Linear Polymers to Inhibit Bacterial Quorum Sensing

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Members of the genus *Aeromonas* are ubiquitous inhabitants of aquatic ecosystems and have been related with diverse fish diseases and human infections. The virulence of *Aeromonas* is multifactorial, which means that it is dependent of several factors. The expression of many of these virulence determinants is associated with high cell density and consequently controlled by quorum sensing. Quorum sensing is a density-dependent regulation of gene expression by self-generated signal molecules, such as the acyl-homoserine lactones (AHLs). This key mechanism controls several cell responses, including virulence expression, antibiotic production, motility and biofilm formation. Different approaches have demonstrated the possibility of inhibiting quorum sensing bacterial phenotypes, such as luminescence, through the presence of Signal Sequestering Polymers (SSPs). In the past decade, different polymers (linear polymers) have been developed with the purpose of adsorbing signal molecules both from Gram-positive and Gram-negative bacteria. Additional studies have provided data regarding polymer binding capacity and their cytotoxicity to mammalian cells. Polymers integration in paint, filter membranes with practical applicability, such as aquacultures, or tanks with fish is also discussed. This chapter gives an overview of the recent progress on the development of polymers capable to sequester quorum sensing signal molecules and consequently decrease or prevent the biofilm formation or other pathogenic phenotypes. Data integration will allow identifying the approaches with greater potential and may represent new solutions to resolve disease outbreaks, not only in aquacultures, but also in both clinical and other non-clinical fields in which biofilm can become a serious problem.

'Linear Polymers to Inhibit Bacterial Quorum Sensing' is a tribute to Professor António Correia. This chapter was being prepared with his collaboration when he suddenly passed away on the 5th January 2016.

Keywords: *Aeromonas hydrophila*; *Vibrio fischeri*; copolymers; quorum sensing; biofilm

1. Quorum sensing (cell-to-cell communication)

Quorum sensing dependent bacterial phenotypes are responsible for the growth and proliferation of some microorganisms in different environments [1]. These phenotypes, both in Gram-negative and Gram-positive bacteria, are regulated by cell-to-cell communication systems [2]. The majority of these phenotypes are density-depend and are therefore influenced by the increase of microbial population [1]. Antibiotic production, bioluminescence, conjugation, nodulation, sporulation, swarming, biocorrosion, biofilm production and expression of several virulence factors, are phenotypes correlated with quorum sensing (QS) [2–4]. Hence bacteria are able to coordinate activities as a multicellular organism; they act as a group through the cell-to-cell communication system (intracellular communication) [7,8]. The name quorum sensing was introduced by Fuqua [1] and describes a refined system of communication that allows sending and receiving chemical messages to and from bacteria in a cell-density-dependent manner [5]. The communication between bacteria is mediated by small diffusible molecules called autoinducers (AIs) [6]. The autoinducers are linked to gene expression as a function of cell density [7]. These signal molecules are produced inside the cells at low levels and their concentration increases with cellular growth. Autoinducers can diffuse outside by crossing the cell membranes. When the concentration outside reaches a critical concentration, the signal molecules across the membrane and inside the cell can bind and activate receptors [5]. As the bacterial population grows there is a proportional increase of extracellular signal molecules. When this happens, bacterial cells detect these signals and respond with an alteration of gene expression [8]. The high specificity of QS is due to the specificity of the interactions between the signal molecules and their receptors. The signal molecules are different in Gram-negative and Gram-positive bacteria [9]. At specific growth and environmental conditions, several bacterial genes involved in pathogenicity are regulated by QS [10]. Some examples are the motility in *Yersinia*, the production of antibiotics in *Erwinia* and the biofilm formation in *Pseudomonas aeruginosa* [7].

1.1 Quorum sensing: intercellular signals communication

Bacteria use different QS systems. The LuxR/I-type system, used by Gram-negative bacteria includes acyl-homoserine lactone (AHL) as the signalling molecule; however these type of bacteria can also use others molecules like long-chain fatty acid derivatives and fatty acid methyl esters, peptides as the 4,5-dihydroxy-2,3-pentandione (DPD) collectively referred to as autoinducer 2 (AI-2) and autoinducer 3(AI-3) [11]. Gram-positive bacteria can also produce AI-2, though these bacteria prefer to use several types of peptides [5,7,11]. Signal molecules used in QS are usually small (<1000Da) organic molecules or peptides with 5-10 amino acids [7] and diffusible allowing an easy communication between cells. These signal molecules generally are: i) produced during specific stages of cell growth or in response to alterations in
the extracellular environment. ii) recognised by a specific cell receptor while there is an extracellular accumulation and iii) responsible for producing a specific response once their extracellular accumulation reaches a critical threshold concentration. Cells respond with physiological alterations to metabolise or detoxify the signal molecules [11].

1.2 Quorum sensing in Gram-negative bacteria

Gram-negative bacteria produce and use N-acyl-L-homoserine lactones (AHLs) as intercellular signal molecules for cell-to-cell communication [12]. The N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-AHL) used by *Vibrio fischeri* was the first AHLs identified as an autoinducer [13]. AHL has a conserved homoserine lactone ring that can be linked to a variable length acyl side chain (4-18 carbon atoms). This chain can undergo variable and extra modifications [7]. Short chain AHLs can pass freely in and out the bacterial cell membrane. Long chain AHLs need an activation mechanism to transport them across the cell membrane [12]. The AHLs signal molecules are synthesised by the LuxI/LuxR system: the LuxI protein, also known as AHL synthase, is responsible for the synthesis of AHLs while the LuxR homologues encode for a transcriptional protein that activates luxI in response to the presence of an AHL. The LuxI synthase uses S-adenosylmethionine (SAM) as substrate and acyl-acyl carrier proteins [14]. The AHLs synthesised are then released outside the cell. When the concentration threshold is reached, cytoplasmic proteins, such as LuxR protein (AHL receptor), detect and bind AHLs [11,15]. This interaction induces LuxR dimerization leading to signal detection. The LuxR proteins have in fact two functions: binding the signal molecule (autoinducer) and binding the promoter of target genes, inducing gene expression [12]. A schematic representation of this mechanism is reported in Fig. 1A.

![Fig. 1](image-url) Quorum sensing mechanisms in Gram-negative and Gram-positive bacteria controlled by N-acylhomoserines (A); autoinducing peptides (B); autoinducer 2 (C) and autoinducer 3 (D) (adapted from [16]).
AHL receptors, such as the LuxR-type, frequently bind only to a specific AHL. However they can bind different AHLs, making them responsible for interspecies communication [16]. Gram-negative autoinducers have similar structural elements, and the main difference being at the 3-position of the N-acyl chain, which sometimes assumes the 3-oxo group as in *Pseudomonas aeruginosa* [17]. The LuxR/LuxI system was identified and described for the first time in the marine species *V. fischeri* and *Vibrio harveyi* [14,18]. The LuxI/LuxR homologues show diversity in their mechanism, with variations between different bacterial species. In *V. fischeri*, bioluminescence is a phenotype resultant of QS, in which the LuxI/LuxR system is essential. As mentioned, LuxI protein is responsible for synthesis of acyl-DSL, and LuxR protein for binding the autoinducer and promoting the transcription of the luciferase operon *luxCDABE* [1]. The QS mechanism of the opportunistic pathogen *P. aeruginosa* relies on two LuxI/LuxR signalling systems: the LasI/LasR and the RhlII/RhlR (with C4-homoserine lactone). Both systems are linked to virulence factors’ expression [14] and biofilm formation [19]. Similar to *V. fischeri*, in *P. aeruginosa* LasI protein synthesizes 3-oxo-C12-homoserine lactone and LasR regulates the expression of target genes. The RhlII/RhlR system has a similar function and also regulates gene expression of the bacterium. Some of the genes involved encode virulence genes, like elastases and proteases, important for the infection process [19].

### 1.3 Quorum sensing in Gram-positive bacteria

Gram-positive bacteria do not use the LuxI/LuxR QS system or AHLs as signal molecules; instead, they produce small peptides as autoinducers through the ATP-binding cassette (ABC) exporter protein [16] (Fig. 1B). These autoinducers are known as auto-inducing polypeptides (AIP). AIP contain between 5-17 amino acids and can be processed with side chain modifications, such as by addition of isoprenyl groups (in *Bacillus* spp.) or thiolactone rings (in *Staphylococcus* spp.). Autoinducing polypeptides precursors are synthesised in the cytoplasm and then they are cleaved, modified and transported to the extracellular environment of the bacterium [16]. At a critical extracellular peptide concentration, kinases (two components system) in cell membranes detect and bind these peptides. Alternatively, some peptides are directly transported into the cytoplasm, where binding to their receptors lead to the activation of transcription. These small signal peptides are commonly specific for the corresponding cognate receptor [14]. This type of QS is known to control the development of several bacterial phenotypes in *Bacillus subtilis* and *Streptococcus pneumoniae*, virulence in *Staphylococcus aureus*, conjugation in *Enterococcus faecalis* and microcin production both in *Lactobacillus* sake and in *Carnobacterium piscicola* [2]. *Staphylococcus aureus* is frequently associated with pneumonia, wound infections and others human diseases and its QS system has been largely studied. Secretion of toxins, haemolysins, enzymes, enterotoxins and other pathogenesis associated genes are regulated by the *agr* (accessory gene regulator) system [20]. The short peptides (5-9 amino acids) are cyclic and have a thiolactone ring structure at the terminal carboxylic group. The linkage between the carboxyl terminus and a conserved cysteine moiety within the peptide sequences generate the formation of the ring [21]. The peptides are recognised by receptors in the cell surface, activating a histidine kinase that causes the transcriptional activation of multiple gene *loci*. At low densities, *S. aureus* expresses proteins required for attachment and colonisation. When cell density increases, the protein expression profile changes, with proteins such as proteases and toxins being synthesised due to QS [20]. The LuxS protein produces the furanosyl borate ester AI-2 (autoinducer 2). The AI-2 was described in Gram-positive and Gram-negative bacteria (Fig. 1C) [9]. The AI-2 binds a periplasmic protein, interacting with a two-component system. After the ligation, a phosphorelay cascade that culminates in the activation or repression of gene expression is activated. The enzymes Pfs and LuxS are responsible for activate reactions of synthesis of AI-2 [22]. Autoinducer 2 precursor is a main methyl donor in bacterial cells, and therefore activates several metabolic processes. The autoinducer AI-2 is recognised as one ubiquitous signalling system used by Gram-negative and Gram-positive bacteria. Xavier and Bassler (2003) have described the auto-inducer AI-2 as a “universal signal” for interspecies communication [34]. The marine bacterium *V. harveyi* uses QS system to control bioluminescence. *Vibrio harveyi* QS mechanism is a mix between components of Gram-positive and Gram-negative systems [23]. *Vibrio harveyi* has a second QS pathway, which is controlled by the luxS gene *locus* and associated homologues. However, many bacteria contains AI-2 receptor and apparently they do not express luxS gene [9, 24]. In *V. harveyi* the periplasmatic LuxP protein is the receptor for AI-2. After the interaction between LuxP and AI-2, the complex binds the LuxQ, a two-component protein that have a sensor kinase domain and a response regulator domain [25].

The autoinducer type 3 (AI-3) QS signalling cascade also allows interspecies communication between both Gram-positive and Gram-negative bacteria and may play an important role in infection [26]. The production of AI-3 has many similarities to the AI-2 system (Fig. 1D), [16]. Both use a two-component receptor kinase-intracellular signalling complex to activate gene expression. However, the AI-3 system is able to use as signal molecules epinephrine or norepinephrine, two compounds known also as human stress hormones [12]. It is still not very clear which molecules can activate AI-3 autoinducers, but there is indication that it might be some type of catecholamines. Nevertheless, in *E. coli* the α-adrenergic receptor can block AI-3 signalling [24, 26]. The virulence genes in *E. coli* are also regulated by QS mechanism. The AI-3 autoinducer seems to be involved in this regulation. The two-component system QseBC has been identified in numerous bacterial species as the receptor/response regulator of AI-3 and epinephrine/norepinephrine. Despite the incomplete knowledge about this QS system, it has been recognised that it is very important for inter-kingdom communication [17, 26].
1.4 Bacterial phenotypes controlled by quorum sensing

1.4.1 Bioluminescence

Several marine Gram-negative bacteria, such as *V. fischeri*, emit light, a behaviour regulated by QS. Organisms like squids have evolved organs to house such bacteria and use their bioluminescence during particular nocturnal behaviours. The mechanisms regulating bacterial bioluminescence are well understood particularly for bacteria in the *Vibrionaceae* family [27]. The LuxCDAEBEG genes, that encode all of the structural components necessary for light production, are regulated by the LuxR-LuxI quorum sensing. The enzyme luciferase is at the core of light production. Luciferase is a heterodimer composed of alpha and beta subunits encoded by LuxA and LuxB respectively. The enzyme produces light by oxidising a long chain aldehyde and at the same time reducing a flavin mononucleotide (FMNH₂). LuxD diverts fatty acids from their biosynthesis pathway and makes them available for luminescence production [28], LuxC activates the acyl group within AMP and LuxE reduces it to the long chain aldehyde. LuxC and LuxE use the same mechanism to recycle the long chain fatty acids, produced by the luciferase reaction. Finally LuxG reduces FMN, also a product of the luciferase reaction [29].

1.4.2 Biofilm formation

Most bacteria can be present in nature either as single free suspended cells or associated with a solid surface – a biofilm [30]. These two states have differences at the physiologic, morphologic and genetic expression levels [6]. Biofilms are sessile microbial communities characterised by adhesion to each other and/or to a solid surface and by the production of a hydrated exopolysaccharide matrix. Usually the structure is present as microcolonies enclosed in a matrix [31]. The matrix is constituted by extracellular polysaccharides (EPS), proteins and nucleic acids, and can assume different structures such as monolayers or multilayer’s [30]. The matrix structure allows transportation of nutrients through circulation in channels, as well as regulation of the waste in and out the biofilm. Inside the biofilm, cells behave more as members of a collective living system rather than as individual cells [31] with diverse gene expression in different structures such as monolayers or multilayers [30]. The matrix structure allows transportation of nutrients through circulation in channels, as well as regulation of the waste in and out of the biofilm. Inside the biofilm, cells behave more as members of a collective living system rather than as individual cells [31] with diverse gene expression in different regions of the biofilm. Biofilm bacterial biofilms are widespread in many environments and almost all species can produce or be part of biofilms. Biofilms offer a preventive and secure environment for bacteria, providing higher tolerance against several extreme conditions like high or low temperature, salinity, pH and environmental agents or stresses in general [32]. Inside the biofilms the matrix conditions are favourable for cell-to-cell interactions. For instance, it is known that sensibility of bacteria to antibiotic is altered within biofilms. In fact, bacterial biofilms are much less sensitive to antibiotics than the ‘free suspended cells’ form [6, 30]. In some cases, biofilms consist on a mixture of diverse bacterial populations, but often may consist of a single bacterial species [31]. The essential steps of formation of biofilms (Fig. 2) are: i) attachment, ii) maturation and iii) detachment and dispersion [33].

i) Attachment: Free and planktonic cells are randomly and reversibly attached to a solid surface or a substratum, through an exopolymeric material formation [30]. The adhesion depends on the surface nature and on several microbial factors such as the bacterial structures (e.g. flagellum) [33].

ii) Maturation: The second step involves cell-to-cell aggregation, proliferation, growing and formation of microcolonies. During maturation biofilms can assume different architectures from flat and homogenous to highly complex films. The type of architecture influences the antimicrobial tolerance of the bacteria present in the biofilm. The architecture factors such as motility or extracellular polymeric substances (EPS) production [30, 33]. Regardless the type of architecture in the maturation step biofilms is formed in a 3-dimensional structure enclosing cells in packages with several channels between them. These channels allow water and nutrients transportation, which maintain the cells’ survival [31].

iii) Detachment and dispersion: At the last step of biofilm formation, the detachment of cells from the biofilm and their dispersion occur. Once detached, these cells can attach to other biofilms or be dispersed as planktonic cells (nonadherent cells). Apparently the dispersed cells might facilitate colonization of new surfaces, reinitializing the biofilm process at a new location or inducing an infection process. The same pathogenic bacteria can use a biofilm as a negative regulation of their virulence factors. It is common for several non-virulent bacteria to grow inside a biofilm. When biofilm dispersion occurs, a high concentration of planktonic bacteria quickly becomes virulent and can be released at the same time [34]. Quorum sensing has been associated with the release of bacteria from the extracellular matrix of the biofilm [31, 35]. Several infections, such as chronic bacterial infections, are linked to biofilm formation and the usual therapy with antibiotics is generally insufficient for their elimination [35, 36]. Consequently, various medical treatments involving the use of regular antimicrobials fail in destroying biofilm and the bacterial infections [36, 37]. Biofilms contribute to the development of antibiotic resistance, becoming a significant key in the virulence of several pathogenic bacteria [38]. Because of all these issues related with biofilms, it is important to find solutions to prevent biofilm formation and promote the eradication of those already formed. One possible approach, which has been already used for *P. aeruginosa*, is exploiting the relation between biofilms and QS It has been shown that when QS was blocked, bacterial pathogenicity declined [32].
1.4.3 Biofilm formation and quorum sensing

As described, QS is a complex regulatory process that is dependent on bacterial cell-density [6]. QS is implicated on gene expression and regulation, and in maturation and maintenance of biofilm formation as a manager of available resources [9, 39]. Cell-to-cell communication is required for bacterial biofilm formation, so QS is inherently linked to the formation of bacterial biofilms [10, 17]. Inside a biofilm, the bacterial cell concentration is high. So it is not surprising that QS is responsible for the control and activation of several cell pathways including the induction of formation of mature and differentiated biofilms [32, 35]. Therefore, QS regulate virulence factors’ production in Gram-negative and in Gram-positive bacteria within biofilms [17, 38]. As it was mentioned above, P. aeruginosa has two different QS systems (LasR/LasI and RhlR/RhlI). These systems were related to biofilm formation and differentiation. QS seems to be associated with all the steps of biofilm formation (Fig. 2). Therefore, controlling QS control could be an approach to mediate biofilm formation, and in this way prevent the adherence of pathogenic microorganisms [33].

![Fig. 2 General scheme of biofilm formation (adapted from [59]).](image)

Several studies have confirmed that, when cell density increases, the probability of a bacterial infection occur also increases. Most pathogens delay their production of virulence factors until a crucial population density is reached, which allows a successful infection process against the host [39]. A better understanding of the chemical nature of QS autoinducers may be useful to identify new compounds that can interfere with QS mechanisms, in order to slow down some processes related to pathogenesis [31]. Indeed the higher levels of resistance to antibiotics shown by bacteria in biofilms suggest the need for the development of new therapies focused in inhibition and dispersal of the biofilm structure. These strategies do not aim to eliminate bacteria; they rather make bacteria/biofilm more susceptible to the action of conventional antibiotics or disinfectants [38].

1.4.4 Antibiotic resistance

The ability of bacteria to evolve quickly and the heavy and sometimes unjustified use of antibiotics have contributed to the development of multi-drug resistant microorganisms [40]. Antibiotics can be classified according to their mechanism of action: interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, inhibition of a metabolic pathway, and disruption of bacterial membrane structure [41]. Bacteria may be intrinsically resistant to one or more classes of antimicrobial agents, but can also acquire resistance by the acquisition of resistance genes from other organisms [40, 42]. The resistance genes can work in many different ways such as enabling a cell to produce enzymes that destroy the antibacterial drug; they may be able to prevent the drug from reaching its intracellular target; they can modify the drug’s target site, and they can promote an alternative metabolic pathway that bypasses the action of the drug [43]. In addition to this, once bacteria have created a biofilm, not only the penetration of the antibiotic is more difficult, but also some mechanisms of resistance are more efficient, making the destruction of the bacterial biofilm nearly impossible [38]. Regardless of the mechanism of resistance developed by the microorganisms, the process is initiated by a survival reaction: bacteria have evolved to learn how to resist to antimicrobial drugs. As explained above, the new approaches to fight bacteria are focusing on the disruption of QS, without necessarily killing the microorganism. Control of QS would allow keeping the bacterial growth low and prevent the microorganisms from becoming virulent and form biofilms [41, 43].

1.4.5 Inhibition of QS – quorum quenching

Different strategies to overcome bacterial resistance to antibiotics that do not imply killing or inhibiting bacterial growth have been investigated. In fact, by controlling the expression of virulence factors, bacterial virulence can be decreased without inducing any bacterial resistance [4]. Controlling cell-to-cell communication – quorum sensing – has been explored as a virulence control strategy [44]. Swift and co-workers (1999) reported a significant decrease in the expression of virulence factors and in the virulence characteristics of pathogens, when QS was inactivated. Each QS
component can be used as a target for an antimicrobial strategy [45]. Frequently, QS is the crossing point between bacterial populations and the host. The mechanism capable of interrupting cell-to-cell communication is described as quorum quenching (QQ) [9] QS inhibitors should be highly specific due to the extreme conservation in different bacteria.

1.4.6 Mechanisms to block quorum sensing

Quorum sensing inhibition has been explored as a measure to block the formation of biofilms, the attenuation of virulence and the adjustment of other mechanisms related to QS. Three strategies have been used: i) blocking signal generators [46], ii) enzymatic degradation of signal molecules [47] and iii) blocking signal receptors [48].

Blocking signal generators: the use of compounds that interfere with the signal molecules is a strategy to disrupt QS. In most Gram-negative bacteria the synthesis of autoinducers (AHLs) is encoded by luxI or homologue genes [49]. The AHL signals precursors are acyl-ACP and SAM (S-adenosylmethionine), which are used as substrates of AHLs signals precursors. The chemical reaction involving LuxI suggests that specific inhibitors for QS can be identified [3,46]. Parsek and co-workers reported that analogues of SAM inhibit 97% of the LuxI homologue (RhII) activity in P. aeruginosa [50]. The reaction between AHL synthase and SAM has shown to be exclusive. This is important as SAM is a frequent intermediate in prokaryotic and eukaryotic pathways, and the exclusivity of the reaction improves the possibility of using SAM analogues as specific inhibitors of QS signal generator, without disturbing eukaryotic enzymes that use SAM as substrate [47]. Degradation of signal molecules: autoinducers have a high specificity to cognate receptors and for this reason a better understanding about their structure and composition is very important to synthesise specific analogues. The acyl side chain of autoinducers in Gram-negative bacteria has a high influence in the structure of the cognate receptor. The interaction between the autoinducer and LuxR-like proteins can be disconnected by differences in the length of the structure or even in substitutions in the acyl side chain groups, resulting in an inhibition of QS [49,50]. This specificity involving cognate receptors and the functional group of the side chain of the signal molecule guarantees that only specific types of signals are formed (or disrupted) in different species [39]. Another approach capable of inhibiting QS involves promoting the degradation of signal molecules. Most Gram-negative bacteria seem to be able to degrade AHLs. Enzymes able to degrade AHLs have been described in several species. Two types of enzymes have been identified: AHL lactonases and AHL acylases [49]. The degradation of AHLs by lactonases – lactonolysis - can occur at alkaline conditions. Lactonases catalyse the hydrolysis of the ester bond in the lactone ring, opening the ring, leading to the production of N-acylhomoserines. Acylases, produced by a small number of bacteria, catalyse the hydrolysis of the amide bond, connecting the lactone ring to the acyl chain, releasing a homoserine lactone and a fatty acid [49,51]. Blocking signal receptors: another strategy of QQ is blocking the signal receptors of the autoinducers. As well as the signal generators, signal receptors are also highly specific for the signalling molecules. Alterations in receptor specificity can result in QS disruption [4]. This specificity is especially important for pathogenic bacteria [39,60]. A strategy to disrupt QS is using small molecules that can compete with the AHLs to the cognate receptor. This can be achieved by the production of antagonists QS molecules that can bind and occupy the receptor [49].

1.5 Aeromonas sp. and quorum sensing

The genus Aeromonas has a widespread distribution in aquatic environments, such as rivers, estuarine and marine waters, and even wastewater and drinking waters [52]. Aeromonas spp. are facultative anaerobic, oxidase positive and Gram-negative bacteria. They can grow between 0 °C and 45 °C but the optimal growth temperature is between 22 °C and 32 °C. Several Aeromonas have been linked to animal diseases, including humans. Aeromonas hydrophila and A. salmonica are responsible for skin lesions, septicaemia and haemorrhagic diseases in fish, reptiles, birds, amphibians and mammals [52,53]. Aeromonas hydrophila has also been linked to motile aeromonad septicaemia and intestinal and extra intestinal infections [53,54]. Aeromonas hydrophila was considered the most important microorganism in the “Contaminant Candidate List” of potential waterborne pathogens [52]. Sequencing of the complete genome of A. hydrophila ATCC 7966 [55] showed that the virulence, infection and transmission of disease of Aeromonas spp. is multifactorial but still not completely understood. Several factors contribute to the virulence, including proteases, toxins, lipases, adhesins (e.g. pili), haemolysins, the S-layer and a variety of hydrolytic enzymes [56]. The first step on the process of infection starts with the evasion to host defences [53,57]. The regulation of virulence factors through the regulation of gene expression can be an advantageous method for the attenuation of infections [1]. Quorum sensing in Aeromonas spp. has been investigated and biofilms structures have been implicated, making Aeromonas less susceptible to environmental stresses [58]. This process is extremely relevant in drinking waters, where Aeromonas spp. are predominant but also in other environments. Lynch and co-workers (2002) have clearly demonstrated that biofilm production in Aeromonas is regulated by QS. Aeromonas pathogenicity is particularly relevant to the aquaculture industry and [59] with A. hydrophila being one of the major responsible for economic losses in farm breeding fish [60]. Infectious diseases are one of the major limiting factors in aquaculture and the control of these diseases has become crucial for a successful fish farming management. The control of several fish diseases in aquaculture is currently achieved using antibiotics with the consequent increase of bacteria resistance to these compounds [45, 61]. A few
studies have reported the use of vaccines in aquaculture, as alternative to antibiotics, in the attempt to reduce diseases and economic losses. However, only limited types of vaccines useful against a minority number of *A. hydrophila* serotypes are available. Alternative solutions, that avoid the use of antibiotics or improve their efficacy, are necessary [49]. Swift and co-workers (1997) identified in *A. hydrophila* a LuxRI homologue and its associated signal molecules. *Aeromonas hydrophila* QS mechanism is similar to that observed in *V. fischeri*. Recently, the LuxS protein, which is responsible for the formation of AI-2 was detected in *A. hydrophila* [62]. The N-butanoxy-L-homoserine lactone (C4-HSL) is the most important AHL (signal molecule) produced by AhI and AsaI (LuxI synthase) in *Aeromonas*; they also produce N-hexanoyl-L-homoserine lactone (C6-HSL) as a second signal, but at a lower concentration [58]. In *A. hydrophila* the recognition of C4-HSL by AhyR (cognate receptor LuxR) protein is dependent on QS. The AhyR/C4-HSL controls both extracellular protease production and biofilm formation, and target genes such as those encoding serine protease and metalloprotease’s production. The LuxR protein expression in *Aeromonas* seems also to be responsible for control of virulence factors’ production [54, 59]. Jangid and co-workers (2007) reported that LuxRI homologues are present in different species of *Aeromonas*. In *A. hydrophila*, LuxRI controls biofilm formation, extracellular proteolytic activity, T6SS and also contributes to *A. hydrophila* virulence in fish and mice [59, 62, 63]. Nevertheless, LuxRI shows no effect on the swimming or swarming motility in *A. hydrophila*. On the contrary, LuxS, also regulated by QS, has shown to exhibit a negative regulation (reduction) of general *A. hydrophila* virulence [64]. In conclusion, QS has an important role in biofilm formation by *Aeromonas*. The study of QS and its relationship with virulence factors can provide a better understanding of *Aeromonas* pathogenicity. The link between QS and biofilms’ formation makes QS directly connected to *Aeromonas* pathogenicity [53, 59].

### 2. Linear Polymers

Since QS is responsible for multiple pathogenic related phenotypes, as well as for increased antimicrobial resistance, quorum quenching may be used to avoid, inhibit or decrease these phenotypes. The use of materials able to sequester QS related molecules can, in theory, allow a simple method for QS disruption. Bellow we give a brief description of some of these materials – linear polymers – that are being used for the inhibition of QS phenotypes. There are two different types of polymers: natural (polysaccharides, proteins) and synthetic polymers (plastics, fibres). The word *polymer* arises from the Greek prefix *poly* (“many”) and *meros* (“part”). Polymers are the product from the accumulation of small and repeated monomers forming long chains. The number of repeat units (monomers) and the architecture of molecules in the polymer, provide different properties to the final polymer [65, 66]. Two types of monomers along the backbone. The polymerisation occurs with low molar mass units (“block”) of homopolymers [65]. Polymers may also be linear or branched. Branching occurs when a monomer with more than one double bond, and therefore more than one point for polymerisation, is used. The monomer composition, the structure and the architecture of molecules in the polymer, provide different properties to the final polymer [65, 66]. Two types...
of classifications have been used for polymers so far. One is based on polymer structure (or composition) and classifies polymers into condensation and addition polymers; the other, based on polymerisation mechanism classifies them into step and chain polymerisations. Most of the times, these classifications are used mutually. Condensation or step represents the same type of polymerisation, and addition or chain corresponds to the other polymerisation type [67]. Step-growth and chain-growth polymerisation have a large number of differences, such as different kinetics and the molecular weight of the resulting polymers [68, 69].

2.1 Step-Growth polymerisation

Step-growth polymerisation relates with condensation polymerisation because during the polymer synthesis small molecules, such as water, are released. As the name suggest, on the step-growth polymerisation, the polymer-chain grows in a step-wise reaction of two functional monomers [68, 70]. As a result, an intermediate molecule with a new functional group is formed. This new intermediate could react with another intermediate or with another monomer producing a larger intermediate [66]. In step-growth polymerisations two distinct polymer groups can be formed based on the type of monomer. One group is related with the presence of two different bifunctional and/or polyfunctional monomers; the other group is associated with a monomer which contains both types of functional groups [64, 107]. The two reaction groups (A and B) are represented in Fig. 4 as different types of functional monomers. The features of these two polymerisation reactions are extremely similar. As we describe below, the synthesis of high molecular weight polymer is achieved more easily through chain-growth polymerisation [67]. An example of step growth polymerisation is the nylon-6,6. The nylon 6,6 is formed by the adipic acid (1,6-hexanedioic acid) reaction with hexamethylenediamine (1,6-diaminohexane), producing poly(hexamethylene adipamide) and water. This polymer is called nylon-6,6 because both the diacid monomer and the diamine monomer provide 6 carbons atoms in the polymer chain [66].

\[
\begin{align*}
\text{nA-A + nB-B} & \rightarrow \text{(A-AB-B)}_n \quad \text{a) } \\
\text{nA-B} & \rightarrow \text{(A-B)}_n \quad \text{b)}
\end{align*}
\]

Fig. 4 Equations a) and b) represented general step-growth polymerisation (adapted from [66]).

2.2 Chain-Growth polymerisation

Chain-growth polymerisation involves monomers containing carbon-carbon double bond (unsaturated compounds). The reactivity of these compounds is higher than those involved in a typical step-growth polymerisation. In the chain-growth polymerisation, intermediates between monomer and polymers do not exist and formation of high molar mass polymers is reached since the beginning of the reaction [65]. In chain-growth polymerisation, the monomers are successively added to a growing chain.

Table 1 Comparison between step-growth and chain-growth polymerisation (adapted from [66]).

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<tr>
<th>Step-Growth Polymerisation</th>
<th>Chain-Growth Polymerisation</th>
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<tbody>
<tr>
<td>Monomers have two reactive functional groups</td>
<td>Monomers have a carbon-carbon double bond</td>
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<tr>
<td>Polymer backbone contains carbon and others atoms (oxygen or nitrogen)</td>
<td>Polymer backbone contains only carbons</td>
</tr>
<tr>
<td>Functional groups react without initiator; reaction may be catalysed; reaction often gives off to small by products</td>
<td>Reaction usually begins with an initiator</td>
</tr>
<tr>
<td>Intermediates are compounds (e.g., oligomers) with the same two reactive functional groups as the monomer</td>
<td>Polymerisation proceeds by chain reaction through short-lived reactive intermediate (free radical, cation, anion); monomer reacts only with the reactive intermediate, not with other monomers</td>
</tr>
<tr>
<td>Polymer develops at slow rate; high polymer is formed only at the very end</td>
<td>High polymer is formed very rapidly</td>
</tr>
<tr>
<td>Reaction mixture contains monomers, oligomers, and polymer</td>
<td>Reaction mixture consists only of monomer and high polymer</td>
</tr>
<tr>
<td>The degree of polymerisation and the molar mass increase as the reaction proceeds</td>
<td>The molar mass of polymer formed early in the reaction is approximately the same as that formed later on, increasing time increases yield, not molar mass</td>
</tr>
</tbody>
</table>
The classification of the polymerisation is defined according the way of initiation and the nature of the propagating chain; this can be cationic, anionic or free radical. For chain-growth polymerisation, three basic processes are generally established: initiation, propagation and termination [71]. The initiator produces an initiating species with a reactive centre. The reactive centre can be an anion, cation or a free radical, determining the way in which polymerisation occurs. Propagation is the second step and is depended on the reactive centre by the addition of several monomers to the chain reaction. The production of a polymer is based on these successive additions of monomers. The polymerisation process is then stopped when one of several terminations reactions destroys the reactive centre [67]. The step-growth polymerisation does not need an initiator to initiate the reaction; if monomers have the appropriate functional groups, they are capable of initiating and continuing the reaction until the formation of large molecules. Chain-growth polymerisation needs an initiator to react with the monomers. For this crucial difference, the conversion of monomer into polymer is highest and faster in chain-growth polymerisation. The most important differences between step-growth and chain-growth polymerisation are reported in Table 1.

2.3 Free radical polymerisation

Free radical polymerisation is probably the most well-known mechanism of polymerisation where successive addition of monomers to the radical chain is involved. Polymerisation with vinyl monomer is the one that represents this mechanism Fig. 5a). Initiation begins with the formation of a free radical and at the same time a monomer is added to this radical. 2,2’-azobisisobutyronitrile (AIBN) has been used as a thermal free-radical initiator in several reactions (Fig. 5b). The propagation is the main step of the entire process. The molecular weight of the final polymer is dependent on the number of monomers that are added to each initiator molecule [67, 68].

As it was mentioned before, free radical polymerisation originates a high molar mass polymer quickly. Sometimes it is convenient to use a chain-transfer agent to control the molecular weight. Usually it is the solvent that exerts this function. The choice of the solvent, where polymers’ synthesis occurs, should consider this characteristic [68]. For the polymerisation reaction, the presence of water is not a concern; however, the presence of anionic species and oxygen might be an issue.

3. Applications of Linear Polymers

The control of bacterial virulence via QS has been strongly encouraged and several natural or synthetic compounds have been selected as a strategy to fight bacterial infections [72]. However, in order to have more pronounced effects in real environments, the applications of these compounds still need improvement [73]. It has been already shown that synthetic antimicrobials can be applied as coatings to protect surfaces from the colonisation of bacteria [73]. Copolymers based on methyl methacrylate were developed to reduce QS-phenotypes, decreasing bioluminescence and biofilm formation [74]. These polymers can contribute to the control of bacterial infections. Piletska and colleagues (2010) have reported the synthesis of rational designed polymers (cross-linked polymers) with capability to reduce QS-phenotypes, namely the bioluminescence of V. fischeri. The monomers itaconic acid (IA) and methacrylic acid (MAA) were chosen among others, from a molecular screening, because they showed to have the highest interactions with the AHLs targets [75]. Cavaleiro and colleagues (2015) developed two linear polymers based on the interactions of the two functional monomers, itaconic acid and methacrylic acid, with AHLs of V. fischeri (3-oxo-C6-HLS) and A. hydrophila (C4-HSL). These two functional monomers were identified by molecular modelling as producing strong non covalent interactions with the bacterial signal molecule. These monomers had been previously used to synthesise polymers for...
drug delivery, and have been incorporated into hydrogels for the prevention of bacterial adhesion on intraocular lens [76]. In fact, methacrylate/acylate polymers have been already shown to be a determinant factor for avoiding bacterial adhesion [77]. The binding between AHLs and these types of monomers has been shown to be mainly due to electrostatic interactions. Cross-linked polymers were synthesised in a bulk format using a cross-linker (EGDMA) in the polymerisation process. The cross-linked polymer with 5% of IA showed to be efficient in the reduction of bioluminescence and biofilm formation of *V. fischeri* [120]. The normal growth of bacteria was not affected by the presence of methacrylate and acrylate linear polymers, evidencing the lack of toxicity for the microorganisms [74, 77]. When evaluating new material, either natural or synthetic, for quorum quenching, it is essential to evaluate the toxicity of the compounds. Compounds that lead to the death of bacterial cells may, in theory, lead to resistance phenotypes. Using the metabolic activity by resazurin (alamar blue) it is possible to evaluate the cytotoxicity associated to the materials [78, 124]. A study using Vero cells, reports that no cytotoxicity was associated to methyl methacrylate linear polymers [74]. Examples of non-cytotoxic synthetic polymers based on methacrylic acid have also been described [79]. The biocompatibility of synthetic polymers, when these are used for coating surfaces, is an essential characteristic: these biocompatible materials can inhibit bacterial films formation without damaging the cells, and therefore, without inducing resistance phenotypes. Jahid and collaborators reported that biofilm formation and exoproteases production, both QS-phenotypes of *A. hydrophila*, were influenced by 0.05% of glucose [19]. In another study, it has been demonstrated that the presence of vanillin in membranes was able to reduce biofilm formation of *A. hydrophila* [80]. More recently, a polymeric material, polyethylene (used in the water industry and in medicine) modified with activated organosilanes was tested to verify the impact on biofilm formation and bacterial cell attachment of *A. hydrophila* [81]. The results showed that surfaces modified with the material demonstrated better antibacterial and anti-adhesive properties for *A. hydrophila* than control surfaces [81]. Compounds such as cinnamaldehyde were used to interfere with the AI-2 signal molecule of *Vibrio*. The results showed an influence on bioluminescence, inhibition of biofilm formation and reduction of protease production, without inhibiting bacterial growth [82].

### 4. Conclusions

Quorum sensing inhibition – quorum quenching – bears the potential to revolutionize the use of antibiotics. New and exciting, biocompatible, natural or synthetic compounds, able to diminish QS phenotypes, will be used in health care equipment and materials but also in aquacultures and industries – where pathogenic, biofilm making bacteria induce large damages. Nonetheless, despite the encouraging results, in most studies, the mechanism of the antibacterial activity was not fully cleared and the QS targets were not identified [72]. Efforts to develop new materials able to inhibit QS phenotypes and to fully understand how QS phenotypes can be controlled should be pursuit. The future against biofilm-mediated infections could be a chemotherapeutic approach, combining conventional antibiotics with drugs possessing anti-biofilm activity.

**Acknowledgements**  This study was partially supported by FEDER funding through COMPETE program and by national funding to CESAM (UID/AMB/50017/2013), AC Esteves (BPD/102572/2014) also acknowledges FCT.

**References**


