DEVELOPMENT OF NOVEL NANOMATERIALS FOR DEGRADATION OF AUTOINDUCERS OF GRAM-NEGATIVE BACTERIA

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Abstract

DEVELOPMENT OF NOVEL NANOMATERIALS FOR DEGRADATION OF AUTOINDUCERS OF GRAM-NEGATIVE BACTERIA

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Bacteria are able to coordinate gene expression to develop different phenotypes such as motility or biofilm formation using quorum sensing (QS) systems. To synchronise its activity, Gram-negative bacteria use N-acyl-L-homoserine lactone as QS signal molecules. Using molecularly imprinted polymers (MIP), we have attempted to synthesise polymeric catalytic nanoparticles (NPs) to hydrolyse AHL. The activity of these MIP NPs relies on the template, a transition state analogue (TSA) of the hydrolysis of AHL. The solid phase synthesis of the MIP NPs has been performed using radical polymerisation.

The hydrolytic capacity of the MIP NPs was analysed by measuring the degradation of N-hexanoyl homoserine lactone (C6-AHL) by HPLC-MS and comparing the degradation degree of C6-AHL between the TSA-NPs, control-NPs and natural degradation. The first approach was accomplished using commercially available monomers, which showed a significant decrease in the concentration of the C6-AHL in the samples containing TSA-NPs.

Despite being the first attempt to use MIPs as attenuating agents of the development of QS in Gram-negative bacteria, these new polymeric materials have demonstrated high efficiency. The NPs obtained using methacrylic acid (MAA) as functional monomer showed the best performance, being able to degrade 41% of C6-AHL in the sample during first 2 h, while the MAA control polymer was able to remove only 18%. Moreover, MIP NPs are more robust, cheaper and more stable than enzymes or antibodies and can be added to paints or be immobilised on the surfaces of medical devices, food containers, water tanks or as a complement for antimicrobial treatments to control resistant bacterial infections.
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List of Abbreviations

- 3-oxo-C12-AHL \(N\)-(3-oxododecanoyl) homoserine lactone
- AHL \(N\)-acyl-\(L\)-homoserine Lactone
- C4-AHL \(N\)-butanoyl homoserine lactone
- C6-AH \(N\)-hexanoyl homoserine
- C6-AHL \(N\)-hexanoyl homoserine lactone
- D Diffusion coefficient
- \(d_{h}\) Hydrodynamic diameter
- DNA Deoxyribonucleic acid
- DPD 4,5-dihydroxy 2,3-pentadione
- IR Infrared spectroscopy
- LRP Living radical polymerisation
- MDRB Multidrug-resistance bacteria
- MIC Molecularly imprinted catalyst
- MIP Molecularly imprinted polymers
- MS Mass spectrometry
- NBS \(N\)-bromosuccinimide
- NCS \(N\)-chlorosuccinimide
- NMR Nuclear magnetic resonance spectroscopy
- NPs Nanoparticles
- PDI Polydispersity index
- QQ Quorum quenching
- QS Quorum sensing
- r.t. room temperature
- RSD Relative standard deviation
- \(t_{R}\) retention time
- SD Standard deviation
- TLC Thin layer chromatography
- TSA Transition state analogue

Abbreviation of chemicals reagents in “Chemicals” (2.1.1).
Chapter 1

General Introduction
1.1. Approach to the topic of study

According to the Assistant Director-General for Health, Security and Environment for the World Health Organization, Dr Keiji Fukuda, in 2015 “A particularly urgent concern is the development of bacteria that are progressively less treatable by available antibiotics. This is happening in all parts of the world, so all countries must do their part to tackle this global threat”.¹ He has highlighted the great need for the discovery of new treatments against bacterial infections, as the number of, effective antibiotics for the new resistant super-bacteria is scarce and the development of new ones has decreased in recent years.²

About fifty years ago, it was observed that *Photobacterium fischeri* (Gram-negative bacteria) is able to coordinate light emission when the cell density in the cell culture is very high.³ Twenty years later, this fascinating behaviour was named Quorum Sensing. The reason that a cell culture of the bacteria *Vibrio fischeri* can glow in the dark is because these bacteria produce and secrete low molecular weight signal molecules which are used by bacteria to sense their population density, or quorum. Once the concentration of signal molecule reaches a threshold, the whole bacterial population starts changing its expression of specific genes to activate the QS-controlled processes, which include luminescence in the case of *V. fischeri*. Other, more clinically-relevant QS phenotypes include the production of virulence factors and biofilm formation.⁴ The formation of biofilms is one of the main sources of resistance in pathogenic bacteria. Firstly, in order to kill bacteria within a biofilm, the concentration of antibiotic needs to be increased dramatically, between ten and one thousand times.⁵ Similarly, biofilms protect bacteria against antibodies and white cells, detergents and disinfectants and act as a source of bacteria ready to colonise more tissues or surfaces when circumstances allow.

It is important to mention that constantly living in competition with other species of microorganism means that bacteria have mechanisms not only to activate Quorum Sensing but also inhibit it. For instance, some enzymes (such as lactonase or acylase) generated by some types of bacteria can break down the signal molecules of other bacteria. It is well known that disrupting the communication between bacteria (removing autoinducers from the medium) makes them incapable of carrying their behavioural traits coordinated by QS. It makes them more sensitive to antibiotics and all the mechanisms that our body uses to fight them because they cannot protect themselves with a biofilm. However, these natural enzymes have limitations; they only work under very specific
conditions and, therefore are not suitable as a technological solution for practical environmental and clinical applications. For this reason, the development of new synthetic antimicrobial agents is of crucial importance, since such a development would provide the tools to keep bacterial infections under control.

In this thesis, a new approach for the development and application of molecularly imprinted polymers (MIPs) as synthetic catalysts is presented. These ‘plastic’ enzymes should, ideally, mimic the activity of the natural lactonase enzyme, being able to deactivate the signal molecules and prevent the bacteria from detecting other bacterial cells in their environment. Furthermore, such plastic enzymes are much more stable, easy to obtain and cheaper than enzymes. As the destruction of the external signal molecules does not kill the bacteria, there is no incentive for bacteria to mutate and develop resistance towards antimicrobial agents that operate in this manner.

1.2. Bacterial Quorum sensing

1.2.1. Quorum Sensing

The ability to communicate with members of the same species does not belong only to multicellular organisms. It was found that various microorganisms, such as bacteria (Gram-positive or Gram-negative), fungi, moulds and even protozoa have the ability to communicate and hence coordinate their behaviour using QS systems. When bacteria interact with any surface (a catheter, water filter or food container) or with the tissue of an organism (plant or animal) their goal is to colonise it and establish their dominance. Nevertheless, as their cell density is especially low, they are quite vulnerable, so at this point they just feed by consuming nutrients from their environment, grow, divide and multiply by binary fission. This behaviour is carried out by even very virulent bacteria such as Vibrio cholera or Mycobacterium tuberculosis. During this growth process, the concentration of small signal molecules, which are detected by other bacteria of the same species, increases until it reaches a concentration threshold. Once this happens the whole bacterial population responds by changing gene expression pathways, leading to significant changes in their phenotypic traits. This mechanism allows bacteria to save resources and coordinate different actions that are impossible accomplish with a low population density. It is necessary to stress that all these mechanisms related to QS are dependent on the concentration of autoinducers (1.2.1.1) in the medium. The most studied QS circuit is the LuxI/LuxR system in the Gram-negative marine bacterium V.
*fischeri*. These bacteria are able to produce bioluminescence, by using the regulatory system LuxI/LuxR (Figure 1) in a process mediated by QS. The LuxI protein is an enzyme that catalyses the biosynthesis of specific acylated homoserine lactone signal molecules, in this case, N-(3-oxohexanoyl) homoserine lactone. The protein LuxR (cytoplasmic autoinducer receptor) is activated by AHL molecules, which interact reversibly with its N-terminal domain. Once it is activated, its C-terminal domain can interact with specific DNA motifs to stimulate transcription of the luxICDABE promoter (luciferase operon). These genes encode for luciferase and encourage the expression of the LuxI autoinducer synthase enzyme. For this bioluminescent bacterium, this means that once the luxICDAB promoter is activated, the autoinducer synthesis and light emission increase exponentially, due to the positive feedback. Nevertheless, the LuxR expression decreases (negative feedback), as a mechanism to control this process.

![Figure 1. Scheme of the QS system LuxI/LuxR of the marine bacteria V. fischeri.](image)

This QS process allows bacteria to offset a small variation in the population density of the colony. There are similar LuxI/LuxR-type systems participating in the QS process of other Gram-negative bacteria, which are adapted to the niche in which they live. For instance, *Pseudomonas aeruginosa* is a Gram-negative opportunistic microorganism, being a pathogen of both, plants and animals (also humans). It is a multidrug-resistance pathogen, being the most common coloniser of medical devices, such as catheters. It is responsible for many diseases, such as pneumonia, septic shock, urinary tract infection, gastrointestinal infection, skin and soft tissue infections, etc.
*P. aeruginosa* has two homologous pairs of QS systems, LasI/LasR and RhlI/RhlR, organised hierarchically (Figure 2). They are responsible for the expression of virulence factors in this bacterium. Both LasI and RhlI are AHL-synthases catalyse the biosynthesis of \(N\)-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-AHL) and \(N\)-butanoyl homoserine lactone (C4-AHL), respectively. These two systems work together as a QS regulatory mechanism.

**Figure 2.** Outline of the QS systems LasI/LasR and RhlI/RhlR in the opportunistic pathogenic bacterium *P. aeruginosa*.

When a *P. aeruginosa* population starts to grow in a certain tissue or surface, it begins to release autoinducers into the environment. Once it reaches a high cell density (high autoinducers concentration) the protein LasR interacts with the signal molecule (\(N\)-3oxo-C12-AHL). This LasR-AHL complex can activate the promoter and initiate the transcription of genes which encode for various virulence factors, such as elastase (protease), an enzyme that is released to destroy the host tissue. Besides, it boosts the expression of LasI to generate the positive feedback loop and increase the synthesis of signal molecules (3-oxo-C12-AHL).\(^{15}\) Moreover, LasR leads to the activation of the second QS system, RhlI/RhlR, by inducing the expression of the RhlR protein. RhlR interacts with C4-AHL and the complex formed activates the promoter to transcribe the genes that encode RhlI, the autoinducer synthase for C4-AHL. It rapidly increases the synthesis of this autoinducer as well. Thus, the LasI/LasR system controls and regulates the RhlI/RhlR system.\(^{16}\)
In Gram-positive bacteria, there are many different mechanisms to regulate their phenotype depending on the cell density. The most studied and common model is presented below. The process begins with the synthesis of the peptide precursor, which must be cleaved, in a modification and maturation process, resulting in the signal molecule (pheromone). Through an ABC transporter (ATP-binding cassette transporter), the signal molecules are released to the environment by an active transport process. The concentration of pheromones in the environment of the bacteria increases as result of the cell population density. When the accumulation of signal molecules reaches a threshold, it is detected by a two-component signalling transduction system, composed of a sensor histidine kinase and a response regulator protein. This system is responsible for the detection and transfer of the signal inside the cell. To undertake this task, the pheromone interacts with the extracellular domain of the sensor kinase, launching an auto-phosphorylation on a histidine residue. Then, the aspartate residue of the cognate response regulator protein gets phosphorylated. Once the regulator protein is phosphorylated, it is able to activate the transcription of the peptide-pheromone that in its turn triggers the activation of the genes that are dependent on the cell-population density (Figure 3).17

**Figure 3.** Diagram of the pheromones-mediated QS in Gram-positive bacteria which consists of two regulatory systems: membrane-bound histidine kinase and a cognate response regulator.

The above examples shown how QS systems work in Gram-negative and Gram-positive bacteria, as well as some phenotypes developed by bacteria using QS systems such as bioluminescence, resistance (1.2.1.3), virulence (1.2.1.4) or biofilm formation (1.2.1.5),
symbiotic interactions, motility, sporulation and numerous other activities. For these reasons, QS is a very interesting target for new therapies. However, we have just started uncovering the details of this amazing mechanism that allows bacteria to act as a multicellular organism and to control the relationship between bacteria and eukaryotic cells.

1.2.1.1. Autoinducers or QS signal molecules

Autoinducers are the molecules that constitute an important part of the QS function. These signal molecules allow bacterial populations to sense their density and act in the most appropriate way, changing and synchronising their phenotypes. A signal molecule has to possess at least these five basic features to be labelled an autoinducer.18

- The bacterial colony produces signal molecules during its growth once the optimal physiological conditions have been reached. These signals are sensed by specific receptors placed in the bacterial wall of the member of the same bacterial strain.
- The autoinducers are released into the bacterial environment by simple diffusion (Only in Gram-negative bacteria). Hence, its concentration is the same inside and outside of the bacterial cell.
- The concentration of these molecules is proportional to the cell density of a bacterial population; when its concentration reaches a threshold, it triggers a synchronised response in the bacterial community.
- These kinds of molecules trigger changes in cellular physiology and activate different metabolic traits in which these molecules are involved.
- The most characteristic feature of QS signals is the fact that they are able to activate the synthase genes used by bacteria for its synthesis, inducing its own synthesis.

There are several types of signal molecules. One of the main representatives of signal molecules in the Gram-negative bacteria is the family of N-acyl homoserine lactones (AHLs). Gram-positive bacteria use post-translational oligopeptides (pheromones) constituted by 5 to 34 amino acids with a very wide range of architectures (Figure 4).
**Figure 4.** Post-translational proteins used as QS signals molecules by the following Gram-positive bacteria: (A) *Lactococcus lactis*; (B) *Staphylococcus aureus*; underneath, schematic structures of autoinducer peptides (AIP) from some species of *Staphylococcus*; (C) *Bacillus subtilis*; (D) *Lactobacillus plantarum* and (E) *Enterococcus faecalis*.

AHLs are the autoinducers of Gram-negative bacteria (Figure 5).\textsuperscript{19,20} They are small hydrophobic molecules and can diffuse freely through the cell membrane; the concentration of autoinducers in the extracellular media and the bacterial cytoplasm are therefore the same. Most of them are of the $N$-acylated homoserine group ($\gamma$-butyrolactone) with a fatty acyl group placed in the $\alpha$-position; this position is a chiral carbon, which gives to all AHL $L$-configuration. The acyl chain is the part of the molecule that changes depending on the bacterial species. Its length is between 4 and 18 carbons, normally it is an even number of carbons, but acyl chains with 7 carbons have also been found.\textsuperscript{21} Moreover, the acyl chain can have a substituent in the third carbon which could
be a hydroxyl group or more likely a ketone\textsuperscript{22} and when they have between 14 and 18 carbons, they can contain one or two double bonds.\textsuperscript{23} These signal molecules are quite susceptible to hydrolysis of the lactone ring; that means that they are very sensitive to high pH and temperature.\textsuperscript{24} With a longer acyl chain, the AHL is stable at a higher pH.

Figure 5. Structures of representative Gram-negative bacterial autoinducers. A) General structure of the most common AHL. In all of them, R is an aliphatic chain with a number of carbons between 1 and 15; also, it may contain one or two double bonds. B) Structure of several autoinducers and the bacteria that produce them, the green line divides the general moiety and the specific part. C) Structure of other less common signal molecules found in bacterial cultures: AI-2, furanosyl borate ester form; A-factor, 2-isocapryloyl-3-hydroxymethyl-$\gamma$-butyrolactone; PQS, 2-heptyl-3-hydroxy-4(1H)-quinolone (Pseudomonas quinolone signal); DSF, \textit{cis}-11-methyl-2-dodecenoic acid (diffusible factor); PAME, hydroxyl-palmitic acid methyl ester.

The hydrolysed form of the AHL (open-ring form) has no biological activity, for this reason, the smallest AHLS found are $N$-butanoylhomoserine lactone (C4-AHL) and $N$-hydroxybutanoylhomoserine lactone (3-hydroxy-C4-AHL).
Table 1. Review of the LuxI/LuxR-type systems used by several Gram-negative bacteria, the autoinducers which they possess QS signal molecules and the phenotype they trigger once they are activated.6,20

<table>
<thead>
<tr>
<th>Organism</th>
<th>Major AHL</th>
<th>LuxR</th>
<th>LuxI</th>
<th>Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas hydrophila</td>
<td>C4-AHL</td>
<td>AhyR</td>
<td>Ahyl</td>
<td>biofilm, exoproteases</td>
</tr>
<tr>
<td>Aeromonas salmonicida</td>
<td>C4-AHL</td>
<td>AsaR</td>
<td>Asal</td>
<td>exoprotease</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>3-oxo-C8-AHL</td>
<td>TraR</td>
<td>Tral</td>
<td>plasmid conjugation</td>
</tr>
<tr>
<td>Agrobacterium vitiae</td>
<td>C14:1-AHL, 3-oxo-C16:1-AHL</td>
<td>AvsR</td>
<td>Avsl</td>
<td>virulence</td>
</tr>
<tr>
<td>Burkholderia cenocepacia</td>
<td>C6-AHL, C8-AHL</td>
<td>CepR, CciR</td>
<td>Cepl, Ccil</td>
<td>exoenzymes, biofilm, swarming motility, siderophore, virulence</td>
</tr>
<tr>
<td>Chromobacterium violaceum</td>
<td>C6-AHL</td>
<td>CviR</td>
<td>CviI</td>
<td>exoenzymes, cyanide, pigment</td>
</tr>
<tr>
<td>Erwinia carotovora ssp. carotovora</td>
<td>3-oxo-C6-AHL</td>
<td>ExpR/CarR</td>
<td>Carl (Expl)</td>
<td>carbapenem, exoenzymes, virulence</td>
</tr>
<tr>
<td>Pantoea (Erwinia) stewartii</td>
<td>3-oxo-C6-AHL</td>
<td>EsaR</td>
<td>Esal</td>
<td>exopolysaccharide</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>C4-AHL; 3-oxo-C12-AHL</td>
<td>LasR, RhlR,</td>
<td>Lasl, Rhl</td>
<td>exoenzymes, secretion, hcn, biofilm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QscR, VqsR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aureofaciens</td>
<td>C6-AHL</td>
<td>PhzR, CsaR</td>
<td>Phzl, Csal</td>
<td>phenazines, protease, colony morphology, aggregation, biofilm</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>3-oxo-C10-AHL, 3-oxo-C12-AHL</td>
<td>PpuR</td>
<td>PpuI</td>
<td>biofilm</td>
</tr>
<tr>
<td>Pseudomonas chlororaphis</td>
<td>C6-AHL</td>
<td>PhzR</td>
<td>Phzl</td>
<td>phenazine-1-carboxamide</td>
</tr>
<tr>
<td>Pseudomonas syringae</td>
<td>3-oxo-C6-AHL</td>
<td>AhlR</td>
<td>AhlI</td>
<td>exopolysaccharide, swimming motility, virulence</td>
</tr>
<tr>
<td>Rhodobacter sphaeroides</td>
<td>7-cis-C14-AHL</td>
<td>CcrR</td>
<td>CcrI</td>
<td>aggregation</td>
</tr>
<tr>
<td>Serratia spp. ATCC 39006</td>
<td>C4-AHL</td>
<td>SmaR</td>
<td>SmaI</td>
<td>antibiotic, pigment, exoenzymes</td>
</tr>
<tr>
<td>Serrana liquefaciens MGI</td>
<td>C4-AHL</td>
<td>SwrR</td>
<td>Swrl</td>
<td>swarming motility, exoprotease, biofilm, biosurfactant</td>
</tr>
<tr>
<td>Serratia marcescens SS-1</td>
<td>C6-AHL, 3-oxo-C6-AHL</td>
<td>SpnR</td>
<td>SpnI</td>
<td>sliding motility, biosurfactant, pigment, nuclease</td>
</tr>
<tr>
<td>Serratia proteamaculans B5a</td>
<td>3-oxo-C6-AHL</td>
<td>SprR</td>
<td>SprI</td>
<td>exoenzymes</td>
</tr>
<tr>
<td>Vibrio fischeri</td>
<td>3-oxo-C6-AHL</td>
<td>LuxR</td>
<td>Luxl</td>
<td>bioluminescence</td>
</tr>
<tr>
<td>Yersinia pseudotuberculosis</td>
<td>C6-AHL, 3-oxo-C6-AHL, C8-AHL</td>
<td>YpsR, YtbR</td>
<td>Ypsl, Ytbl</td>
<td>motility, aggregation</td>
</tr>
</tbody>
</table>
When the concentration of AHLs reaches a level of usually 1 to 10 µg/mL, the LuxR-type protein starts to interact with the autoinducers and initiates the development of different phenotypes synchronously in all the population. However, the threshold could change depending on the bacteria or signal molecule; likewise, the phenotype that the same signal molecule could develop into a different organism could be different, as is shown in Table 1.

1.2.1.2. Interspecies communication

QS is not just a mechanism to control gene expression and hence the behaviour of a colony of just one bacterial species. Any bacterial species has its own mix of autoinducers to synchronise its phenotype and sense its cell density. This is intra-species communication. However, bacteria live in niches with many other different types of bacteria, thus, there must be a mechanism that permits bacteria to sense the cell density of other species, and hence decide what task they should carry out depending on the majority species and the minority species. In other words, inter-species communication. It has been observed that there are several bacterial species that need to grow within the same colony to develop different functions in symbiosis, such as biofilms or virulence. This is the case for the community formed by *Streptococcus gordonii* and *Porphyromonas gingivalis*, which contribute together to the formation of a biofilm in dental plaque.

Bacteria have several QS systems, performed by different mechanisms; those shown above (1.2.1) are mainly intra-species QS systems. Nonetheless, bacteria have more QS mechanisms beyond those. LuxS/LuxP is the most studied inter-species QS system, found in a wide range of Gram-positive and negative bacteria. This system is able to produce, sense and respond to Autoinducer-2 (AI-2) (Figure 11), which is the autoinducer generated in this mechanism.

This autoinducer, AI-2, has something special: depending on the medium where is released it can have different structures. The process begins with the biosynthesis of 4,5-dihydroxy-2,3-pentanedione (DPD), which is the autoinducer precursor. In the extracellular medium the molecule reacts spontaneously to produce (2S,4S)-2-methyl-2,3,3,4-tetrahydroxy-tetrahydrofuran-borate (S-THMF-borate) when there is boron present or, (2R,4S)-2-methyl-2,3,3,4-tetrahydroxy-tetrahydrofuran (R-THMF) when there is not (Figure 6).
The formation of one structure or the other is controlled by fast equilibrium, which could be observed in a cell culture of *Salmonella typhimurium* and *Vibrio harveyi*. *S. typhimurium* can sense AI-2 when the culture medium is depleted in boron, because of the formation of R-THMF; however, if boron is added to the cell culture, the equilibrium shifts to S-THMF-borate and *V. harveyi* can sense AI-2 and develop a process controlled by QS, such as bioluminescence. This is understandable because *V. harveyi* is a marine bacterium. Therefore, accustomed to living in an environment rich in boron, while *S. typhimurium* is not.

The mechanism shown by this system is similar to the mechanism carried out by pheromones in Gram-positive bacteria. The autoinducer precursor, DPD is synthesised by the LuxS enzyme and released by diffusion to the environment. DPD becomes the signal molecule, AI-2 by spontaneous reactions. It is recognised by the LuxP binding-protein which transfers the signal by the phosphorylation of a two-protein component in the signal-transduction system. This process ends with the activation or suppression of different genes, but this autoinducer usually acts in conjunction or under a hierarchy with other signal molecules. For example, in *Vibrio cholerae* there are three parallel QS systems which regulate the virulence cascade. Furthermore, the phenotype produced varies depending on the organism. For instance, in *Actinobacillus actinomycetemcomitans*, AI-2 activates virulence factors, but in *Vibrio harveyi*, it activates bioluminescence.
1.2.1.3. Resistance development

The number of multidrug-resistance bacteria (MDRB) has risen to a very alarming level; several bacteria such as *P. aeruginosa, Staphylococcus aureus, Clostridium difficile, Salmonella enterica*, some strains of *Escherichia coli, V. cholerae* or *Mycobacterium tuberculosis*, are on the top of the list of super-resistant bacteria. This is because, once they have infected a human being, there is no easy treatment and also, they are widely spread in the biosphere. For instance, there are close to two million cases per year of infection by MDRB in U.S., involving a cost of around $8 billion to the health care system of which, nearly 99,000 patients do not survive the treatment.\(^3\)\(^2\) There is a number of reasons which have caused this situation and we can find these reasons all over the world. Since antibiotics were commercialised, they have been overused, prescribed to treat diseases for which they were useless or taken without a doctor’s prescription. Their production has been optimised to the limit that they are produced in huge amounts industrially, frequently, disposed of in the environment due to the lack of proper waste management. Moreover, their massive use in agriculture and livestock production has increased the exposure of bacteria to antibiotics and, hence, the survival of only resistant bacterial strains,\(^3\)\(^3\) facilitating the spread of resistant genotypes.

**Figure 7.** Summary of the main mechanisms used by bacteria to obtain resistance against antibiotics. **A**) Entrance of antibiotics into the bacterial cell by porins. **B**) Efflux pump,
a three-protein active transport system. C) Random mutations in the DNA that lead to modification in the binding site of proteins, either membrane (decreasing access to the cell) or cytoplasmic (nullifying the adverse effect) proteins. D) Production of specific enzymes for the degradation of antibiotics, such as acetyltransferases or monoxygenase. E) Changes in the cell membrane receptors and porins as consequence of DNA mutations, modifying the permeability of the cell membrane and its interaction with the antibiotics.\textsuperscript{34}

One such example is \textit{P. aeruginosa}. This opportunistic pathogen is able to pump substances (such as antibiotics), which can pose a threat for its integrity, out of the cell. When one drug is recognized by this three-protein mechanism, this bacterium becomes resistant to it.\textsuperscript{35} Additionally, \textit{P. aeruginosa} is able to produce enzymes that break down \(\beta\)-lactam antibiotics (\(\beta\)-lactamases).\textsuperscript{36} Curiously, an interaction between QS systems and the increase of resistance has been observed, notably, in the encouragement of the protein complex involved in the efflux pump system that pumps the antibiotics out of bacteria.\textsuperscript{37}

At the moment there is no solid foundation of how this relationship works and all the mechanisms behind this finding. As well as \textit{P. aeruginosa}, there is a plethora of bacteria that are becoming resistant to the current antibacterial therapies,\textsuperscript{11} using a wide variety of mechanisms (Figure 7), which continue to rise due to random mutations generating new resistant strains and the activation or exchange (mainly plasmid or recombination) of already existing genes.

\textbf{1.2.1.4. Pathogenesis and virulence}

Pathogenesis is the capacity of a microorganism (pathogen) to proliferate in a specific tissue of an organism. The pathogen develops its biological activity at the expense of the colonised organism; depending on how this affects the host organism, there are different grades of virulence. In the case of opportunistic pathogens, these organisms can live in the host without causing harm or having a low virulence. However, in a time of weakness of the host organism or when the population of pathogens is high enough, the pathogen can take advantage of the situation and became virulent, being able to kill the host. In general, the development of virulence by a bacterium starts with the adherence of the bacteria to the cells of a tissue and the consequent development of a synchronised bacterial community through QS mechanisms. The activation of virulence factors does not happen until the bacterial community has enough members to accomplish an effective and coordinated change of genetic profile and has sufficient strength to attack the
defences of the host organism. At this point, bacteria start changing the molecular composition of their bacterial wall and capsule, cell membrane, superficial appendages (flagella or fimbriae) and many other cellular features. They also change their metabolism, for example releasing siderophores (hydroxamates and catechols) in order to obtain iron from the host, developing some protective phenotypes, like the generation of flavohaemoglobin to neutralise reactive nitrogen intermediates, used as an antibacterial agent by the host or producing toxins, the function of which is to disturb and/or weaken the host cells and tissues.

Table 2. Bacterial pathogens isolated from the biofilm formed on the surface of medical devices commonly used.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Has been found in biofilms from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>artificial voice prosthesis, central venous catheter, intrauterine device</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>artificial hip prosthesis, artificial voice prosthesis, central venous catheter, intrauterine device, prosthetic heart valve, urinary catheter</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>artificial hip prosthesis, central venous catheter, intrauterine device, prosthetic heart valve, urinary catheter</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>central venous catheter, urinary catheter</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>artificial hip prosthesis, central venous catheter, urinary catheter</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>artificial hip prosthesis, central venous catheter, intrauterine device, prosthetic heart valve</td>
</tr>
</tbody>
</table>

It is well known that the majority of these processes in any bacterium are controlled by QS. One of the examples of a QS-triggered pathogenic phenotype is biofilm formation (1.2.1.5), which functions as a very versatile and efficient defence system. Once the biofilm is matured, it is very difficult to treat because it protects, feeds and acts as a source of bacteria, either in a host organism or for instance on the surface of medical devices as is shown in Table 2.

1.2.1.5. Bacterial biofilms

Bacteria and fungi are microorganisms that are used to living as single planktonic cells; however, they can become sessile using QS mechanisms to coordinate their behaviour.
and create a colony. They can colonise a surface or a tissue, growing on top of it as an organised and coordinated society whose members can be from the same (mainly) or different species; being able to accomplish multicellular processes such as the formation of a bacterial biofilm or production of toxins (Figure 8).

**Figure 8.** Biofilm formed by *P. aeruginosa* in the surface of a thread from a surgical suture (photograph is taken from reference 44).

Formation of bacterial biofilm is the main virulence factor in chronic infectious diseases in humans. It is important to note that once the microorganism has settled in the biofilm it is very difficult to kill it. Some of the examples include fatal outbreaks of *S. aureus*, *V. cholera* or *P. aeruginosa*\textsuperscript{45,46} It happens because the biofilms provide a favourable environment for the protection of bacteria, for example stronger attachment to the host cells, easier access to water and nutrients and exchange of information (signal molecules) or the perfect environment to exchange plasmids (extrachromosomal DNA) by conjugation. Besides, it provides protection against different kinds of threats, either environmental, chemical (disinfectants, drugs) or host defences (antibodies or macrophages), promoting the generation of persister cells (cells especially resistant embedded in a biofilm) as a source of very resistant inactive cells to activate once the threat has ceased, to rebuild the colony.\textsuperscript{47,48} In the case of *P. aeruginosa*, the formation and maturation of biofilm is mediated by the QS systems LasI/LasR, RhlII/RhlIR (1.2.1)
among others. These systems use the mechanisms that modify the phenotype in bacterial cells, which is revealed mainly in metabolic and physiological changes, allowing them to remain in a sessile state (Figure 9). The main reason why this biostructure has all these benefits for a bacterial community is because of its architecture and composition. Commonly the bacterial biofilm is composed of exopolysaccharides (main structural component), extracellular DNA, proteins, up to 97% of water, lipids, ions (Mg (II) or Ca (II)), surfactants and extracellular membrane vesicles.\textsuperscript{49}

**Figure 9.** Summary of the formation and maturation of bacterial biofilm, using as example *P. aeruginosa.* A) Bacterial cells enter the host organism as planktonic cells with flagella which aid the motility. B) The flagella mediate the adhesion to the tissue, leading to a sessile life. C) Bacteria start spreading on the surface and grow in layers of bacteria, forming microcolonies and releasing QS signal molecules. D) Once the population density is enough, bacteria start to develop pre-biofilm matt generating an exopolysaccharide matrix that encloses them, losing the flagella and changing its phenotype synchronously. E) The matured biofilm starts to form mushroom-like shapes by producing enzymes that break down the biofilm. F & G) The broken biofilm parts, release planktonic bacteria able to create new colonies.

Bacterial biofilms are a widespread problem, common in many areas aside from healthcare, and whose solution is quite difficult, costing huge amounts of money to companies and governments.\textsuperscript{50} They create problems in aquaculture,\textsuperscript{51} agriculture, biofouling in pipes used for drinking water and for wastewater.\textsuperscript{52} Additionally, bacterial
biofilms may affect historical monuments and buildings in general, accelerating their deterioration and ruin.53,54

1.2.2. Quorum Quenching

Just like all biological processes, QS has its own antagonist called Quorum quenching (QQ). There is a wide variety of QQ mechanisms, the goal of which is to block the QS systems used by any microorganism.55 Nowadays, there are a lot of studies that support how great an advantage can be gained by any given bacterial population, due to its QS mechanisms, over the rest of the organisms in its environment. However, many kinds of organisms have developed mechanisms to block systems involved in the QS process of a rival and stop this powerful machinery. Therefore, QQ is similar to QS resistance, because its aim is to protect an organism against QS, and, as with every other kind of resistance, it comes from the interaction of an organism with something that would cause it damage. In this case, the accumulation of autoinducers in the environment around a bacterial colony can generate a response from others kinds of cells such as mammalian cells, plants or algae.56,57

Focusing on the autoinducers generated by the Gram-negative bacteria (AHLs) which are one of the central themes of this thesis; it is important to say that each bacterial species uses its own mix of autoinducers, but a wide range of bacteria have one if not more signal molecules in common, which is quite a frequent occurrence. Thus, bacteria from different genera can sense the AHL of its neighbours, e.g. the autoinducer C4-AHL is used by *Serratia liquefaciens*, *P. aeruginosa* and *Aeromonas hydrophila*. Nonetheless, it has been observed that AHL can interact with Gram-positive bacteria and eukaryotic cells as toxins. For instance, the addition of 3-oxo-C12-AHL in a culture of the Gram-positive bacteria, *S. aureus*, suppresses the QS system, which triggers the expression of several membrane proteins, as well as virulence factors.58 In the case of eukaryotic organisms, there are various types of response to the signal molecules.59,60 Using as examples mammalian cells and the autoinducer 3-oxo-C12-AHL it has been observed that this signal molecule can induce apoptosis in several kinds of human vascular endothelial cells due to the blockade of calcium release from the cytosol.61 This is just one example of the interaction between autoinducers and eukaryotic cells; which highlights how important it is to know all the mechanisms behind this interaction and even more importantly how to fight against them. Thus, one of the best ways to accomplish the purpose is to analyse and
study the natural mechanisms of QQ that cells have developed throughout the millennia to efficiently deal with this aggressive bacterial weapon called QS.

1.2.2.1. **Natural Quorum Quenching**

This section describes how the QQ process summarised above affects the QS systems of Gram-negative bacteria. These processes can manifest in nature mainly in four ways:

a) Blocking the activity of the cognate proteins responsible for detecting the AHL and transferring the signal to develop a response.

b) Reducing or inhibiting the synthesis of autoinducers at any step of the process.

c) Modification of AHL to remove its biological activity.

d) Use of analogues of autoinducers which mimic their structure and are able to interact with the receptor, but without any signal transduction.

The most common prokaryotic QQ mechanisms found in wild organisms are based in the modification and hence the inhibition of AHL using enzymes. Hitherto, five kinds of enzymes have been found for this purpose: lactonases and decarboxylases which affect the lactone ring, whereas deaminases, acylases and oxidoreductases affect the acyl chain (Figure 10).

![Figure 10](image_url) **Figure 10.** Diagram of a generic AHL with the enzymes used by prokaryotic cells to deactivate it by the modification of different functional groups or bonds (tagged in red).

i. **Lactonase and decarboxylase.** These families of enzymes catalyse the hydrolysis of the lactone ring of a wide range of AHL from C4-AHL to 3-oxo-C12-AHL\(^6^2\) (Figure 11). However, some organisms only produce this enzyme for a more specific range of AHL such as short acyl chain or long acyl chain.\(^6^3\)
ii. **Acylase (amidohydrolase) and deaminases.** In contrast to the reaction of hydrolysis mentioned above, the reaction catalysed by these other families of enzymes is irreversible. The reaction results in the cleavage of the bond that joins the lactone ring and the acyl chain, releasing homoserine lactone and a fatty acid\(^{64}\) (Figure 11).

iii. **Oxidoreductase.** The aim of this family of enzymes is the reduction of the 3-oxo group in AHL to generate a 3-hydroxy-AHL, but this molecule still has QS activity; for this reason, the deactivation of the AHL requires a deamination by an amidohydrolase after the reduction of the carbonyl group\(^{65,66}\) (Figure 11).

![Figure 11](image-url) This outline shows the catalytic degradation of an AHL to products without biological activity. These kinds of enzymes are the most widespread QQ enzymes used by prokaryotic cells.

In the eukaryotic organism, we find a huge diversity of QQ systems. The most studied and highlighted are pointed out below. It has been observed that human epithelial cells are able to inhibit QS of *P. aeruginosa*.\(^{67}\) It was also noticed in porcine kidney cells, where the enzyme acylase was found to be responsible for the degradation of 3-oxo-C12-AHL and C6-AHL.\(^{68}\) Other examples include food products such as beef steak, chicken breast or turkey patties, which have also shown a high inhibition of AI-2, the interspecies autoinducer; even though the source of this QQ activity remains unknown, the ratio of inhibition was quite high: 90.6%, 97.5% and 99.8% respectively.\(^{69}\)

Numerous plant extracts have demonstrated QS inhibition; notably, degrading QS protein receptors such as LuxR and LasR from *P. aeruginosa* and *E. coli*\(^{70}\) and blocking the receptors because of the similar structure of some compounds, such as lumichrome, found in...
in the extracts.\textsuperscript{71} Another encouraging finding is the anti-QS properties of grapefruit juice, which is able to inhibit biofilm formation in bacterial pathogens such as \textit{E. coli}, \textit{S. typhimurium} and \textit{P. aeruginosa} due to the presence of furocoumarins.\textsuperscript{72} Apart from the well-known penicillin antibiotic produced by the fungus \textit{Penicillium}; organisms from the fungi kingdom are able to inhibit QS. Some fungi from the Penicillium species produce penicillic acid and patulin which affect the expression of QS genes in \textit{P. aeruginosa}.\textsuperscript{73} Furthermore, the fungus from the kingdom division \textit{Ascomycota} and \textit{Basidiomycota} release into the plant rhizosphere, where they dwell, lactonase-type enzymes with AHL-hydrolase activity.\textsuperscript{74}

The marine alga \textit{Delisea pulchra} produces halogenated furanones, which are compounds with a similar structure to that of AHL. These molecules are able to interact with the RhlR/LuxR receptor proteins and inactivate its functional site,\textsuperscript{75} being able to inhibit biofilm formation and swarming in \textit{E. coli}.\textsuperscript{76}

\textbf{1.2.2.2. Artificial Quorum Quenching}

Nowadays more than ever, new artificial QQ systems, usually based on natural QQ systems, are being developed.\textsuperscript{77} One of the main types of synthetic QS inhibitors are analogues of AHL where either the acyl chain or the lactone ring has been modified. The target of these molecules is to bind the receptor proteins to avoid its interaction with bacterial autoinducers. One of the most common strategies is to change the length of the acyl chain. This characteristic is crucial for its biological activity,\textsuperscript{78} for example, the increase in one or two methylene groups involves a loss of 50\% and 90\% of activity respectively.\textsuperscript{79} Similar results have been achieved by many researchers using analogues of AHL with extra functional groups in the acyl chain such as methyl, sulphonamide or phenyl group.\textsuperscript{80,81} \textit{N}-acyl cyclopentylamides are the most effective analogues of AHL inhibiting QS by interaction with LasR and RhlR systems (Figure 12).\textsuperscript{82}

The rise of new materials and techniques has allowed us to design new methods to prevent QS. Apart from the mechanisms mentioned above, there are researchers who venture for other QQ systems to inhibit different stages in the QS process. A different approach is the depletion of iron (Fe (III)) from the bacterial environment. For this purpose, desferri-oxamine gallium as an ion sequester and the antibiotic gentamicin were used in rabbit models infected with \textit{P. aeruginosa}; achieving the inhibition of the biofilm formation and killing planktonic bacteria.\textsuperscript{83} Another strategy is the use of polymers to sequester the
autoinducers released by Gram-negative bacteria to their environment and make a passive regulation of the development of QS phenotypes. This resulted in a decrease of the bioluminescence and biofilm formation in *V. fischeri*<sup>84</sup> and a significant attenuation of biofilm formation by *P. aeruginosa* using a rationally designed MIP.<sup>85</sup>

Natural halogenated furanones are one of the most effective QS inhibitors, for this reason, they have been synthetically modified to improve their performance. One example is C30 (Figure 12) which is an artificial brominated furanone and the best QS inhibitor known of its family, however, *P. aeruginosa* has developed resistance to C30,<sup>86,87</sup> which means that bacteria are able to generate resistance against any substance which interacts with them, regardless of the manner in which it does so.

Another group of anti-QS agents are antibodies, the proteins that are part of the immune system of mammals. They are produced as a response for presence of a specific antigen which could be polysaccharide, peptide or lipid. For this reason it is not likely that an organism will generate antibodies for a small molecule of AHL autoinducer, which does not belong to any of the above mentioned types of antigens. However, using an active immunisation strategy and squaric monoester monoamide as hapten (a small molecule capable of triggering an immune response once it is linked to a big carrier molecule such as a protein) (Figure 12), it was possible to generate antibodies with catalytic activity for the hydrolysis of 3-oxo-C12-AHL,<sup>88</sup> this goal was also achieved using as hapten a transition state analogue of the reaction of hydrolysis of the lactone ring (Figure 12).<sup>89</sup>

![Figure 12. Structures of some of the most successful synthetically obtained quorum sensing inhibitors.](image-url)
Taking into account the evidence shown above, it is evident that at the moment when there is an interference with some important bacterial process, there is a very high risk that the bacterium develops resistance; regardless of whether it is natural or synthetic. For this reason, the most convenient QQ system is the one that does not interact at all with the microorganism in question. Consequently, the use of sequestering polymers seems to be the best choice to look for a solution without causing the development of resistance.

1.3. Molecularly imprinted polymers

Our biggest concern when dealing with a bacterial infection is antibiotic resistance. Consequentially, one of the best approaches to overcome this problem is the use of polymers to sequester the QS signal molecules. However, the polymer’s capacity is limited: once the binding sites of the polymer are saturated, the polymer cannot inhibit QS further and it is possible that the polymer can then act as a source of AHL, triggering the bacterial response that it initially suppressed. One of the solutions could be the use of QQ enzymes (1.2.2.1). Unfortunately, their manufacture is not straightforward, they have short lifetimes and require special storage conditions. Furthermore, their activity is subject to conditions such as temperature or pH. To overcome these problems we propose the use of molecularly imprinted polymers (MIP) with catalytic activity, also known as molecularly imprinted catalysts.22

MIPs are polymers prepared from cross-linkers and functional monomers, whose main characteristic is that the polymerisation takes place in the presence of a template. After the polymerisation, the template is removed from the polymer. This leaves special cavities in the polymer matrix mirroring the structure of the template. In this cavity, the monomers are arranged in an exact position with their functional groups orientated in a complementary arrangement to functional groups of the template. This specific binding site makes MIP the perfect synthetic material that is capable of recognising and interacting with a specific molecule (for instance, an autoinducer) and be able to rebind specifically the template. The recognition properties of MIPs make them valuable materials in solid phase extraction90 and sensors.91

1.3.1. Synthesis of molecularly imprinted polymers

The synthesis of MIPs is accomplished by two different pathways considering the linkage between the template and the functional monomers. G. Wulff and co-workers presented
the *covalent imprinting*, where the interactions between template and monomers are reversible covalent bonds. In contrast, K. Mosbach and co-workers developed the *non-covalent imprinting*, where the interactions are non-covalent, mostly hydrogen bonds, but also, hydrophobic and electrostatic interactions.\textsuperscript{92} The latter method is more convenient for our purposes because even though the interaction between the monomers and the template is more specific in the covalent imprinting, the templates would have needed to be chemically derivatised to be suitable for this kind of synthesis. On the other hand, the non-covalent method is easier and more versatile and uses an excess of monomers to ensure the formation of the complex template-monomer. There are many different techniques to synthesise MIP:\textsuperscript{93}

a) **Bulk polymerisation.** This is the easiest way to synthesise a MIP. The polymer is obtained as a block. Thus, it must be ground and sieved in order to be used which means that there will be heterogeneity of the binding sites.

b) **Precipitation polymerisation.** This process is fast and has a high yield. This process requires a lower concentration of monomer in solution which may reduce the interaction between monomer and template compared with other techniques.

c) **Emulsion polymerisation.** This process requires surfactants to emulsify the organic compounds in water solution. As a result, it generates nanoparticles with size about 50 nm. Unfortunately, the use of surfactants can interfere with the imprinting process.

d) **Solid phase radical polymerisation.** Characterised by the use of a silica solid phase (glass beads) and initiators such as dialkyldithiocarbamate esters, and UV light to trigger the polymerisation. This reaction is ideal for thermolabile templates but not suitable for photolabile templates. Despite the low yields because of the high concentration of monomer used, this polymerisation allows a better control of the size of the polymeric particles (30-300 nm) and the polydispersity.

MIP can by synthesised by several methods (shown above) and in different formats such as membranes, microparticles or nanoparticles. MIP nanoparticles (MIP NPs) synthesised by living radical polymerisation (LRP) appear to be the most convenient format and method to obtain the best performance due to their properties. Nanoparticles have the highest surface-volume ratio in comparison with other formats and the imprinted cavity is more available for the substrate, which enhances its performance.\textsuperscript{94} By synthesising the
polymeric nanoparticles by LRP the size of the nanoparticles can be easily controlled. In this work $N,N'$-diethyldithiocarbamic acid benzyl ester is used as an iniferter (Figure 14). The reaction will be triggered by UV light; the iniferter acts as initiator, chain transfer agent and terminator, and it is used to avoid autoacceleration; which means that there is a fast termination reaction which happens straight away once the UV light is switched off.\(^9_5\)

Purification is a very important step in the process of synthesising of MIPs because it is crucial to remove the template and the unreacted reactants of the obtained polymer. For this reason, polymerisation is followed by a purification step which is done using dialysis filtration or Soxhlet extraction. Leicester Biotechnology Group (LBG) has developed a solid phase synthesis protocol which does not require additional purification steps and allows nanoparticles to be obtained virtually free of template, other reactants, non-imprinted polymers and low affinity polymers for the used template (Figure 13).\(^9_6\)

**Figure 13.** Outline of the followed protocol to synthesise MIP NPs using a template linked covalently to a solid support (silica glass beads). UV light is used to trigger the polymerisation reaction. Washing at low temperature is used to remove the undesired side products, unreacted monomer and polymer fragments. The high-affinity MIP NPs remain bound to the solid phase (affinity purification). The high-affinity MIP NPs are then eluted at elevated temperature.
Figure 14. Mechanism of LRP. A) Initiation. The radical polymerisation is triggered by UV light, using as initiator the iniferter $N,N'$-diethyldithiocarbamic acid benzyl ester. The benzyl radical act as initiator, the dithiocarbamyl radical acts as chain transfer agent and terminator due to the fact that the S-C bond that forms with the monomers which can be reversibly cleaved homolytically by UV light. B) Propagation. Reaction between the radicals generated by the iniferter and the monomers. C) Propagation. Elongation of the polymeric chains by radical reaction between monomers and cross-linkers. D) Termination. Once the UV light is swift off the radicals react to each other to end the polymerisation reaction. E) Reactivation of the dithiocarbamyl radical, allowing further polymerisation to be performed.
1.3.2. MIP with catalytic activity

In 1986, R. A. Lerner and collaborators presented antibodies with catalytic activity, generated using transition state analogue (TSA), which were able to hydrolyse carboxylate esters. Three years later in 1989, K. Mosbach and his group developed the first TSA-imprinted MIP able to catalyse the hydrolysis of the ester group of 4-nitrophenyl acetate, using bulk polymerisation and a 4-nitrophenol methylphosphonate as TSA template. Since these works were published, the use of TSA as template has been the most commonly used technique to synthesise MIPs with catalytic activity. For the moment, one of the most efficient molecularly imprinted catalyst has been achieved by G. Wulff, mimicking the activity of carboxypeptidase A using a molecule with two amidine groups as functional monomer in order to stabilise the transition state of the reaction and also forming a complex with a Cu(II) ion between them helping to trigger the hydrolysis (Figure 15). However, its catalytic proficiency is still surpassed by a natural enzyme. Nevertheless, there are more examples of MIP, which were used to mimic the catalytic activity of natural enzymes such as an aldolase to catalyse a cross-aldol reaction between 4-nitrobenzaldehyde and acetone.

![Figure 15](Diagram of the stabilisation process of the transition state of hydrolysis of a carbonate ester carried out by the amidine group and the Cu (II) ion present in the functional monomer of a MIP with carboxypeptidase A activity.)

1.3.2.1. Mimicking the catalytic activity of AHL-lactonase

The hydrolysis of AHL (Gram-negative autoinducers) is catalysed by the natural enzyme AHL-lactonase (1.2.2.1). This metalloenzyme has a binuclear zinc ion cluster in its catalytic site, which is essential for the catalytic activity. The active part of the catalytic
site is formed by the following amino acids His104, His106, His169, Asp191 which are penta-coordinated to a Zn (II) and another penta-coordinated Zn (II) is joined to Asp191, His235, His109 and Asp108. Both zinc ions are linked by a hydroxide ion bridge (OH⁻); this hydroxide ion is responsible for the hydrolysis (the ring-opening reaction). The zinc ions are involved in the stabilisation and polarisation of the ester bond in the lactone ring, increasing the electrophilicity of the carbonyl carbon, while the OH group from Try194 stabilises the transition to trigger the reaction (Figure 16).¹⁰¹,¹⁰² Moreover, some members of this family of enzymes responsible for the hydrolysis of long chain AHLs (with seven or more carbons in the acyl chain) have a second binding site composed of two phenylalanines. They act as a clamp that binds, the acyl chain of AHL by hydrophobic interactions.¹⁰³

**Figure 16.** Mechanism of the hydrolysis of an AHL by an AHL-lactonase (picture is taken from reference 103).

During the hydrolysis of the lactone ring of AHL a tetrahedral transition state is generated (Figure 17), regardless of the triggering cause of the process (e.g. enzyme (Figure 16) or low pH.²⁴
Figure 17. Mechanism of hydrolysis reaction of C6-AHL, the transition state and open-ring product which has lost the biological activity are shown.

For this reason, the template used for the synthesis of MIP NPs is a TSA of the hydrolysis of the lactone ring from C6-AHL, N-(1,1-dioxidotetrahydrothiophen-2-yl) hexanamide (Figure 18).

Figure 18. Molecular structures of the bacterial autoinducer, N-hexanoyl homoserine lactone (C6-AHL) (1) and the template, N-(1,1-dioxidotetrahydrothiophen-2-yl)hexanamide (TSA) (2).

The TSA used as template has a sulfone ring, which has the same size as the γ-lactone ring of the AHL, the sulfone group has a tetrahedral structure and similar electron density distribution to the carbonyl carbon in the transition state of the hydrolysis. This TSA was proposed by Kapadnis and co-workers as a hapten used to generate antibodies with catalytic activity against AHLs.89

1.4. Aims and Objectives

The fight against bacteria is a very important worldwide issue of our time, which affects all mankind. The discovery of new antibiotic drugs has slowed during recent decades. Furthermore, the generation of drug resistance in pathogens is developing much faster than before, creating new resistance traits with a wide range of mechanisms to deal with antibiotics, apart from a plethora of genes ready to activate and protect their host.
Additionally, bacteria have shown an ability to develop resistance to QQ compounds. Now, the new QQ approach based on halogenated furanones is manifesting the same problem, resistance.

As mentioned in “Approach to the topic of study” and subsequently justified, the best option to disrupt the QS mechanisms in Gram-negative bacteria seems to be the use of QQ enzymes. Because there is no direct interaction with the target bacteria, the chance that resistance will be generated decreases dramatically. For this reason, the goal of this project is to develop new polymeric nanomaterials to mimic the catalytic activity of QQ enzymes. This is the first time that this has been attempted, the target being to synthesise a mimic with the catalytic activity of lactonase enzymes to degrade the common motif of all AHL autoinducers. In addition, hydrolysis of the lactone ring requires less energy than cleavage of an amide bond (reaction catalysed by an acylase enzyme) to remove the N-acetyl side-chain. Apart from this, the reaction proceeds via a transition state which could be easily mimicked using different functional groups such as sulfone or phosphate in a TSA template (1.3.2.1). With the MIP technology, we can obtain polymeric nanoparticles, which have an imprinted cavity where the functional groups of the monomers are in the right orientation to stabilise the transition state in the hydrolysis of AHL and hence, produce a hydrolysis product which has no biological activity.

Therefore, using MIP NPs with the capacity to hydrolyse the QS signal molecules can be a complement to antibiotic therapies, by inhibiting pathogenic phenotypes and preventing biofilm-formation, allowing the bacteria to be killed using antibiotics. The effectiveness of these therapies has been proven in vitro and in small animal models to demonstrate that the use of antibiotics with a QS inhibitor is significantly more efficient that using antibiotics alone. Furthermore, recent studies have shown that nanoparticles of this type are biocompatible with mammalian cells such as fibroblast and macrophages, whose metabolism was not affected by MIP NPs which supports the possibility to use such materials in a new QQ approach.

The main aim of this work is to synthesise MIP NPs as a QQ tool for the degradation of Gram-negative bacterial autoinducers. Notably, as a catalyst of the hydrolysis of the γ-lactone ring of AHL. For this purpose, an innovative solid phase synthesis has been used in the synthesis of the MIP NPs and commercially available monomers have been chosen.
as functional monomers, due to this being the first approach to generate MIP NPs with catalytic activity for the degradation of bacterial autoinducers.
Chapter 2

Experimental Part
2.1. Materials and Methods

2.1.1. Chemicals

- (3-glycidyloxypropyl)trimethyloxysilane (GLYMO), Sigma-Aldrich, UK.
- 2-(dimethylamino)ethyl methacrylate (DEAEM), Sigma-Aldrich, UK.
- 2-(diphenylphosphino) phenol, Sigma-Aldrich, UK.
- 3-aminopropyltrimethyloxysilane (APTM), Sigma-Aldrich, UK.
- 4-Dimethylaminopyridine (DMAP), Sigma-Aldrich, UK.
- Acetic acid (AcOH), Fisher Scientific, UK.
- Acetone, HPLC grade, Fisher Scientific, UK.
- Acetonitrile (MeCN), HPLC grade, Fisher Scientific, UK.
- Boc-6-aminocaproic acid, Sigma-Aldrich, UK.
- Bromine, Sigma-Aldrich, UK.
- Chloroform deuterated (CDCl₃), Cambridge Isotope Laboratories, UK.
- Cyclohexane, Fisher Scientific, UK.
- Deuterium Oxide (D₂O), Sigma-Aldrich, UK.
- Dichloromethane (DCM), Fisher Scientific, UK.
- Ethanolamine, Sigma-Aldrich, UK.
- Ethyl acetate (EtOAc), Fisher Scientific, UK.
- Ethylcarbodiimide hydrochloride (EDC.HCl), Sigma-Aldrich, UK.
- Ethylene glycol dimethacrylate (EGDMA), Sigma-Aldrich, UK.
- Formic acid (FA), BDH Chemicals, UK
- Glass beads (diameter, 70 - 100 μm) (Potters, Spheriglass A-Glass cat. no. 2429 CP 00), Blagden Chemicals, UK.
- Glutaraldehyde (GA), Sigma-Aldrich, UK.
- Hydrochloric acid (HCl), Fisher Scientific, UK.
- Itaconic acid (IA), Sigma-Aldrich, UK.
- Lithium chloride (LiCl), BDH Chemicals, UK
- Magnesium monoperxyphthalate (MMPP), Sigma-Aldrich, UK.
- Magnesium sulfate – dried, Sigma-Aldrich, UK.
- Melamine (MEL), Sigma-Aldrich, UK.
- Methacrylic acid (MAA), Sigma-Aldrich, UK.
- Methanol (MeOH), HPLC grade, Fisher Scientific, UK.
- $N,N'$-diethyldithiocarbamic acid benzyl ester (iniferter), TCI Europe, Belgium.
- $N,N'$-diisopropylethylamine (DIPEA), Sigma-Aldrich, UK.
- $N,N'$-dimethylformamide (DMF), Fisher Scientific, UK.
- $N$-hexanoyl homoserine lactone (C6-AHL), Sigma-Aldrich, UK.
- Pentaerythritol-tetrakis-(3-mercaptopropionate) (PETMP), Sigma-Aldrich, UK.
- Phosphate buffered saline (PBS), Sigma-Aldrich, UK.
- Silica gel (35 – 70 μm, 60Å), Arcos Organics, Belgium.
- Sodium azide, Sigma-Aldrich, UK.
- Sodium bicarbonate (NaHCO$_3$), Fisher Scientific, UK.
- Sodium hydroxide (NaOH), Sigma-Aldrich, UK.
- Sodium sulfite (NaSO$_3$), Fisher Scientific, UK.
- Tetrahydrofuran (THF), Fisher Scientific, UK.
- Tetrahydrothiophene (THT), Sigma-Aldrich, UK.
- Thionyl chloride, Fisher Scientific, UK.
- Toluene, Fisher Scientific, UK.
- Trifluoroacetic acid (TFA), Sigma-Aldrich, UK.
- Trimethylolpropane trimethacrylate (TRIM), Sigma-Aldrich, UK.
- Water, HPLC grade, Fisher Scientific, UK.

All chemicals were used without further purification. Millipore (MilliQ) purification system was used to obtain deionised water with a resistivity of 18.2 MΩ cm. Dry solvents were prepared using molecular sieves 4Å (beads) purchased from Sigma-Aldrich, UK.

### 2.1.2. Equipment and Techniques

- High performance liquid chromatography–mass spectrometry (HPLC-MS). WATERS 2795, Luna reverse phase C18 (2) column Shim-pack XR-ODC, 3.0 x 50 mm, 3 μm; Quattro Micro Mass spectrometer detector, electrospray, positive ionisation mode.
- IR spectroscopy. Spectrophotometer Avatar 370 FTIR ThermoNicolet, UK.
- Nuclear magnetic resonance (NMR). Instrument Bruker DPX 400, 400MHz. The NMR spectra were recorded with Chemical shifts quoted in ppm.
- Ultraviolet–visible spectroscopy (UV-Vis). Instrument spectrophotometer UV 1800 Shimadzu, Japan.

2.2. Experimental procedures

2.2.1. Synthesis of the Template

2.2.1.1. Synthesis of 2-(diphenylphosphino) phenyl 6-(Boc-amino)-hexanoate (3)

In a 100 mL two neck round bottom flask with magnetic stirring was added anhydrous DCM (25 mL), 2-(diphenylphosphino) phenol (0.5 g, 1.8 mmol, 1 eq.), Boc-6-aminocapric acid (0.5 g, 2.16 mmol, 1.2 eq.), DIPEA (0.43 mL, 0.32 g, 2.5 mmol, 1.4 eq.), EDC.HCl (0.48 g, 2.5 mmol, 1.4 eq) and DMAP (25 mg). The reaction was carried out under N₂ at room temperature (r.t.) for 2 h. After TLC analysis showed completion of the reaction, DCM (75 mL) was added to the reaction mixture. The organic layers were washed with aqueous HCl 10% (2 x 30 mL). The organic phase was dried over magnesium sulfate, filtered and the solvent removed by rotary evaporation. The crude product was purified by column chromatography (SiO₂; EtOAc:DCM 1:20). Product 3 was obtained as a colourless oil (0.714 g, 83%).

\(^{1}H\text{-NMR}\) (CDCl₃): \(\delta = 7.22-7.16\) (11 H, m, aryl CH), 7.02-6.99 (1 H, ddd, \(J = 8.12\) Hz, 4.11 Hz, 0.88 Hz, 5.0 Hz, aryl CH), 6.68 (2 H, m, \(J = 7.63\) Hz, 4.3 Hz, 1.57 Hz, aryl CH), 4.78 (1 H, br s, NH), 3.59 (2 H, br m, CH₂NH), 2.12 (2 H, t, \(J = 7.43\) Hz, C(=O)CH₂), 1.57-1.68 (2 H, m, CH₂CH₂NH), 1.33 (11 H, m, C(=O)CH₂CH₂ and 3CH₃), 1.32-1.18 (2 H, m, CH₂CH₂CH₂NH). \(^{13}C\text{-NMR}\) (CDCl₃): \(\delta = 171.34\) (C=O ester), 152.87 (C=O, carbamate), 144.14, 135.70, 135.55, 134.11, 133.84, 133.70, 129.89, 129.04, 128.65, 128.55, 126.07, 122.53, 76.06, 40.36, 33.85, 29.67, 28.45, 26.20, 24.12. \(\text{IR } \nu_{\text{max}} \text{ cm}^{-1}\):
In a 1 L round bottom flask containing a magnetic stirring bar and a fitted rubber cap, dry Silica gel (100 g) was placed followed by water (50 mL) added very slowly while shaking the mixture until a free-flowing powder was obtained. DCM (500 mL) was added and the mixture was stirred vigorously, then THT (6 g, 68.18 mmol, 1 eq.) in DCM (10 mL) was added to the stirred heterogeneous mixture. A solution of bromine (13.1 g, 82.0 mmol, 1.2 eq.) in DCM (30 mL) was added dropwise to the mixture. The colour of the mixture turned slightly green. The mixture was stirred at r.t. for 90 min. The mixture was then filtered through a 3A sintered glass funnel, the silica gel was washed with DCM (5 × 100 mL) and the washings were added to the filtrate. The DCM was removed by rotary evaporation. The crude product did not require further purification. Product 10 was obtained as a light yellow liquid (4.61 g, 65%).

$^1$H-NMR (CDCl$_3$): δ = 2.62 (4 H, br s, 2 S(=O)CH$_2$), 2.17 (2 H, br s, CHH'CHH'), 1.80 (2 H, br s, CHH'CHH'). $^{13}$C-NMR (CDCl$_3$): δ = 54.22, 25.25. IR $\nu_{\text{max}}$ cm$^{-1}$: 2945 and 2871 (alkane), 1023 (S=O). MS (ESI$^+$) m/z found [M+H]$^+$ 105, [M+MeOH+H]$^+$ 137. (Spectra in Chapter 5 Appendix)

2.2.1.3. Synthesis of 2-azidotetrahydrothiophene (9)

A 25 mL two neck round bottom flask containing a magnetic stirring bar and anhydrous DMF (2 mL) was placed in an ice bath. Sodium azide (1.25 g, 19.23 mmol, 2 eq.) and 10 (1.0 g, 9.62 mmol, 1 eq.) were added followed by the slow, dropwise addition of thionyl chloride (1.62 g, 10.58 mmol, 1.1 eq.). The mixture was stirred for 90 min under N$_2$. After this time, water (20 mL) was added and the resultant mixture was extracted with diethyl
ether (2 × 50 mL). The organic layer was washed with 10% LiCl (15 × 30 mL), once with brine (30 mL) and then dried over magnesium sulfate. The solvent was removed through rotary evaporation. The product did not require further purification. Product 9 was obtained as a yellow liquid (0.75 g, 5.82 mmol, 58%).

$^1$H-NMR (CDCl$_3$): $\delta$ = 5.08 (H, dd, $J_{4.79}$ Hz, 1.89 Hz, CHN$_3$), 3.01 (H, m, SCHH'), 2.80 (H, m, SCHH'), 2.10 (1 H, m, CHN$_3$CH$_2$), 1.88 (1 H, m, SCH$_2$CH$_2$). $^{13}$C-NMR (CDCl$_3$): $\delta$ = 69.49, 37.93, 31.63, 27.46. IR $\nu_{\text{max}}$ cm$^{-1}$: 2957 and 2865 (alkane), 2105 (azide). MS (ESI$^+$) m/z found [M]$^+$ 129, [M-N$_2$]$^+$ 102, [M-N$_3$]$^+$ 87, [SC$_3$H$_6$]$^+$ 74. (Spectra in Chapter 5 Appendix)

2.2.1.4. Synthesis of 1,1-dioxide-2-azidotetrahydro-thiophene (5)

A 50 mL round bottom flask containing a magnetic stirring bar and fitted with a reflux condenser was charged with dry silica gel (2.6 g). Water (1.3 mL) was added the mixture shaken until a free-flowing powder was obtained. MMPP (1.6 g, 2.58 mmol, 2 eq.) was added and the mixture was shaken further. DCM (15 mL) was added and the mixture was stirred vigorously, before the addition of 9 (0.17 g, 1.29 mmol, 1 eq.) in DCM (1 mL) to the stirred heterogeneous mixture. The mixture was stirred at 50 ºC (reflux) for 90 min. The mixture was then filtered through a 3A sintered glass funnel, the silica gel was washed with DCM (3 × 20 mL) and the combined washings added to the filtrate. The organic phase was washed with 1M NaOH (3 × 30 mL) before drying over magnesium sulfate. The DCM was removed by rotary evaporation. The crude product was purified by column chromatography (SiO$_2$; DCM). Product 5 was obtained as a pale yellow very viscous oil (0.15 g, 78%).

In a 250 mL round bottom flask, 9 (1.29 g, 10 mmol, 1 eq.) was added to glacial acetic acid (25 mL). 30% H$_2$O$_2$ (3.6 g, 30 mmol, 3 eq.), diluted in glacial acetic acid (25 mL), is dropwise very slowly into the 9 solution and stirred for 48 h at r.t. Then, reactions mixture was diluted with water (50 mL) and extracted with DCM (3 x 50 mL). The organic layer was washed with 10% Na$_2$S03 (3 x 50 mL), saturated NaHCO$_3$ (3 x 50 mL), and water (50 mL). Then, it was dried over magnesium sulfate and the DCM was removed.
by rotary evaporation. The crude product was purified by column chromatography (SiO<sub>2</sub>; DCM). Product 5 was obtained as a pale yellow very viscous oil (1.34 g, 83%).

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): δ = 4.40 (H, dd, J 5.97 Hz, 4.21 Hz, CHN<sub>3</sub>), 3.09 (2 H, m, S(=O)<sub>2</sub>CH<sub>2</sub>), 2.39 (H, m, CHN<sub>3</sub>CHH'), 2.24 (H, m, CHN<sub>3</sub>CHH'), 2.16 (H, m, CHN<sub>3</sub>CH<sub>2</sub>CHH'), 2.03 (H, m, CHN<sub>3</sub>CH<sub>2</sub>CHH'). <sup>13</sup>C-NMR (CDCl<sub>3</sub>): δ = 73.61, 48.01, 27.11, 17.92. IR ν<sub>max</sub> cm<sup>-1</sup>: 2956 (alkane), 2111 (azide), 1312 and 1133 (S=O). MS (ESI<sup>+</sup>) m/z found [M-N<sub>2</sub>]+ 134.0, [M-N<sub>3</sub>]+ 119.1, [C<sub>4</sub>H<sub>7</sub>NSO]+ 116.9, [C<sub>3</sub>H<sub>5</sub>SO<sub>2</sub>]+ 107.0, [C<sub>3</sub>H<sub>5</sub>SO]+ 89.0. (Spectra in Chapter 5 Appendix)

2.2.1.5. Synthesis of tert-butyl (6-((1,1-dioxidotetrahydrothiophen-2-yl)amino)-6-oxohexyl)carbamate (2)

A 50 mL round bottom flask equipped with a magnetic stirrer was added anhydrous DMF (10 mL) and 5 (160 mg, 1.0 mmol, 1.5 eq.). When 5 was completelly dissolved, 3 (321.9 mg, 0.66 mmol, 1 eq.) was added. The resulting mixture was stirred under N<sub>2</sub> at 70 °C for 1 h, water (5 mL) was then added and the mixture left to stir overnight. The solvent was removed by rotary evaporation. The crude product was purified by column chromatography (SiO<sub>2</sub>; 0.1:10, MeOH:CHCl<sub>3</sub>). Product 2 was obtained as a white solid (161 mg, 71%).

<sup>1</sup>H-NMR (CDCl<sub>3</sub>): δ = 6.98 (H, br d, J 8.41 Hz, CHNH), 5.12 (H, q, J 8.61 Hz CHNH), 4.79 (H, br s, CH<sub>2</sub>NH), 3.18-3.07 (3H, m, CH<sub>2</sub>NH, CHH'SO<sub>2</sub>), 3.00 (H, ddd, J 13.01 Hz, 10.07 Hz, 7.63 Hz, CHH'SO<sub>2</sub>), 2.51 (H, m, CHH'CHNH), 2.29 (2H, t, J 7.73 Hz, COCH<sub>2</sub>), 2.22 (H, m, SO<sub>2</sub>CHCHH'), 2.11 (H, m, SO<sub>2</sub>CHCHH'), 2.00 (H, m, CHH'CHNH), 1.68 (2H, m, C(=O)CH<sub>2</sub>CH<sub>2</sub>), 1.45 (11H, m, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.36 (2H, m, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>). <sup>13</sup>C-NMR (CDCl<sub>3</sub>): δ = 173.64 (C=O amide), 156.14 (C=O Carbamate), 79.12, 66.80, 50.12, 40.26, 35.89, 29.54, 29.00, 28.41, 26.08, 24.89, 18.50. IR ν<sub>max</sub> cm<sup>-1</sup>: 3349, 2937 (alkane), 1688 (C=O amide), 1659 (C=O amide).

### 2.2.1.6. Synthesis of 6-amino-N-(1,1-dioxidotetrahydro-thiophen-2-yl)hexanamide, TSA (I)

![Chemical structure of TSA (I)](image)

To a mixture of EtOAc (5 mL) and 1M HCl (5 mL), 2 (100 mg, 0.287 mmol) was added at r.t. The mixture was stirred for 5 h until TLC shown completion of the reaction. Product 1 (template) was obtained as a white solid (64.1 mg, 90%).

**1H-NMR** (D₂O): δ = 4.97 (H, t, J 7.92 Hz, CHNH), 4.70 (H, CHNH), 3.29-3.23 (H, m, CH’HSO₂), 2.59-2.51 (H, m, SO₂CHCH’HCH₂), 2.34 (2H, td, J 7.19 Hz, 2.45 Hz, COCH₂), 2.26-2.15 (2H, m, COCH₂CH₂), 2.16-2.06 (2H, m, SO₂CHCH’HCH₂), 2.06-1.96 (H, m, SO₂CHCH’HCH₂), 1.68-1.55 (2H, m, CH₂CH₂CO), 1.43-1.30 (4H, m, NH₂CH₂CH₂CH₂CH₂). **13C-NMR** (D₂O): δ = 177.79, 139.68, 68.39, 50.50, 39.32, 34.80, 18.71. **IR** ν max cm⁻¹: 3198 (NH, NH₂), 1677 (C=O amide), 1529 (NH amide), 2940 (alkane), 1529 (NH amide), 1258 and 1113 (S=O). **MS (ESI+)** m/z found [M+H]+ 249, [C₄H₁₀NO₃S]+ 136, [C₆H₁₂NO]+ 114. (Spectra in Chapter 5 Appendix)

### 2.2.2. Synthesis of MIP NPs

#### 2.2.2.1. Functionalisation of the glass beads

Glass beads (600 g) were placed in a 1 L one-neck round bottom flask and boiled with 1M NaOH (250 mL) solution for 15 min, then rinsed with deionised water until the washings were at pH 9, followed by 0.1 M HCl until pH 7. The beads were rinsed with acetone and oven dried at 80 °C for three hours.

To functionalise the glass beads for the synthesis of TSA-NPs, they were placed in a bottle and incubated for 24 h with a 2 % v/v solution of GLYMO in dry toluene with DIPEA (2 mg/mL) at 65 °C. The following steps were followed in order to immobilise the template. The silanised beads (90 g) were placed in a bottle with a solution of TSA (1 mg/mL) and DIPEA (1 mg/mL) in dry DMF (60 mL). The mixture was incubated at 60
°C overnight. After functionalization, ethanolamine (1 eq. to GLYMO) was added to the reaction mixture to block the unreacted epoxy groups for 3 h at 60 °C. The functionalised glass beads were washed five times with acetone and three times with methanol, dried under vacuum and stored in a dry brown bottle under N₂ at -20 °C.

In the case of the solid phase used to prepare control-NPs (melamine-imprinted), the glass beads were incubated with 2 % v/v APTMS in dry toluene at r.t. overnight. Once the glass beads were silanised, they were incubated in GA/ PBS solution (7 % v/v) at pH 7.2 for 2 h and then washed eight times with deionised water to remove excess GA. Then, they were incubated in PBS solution (pH 7.2) with melamine (1 mg/mL) overnight to perform the immobilisation. After the reaction, the functionalised glass beads were washed three times with deionised water, dried under vacuum and stored in a dry bottle under N₂ at 4 °C.

2.2.2.2. Polymerisation of the MIP NPs

In a 30 mL glass vial were placed: cross-linkers TRIM (1.62 g) and EGDMA (1.62 g); iniferter N,N’-diethyldithiocarbamic acid benzyl ester (0.37 g); chain transfer agent PETMP (0.09 g) and solvent MeCN (5.26 g, 6.69 mL; for MAA or DEAEM) or DMF (5.26 g, 5.57 mL; for IA). This mixture was common to all the polymerisations, the only change was the functional monomer, using always the same molar quantity (16 mmol): MAA (1.44 g), DEAEM (2.99 g) or IA (2.10 g). The polymerisation mixture was purged by bubbling nitrogen for 10 min to remove oxygen. Glass beads functionalised with the TSA template (30 g) were placed in a 200 mL crystallising dish, homogeneously spread over the bottom of the vessel. The dish was covered with a watch glass and placed in a desiccator to remove oxygen and moisture by using vacuum and nitrogen flow alternatively for 30 min. Just before the polymerisation reaction, the polymerisation mixture was poured into the crystalliser with the glass beads, covered with the watch glass, nitrogen flow and placed between two UV-lamps (Philips HB/171/A, each with 4 × 15 W tubes, one above and one below), for 1.5 mins.

After the polymerisation, all the mixture reaction was poured into a solid phase extraction cartridge with a polyethylene frit (20 μm porosity). In order to obtain the high-affinity MIP NPs in high purity, the reaction mixture was first washed in the cartridge with MeCN at 0 °C (8 × 30 mL) to remove monomers and low-affinity polymers. Secondly, to break the strong interaction between template and MIP NPs and to elute them, the cartridge was
placed in a water bath at 65 °C until the temperature in the cartridge reached 65 °C. The column was then eluted with MeCN at 65 °C (8 × 30 mL) to obtain a solution containing the MIP NPs (1.3.1, Figure 14). The MIP NPs obtained were stored in MeCN at -20 °C before use.

2.2.2.3. Analysis of size and concentration of MIP NPs

In order to determine the size of the synthesised nanoparticles and be sure that the synthesis was successful, all the samples were measured using Dynamic Light Scattering (DLS). This technique is based on the interaction between a laser source and the particles to be measured. The instrument measures the light scattered by the nanoparticles. Since the particles are subjected to constant Brownian movements which depend on their size, there are fluctuations in the intensity of the scattered light. The DLS apparatus compares the timing of these fluctuations to determine the velocity of Brownian motion which is related to the diffusion coefficient (D). Using the Stokes-Einstein equation \( k_B = \text{Boltzmann's constant; } \eta = \text{viscosity and } T = \text{absolute temperature} \) we can determine the hydrodynamic diameter \( (d_h) \) of the particles as:

\[
d_h = \frac{kT}{3\pi\eta D}
\]

All the measurements were taken the day after the synthesis, 2 mL of the MIP NPs in MeCN were placed in a 4 mL glass vial and sonicated for 2 min. Then, a 1 mL aliquot was transferred to a 3 cm³ glass cuvette and analysed in the DLS equipment at 25 °C, recording 7 measurements per sample; the instrument automatically sets up the attenuator position, number of runs and measurement duration, obtaining the particle size as an average of the hydrodynamic diameter and the polydispersity index (PDI) which is an indication of the heterogeneity of the particle sizes in the sample. In a monodisperse sample, PDI tends to 0, and the acceptable values are between 0 and 0.7. Each sample was analysed at least five times.

The concentration of MIP NPs in each sample was measured using a UV-VIS spectrophotometer by recording the absorbance at \( \lambda = 197.4 \text{ nm} \). The MIP NPs were analysed the day after the synthesis, a few millilitres of the MIP NPs (from MeCN solution) were transferred to water and placed in a 4 mL glass vial and sonicated for 2 min. Then, a 1 mL aliquot was transferred to 3 cm³ quartz cuvette for the analysis. To
calculate the concentration of each sample, the measurement was extrapolated in a calibration curve shown in the Appendix (5.2.1).

2.2.3. Degradation of C6-AHL by MIP NPs

The degradation of C6-AHL was quantified using HPLC-MS. Three types of samples were analysed, all of them containing C6-AHL (500 ng/mL) with HPLC water as the solvent. The blank sample which only contained C6-AHL and water was used to analyse the natural degradation of the analyte. The samples with MIP NPs contained 1 mg/mL of either TSA-NPs or control-NPs. To prepare the samples, the necessary amount of nanoparticle solution (after 2 min sonication) was placed in a 4 mL glass vial and MeCN was removed by nitrogen flow; when there was 1 mL of MeCN remaining in the vial, 1 mL HPLC water was added and the mixture sonicated for 2 min. The sample was evaporated under a flow of nitrogen. Once the volume was 0.5 mL, another 0.5 mL of aqueous solution of C6-AHL was added.

The incubation of the samples was carried out in a temperature-controlled room at 25 °C. All the samples were filtered through 0.1 μm PTFE syringe filters (Whatman, 6798-2501) to remove any nanoparticle or polymer traces from the aliquot before being placed in the 100 μL plastic vial used for HPLC-MS measurement.

HPLC method: Injection volume 10 μL; flow 0.2 mL/min; solvents 100% MeOH with 0.1% FA (isocratic); run time 5.0 min; Luna reverse phase C18 (2) column Shim-pack XR-ODC, 3.0 x 50 mm, 3 μm.

MS method: Solvent delay 1.5 min; collision energy 10.0 eV; cone 20.0 V; capillary 4.50 kV; source temperature 120 °C; desolvation temperature 350 °C; pump flow 2.0 mL/min; parent and daughter masses analysed (m/z): 200.0 and 102.0.

To calculate the concentration of each sample, the measurements were extrapolated according to a calibration curve (shown in the Appendix, 5.2.2).
Chapter 3

Results and Discussion
3.1. Synthesis of the Template

![Chemical structure](image)

**Figure 19.** Outline of the synthetic pathway followed to obtain the TSA (1); 2-(diphenylphosphino)phenyl 6-(Boc-amino)hexanoate (2); 2-(diphenylphosphino)phenyl 6-((Boc-amino)hexanoate (3); Boc-6-aminocaproic acid (4); 1,1-dioxide-2-azidotetrahydrothiophene (5); THT (6).

A simple and effective way to synthesise 6-amino-N-(1,1-dioxidotetrahydrothiophen-2-yl)hexanamide (TSA, 1) was developed to use as template in the synthesis of MIP NPs. Tetrahydrothiophene (THT) and Boc-6-aminocaproic acid were used as starting materials (Figure 19).

3.1.1. Synthesis of 2-(diphenylphosphino)phenyl 6-(Boc-amino) hexanoate (3)

![Chemical structure](image)

**Figure 20.** The reaction is based on the activation of the carboxylic acid by 4-Dimethylaminopyridine (DMAP) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydro-chloride (EDC.HCl), followed by a nucleophilic attack on the carbonyl carbon by hydroxyl group.

The synthesis of 2-(diphenylphosphino)phenyl 6-(Boc-amino)hexanoate (3) was carried out in order to attach the triphenylphosphine group to the carboxylic acid (Figure 20).108
Depending on the length of the acyl chain and the presence of functional group (=O, -OH or -H) at the third carbon we can distinguish different types of AHL. This synthetic route is suitable to obtain AHL without functional group at the third carbon of the acyl chain. The triphenylphosphine group is necessary for the compound to couple the sulfur-containing ring by an amide bond, using Staudinger Ligation. For easier purification of the 2-(diphenylphosphino)phenyl 6-((tertbutyloxycarbonyl)amino)-hexanoate (3), Boc-6-aminocaproic acid (4) has to be used in 1.2 equivalents to 2-(diphenylphosphino) phenol (7).

3.1.2. Synthesis of 1,1-dioxide-2-azidotetrahydrothiophene (5)

Figure 21. Outline of the synthetic pathways followed to obtain 5.

To obtain 1,1-dioxide-2-azidotetrahydrothiophene (5) using 6 as starting material, different methods were developed since the literature methods were found to be non-productive (Figure 21). After many attempts with no results, the conditions of the reaction were altered i.e. temperature variations between -10 °C and 50 °C, variations in reaction time and solvents: benzene, toluene, DCM, acetone, chloroform and DMF. Moreover, instead of \(N\) chlorosuccinimide (NCS), \(N\) bromosuccinimide (NBS) was used. One of the main problems with this pathway is the large amount of side products generated in the synthesis of 2-chlorotetrahydrothiophene (8) and the following reaction with sodium azide to obtain 2-azidotetrahydrothiophene (9). At the end of the reaction, there are many byproducts which were complicated to remove in the attempted of purification of 5 (Experimental procedure described in Appendix: 5.3.1).

The following synthetic route was carried out using 1-oxidotetrahydrothiophene (10) as starting material in a Pummerer reaction. The first approach to obtain 10 was to use \(\text{NaIO}_4\) as the oxidising agent. This procedure was used previously to synthesise similar 58
sulfoxides,\textsuperscript{109} unfortunately, it did not work (Experimental procedure described in Appendix: 0). The best method found to obtain 10 was to use bromine as the oxidising agent and H\textsubscript{2}O as the oxygen source (Figure 22).\textsuperscript{110} The role of the silica gel in this reaction is fairly important as it provides just the necessary amount of water for the reaction and it absorbs the HBr produced, avoiding possible side reactions. After filtration to remove the silica gel, no further purification was necessary.

![Figure 22](image.png)

**Figure 22.** Oxidation of THT (6) to 1-oxidotetrahydrothiophene (10) using a versatile procedure to oxidise thioethers to sulfoxides.

The sulfoxides are common functional groups used in the Pummerer rearrangement (Figure 23), this reaction allows for the attachment of different groups such as leaving groups, to the alpha carbon. The reaction to obtain 2-acetoxytetrahydrothiophene (11) from 10 was also non-productive, in spite of reports that this reaction can functionalise similar compounds to 10.\textsuperscript{111,112} To attach the acetoxy group, acetic anhydride was used at a range of temperatures (from 50 °C to 120 °C) and times (from 12 to 48 hours). Trichloroacetic anhydride, which has more electrophilic carbonyl carbons, was also used without success (Experimental procedure described in Appendix: 5.3.3).

![Figure 23](image.png)

**Figure 23.** Reaction mechanism of the Pummerer rearrangement, using 10 and acetic anhydride to obtain 11.

Another common chemical in the Pummerer rearrangement is thionyl chloride. The reaction between thionyl chloride and 10 is very rapid. At r.t. it gave the same problems as NCS due to the generation of 8. However, when this reaction was carried out at 0 °C, with slow, dropwise addition of thionyl chloride to a solution of 10 and sodium azide in
DMF, 9 was obtained in low yield but without the need for additional purification (Figure 24).

![Figure 24](image)

**Figure 24.** Outline of the synthesis to obtain 9. In this case, the reaction starts as a typical Pummerer reaction followed by the nucleophilic attack on the α-carbon that is carried out by sodium azide instead of the chloride ion from the thionyl chloride.

To obtain 5, two methods were used (Figure 25). Firstly, the oxidation was done using hydrogen peroxide (H$_2$O$_2$) as the oxidising agent in acetic acid over 48 hours. The second option used magnesium monoperoxyphthalate hexahydrate (MMPP) as the oxidising agent with hydrated silica gel in DCM as solvent. In this case, the reaction is complete in just 1 hour. Both reactions give product 5 in high yield, nevertheless, the best choice is MMPP because of the shorter reaction time.

![Figure 25](image)

**Figure 25.** Reactions to oxidise 9 (sulphide) to 5 (sulfone).

### 3.1.3. Synthesis of the TSA of C6-AHL: 6-amino-N-(1,1-dioxido-tetrahydro-thiophen-2-yl)hexanamide (1)

![Figure 26](image)

**Figure 26.** Coupling of 3 and 5 by Staudinger ligation to obtain 2.
The compound 5 can be used to synthesise different TSAs because the part of the molecule which changes in the AHL is the side chain attached to the triphenylphosphine of the molecule 3. The Staudinger ligation was used to join 3 and 5 together (Figure 26); the reaction happens between the triphenylphosphine group of 3 and the azide group of 5 (Figure 27).

![Figure 27. Reaction mechanism of the Staudinger ligation between 3 and 5.](image)

To obtain 2,\textsuperscript{108} the reaction was performed using anhydrous DMF under an inert atmosphere to couple the azide group with the phosphorus. When the azide derivative was consumed, water was added to the reaction mixture to hydrolyse the iminophosphorane bond.

![Figure 28. Cleavage of the protective Boc group in acid media to obtain the TSA template (1).](image)

To use this compound as template, the protecting group (Boc group) needs to be removed; this is easily cleaved using hydrochloric acid in ethyl acetate (Figure 28). The overall yield of this multistep reaction is 19%.

### 3.2. Synthesis of MIP NPs

The protocol used for the synthesis of MIP NPs is an adaptation of the procedures used by Canfarotta \textit{et al.}\textsuperscript{96} and Poma \textit{et al.}\textsuperscript{113} This protocol begins with the functionalisation
of the solid phase, silica glass beads, whose mean diameter is 75 μm, with the template. For this purpose, the glass beads were activated by boiling them in a 1 M NaOH solution, thus the number of silanol groups (Si-OH) will be higher, increasing the reactivity of the surface. The activated glass beads were silanised with an epoxy silane (3-glycidyloxypropyl)trimethoxysilane (GLYMO). The remaining epoxy group will react with the primary amine group of the TSA template, thus the TSA will be linked to the solid phase by a covalent bond (Figure 29).

This strong attachment (covalent bonding) is the main reason why the solid phase, which was successfully obtained using this protocol to functionalise the glass beads, can be used more than once. According to the protocol followed to carry out the polymerisation, acetonitrile is the solvent chosen due to the molecular weight (Mw < 500 Da) of the TSA. Moreover, solid phase synthesis of MIP NPs and the synthesis by LRP allow us to conduct a post-polymerisation modification of nanoparticles and functionalise them by adding an external layer (shell) that allows us to change their properties, i.e. solubility/surface reactivity, without affecting the binding site.

Figure 29. Scheme for the functionalisation of glass beads with epoxy silane and the immobilisation of the TSA template.

To ensure that the degradation of the AHL is performed by nanoparticles specifically and not due to the adsorption on the surface of the polymer or other interactions between the polymer and the AHL, control MIP NPs were synthesised using exactly the same protocol and composition, but for the different template which is not specific for the QS process. Melamine was chosen as template for the preparation of control-NPs. The protocol to
functionalise the glass beads with melamine is different because the reactivity of the primary amino groups in melamine is lower than that of the TSA. For this reason, the template was attached to the glass beads using 3-aminopropyl-trimethyloxysilane (APTMS) as silane and glutaraldehyde (GA) (Figure 30). Thus, the GA will be linked to the silane and the template (melamine) by double bonds between carbon and nitrogen (Schiff’s base).

![Diagram of functionalisation process](image)

**Figure 30.** Scheme for the functionalisation of glass beads with amino silane and the immobilisation of the control template using GA.

The template was linked to the solid phase by strong covalent bonds generated in the reaction between the primary amino group of the template and the epoxy group from the functionalised glass beads. This ensured that the cold washes (MeCN, 0 °C) removed unreacted monomers and low-affinity polymers and the hot washes (MeCN, 60 °C) only extracted the high-affinity MIP NPs from the solid phase by breaking the non-covalent interaction between the polymer and the template to obtain virtually clean MIP NPs.

To exert good control over the polymerisation reaction, an iniferter (N,N'-diethylthiocarbamic acid benzyl ester) was employed as initiator due to the absence of autoacceleration in polymerisation possible with other radical initiators. The dithiocarbamyl radical will recombine with the propagating polymer chain when the UV light used to trigger the polymerisation reaction is switched off. The resultant new macroiniferter can be cleaved again homolytically by reapplying UV light, initiating a
new polymerisation (living polymerisation).\textsuperscript{115,116} This “living” property can allow us to perform a further polymerisation, with a new monomer or monomer mixture, to cover the bare nanoparticles with a shell in order to bestow a wide variety of properties and functionalities to the surface of MIP NPs without affecting their recognition or catalytic properties.

A high degree of cross-linking will confer rigidity to the polymeric NPs which is important to maintain the integrity of the active cavity of the nanoparticle, for this reason, the polymerisation mixture contained two cross-linkers, TRIM and EGDMA (30% w/w); a chain transfer agent, PETMP was added to increase the homogeneity of the molecular weight of the polymer. All the functional monomers tested were inexpensive and commercially available, the MIP NPs described here were synthesised using MAA, DEAEM or IA.

The MIP NPs obtained were analysed firstly by DLS to determine the average hydrodynamic diameter ($d_h$) of the NPs and its PDI; these parameters will show if the synthesis of NPs was successful. Then, the concentration of NPs in MeCN solution and hence the amount obtained was determined by UV-Vis spectrophotometry, using the calibration curve shown in Appendix (5.2.1) (Table 3).

Table 3. List of the parameters measured by analysis of MIP NPs synthesised.

<table>
<thead>
<tr>
<th>Template</th>
<th>TSA-NPs</th>
<th>Control-NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>MAA</td>
<td>DEAEM</td>
</tr>
<tr>
<td>$d_h$ (nm)</td>
<td>204 ± 7</td>
<td>175 ± 4</td>
</tr>
<tr>
<td>PDI</td>
<td>0.193 ± 0.005</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>NPs (mg/mL)</td>
<td>9.0</td>
<td>12.9</td>
</tr>
</tbody>
</table>

The results presented above (Table 3) show the size distribution of NPs synthesised with different monomers: MAA, DEAEM and IA. There were no significant differences in the average hydrodynamic diameter obtained for the MIP NPs synthesised with different monomer and template (TSA and melamine for the control-NPs). The size distribution according to PDI found its value is between 0.08 and 0.7 in all the samples, being within the acceptable range for this measurement.
Analysing the size distribution by intensity graphs (Figure 31), we can observe that the size distribution is quite homogenous, taking into account the absence of other populations of NPs with different sizes, in the samples with MAA-NPs and IA-NPs. However, a second population can be observed in the sample prepared with DEAEM-NPs. It is though important to note that small NPs scatter light with lower intensities than larger NPs and hence its incidence in the results is smaller. In this case, the second population has a $d_h$ of $43 \pm 25$ which was only detected in two of the five measurements.

**Figure 31.** Correlation curves and size distribution by intensity graph from the analysis of TSA-NPs with DLS.

From the correlation curves (Figure 31), the main information extracted is if the concentration of NPs in the sample is appropriate to obtain valid measurements in DLS.
and whether the NPs in the sample readily aggregate. When the values of the correlation coefficient at low delay times are between 0.5 and 1.0, the sample has the right concentration to perform the measurements as shown in the correlation curves below. When the value of the correlation coefficient at high delay times arise from the baseline giving place to a “step” or small fluctuations, it means that the NPs in the sample can aggregate easily, which happens due to an increase in non-random movements (Brownian movements) when the NPs aggregate or sediment.

Thus the NPs synthesised using DEAEM and IA tend to form aggregates and this process appears to be more pronounced in the IA-NPs. All the data acquired by DLS were obtained directly from the Malvern-Zetasizer software, with no further modifications, following the procedure recommended by the manufacturer.

### 3.3. Tests for the evaluation of degradation of C6-AHL using MIP NPs

In order to measure the degradation of the bacterial autoinducer C6-AHL in the presence of MIP NPs, the following test has been conducted. The samples were incubated under the same conditions and filtrated using a syringe filter before analysis. DLS confirmed the absence of NPs in the analysis samples after filtration. The measurements were taken in triplicate after the same incubation time to monitor the decrease in the concentration of autoinducer in the samples. The method used for this analysis was optimised to minimise the retention time ($t_R$). The best mobile phase found was isocratic elution using MeOH with 0.1% FA on a Luna reverse phase C18 (2) column, 3.0 x 50 mm, 3 µm. Reaching a $t_R$ of 2.28 min, it is important to notice that there was a solvent delay of 1.5 min in the input of each sample to the mass spectrophotometer. During the analysis, it was observed that when the MS spectrometer analysed positive ions (ES+ mode) the sensitivity for C6-AHL was quite good while the sensitivity for C6-AH decreased dramatically; however, when analysing negative ions (ES- mode) C6-AHL was undetectable while the sensitivity for C6-AH increased. It was not possible therefore to accurately measure both the AHL and its hydrolysis product simultaneously as the MS conditions were incompatible.

Data obtained by HPLC-MS for the degradation of C6-AHL is presented in Table 4. These results show a clear increase in the degradation process of C6-AHL which is greater
in the samples containing TSA NPs. The best performance is exhibited by the TSA-NPs synthesised using MAA as functional monomer, with the highest initial rate in the degradation reaction, 1.7 ng/min, compared to 0.8 ng/min for DEAEM-NPs and 0.7 ng/min for IA-NPs.

In the following graphs (Figure 32. A-C) the hydrolysis of the autoinducer (ng/mL) at different times is plotted, comparing the natural degradation with that shown in the presence of TSA and control-NPs, synthesised with the same functional monomer. In all cases, the degree of hydrolysis was significantly higher with the TSA-NPs. The initial rates for hydrolysis in the presence of NPs synthesised with IA and DEAEM were however much lower than that obtained with MAA-NPs. In the samples with IA and DEAEM-NPs, it is noted that after 2 h the decrease of C6-AHL is very similar in the samples with TSA and control-NPs; however, after 20h, the amount of C6-AHL is significantly lower in the samples with TSA-NPs as compared with the samples with control-NPs. This could be the result of an initial activation step of the NPs, which explains why the initial rates are similar for both types of NPs at the beginning of the incubation.
Table 4. The concentration of C6-AHL in the samples with TSA-NPs, control-NPs and without polymer to compare the natural degradation of C6-AHL and the degradation in the presence of MIP NPs.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>MAA-TSA C6-AHL (ng/mL)</th>
<th>SD</th>
<th>MAA-TSA C6-AHL (ng/mL)</th>
<th>SD</th>
<th>IA-TSA C6-AHL (ng/mL)</th>
<th>SD</th>
<th>DEAEM-TSA C6-AHL (ng/mL)</th>
<th>SD</th>
<th>Without MIP NPs C6-AHL (ng/mL)</th>
<th>SD</th>
<th>MAA-control C6-AHL (ng/mL)</th>
<th>SD</th>
<th>IA-control C6-AHL (ng/mL)</th>
<th>SD</th>
<th>DEAEM-control C6-AHL (ng/mL)</th>
<th>SD</th>
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<tbody>
<tr>
<td>0</td>
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Figure 32. Degradation of C6-AHL (non-catalysed) compared with catalysis with MIP NPs over 22 hours. A) MIP NPs synthesised with MAA as the functional monomer. B) MIP NPs synthesised with IA as the functional monomer. C) MIP NPs synthesised with DEAEM as the functional monomer.

Comparing the results obtained from the TSA-NPs and control-NPs, synthesised with a different functional monomer (Figure 33) at 2 and 20 h, we can say that all the control-
NPs tested had a similar performance, while the TSA-NPs showed different activities ending in a higher degradation of C6-AHL.

![Figure 33. Diagram showing decay in the concentration of autoinducer C6-AHL after 2 h and 20 h of incubation: A) in the samples containing TSA-NPs synthesised with different functional monomers; B) in the samples containing control-NPs synthesised with different functional monomers.]

Based on the data shown in the graphs above, we can say that the degradation of the bacterial autoinducer C6-AHL in the samples with control-NPs is due to a non-specific catalysis from the functional groups placed on the polymer surface, leading to an approximate decrease of 18% in the concentration of C6-AHL after 2 h. Nevertheless, the MIP NPs imprinted with the TSA are able to achieve a higher degree of hydrolysis due to the catalytic activity of its imprinted cavity; reaching a 41%, 17% and 19% of degradation in 2 h in the case of MAA, IA and DEAEM-TSA, respectively. Confirming that the MIP NPs synthesised using a TSA as template increase the hydrolysis rate of the Gram-negative bacterial autoinducer C6-AHL significantly.
Chapter 4

Conclusion and Future work
4.1. Conclusion

The formation of bacterial biofilms is more than just a health issue; this problem has a notable effect in numerous other areas of society, with the worst affected areas likely being those connected to agriculture and water treatment. The formation of biofilm is triggered by the chemical language of QS used by microorganisms; in the case of Gram-negative bacteria, QS is mediated by AHL autoinducers. These small signal molecules are used to sense the bacterial population density and develop a phenotype (such as biofilm, motility or virulence) in response. Biofilm is the main biostucture that, once mature, helps bacteria to be resistant even to detergents and disinfectants. Herein, is proposed a new approach to attenuating the biofilm formation by Gram-negative bacteria, based on the degradation of the autoinducers AHL using MIP NPs imprinted with a TSA. Moreover, since this process occurs completely out of the cell, bacteria cannot develop resistance against something that they cannot sense.

The synthesis of the MIP NPs was accomplished using an adaptation of the innovative solid phase synthesis. As the NPs were obtained virtually free of the template, unreacted monomers and low-affinity polymers, further purification was not required. The template used in the polymerisation was a transition state analogue of the hydrolysis of the γ-lactone ring of the AHL. As all of the AHL have the γ-lactone ring in common, this synthetic route to obtain C6-TSA could be adapted for the synthesis of many other TSA by changing the carboxylic acid used.

In addition, these nanomaterials are potentially biocompatible, resistant, and cheap. Furthermore, they are easy produced and the surface can be readily modified with a polymeric shell that bestows them with different properties and functionalities. Therefore, the possibility to add them to other materials like paints, contact lenses or polymers to protect them from the bacterial biofilm.

In this project, three TSA-MIP nanoparticles were synthesised using inexpensive commercial available monomers: MAA, DEAEM and IA. The catalytic activity of the NPs was compared with control-NPs synthesised with the same monomers and protocol but with different template (melamine). There are significant differences in the rates of degradation of C6-AHL in the presence of TSA-NPs. In the first 2 h of incubation, there is no appreciable natural degradation; however, in the sample containing TSA-NPs (MAA), there is a 41% reduction in C6-AHL. Comparing these results with those
achieved using the control-NPs, we can conclude that this is a promising new technology that will help to fight bacteria without the risk of developing the resistance.

4.2. Future work

4.2.1. Synthesis of new templates

Taking into account that the vast majority of autoinducers used by Gram-negative bacteria are acylhomoserine lactones as signal molecules (1.2.1.1), the synthetic routes suggested below are focused on the synthesis of a wide range of TSAs of these autoinducers. The main differences between both synthetic routes are the use of traceless Staudinger ligation to couple an aryl diphosphine with an α-azide sulfone ring in the synthesis of Cn-TSA, and the use of Meldrum’s acid and a α-amino sulfone ring in the synthesis of 3-oxo-Cn-TSA.

Both types of TSA have two parts joined by an amide bond, one is the acyl hydrocarbonated chain with or without a carbonyl group in the third carbon, this part is characteristic of each AHL; the second part is a sulfone ring which mimics the geometry of the carbonyl carbon of the lactone ring during its hydrolysis; for this reason, this part is common in all the TSA chosen as templates.

4.2.1.1. Synthesis of Cn-TSA

An outline of the generic synthesis to obtain TSA of the hydrolysis of the γ-lactone ring of Cn-AHL and 3-oxo-Cn-AHL to use as the template for the generation of MIP is proposed below. The synthetic route shown in 3.1.2 was used in order to obtain 2-azidotetrahydrothiophene 1,1-dioxide (5). To generate different AHL the acyl chain must change, following the synthetic route is shown in 3.1.1, we can synthesise diverse analogues of 3, just changing the Boc-amino fatty acid used in the reaction, and hence obtain other TSA to use as templates.

4.2.1.2. Synthesis of 3-oxo-Cn-TSA

The main differences between the synthesis of Cn-TSA and 3-oxo-Cn-TSA are the coupling between the sulfone ring and the Boc-amino fatty acid, which is shown below (Figure 34). The proposed synthetic method to obtain 2-aminotetrahydrothiophene 1,1-dioxide (12) follows the same reactions used to synthesise 5, but in this case, the procedure is continued with the reduction of the azide group to an amine group using the Staudinger reduction. Therefore, after the synthesis of 5, the product is reduced by
triphenylphosphine and water in THF. The synthesis followed in order to obtain tert-butyl-(4-(2,2-dimethyl-4,6-dioxo-1,3-dioxan-5-yl)-4-oxobutyl)-carbamate (13) has been reported by Chhabra and co-workers. In dry DCM we dissolve the desired Boc-amino fatty acid, depending on the TSA we want to mimic, which is activated by DMAP and DCCI to react with Meldrum’s acid.

**Figure 34.** Outline of the suggested reaction to synthesise 3-oxo-Cn-TSA, in this case, the synthesis of 3-oxo-C6-TSA is shown.

The compound synthesised (13) must be deprotected to be suitable as the template, the protection group (Boc group) is easily cleaved using TFA (3.1.3), releasing a primary amine group, which is essential for the coupling with the solid phase. To obtain TSA-template, 6-amino-N-(1,1-dioxidotetra-hydrothiophen-2-yl)-3-oxohexan-amide (14); the previous product (13) is dissolved in MeCN with trimethylamine and the 5. The mixture reaction is stirred for 2 hours and then heated under reflux for 3 hours according to the synthetic route used by Chhabra and co-workers to carry out this coupling. After the reaction, the product is purified by column chromatography. The following step is the deprotection of the amino group which could be accomplished using the same protocol shown in 3.1.2 to remove the Boc group from 2.

### 4.2.2. Synthesis of new functional monomers

#### 4.2.2.1. Analogues of amino acid monomers

The functional monomers chosen are analogues of the most common amino acids found in the catalytic site of natural enzymes: serine (Ser), aspartic acid (Asp), arginine (Arg) and histidine (His). These amino acids have chemical properties such as nucleophilicity,
basicity and acidity, which allows them to be perfect to stabilise the transition states and carry out the reactions. Nevertheless, we can find other amino acids in the catalytic site such as tyrosine (Tyr), cysteine (Cys) or asparagine (Asn), which add more chemical properties to the catalytic site depending on the necessities of the reaction catalysed by any given enzyme. These amino acids will be transformed into their monomer analogues, in reactions between the natural amino acid or an analogue (e.g. histamine instead of histidine) and monomers such as 4-vinylbenzyl chloride or methacryloyl chloride (Figure 35).

Figure 35. Amino acid and their functional monomer analogues.

4.2.2.2. Non-analogues of amino acid monomers

The chosen non-analogues of amino acid monomers are shown in Figure 36. All of them have in their structures two amine groups separated by a $\text{Sp}^2$ carbon, resulting in a pair of electrophilic sites that allows them to interact the substrate and the polymer. The
monomers with the vinylbenzamidine structure have been designed by G. Wolff and co-workers.\textsuperscript{99}

![Figure 36](image-url)  
**Figure 36.** Functional monomers and the different substituents that will be used to change their properties.

To mimic the carboxypeptidase (A) activity this type of functional monomer was used because it is able to stabilise the transition state analogue of the hydrolysis reaction; being until now the best example of catalytic activity carried out by MIPs. One other interesting functional group which can help to stabilise the transition state is thiourea (B) which has already shown the capacity to interact strongly with nitro groups by hydrogen bonding.\textsuperscript{120}

![Figure 37](image-url)  
**Figure 37.** Different possible stabilisations of AHL, depending on the interaction between the functional monomer and the AHL: A) stabilisation of the transition state of the hydrolysis of C6-AHL; B) hydrophilic interaction between the lactone ring and the thiourea group.
One of the most important features of the functional monomers is the capacity to stabilise the transition state of the reaction of hydrolysis. Other interactions that may happen using this kind of functional monomers is between the carbonyl and lactone oxygens, and the thiourea group (Figure 37). This situation is more similar to the interaction that happens between the AHL and AHL-lactonase hydrolase, where both oxygens are interacting with the Zn (II) ions during the catalytic process.

Some of them ((E)-N-(2-(bis(2-aminoethyl)amino)ethyl)-N’-ethyl-4-vinylbenz-imidamide and (E)-N,N’-bis(3-(bis(2-aminoethyl)amino)propyl)-4-vinylbenzimidamide) are designed to coordinate one or two metal ions, Zn (II) or Cu (II) which will be supplied using anhydrous chlorides or acetates of the desired metals.

4.2.3. Degradation of C6-AHL by MIP NPs

4.2.3.1. Degradation test in aqueous solution
The main characteristic of these MIP NPs is their catalytic activity. Various kinetics studies of AHL hydrolysis by nanoparticles will be done to calculate the constants of the catalysis (Km, Vm and Kcat) by recording measurements of the reaction at different times to see the advance of the reaction, using same concentration of MIP NPs and different concentration of the substrate. These tests will be carried out in aqueous solution. The same experiments will be carried out in control-NPs.

4.2.3.2. Catalytic test in bacterial cultures
The MIP NPs that show catalytic activity in the experiments mentioned above will be analysed in bacterial cultures. The nanoparticles will be immobilised on solid surfaces and their ability to inhibit biofilm formation will be tested with various Gram-negative bacteria such as Chromobacterium violaceum, V. fischeri or P. aeruginosa. These bacteria use as an autoinducer different AHL, C. violaceum use C6-AHL whose transition state during the hydrolysis is the most similar to our template, V. fischeri use 3-oxo-C6-AHL letting us know if it also works in the AHL with a carbonyl group in the third carbon of the chain and P. aeruginosa use C4-AHL whose chain in shorter, letting us know if the chain length affects the catalytic activity of our MIP NPs.
Chapter 5

Appendix
5.1. Spectra: NMR, IR and MS

5.1.1. Synthesis of 2-(diphenylphosphino) phenyl 6-(Boc-amino)-hexanoate (3)

IR. Using NaCl discs as support.

$^1$H-NMR. Using chloroform deuterated as solvent.
$^{13}$C-NMR. Using chloroform deuterated as solvent.

MS (ESI+). Using MeOH as solvent.
5.1.2. Synthesis of 1-oxotetrahydrothiophene (10)

IR. Using NaCl discs as support.

$^1$H-NMR. Using chloroform deuterated as solvent.
$^{13}$C-NMR. Using chloroform deuterated as solvent.

MS (ESI+). Using MeOH as solvent.
5.1.3. Synthesis of 2-azidotetrahydrothiophene (9)

IR. Using NaCl discs as support.

!H-NMR. Using chloroform deuterated as solvent.
$^{13}$C-NMR. Using chloroform deuterated as solvent.

MS (ESI+). Using MeOH as solvent.
5.1.4. Synthesis of 1,1-dioxide-2-azidotetrahydrothiophene (5)

IR. Using NaCl discs as support.

$^1$H-NMR. Using chloroform deuterated as solvent.
$^{13}$C-NMR. Using chloroform deuterated as solvent.

MS (ESI+). Using MeOH as solvent.
5.1.5. Synthesis of tert-butyl (6-((1,1-dioxidotetrahydrothiophen-2-yl)amino)-6-oxohexyl)carbamate (2)

IR.

\[\text{IR.}\]

\[^1\text{H-NMR. Using chloroform deuterated as solvent.}\]

\[\text{^1H-NMR. Using chloroform deuterated as solvent.}\]
$^{13}$C-NMR. Using chloroform deuterated as solvent.

MS (ESI+). Using MeOH as solvent.
5.1.6. Synthesis of 6-amino-\textit{N}-(1,1-dioxidotetrahydrothiophen-2-yl)hexanamide, TSA (1)

\begin{center}
\includegraphics[width=0.5\textwidth]{synthesis.png}
\end{center}

IR.

\begin{center}
\includegraphics[width=0.8\textwidth]{IR.png}
\end{center}

\textsuperscript{1}H-NMR. Using deuterium oxide as solvent.
$^{13}$C-NMR. Using deuterium oxide as solvent.

MS (ESI+). Using MeOH as solvent.
5.1.7. Analysis of C6-AHL

MS. Using MeOH with 0.1% FA as solvent.

5.1.8. Analysis of C6-AH

MS. Using MeOH with 0.1% FA as solvent.

5.2. Calibration Curves

5.2.1. UV-VIS: calibration curve for MIP NPs

The MIP NPs synthesised interacted with the UV-Vis light of the spectrophotometer with the same behaviour (Figure 38). It means that the main chromophore activity relies on the cross-linkers, which are the only common component of the MIP NPs. Otherwise, the light could be scattered proportionally to the concentration of the polymer in the sample.
Figure 38. Compilation of the UV-Vis spectra obtained from the spectrophotometric analysis of MIP NPs synthesised with different functional monomers MAA, DEAEM and IA.

To build this calibration curve, several samples of MIP NPs with different absorbance within 0.164 and 0.005 were measured spectrophotometrically at $\lambda = 197.4$ nm. Then, the samples were dried in a small aluminium foil boat to weight the amount of polymer in the sample and plot it with the absorbance obtained previously.

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5.2.2. HPLC-MS: calibration curve for C6-AHL

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Calibration Curve MIP NPs

\[ y = 7.1251x + 0.0194 \]
\[ R^2 = 0.9998 \]

Calibration Curve C6-AHL

\[ y = 45.533x + 322.17 \]
\[ R^2 = 0.9995 \]
5.3. Unsuccessful attempts to the synthesis of 1,1-dioxide-2-azidotetrahydrothiophene (5)

The first attempts to synthesise 1,1-dioxide-2-azidotetrahydrothiophene (5) were unproductive although the synthetic pathways were already published. These approaches were modified several times; using different solvent, temperature or reaction time in order to achieve the product. Finally, they were rejected by useless.

5.3.1. Synthesis of 2-chlorotetrahydrothiophene (8)

In a 50 mL round bottom flask THT (1.0 g, 11.34 mmol, 1 eq.) is added with either benzene, DCM, toluene, chloroform, DMF or acetone (20 mL). The stirred solution either NCS (1.5 g, 11.34 mmol, 1 eq.) or NBS (2.0 g, 11.34 mmol, 1 eq.) was added very slowly. Once the solid has been added, the reaction mixture was stirred for 30 mins. The mixture was then filtered and the solvent removed by rotary evaporation. Obtaining product 8 which due to its instability was use straightaway in the following reaction.

In a 50 mL round bottom flask, NaN₃ (3.47 g, 53.4 mmol) was dissolved in water (15 mL) with 5 drops of Adogen® 464. Once the azide is completely dissolved the crude product 8 was added to the mixture and stirred vigorously for 21 h (trying until 72 h) at r.t. (trying at 30 and 40 °C). The reaction mixture was extracted with DCM (3 x 20 mL). The organic layers were dried over magnesium sulfate and the solvent was removed by rotary evaporation obtaining a dark brownish yellow liquid.

Product 9 was impossible to purify from the crude product by chromatographic techniques due to the high amount of byproducts generated during the synthesis.
5.3.2. Synthesis of 1-oxotetrahydrothiophene (10)

In a 500 mL round bottom flask, NaIO₄, (2.9 g, 13.6 mmol, 1.2 eq.) was dissolve in water (200 mL); THT (1.0 g, 11.34 mmol, 1 eq.) was added to a solution and the reaction mixture was stirred at 0 °C (trying at r.t. and at 30 °C) for 20 h (trying until 72 h). Then, the crude mixture was filtered and extracted with chloroform (3 x 50 mL). The crude product was analysed by NMR and TLC; showing that the reaction did not take place.

5.3.3. Synthesis of 2-acetoxytetrahydrothiophene (11)

In a 250 mL round bottom flask, 1-oxotetrahydrothiophene (10) (1 g, 9.6 mmol, 1 eq.) was added to acetic anhydride (80 mL); the mixture reaction was stirred for 6 h (trying until 72 h) at 100 °C (trying at r.t. and at 150 and 200 °C). The solvent evaporated and the residue was diluted in EtOAc (50 mL) and washed with water (3 x 20 mL), saturated NaHCO₃ (5 x 30 mL) and brine (30 mL). Then died over MgSO₄, filtered, and evaporated by rotary evaporation. The crude product was analysed by NMR and TLC; showing that the reaction was unsuccessful.
Chapter 6

Bibliography


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