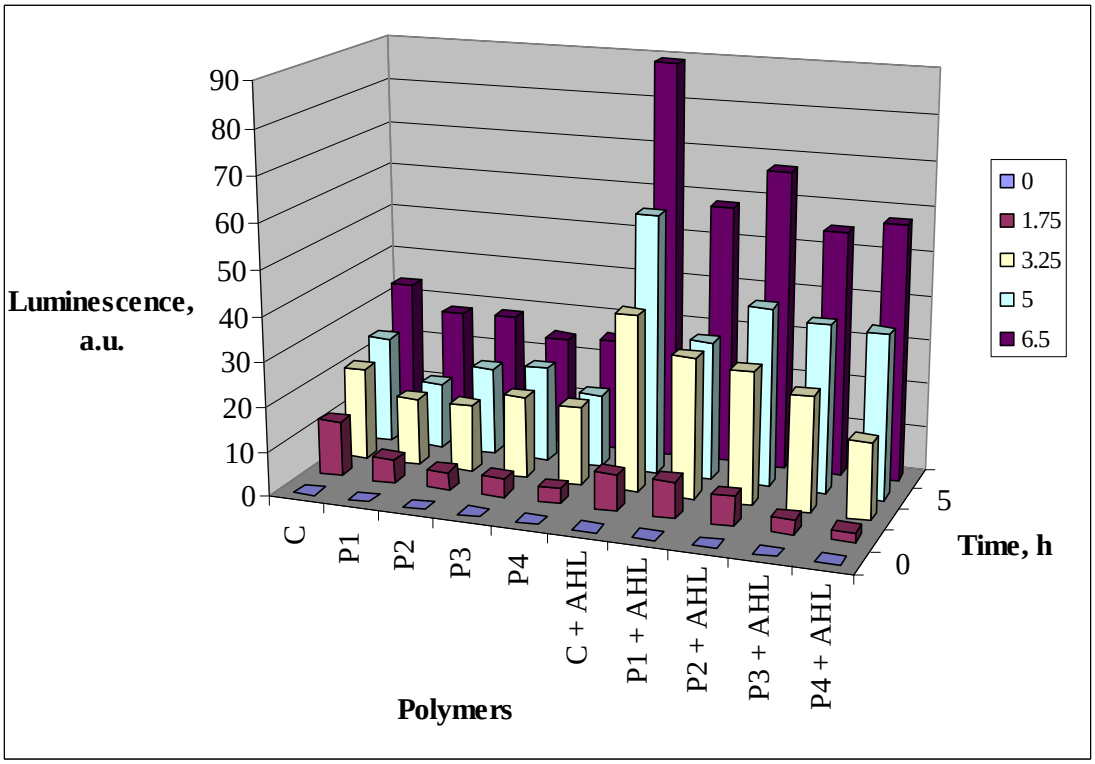


Figure 4.9: The effect of the polymers and external C_4-AHL ("AHL") on the bioluminescence of *P. aeruginosa* PAO1 *lecA::lux*. P1- 10% MAA-based polymer, P2- 10% IA-based polymer, P3- 10% MBAA-based polymer, P4- 10% AA-based polymer, C- control without polymers.

4.4 Conclusions

In this study the development of rationally designed MIP which could recognise and specifically sequester the signal molecule of *P. aeruginosa* (3-oxo-C_{12}-AHL) was reported. The affinity and specificity of MIP and corresponding blank polymers towards 3-oxo-C_{12}-AHL was assessed. MIP demonstrated higher binding towards 3-oxo-C_{12}-AHL (binding capacity- 6.7 mg AHL per g of polymer) than corresponding blank polymer (binding capacity- 4.2 mg AHL per g of polymer). As observed by laser scanning confocal microscopy of WGA- stained biofilms and Alcian Blue staining, addition of MIP specific for 3-oxo-C_{12}-AHL significantly inhibited the biofilm formation of *P. aeruginosa*. Quantification of the biofilm growth using Crystal Violet staining showed that the imprinted polymer reduced biofilm formation by 80% compared to control samples of the biofilm which was grown without polymers as opposed to 40% reduction by the blank polymer. The affinity and capacity of polymers towards C_4-AHL was also evaluated. Polymers could partially inhibit a QS-controlled phenotype, i.e. bioluminescence in *P. aeruginosa* PAO1 *lecA::lux*, by sequestering external C_4-AHL.

Resulting polymers could be considered as very promising materials for future integration as a grafted layer for a variety of medical devices (e.g. catheter sleeves, membranes, contact lenses, etc.) which could make them resistant against biofilm formation and protect the patients from possible medical complications related to *P. aeruginosa* infections. In contrast to traditional antibiotic therapy, treatment with signal sequestering imprinting polymers (or suitable blank) is less likely to induce bacterial resistance due to their non-toxic properties. Over the past years a number of treatments have been used to suppress biofilm formation in *P. aeruginosa*. Gillis and Iglewski (2004) reported that a sub-MIC of azithromycin could retard but not inhibit

biofilm production of *P. aeruginosa*. However other researchers were unable to reproduce effects on QS-controlled phenotypes (i.e. biofilm growth) without using concentrations that also affected growth rate (Bjarnsholt and Givskov, 2007). In another study by Ozer *et al*. (2005) it was shown that 0.25% of wild-type mouse serum containing paraoxonases could inhibit biofilm formation of *P. aeruginosa* by hydrolising 3-oxo-C_{12}-AHL. Furthermore, bovine and human serum was found to possess biofilm growth-inhibitory properties on plastic surfaces and intravenous catheters (Hammond *et al*., 2008). Yet, proteinaceous drugs are more likely to produce undesirable side effects and have considerably low stability compared to the rationally-designed polymers. Finally, garlic affected the architecture of the *P. aeruginosa* biofilm and rendered it susceptible to tobramycin (Bjarnsholt *et al*., 2005). Unfortunately, the previous acknowledged antimicrobial activities of garlic have been related to the presence of growth inhibitory compounds, such as allicin (Ankri and Mirelman, 1999; Bjarnsholt and Givskov, 2007). In the light of this, it is necessary to point out that the attenuation of biofilm formation by *P. aeruginosa* using rationally-designed polymers could be considered as a generic approach and, depending on the selection of target signal molecules, could be applied to a wide range of different microorganisms and applications.

CHAPTER 5

RATIONALLY DESIGNED POLYMERS FOR ATTENUATION OF *BURKHOLDERIA CEPACIA* QUORUM SENSING

5.1 Introduction

The gram-negative bacterium *Burkholderia cepacia* was first discovered as a plant pathogen related to bacterial rot of onion bulbs (Burkholder, 1950). *B. cepacia* refers to a complex which comprises a group of closely related strains. Strains of the *B. cepacia* complex can be found in soil, water, industrial settings, infected plants and animals (Miller *et al*., 2002). In humans it is most well known for its life-threatening lung infections which affect the cystic fibrosis (CF) patients as well as patients requiring mechanical ventilation and individuals with chronic granulomatous disease or compromised immune systems (Govan and Deretic, 1996; Mahenthiralingam *et al.*, 2002). The clinical outcome of infection with *B. cepacia* complex in CF patients is varied and unpredictable. Many patients develop a fatal necrotising pneumonia known as cepacia syndrome, which comes with a high mortality rate (about 20%) and is currently untreatable (Isles *et al.*, 1984; Saiman and Siegel, 2004).

Like most Gram-negative bacteria, *B. cepacia* regulates expression of various extracellular factors via an AHL-dependent quorum sensing (QS) system. This regulatory system consists of the AHL synthase CepI (homologous to LuxI), which synthesises *N*-octanoylhomoserine lactone (C_8-AHL) and *N*-hexanoylhomoserine lactone (C_6-AHL), and the transcriptional regulator CepR (homologous to LuxR) (Lewenza *et al*., 1999; Gotschlich *et al*., 2001; Williams, 2007). It was shown that expression of extracellular proteases and chitinases, swarming motility, and biofilm formation of *B. cepacia* is QS-controlled (Lewenza *et al*., 1999; Huber *et al*., 2001). It was demonstrated that *B. cepacia* mutants which are defective in QS could be easily attenuated in a *Caenorhabditis elegans* model (Köthe *et al*., 2003) as well as in a short-term intranasal colonisation mouse model and a chronic agar bead infection rat model (Sokol *et al*., 2003). Several genes were found to be controlled by the *cep*

system (Aguilar *et al*., 2003), which represents a highly attractive target for therapeutic intervention of *B. cepacia* infections.

As efficient treatment of *B. cepacia* infections is hampered by the formation of biofilms which are resistant to a large range of antibiotics, novel strategies for fighting this pathogen need to be developed. One of the strategies, which is presented here, is an application of the rationally designed synthetic polymers for the sequestration of the signal molecules C_6-AHL and C_8-AHL, aiming at the attenuation of QS-controlled phenotypes of *B. cepacia* (e.g. biofilm formation) similarly to attenuation of the bioluminescence and biofilm formation of *Vibrio fischeri* (Chapter 3), and biofilm formation of *Pseudomonas aeruginosa* (Chapter 4).

5.2 Materials and Methods

5.2.1 Materials

N-hexanoyl-DL-homoserine lactone (C_6-AHL), *N*-octanoyl-DL-homoserine lactone (C_8-AHL), methacrylic acid (MAA), itaconic acid (IA), *N*,*N*-methylene bisacrylamide (MBAA), acrylamide (AA), acrylamido-2-methyl-1-propanesulfonic acid (AMPSA), ethylene glycol methacrylate phosphate (EGMP), ethylene glycol dimethacrylate (EGDMA), 1,1'-azobis (cyclohexanecarbonitrile), *N*,*N*-dimethyloformamide (DMF), phosphate buffered saline (PBS), pH 7.4, acetonitrile were purchased from Sigma (Sigma-Aldrich, Gillingham, UK). Methanol was obtained from Acros Organics (Fisher, UK).

5.2.2 Computational modelling

Molecular modeling and computational screening of the templates (C_6-AHL and C_8-AHL) were carried out as described before (section 3.2.2).

5.2.3 Polymer preparation

According to the computational modelling data, six functional monomers were selected as best candidates for polymer preparation: MAA, IA, MBAA, AA, AMPSA, and EGMP. The polymer compositions are shown in Table 5.1.

Table 5.1: Polymer compositions (for abbreviations see 5.2.1.Materials section).

Polymer	Masses of monomer, cross-linker, solvent and initiator (g)			
	Functional monomer (g)	EGDMA (cross-linker)	DMF (solvent)	Initiator*
P1	10% MAA (1, 0.012)	9, 0.045	10	0.1
P2	10% IA (1, 0.008)	9, 0.045	10	0.1
P3	10% MBAA (1, 0.006)	9, 0.045	10	0.1
P4	10% AA (1, 0.014)	9, 0.045	10	0.1
P5	10% AMPSA (1, 0.005)	9, 0.045	10	0.1
P6	10% EGMP (1, 0.005)	9, 0.045	10	0.1
P7	5% EGMP (0.5, 0.002)	9.5, 0.048	10	0.1
P8	30% IA (3, 0.023)	7, 0.035	10	0.1
P9	5% IA (0.5, 0.004)	9.5, 0.048	10	0.1
P10	5% MAA (0.5, 0.006)	9.5, 0.048	10	0.1
P11	20% MBAA (2, 0.013)	8, 0.04	10	0.1

*1,1'-azobis (cyclohexanecarbonitrile)

All polymers were prepared as described before (section 3.2.3).

5.2.4 Screening of polymers against C_6- and C_8-AHLs

In order to perform the SPE screening of the polymers, C_6-AHL and C_8-AHL were dissolved in PBS containing 5% acetonitrile and PBS containing 15% acetonitrile, respectively, at 0.1 mg mL^{-1} concentration. 100 mg of each polymer prepared with 10% of functional monomers (P1 – P6) were packed in empty 1-mL SPE cartridges. The testing was conducted following the procedure which was described earlier (section 4.2.4).

5.2.5 Quantification of C_6- and C_8-AHLs using HPLC-MS set-up

C_6-AHL and C_8-AHL were quantified using HPLC-MS as it was described earlier (section 4.2.5). The studies were carried out by Dr Elena Piletska.

5.2.6 Determination of binding capacity of polymers

The binding capacity of the polymers which contained 10% of MAA (P1) and 10% of IA (P2) towards the binding of C_6- and C_8-AHLs were assessed using HPLC-MS quantification method and spectrophotometrically. The solutions of C_6-AHL in PBS containing 5% acetonitrile and C_8-AHL in PBS containing 15% acetonitrile in the concentration range from 10 to 1000 ng mL^{-1} were used for the HPLC-MS quantification. The binding of C_6-AHL or C_8-AHL towards IA-based polymers which contained 5% (P9), 10% (P2) and 30% (P8) of functional monomer (0.1 mg mL^{-1}) were assessed spectrophotometrically. The measurements were carried out as described in section 4.2.6.

5.2.7 Equilibrium binding studies of polymers designed for the signal molecules of *B. cepacia*

10 mg of polymer contained 10% AA (P4), 5% EGMP (P7), 5% IA (P9), 5% MAA (P10) and 20% MBAA (P11) were suspended into 1 mL of C_6-AHL or C_8-AHL solution (0.1 mg mL^{-1} in 10% acetonitrile). Thereafter, the same procedure as used before (section 4.2.6) was followed.

5.3 Results and discussion

5.3.1 Computational and SPE screening

a) C_6-AHL modelling

The minimised molecular structure of C_6-AHL is shown in Figure 5.1. a)

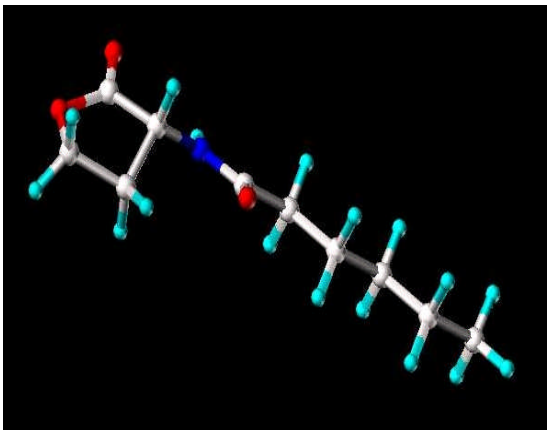
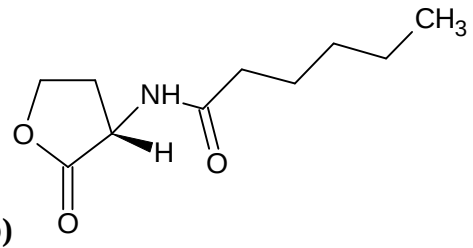
a)  **b)** 

Figure 5.1: 3D molecular structure of C_6-AHL which was minimised in vacuum (a), 2D molecular structure of C_6-AHL (b).

The screening of the library of the functional monomers against C_6-AHL identified AA, MAA, IA, MBAA, AMPSA and EGMP as the monomers with the highest binding score (kcal mol^{-1}) (Table 5.2).

Table 5.2: Computational screening of the functional monomers (binding energy between C_6-AHL and corresponding functional monomer, in kcal mol^{-1}) and SPE screening of the polymers prepared with corresponding monomers (binding from 0.1 mg mL^{-1} of C_6-AHL solution in PBS containing 5% acetonitrile) (in the brackets are the names of the corresponding polymers).

Monomers	Binding energy, kcal mol^{-1}	Adsorption, %
MAA (P1)	-26.74	95±4
IA (P2)	-33.79	98±2
MBAA (P3)	-30.28	85±3
AA (P4)	-29.20	68±2
AMPSA (P5)	-37.70	32±1
EGMP (P6)	-30.93	90±3

It is necessary to report that in accordance with molecular modelling MAA and IA formed two-point interactions and EGMP formed three-point interactions with the template close to the lactone ring (Figure 5.2 and 5.3) and, therefore, these monomers were expected to possess high binding properties towards C_6-AHL. The results of primary SPE screening of six polymers confirmed this hypothesis. Polymers based on MAA, IA and EGMP demonstrated a high retention of C_6-AHL, with IA-based polymer exhibiting the highest affinity (Table 5.2) and was selected for further testing.

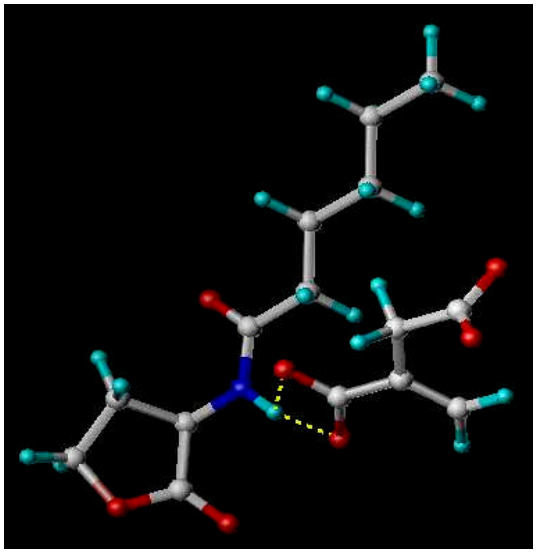


Figure 5.2: Molecular **c**omplex formed by C_6-AHL and charged IA.

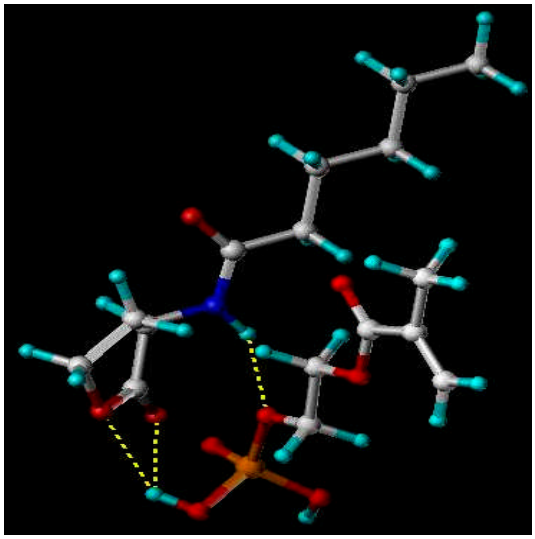
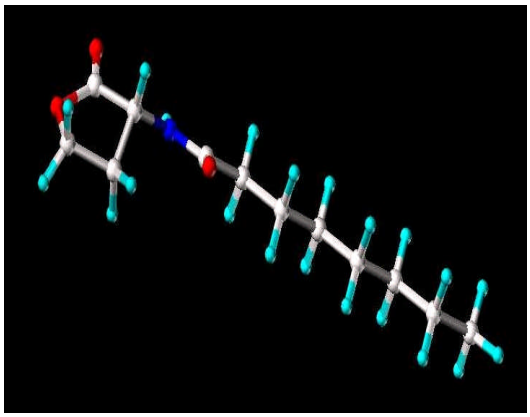


Figure 5.3: Molecular **c**omplex formed by C_6-AHL and EGMP.

b) C_8-AHL modelling

A minimised structure of C_8-AHL is shown in Figure 5.4.a)



b)

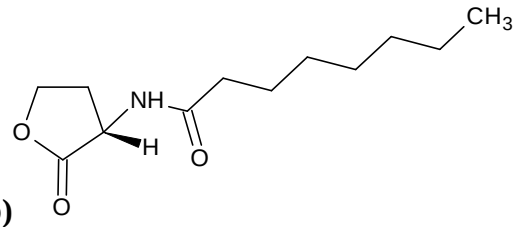


Figure 5.4: 3D molecular structure of C_8-AHL which was minimised in vacuum (a), 2D molecular structure of C_8-AHL (b).

Screening of the C_8-AHL against the library of functional monomers resulted in a table ranking the monomers with the highest binding score (kcal mol^{-1}), which were identified as MAA, IA, MBAA, AA, AMPSA and EGMP (Table 5.3).

Table 5.3: Computational screening of the functional monomers (binding energy between C_8-AHL and corresponding functional monomer, in kcal mol^{-1}) and SPE screening of the polymers prepared with corresponding monomers (binding from 0.1 mg mL^{-1} of C_8-AHL solution in PBS containing 15% acetonitrile) (in the brackets are the names of the corresponding polymers).

Monomers	Binding energy, kcal mol^{-1}	Adsorption, %
MAA (P1)	-26.85	90±5
IA (P2)	-36.37	99±1
MBAA (P3)	-38.73	98±1
AA (P4)	-28.94	79±4
AMPSA (P5)	-34.64	50±1
EGMP (P6)	-36.85	96±4

Among all best monomers, IA, MAA and MBAA monomers formed two-point interactions with C_8-AHL and EGMP formed three-point interactions with the

template close to the lactone ring (Figure 5.5, 5.6 and 5.7). All these functional monomers were suggested to possess high affinity towards C_8-AHL. The results of primary SPE screening confirmed that polymers based on corresponding monomers demonstrated strong binding of C_8-AHL (Table 5.3).

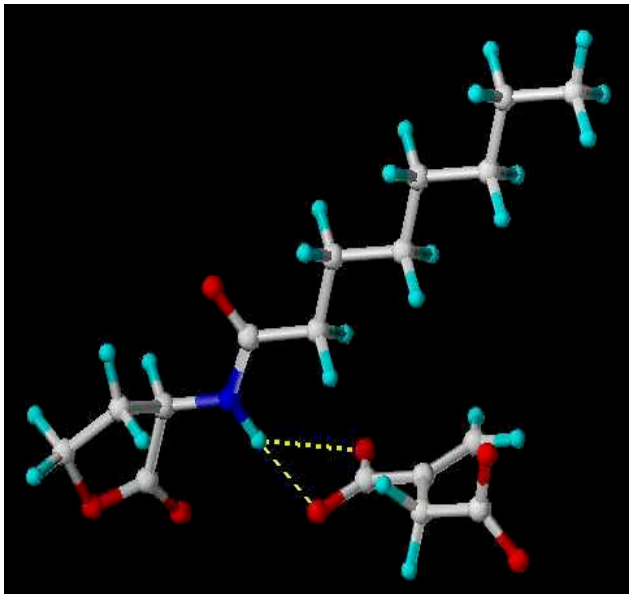


Figure 5.5: Molecular **c**omplex formed by C_8-AHL and charged IA.

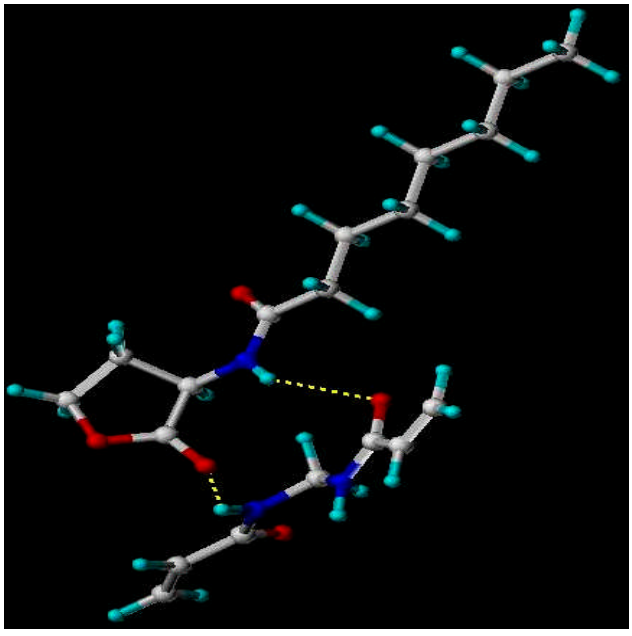


Figure 5.6: Molecular **c**omplex formed by C_8-AHL and MBAA.

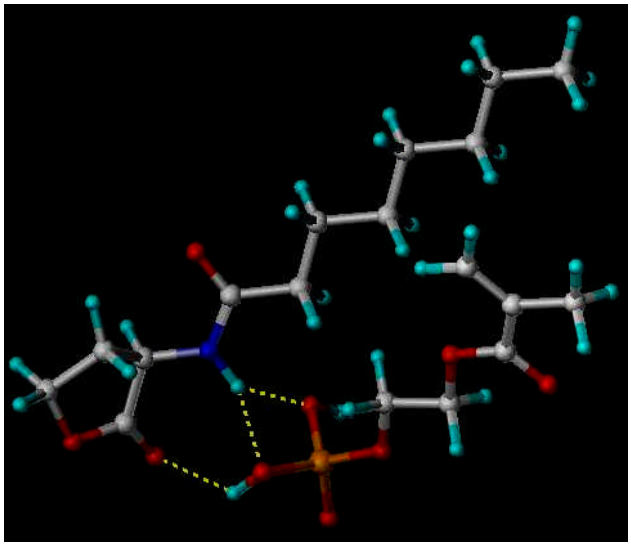


Figure 5.7: Molecular **c**omplex formed by C_8-AHL and EGMP.

5.3.2 Determination of binding capacity of polymers towards C_6- and C_8-AHLs

The binding capacity towards C_6-AHL and C_8-AHL of the polymers based on 30% (P8), 10% (P2), and 5% IA (P9) was tested in PBS containing 5% acetonitrile and PBS containing 15% acetonitrile, respectively, using a spectrophotometer at 204 nm.

Table 5.4: The binding capacity of the polymers towards C_6-AHL and C_8-AHL (0.1 mg mL^{-1}) in PBS containing 5% and 15% acetonitrile, respectively.

Polymers	**Capacity (mg AHL per g of polymer)**	
	C_6-AHL	**C_8-AHL**
P8 (30% IA)	4.0±0.1	5.8±0.3
P2 (10% IA)	4.8±0.2	7.2±0.4
P9 (5% IA)	6.3±0.4	9.4±0.3

It was observed that the binding capacity of polymers towards both signal molecules was sufficient for practical application (Table 5.4).

In order to measure more precisely the adsorption of AHL all the following studies were carried out using the HPLC-MS quantification method. Solutions of C_6-AHL or C_8-AHL in PBS containing 5% or 15% acetonitrile, respectively, were filtered through polymer-packed SPE columns and the presence of the AHLs in the eluent was assessed. It was shown that 10% MAA polymer (P1) sequestered 100 %, 99.4 % and 98.3 % of C_6-AHL from 10, 100 and 1000 ng mL^{-1} sample, respectively. The corresponding values for 10% IA polymer (P2) were 100 %, 99.9 % and 99.1 % adsorption of C_6-AHL. Moreover, both polymers could quantitatively adsorb C_8-AHL at all tested concentrations (10, 100 and 1000 ng mL^{-1}).

5.3.3 Equilibrium binding studies of polymers designed for the signal molecules of *B. cepacia*

Different polymers were tested on adsorption of C_6-AHL and C_8-AHL from 10% acetonitrile using equilibrium binding. The polymer suspensions were incubated overnight in the corresponding AHL solutions, and were then filtered through a syringe filter and retention of the signal molecules was evaluated using HPLC-MS (Table 5.5).

Table 5.5: The binding capacity of the polymers towards C_6-AHL and C_8-AHL (0.1 mg mL^{-1}) in 10% acetonitrile.

Polymers	**Capacity (mg AHL per g of polymer)**	
	C_6-AHL	**C_8-AHL**
P4 (10% AA)	2.1±0.5	2.0±4.0
P7 (5% EGMP)	2.2±0.2	4.5±0.3
P9 (5% IA)	2.2±0.3	4.7±0.1
P10 (5% MAA)	2.6±0.2	5.3±0.2
P11 (20% MBAA)	2.1±0.2	3.4±0.4

It was observed that binding of C_6-AHL to the polymers was moderate due to the stronger affinity of the signal molecule towards the polar solvent (10% acetonitrile) in comparison to the hydrophobic polymer. Interestingly, the resins showed higher binding of C_8-AHL than of C_6-AHL with the exception of 10% AA polymer (P4). In particular, polymers with 5% concentration of functional monomer (P7, P9 and P10) sequestered twice as much C_8-AHL under the same conditions. This could be explained by relative hydrophobic properties of C_8-AHL.

5.4 Conclusions

In this study the design and development of synthetic polymers which are able to recognise and specifically sequester the signal molecules which are used by *B. cepacia* was described. The affinity and capacity of the polymers towards the corresponding signal molecules was assessed using SPE and HPLC-MS. Equilibrium binding studies were also conducted, in conditions close to the biological environment. According to modelling, SPE screening and equilibrium binding data, polymers based on MAA, IA and EGMP with 5% concentration of functional monomer demonstrated sufficiently high capacity towards C_8-AHL (5.3, 4.7 and 4.5 mg AHL per g of polymer, respectively), the major signal molecule produced by *B. cepacia* (Lewenza *et al*., 1999). Thereby, rationally-designed polymers could be regarded as a promising tool for testing with *B. cepacia* infections, and possibly stopping it from forming biofilms.

CHAPTER 6

GRAFTING OF THE QS-SEQUESTERING POLYMERS TO HYDROPHILISED POLYPROPYLENE MEMBRANES

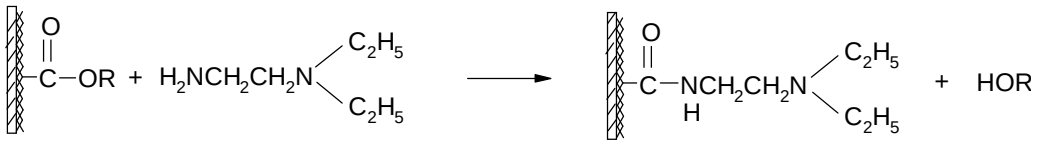
6.1 Introduction

A wide variety of membranes has been studied in various fields of industrial applications, environmental protection, medicine and biotechnology (Ho and Sirkar, 1992). In order to meet the requirements for more specialised applications or innovative technologies, the surface-selective functionalisation of already established membranes is required to prepare novel membranes with improved properties (Ulbricht *et al*., 2006). It is important that the membrane surface is functionalised without significantly changing the properties of the bulk polymer attached to the surface. Reactions initiated by UV irradiation are the basis for techniques that has proven to be highly successful due to their significant advantages: easy and controllable operation, low cost, reducing or even avoiding negative effects on the bulk polymer and permanent chemical stability (He and Ulbricht, 2006 and 2007).

Porous polypropylene (PP) membranes are widely used for microfiltration and other separation technologies due to their chemical and mechanical stability and various surface modifications of such membranes had been investigated (Piletsky *et al*, 2000; Ulbricht and Yang, 2005; Ma *et al*, 2000). In order to enhance their non-fouling properties and improve their wetting performance, membrane manufacturers performed surface modifications of PP membranes making them hydrophilic. Two different photo-grafting polymerisation methods were developed by the group of Prof. Ulbricht in order to functionalise such hydrophilised microfiltration membranes (He and Ulbricht, 2006). The first method was an immobilisation method based on the immobilisation of tertiary amine groups as synergists for the photo-initiator benzophenone (BP) and consisted of two steps: introduction of the tertiary amino groups to the membrane surface by an aminolysis reaction between the ester groups on the surface of the hydrophilised PP membranes and *N*,*N*-diethylethylenediamine

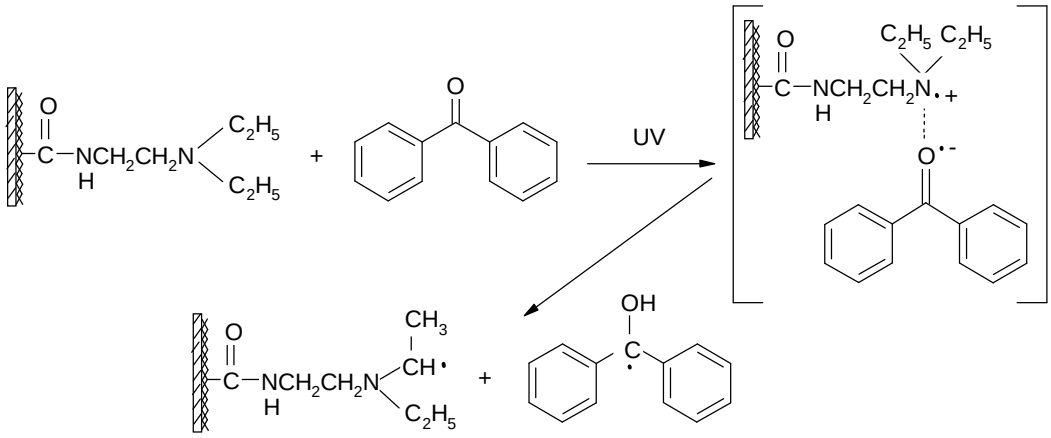
(DEEDA) followed by grafting of the functional monomer by UV irradiation (Figure 6.1) (He and Ulbricht, 2006). The second method involved an immobilisation of iniferter onto the surface of the polypropylene membrane. The modification of the membrane surface was carried out in a similar manner to that of the first method, i.e. introduction of tertiary amino groups to the surface using tetraethylenepentamine (TEPA). Then functional monomer was UV-grafted to the membrane from a DMF solution which also contained photo-iniferter diethyldithiocarbamate (DTC) (Figure 6.2) (He, 2008). Subsequently, the polymer-grafted PP membranes were tested for the sequestration of the signal molecules of *Pseudomonas aeruginosa* (C_4-AHL and 3-oxo-C_{12}-AHL) and attenuation of biofilm formation.

1st step: Aminolysis of membrane



2nd step:

(a) Formation of starter radical



(b) Graft polymerisation

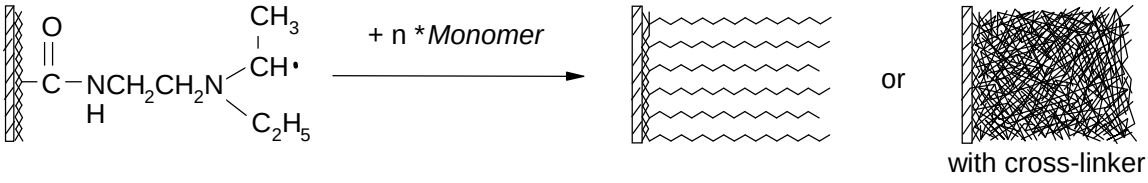


Figure 6.1: Synergist immobilisation method for photo-grafting (He and Ulbricht, 2006).

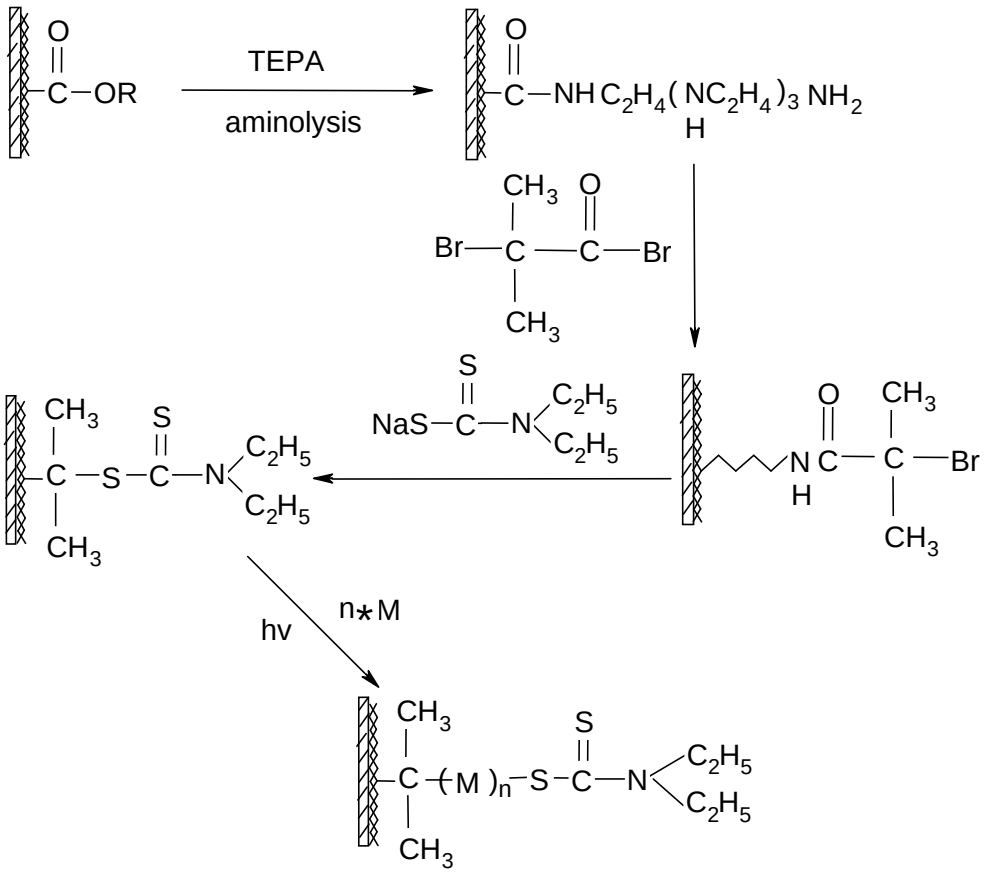


Figure 6.2: Photo-grafting method using the immobilisation of the iniferter (He, 2008).

6.2 Materials and Methods

6.2.1 Materials

The wild strain of *P. aeruginosa* PAO1 was used for testing. Hydrophilised polypropylene (PP) microfiltration membranes with a nominal cut-off pore diameter of 0.2 µm and with a thickness of 101 µm were from Pall Corporation (New York, USA). *N*,*N*-diethylethylenediamine (DEEDA; 98%) was purchased from Acros (Geel, Belgium). Benzophenone (BP) was obtained from Fluka (Poole, UK). Methanol (HPLC grade) was purchased from VWR (Lutterworth, UK). Tetraethylenepantamine (TEPA), 2-Bromoisobuturyl bromide, triethylamine, 4-Dimethylaminopyridine (DMAP), acetonitrile (anhydrous; 98%), sodium *N*,*N*-diethyldithiocarbamate

trihydrate (DTC), ethanol, *N*-butyryl-DL-homoserine lactone (C_4-AHL), itaconic acid (IA), ethylene glycol dimethacrylate (EGDMA) and *N*,*N*-dimethyloformamide (DMF) were purchased from Sigma (Sigma-Aldrich, Gillingham, UK). *N*-(3-oxododecanoyl)-DL-homoserine lactone (3-oxo-C_{12}-AHL) was obtained from GLSynthesis Inc. (Worcester, USA). Wheat germ agglutinin-Alexa Fluor 488 conjugate (WGA) was purchased from Invitrogen (Carlsbad, CA, USA). Luria-Bertani broth (LB) and agar were bought from Merck (Poole, Dorset, UK). HPLC quality water which was purified using a Milli-Q system (Millipore, USA) was used in all experiments.

6.2.2 Preparation of polymeric membranes using the synergist immobilisation method

a) Synergist immobilisation by aminolysis

Circular membrane samples with a diameter of 47 mm were placed into sealed glass vials and 4 mL DEEDA was added. After equilibrating for 10 min at room temperature, the reaction was conducted for 2.5 h at constant temperature (60 ºC) in a thermostat. Then, the samples were taken out, rinsed with methanol, washed twice on a shaker (30 min each time) and finally dried at 45 ºC overnight.

b) Photo-graft polymerisation

A UV illumination system (UVA Print, Hönle AG, Gräfelfing, Germany) equipped with a high-pressure mercury lamp and a glass filter ($\lambda > 300$ nm) was used. The intensity was 40 mW cm^{-2} (measured with the UVA meter, Hönle AG). Pre-weighed aminolysed membrane samples were immersed into 3 mL monomer solution in DMF (54 g L^{-1}), containing the photo-initiator BP (1mM), in a Petri dish (diameter 90 mm) and fixed between two sheets of filter paper (diameter 90 mm; Whatman).

The monomer mixture consisted of 8.1 mg IA and 153.9 mg EGDMA. After 10 min equilibration, membranes were illuminated with UV light for 15 min (effective UV intensity was 7.5 mW cm^{-2}). Thereafter, the samples were taken out and washed with methanol three times (each for 30 min) to remove unreacted monomer, residual initiator and homopolymer. Then membrane samples were dried in vacuum at 45 ºC overnight. The degree of grafting (DG) was evaluated gravimetrically from the weight of each sample before and after polymerisation and calculated using the following equation, where W_0 and W_1 stand for the sample's weights before and after modification, respectively:

$$DG = (W_1 - W_0 / W_0) \times 100\%$$

6.2.3 Preparation of polymeric membranes using the iniferter immobilisation method

a) Iniferter immobilisation

Circular membrane samples with a diameter of 47 mm were placed into 4 mL sealed glass vials which contained 25 vol% TEPA solution in DMF. After equilibrating for few minutes at room temperature, the system was kept for 3 h at 70 ºC. Then, membranes were removed from the vial, washed three times with DMF and once with methanol on a shaker (for 30 min each washing step) and finally dried at 45 ºC overnight. Thereafter, the samples were placed into 10 mL of dry acetonitrile solution which contained 50 mM 2-bromoisobutyryl bromide, 55 mM triethylamine and 5 mM DMAP and incubated at room temperature for 4 h. Then membranes were washed three times with acetonitrile and once with methanol and dried overnight at 45 ºC. For the immobilisation of photo-iniferter (dithiocarbamate group), the samples

were incubated in the ethanol solution which contained 0.3 mol L^{-1} DTC at 30 ºC overnight. Afterwards membranes were rinsed with methanol, thoroughly washed with water and dried overnight at 45 ºC.

b) Photo-graft polymerisation

The membranes with immobilised iniferter were weighed and immersed in 3 mL monomer solution (30 and 40 g L^{-1}) in DMF which was degassed by bubbling argon for 2 min where they were fixed between two sheets of filter paper. MIP-modified membranes were prepared using C_4-AHL and 3-oxo-C_{12}-AHL as templates, IA as a functional monomer and EGDMA as a cross-linker. Reference polymeric membranes were also prepared in the absence of templates as well as membranes made in the absence of functional monomer (100% EGDMA) as negative control. The compositions of the grafted polymers are shown in Table 6.1. To perform the polymerisation, the membranes were exposed to UV light (315 – 380 nm) using a UV illumination system (Hönle UV Technology, Germany) for 7.5 min. After being grafted, the membranes were washed three times with methanol, dried and finally weighed. The template molecules were extracted with methanol in a Soxhlet apparatus until the concentration of the template in the eluent became below quantification level (>2 ng mL^{-1}) as it was measured using HPLC-MS.

For the characterisation of the grafted membranes, FT-IR microscopic technique was used in ATR mode using Bruker IR-Scope-II microscope (Bruker Optics, UK) equipped with attenuated total reflection (ATR) objective and controlled by OPUS/IR optic software.

Table 6.1: Compositions of the polymers grafted to the membranes.

Monomer mixture	Monomer concentration ($g\ L^{-1}$)	Masses of monomer, cross-linker and template (mg) per 3 mL of solvent (DMF)		
		IA	EGDMA	AHL
EGDMA/IA 95:5 (w/w)	30	4.5	85.5	-
	40	6	114	-
EGDMA/IA 95:5 (w/w) MIP1 and MIP2 (1 w/w%* C_4-AHL and 3-oxo-C_{12}-AHL)	30	4.5	85.5	0.9
	40	6	114	1.2
EGDMA	30	-	90	-
	40	-	120	-

* of the total monomer mixture

6.2.4 Equilibrium binding studies of polymeric membranes towards the signal molecules of *P. aeruginosa*

In order to evaluate the binding capacity of the grafted membranes, corresponding disks with a diameter of 6 mm were put into 0.5 mL of C_4-AHL or 3-oxo-C_{12}-AHL solution (100 ng mL^{-1} in water and 20% acetonitrile, respectively). The vials were incubated overnight on an orbital shaker (Yellowline OS 2 basic shaker, Fisher). After incubation the concentration of bound AHL was assessed using HPLC-MS.

Additionally, a similar study to the above was undertaken using disks with a diameter of 20 mm, which were placed into 1 mL of 3-oxo-C_{12}-AHL solution (10 μg mL^{-1} in 20% acetonitrile). Then, the same procedure as before was followed.

6.2.5 Static biofilm assay and laser scanning confocal microscopy (LSCM)

In order to prepare the microscope slides with biofilms, 1 mL of overnight culture of *P. aeruginosa* PAO1 which was diluted 20 times with LB, was added to 28.25-mL vials which contained glass slides (25 mm × 15 mm) and, where required, corresponding membrane disks with a diameter of 20 mm. The testing was carried out following the procedure which was described earlier (section 4.2.10).

6.3 Results and Discussion

6.3.1 Synergist immobilisation method

The photo-grafting functionalisation of aminolysed membranes was performed in a DMF solution which contained EGDMA/IA 95/5 (w/w) and BP under UV irradiation. For comparative purposes, the same polymer composition was used for the photo-grafting as it was used as for the preparation of the bulk polymer (section 4.3.2). It was found that DG was very low (3.2±0.4) possibly due to the reaction between the functional monomer (IA) and the tertiary amino group of DEEDA. Therefore, we concentrated on the development and optimisation of the second method (iniferter immobilisation method).

6.3.2 Iniferter immobilisation method

In order to perform the immobilisation the photo-iniferter (DTC) was immobilised onto the aminolysed membranes. Thereafter, functionalisation of the membranes was carried out in a DMF solution which contained monomer mixtures

which contained functional monomer IA. The effect of monomer concentration on the DG value was studied.

Effect of monomer concentration

Monomer concentration was varied to 30 and 40 g L^{-1}. The UV exposure time was 7.5 min. The synthesised photo-grafted membranes and corresponding DG values are shown in Table 6.2. As expected, the reduced monomer concentration resulted in lower DG values.

6.3.3 Equilibrium binding studies of polymeric membranes designed for the signal molecules of *P. aeruginosa*

MIP1 (5 – 8) and MIP2 membranes (9 – 12), corresponding reference membranes (1 – 4) as well as EGDMA-grafted (15 – 18) and unmodified samples (19) were tested using equilibrium binding of C_4-AHL and 3-oxo-C_{12}-AHL from water and 20% acetonitrile, respectively. The binding of the signal molecules to the polymeric membranes was assessed using HPLC-MS (Table 6.2).

Table 6.2: Characterisation of the polymers grafted to the membranes. Key: DG = (W_1 - W_0 / W_0) x 100%, W_1, W_0 weights after and before modification.

Monomer mixture	Sample (47 mm diameter disk)	Monomer concentration ($g\ L^{-1}$)	DG (%) w/w	Binding capacity* of 6 mm diameter disk (mg AHL/g of grafted polymer)	
				100 ng mL^{-1} C_4-AHL (water)	100 ng mL^{-1} 3-oxo-C_{12}-AHL (20% acetonitrile)
EGDMA/IA 95:5 NIP	1	30	8.2	-	-
	2		7.4	0.3	1.1
	3	40	8.2	0.2	0.9
	4		12.5	0.2	0.8
EGDMA/IA 95:5 MIP1 (1 w/w% C_4-AHL)**	5	30	6.8	-	-
	6		6.1	0.3	-
	7	40	15.1	0.2	-
	8		13.8	0.3	-
EGDMA/IA 95:5 MIP2 (1 w/w%3-oxo-C_{12}-AHL)**	9	30	4.9	-	1.8
	10		6.5	-	1.3
	11	40	8.2	-	-
	12		8.4	-	1.2
EGDMA	15	30	6.4	-	-
	16		9.1	-	-
	17	40	11.1	-	-
	18		11	0.2	0.7
Unmodified	19	0	0	no sorption	no sorption

* The standard deviations of these measurements were under 5%
** of the total monomer mixture
- : this value was not determined

It was observed that MIP membranes possessed higher binding capacity values towards 3-oxo-C_{12}-AHL in comparison to reference membranes, which could be an indication of an imprinting effect. Furthermore, both MIP and reference membranes demonstrated stronger binding of 3-oxo-C_{12}-AHL compared to EGDMA-grafted membranes. This could be explained by the fact that the introduction of the functional monomer (IA) increases the affinity of the binding.

A similar study to the above was undertaken but this time larger samples were used (20 mm of diameter). Corresponding samples were tested using equilibrium

binding of 3-oxo-C_{12}-AHL from 20% acetonitrile. The strength of binding was again evaluated using HPLC-MS (Table 6.3).

Table 6.3: The binding capacity of 20 mm diameter disks towards 3-oxo-C_{12}-AHL (10 µg mL^{-1}) in 20% acetonitrile. Key: DG = (W_1 - W_0 / W_0) x 100%, W_1, W_0 weights after and before modification.

Monomer mixture	Sample (47 mm diameter disk)	DG (%)	Binding capacity (mg AHL/g of grafted polymer)
EGDMA/IA 95:5	4	12.5	5.8±0.1
EGDMA/IA 95:5 MIP2 (1 w/w%* 3-oxo-C_{12}-AHL)	10	6.5	15.4±0.5
	11	8.2	14.9±0.1
	12	8.4	11.0±0.3
EGDMA	17	11.1	5.4±0.2

* of the total monomer mixture

As before, MIP membranes exhibited the highest capacity values for 3-oxo-C_{12}-AHL while the EGDMA-grafted membranes demonstrated low binding of the template. This is another indication of an imprinting effect and also highlights the importance of the functional monomer (IA) for the provision and specificity of the binding.

6.3.4 Characterisation of polymeric membranes using FT-IR microscopy

In order to prove the grafting of the membranes, samples were tested using FT-IR microscopy (Figure 6.3). For iniferter-immobilised membrane, the intensity of the peak at 1728 cm^{-1}, which corresponds to ester group, was reduced compared with that for unmodified membrane. On the contrary, the same peak for the MIP membrane intensified due to the presence of IA and EGDMA, which indicated successful

grafting of polymer to the surface. The same phenomenon was observed for reference polymeric membranes that were prepared without template (C_4-AHL or 3-oxo-C_{12}-AHL).

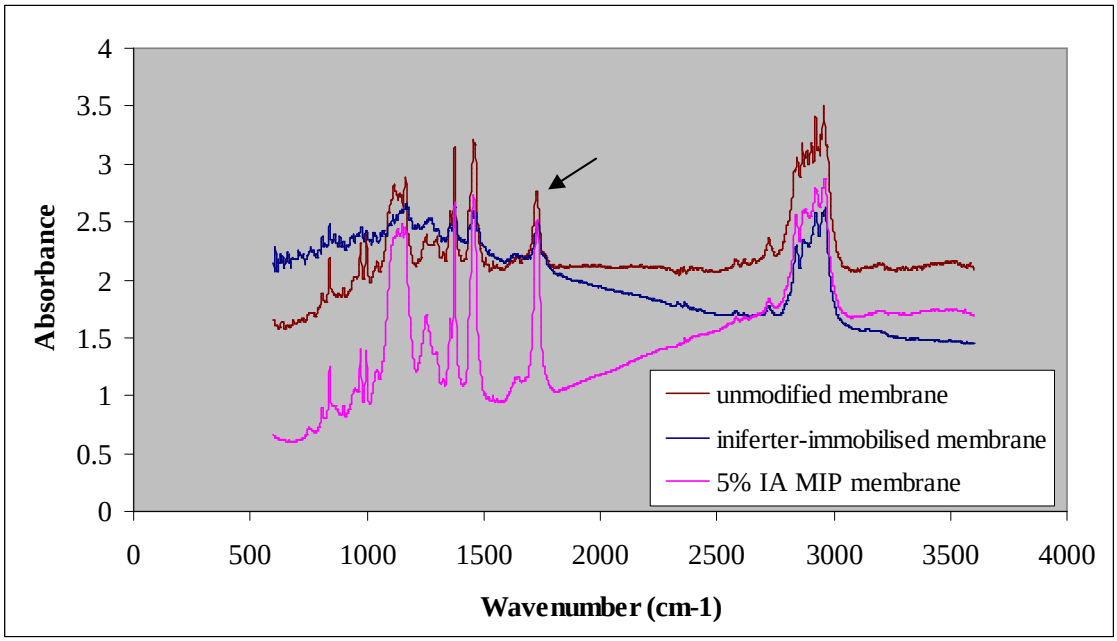


Figure 6.3: FT-IR spectra of unmodified and grafted membranes. The arrow indicates the peak, which corresponds to ester group (1728 cm^{-1}).

6.3.5 *P. aeruginosa*: static biofilm assay

It was shown before (section 4.3.5) that bulk MIP polymer specific for 3-oxo-C_{12}-AHL and based on 5% IA significantly inhibited biofilm formation of *P. aeruginosa*. It was interesting to find out if the corresponding polymeric membrane grafted with polymer of the same composition with the bulk could have the same effect. The corresponding reference membrane was also tested in order to evaluate the specificity of the binding.

Testing of the grafting effect on biofilm formation was conducted using the static biofilm assay (De Kievit *et al.*, 2001). Cells were stained with fluorescent wheat

germ agglutinin (WGA) conjugate, which emits green fluorescence, and visualised using laser scanning confocal microscopy (LSCM).

It was observed that grafting to the MIP membrane partially contributed to inhibiting of biofilm formation (Figure 6.4c and 6.4f) while the presence of corresponding reference membrane did not have much of an effect (Figure 6.4b and 6.4e) in comparison with control biofilm, which was grown in the absence of any grafted membrane (Figure 6.4a and 6.4d). It is necessary to note that the small inhibitory effect on biofilm was achieved by the MIP membrane which was prepared in presence of 1 w/w% (of the total monomer mixture) of 3-oxo-C_{12}-AHL (sample 10). Still optimisation needs to be done with respect to the concentration of the template in the monomer mixture in order to produce polymeric membranes with sufficiently high biofilm growth-inhibitory properties.

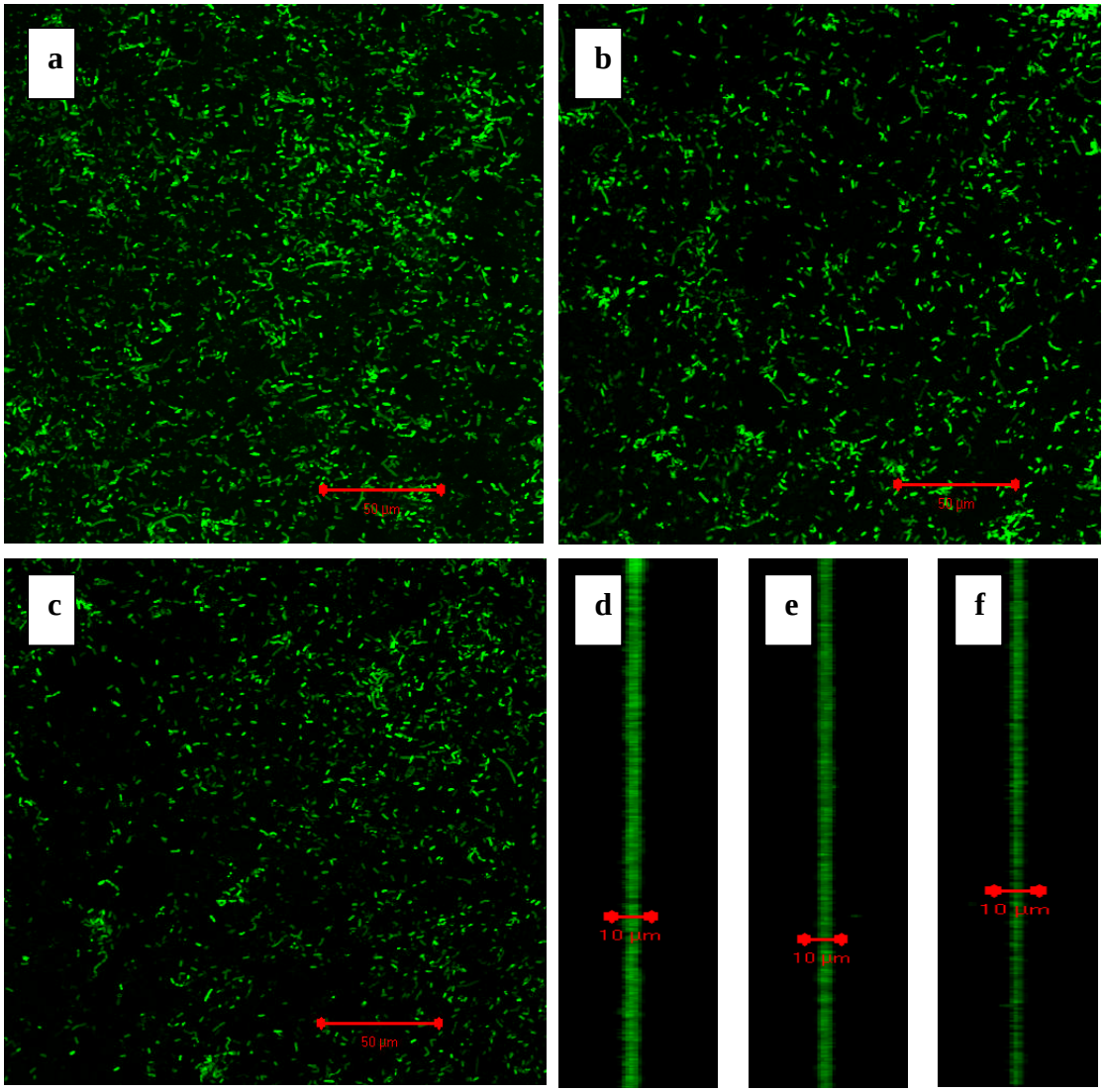


Figure 6.4: Effects of the polymer grafted onto the hydrophilic PP membranes on *P. aeruginosa* biofilm formation on the glass slides after 24 h incubation. Surface distribution of biofilm in control (a), in the presence of 20 mm diameter of reference polymeric membrane (b) and in the presence of 20 mm diameter of MIP membrane (for 3-oxo-C_{12}-AHL) (c). Scale bars are 50 µm. Corresponding biofilm thickness shown by z-stack in control (d), in the presence of 20 mm diameter of reference polymeric membrane (e) and in the presence of 20 mm diameter of MIP membrane (for 3-oxo-C_{12}-AHL) (f). Scale bars are 10 µm.

6.4 Conclusions

In this work a photo-graft polymerisation method aimed at functionalisation of the commercial polypropylene (PP) microfiltration membranes with cross-linked

polymers based on itaconic acid (IA) was developed and the effect of monomer concentration on the DG value was studied. The polymeric membranes were tested for adsorption of C_4-AHL and 3-oxo-C_{12}-AHL using equilibrium binding and evaluated by HPLC-MS. Generally, MIP membranes demonstrated higher binding capacity in comparison to reference polymeric membranes. Characterisation of the membrane samples using FT-IR was a proof of successful grafting. As observed by laser scanning confocal microscopy of WGA- stained biofilms, addition of MIP membrane specific for 3-oxo-C_{12}-AHL partially suppressed the biofilm formation of *P. aeruginosa*. Grafting the commercial membranes with a polymer layer which would be biofilm- and biofouling-resistant could be very important for the variety of the practical applications, and this success is a first step towards achieving this goal.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1 General Conclusions

The current work was aimed at design and development of the synthetic polymers which could disrupt the communication between Gram-negative bacteria *Vibrio fischeri*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* by sequestration of the small signal molecules *N*-acyl-L-homoserine lactones (AHLs). The AHLs are produced and used by bacteria for quorum sensing (mechanism which starts biofilm formation process in response to increased population density). When the bacterial population has reached the threshold density, the bacteria would amass the attack and start production of the virulence factors and biofilms.

Gram-negative, marine bacterium *Vibrio fischeri* which has a bioluminescence as a QS-controlled phenotype was selected as a model system. Molecular modeling was applied to design the polymers which are able to attenuate bioluminescence of *V. fischeri* by sequestering its signal molecule *N*-(β-ketocapryloyl)-L-homoserine lactone (3-oxo-C_6-AHL). Six rationally-designed polymers were produced. *In vitro* tests demonstrated that some of these polymers had a sufficient affinity to 3-oxo-C_6-AHL and could inhibit the development of bioluminescence. *V. fischeri* culture, which was incubated with polymers prepared with 5% IA and 5% MAA, showed significant reduction of the bioluminescence equal to 85 ± 8% and 84 ± 6%, respectively. At the same time it was shown that the growth of bacteria was not affected by the polymers and that the quenching of the bioluminescence could not be due to sequestration of the nutrients or general toxicity.

The successful attenuation of *V. fischeri* QS-controlled phenotype by signal-sequestering polymers was followed by the development of the resins for more problematic, pathogenic microorganism, such as *P. aeruginosa*. The study continued

with the development of rationally-designed polymers which could specifically sequester the signal molecules of *P. aeruginosa* (3-oxo-C_{12}-AHL and C_4-AHL).

Resins with affinity to C_4-AHL were produced and their binding capacity towards the signal molecule measured. The polymers based on 10% MAA, IA, acrylamide and MBAA were tested *in vitro* by using QS-responsive bioluminescent variant of *P. aeruginosa*, namely PAO1 *lecA::lux*. It was found that bacterial growth was not affected by the presence of the polymeric materials therefore some reduction of the luminescence which was observed in our experiments could be explained by the removal of the signal molecule (C_4-AHL) from the system and not by the depletion of the nutrients.

Five polymers based on MAA, IA, MBAA, AA and AMPSA were produced for the specific sequestration of 3-oxo-C_{12}-AHL. The affinity, selectivity and capacity of the resins were characterised. Polymer which contained 5% IA possessed the highest binding capacity of 3-oxo-C_{12}-AHL, which was evaluated as 42.3±2.7 mg AHL per g of polymer.

MIP for a key signal molecule of *P. aeruginosa* 3-oxo-C_{12}-AHL based on 5% IA was produced and tested. It was found that the MIP possessed higher affinity for 3-oxo-C_{12}-AHL (K_d=145±2 µM) than Blank polymer (K_d=312±3 µM). The overall binding capacity of the polymers was estimated at 22.5 µmol/g (MIP) and 14.1 µmol/g (Blank) which could be considered as sufficient for the practical application. Furthermore, it was demonstrated that the presence of the MIP inhibited the biofilm formation of *P. aeruginosa* by 80% as compared with control sample which was grown without polymer and by 40% as in comparison with a sample which was incubated with corresponding blank polymer. This could be due to the sequestration of 3-oxo-C_{12}-AHL and not as a result of depletion of nutrients as none of the

polymers had any inhibitory effect on bacterial growth. This finding, based on the Crystal Violet staining, which is considered as a standard method for the biofilm quantification, was further confirmed by two other methods: by staining with Alcian Blue and also by confocal microscopy of the biofilms which were stained with fluorescent conjugate WGA.

The importance of the ability to control the microbial biofilm growth is difficult to overestimate. By significantly reducing biofim formation it is possible to fight bacteria infection much easier, without applying the high concentrations of the antibiotics and disinfectants. Thereby, 3-oxo-C_{12}-AHL-MIP could be considered as a very promising material towards the eradication of *P. aeruginosa* infections, considering that these bacteria are known for their resistance to many antibiotics.

Another set of the synthetic polymers was developed for the specific sequestration of the signal molecules of *B. cepacia* (C_6- and C_8-AHLs). All polymers were tested for the binding of C_6- and C_8-AHLs using SPE. SPE screening and equilibrium binding showed that 5% MAA-, 5% IA- and 5% EGMP-based polymers possessed sufficiently high binding capacity towards C_8-AHL, which was calculated as 5.3, 4.7 and 4.5 mg AHL per g of polymer, respectively. The corresponding values for C_6-AHL were 2.6, 2.2 and 2.2 mg AHL per g of polymer.

The first step towards the potential practical application of the selected polymers was made by optimisation of the grafting procedure for introduction of the signal-molecule-sequestering polymers onto the commercial membranes. A photo-graft functionalisation protocol for the hydrophilised polypropylene microfiltration membranes with signal molecule-sequestering polymers was developed. It was found that the MIP- modified polymeric membranes exhibited higher binding capacity in comparison to reference polymeric membranes and ability to partially suppress

biofilm formation of *P. aeruginosa*, as it was observed by confocal microscopy. This work would lead to the development of a new generation of membranes which would be resistant to biofilm formation and therefore to biofouling. The developed photo-grafting protocol could also be applied for the modification of various bacteria-susceptible surfaces, e. g. catheters and contact lenses.

7.2 Future work

In order to proceed further towards the practical applications of the rationally-designed polymeric materials, the following future studies could be carried out:

- Production and testing of MIPs for *B. cepacia* based on methacrylic acid as a functional monomer.
- Testing of bulk polymers or polymers grafted onto commercial PP membranes for attenuation of biofilm formation for *B. cepacia*.
- Optimisation of the composition and quantity of the polymer layer which could be grafted onto the PP membranes in order to achieve higher binding capacity towards the signalling molecules of *P. aeruginosa* (C_4- and 3-oxo-C_{12}-AHLs) and further testing of the resulting membranes for attenuation of biofilm formation.
- Different formats of the signal-sequestering polymers could be prepared and tested in order to develop bacteria-resistant materials for wound dressings and grafting of the specific polymer layer onto the surface of medical tools for prevention of biofilm formation (e.g. catheter sleeves, contact lenses). These applications will be explored in close collaboration with hospital researchers.
- Future study will be interesting to perform aimed at the development of slow release materials which will be based on the molecularly imprinted materials.

Selected AHLs (C_4-AHL, 3-oxo-C_{12}-AHL, C_6-AHL and C_8-AHL) could be used as templates for MIPs which will be tested for the possibility to induce the expression of the virulence at the early stage of the bacterial development. This is very important aspect because it gives the infected organism better chance to clear the infection when the bacterial population is still small.

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