Novel linear polymers able to inhibit bacterial quorum sensing

Eliana Cavaleiro[1,2], Ana Sofia Duarte[1], Ana Cristina Esteves[1], António Correia[1], Michael J. Whitcombe[3], Elena V. Piletska[3], Sergey A. Piletsky [3], Iva Chianella[2].

[1] Department of Biology & CESAM, University of Aveiro, Aveiro, Portugal.

[2] Centre for Biomedical Engineering, SATM, Cranfield University, Cranfield, Beds, UK

[3] Department of Chemistry, University of Leicester, Leicester, UK

Abstract

Bacterial phenotypes such as biofilm formation, antibiotic resistance, virulence expression are associated with Quorum Sensing (QS). QS is a density-dependent regulatory system of gene expression controlled by specific signal molecules, such as N-acyl homoserine lactones (AHLs), produced and released by bacteria. This study reports the development of linear polymers capable to attenuate QS by adsorption of AHLs. Linear polymers were synthesized using methyl methacrylate as backbone monomer and methacrylic acid and itaconic acid as functional monomers.

Two different QS-controlled phenotypes, *Vibrio fischeri* bioluminescence and *Aeromonas hydrophila* biofilm formation, were evaluated to test the polymers' efficiency. Results showed that both phenotypes were significantly affected by the polymers, with the itaconic acid-containing material more effective than the methacrylic acid one. The polymer inhibitory effects were reverted by addition of lactones, confirming attenuation of QS through sequestration of signal molecules. The polymers also showed no cytotoxicity when tested using a mammalian cell line.

1 Introduction

Quorum sensing (QS) is a refined system of communication, mediated by small diffusible molecules called autoinducers.[1-3] Autoinducers allow the chemical communication between bacteria in a cell-density-dependent manner.[4,5] These molecules are produced inside the cell at low levels and diffuse outside by crossing cell membranes. When the concentration of signal molecules in the extracellular medium reaches a critical value, these molecules re-enter the cells, affecting their behavior.[3,5,6] To summarize, autoinducers regulate gene expression as a function of cell population density.[4,7]

QS is a highly specific process due to the specificity of the interactions between the signal molecules and their receptors. N-acylhomoserine lactones (AHLs) are the most commonly produced autoinducers of Gram-negative bacteria.[8] The first AHL identified as an autoinducer was N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C_6-AHL) expressed by *Vibrio fischeri*.[9] QS controls several bacterial phenotypes: bioluminescence[10,11], conjugation [12], expression of several virulence factors, such as toxins production[13,14], and development of mature antibiotic-resistant biofilms.[15,16].The development of biofilms is an important feature for bacterial pathogens as it provides their protection and, therefore, makes their elimination difficult. [17,18,19].

Bacterial infections are routinely treated using antibacterial compounds that target cellular processes such as bacterial DNA replication and repair, cell wall biosynthesis and/or the protein synthesis.[16] Nevertheless, bacteria can acquire resistance to these molecules, a phenomenon increasingly reported both for the clinical and natural

environments.[20,21] Consequently, new resistant strains and even superbugs have emerged with serious consequences for human health.[22]

It would be desirable to be able to control the expression of virulence factors and decrease bacterial virulence without inducing phenotypes of resistance.[16] Since bacteria use QS to regulate the genes, responsible for virulence and toxins production[23], quenching of QS, also known as quorum quenching (QQ) may be considered as a potential therapeutic strategy. Quorum Quenching (QQ) could be used not only to block the formation of biofilms, but also for prevention of bacterial virulence and for the control of any other QS-mediated mechanisms. The use of QS inhibitors offers a new tool to fight bacterial diseases by sequestration of signal molecules at early stages of the bacterial infections.[24] An important advantage is that QS inhibition does not impose selective pressure for the development of bacterial resistance, as with antibiotics.

Synthetic polymers based on methacrylates (e.g. PMMA) were successfully used to reduce biofilm formation by *Pseudomonas aeruginosa*.[25] In fact, Gottenbos and colleagues have shown that by immobilizing positively charged methacrylates polymers on microscope slides, the growth of Gram-negative bacteria and biofilm formation could be prevented. Nevertheless, the effect was attributed to the positive charge of the polymers and not to QQ.

In this work, we describe the development of biocompatible, non-cytotoxic methacrylates polymers, which are able to sequester AHLs and interfere with QS of two different test species: *Vibrio fischeri* ATCC 7744 and *Aeromonas hydrophila* strain IR13. [21] A set of itaconic-acid/methyl methacrylate (IA-MMA) and methacrylic acid/methyl methacrylate (MAA-MMA) copolymers were synthesized by free radical polymerization (FRP). The study also includes the evaluation of biocompatibility and

cytotoxicity of the developed polymers by *in vitro* cytotoxicity tests using a mammalian cell line (Vero cells).

2 Material and methods

2.1 Materials

Itaconic acid (IA 99 %), methacrylic acid (MAA 99 %, containing 250 ppm monomethyl ether hydroquinone as inhibitor), methyl methacrylate (MMA 99 %, containing ≤ 30 ppm monomethyl ether hydroquinone as inhibitor), ethylene glycol dimethacrylate (EGDMA), acetonitrile, ethyl acetate 2-butanone, 2-methoxyethanol, N,N-dimethylformamide (DMF), α, α'-azoisobutyronitrile (AIBN) , N-(β-ketocapryloyl)-DL-homoserine lactone (3-oxo-C6-AHL), N-hexanoyl-DL-homoserine lactone (C6-HSL), N-butyryl-DL-homoserine lactone (C4-HSL), Phosphate Buffered Saline, pH 7.4 (PBS), and anti-bumping granules were purchased from Sigma (Gillingham, UK). Nutrient Broth Nº 2 (NB), Luria-broth (LB) and Agar Bacteriological (Agar Nº 1), were purchased from Oxoid (Basingstoke, UK).

2.2 General procedure for copolymer synthesis

A 3:1 monomer ratio was used to synthesized copolymer (or linear polymers) by free radical polymerization: 3:1 (MMA: IA/MAA) as described in **Erro! Autorreferência de marcador inválida.**. As a polymerization solvent a mixture of 2-butanone and 2-methoxyethanol (1:1; v:v) was used. MAA and MMA were distilled under vacuum. Purified monomers were kept at 4 ºC and used without further purification. The initiator α, α'-azoisobutyronitrile (AIBN) was purified by fractional crystallization from ethanol (m. p. = 104 ºC). Other reagents (extra-pure grade) were used without purification.

Polymerization reactions were prepared as described (Table 1). The monomers were poured into a 250 ml three-necked round-bottom flask. The solvent mixture was added, and a condenser and a thermometer were connected. The flask was placed on a magnetic stirrer/heater, immersed into an oil bath and degassed with nitrogen. At 60 ºC,

conventional radical copolymerization started by the addition of the initiator AIBN, and carried out for 20 h. The copolymers were precipitated, drop by drop, in ultra-pure water, recovered by vacuum filtration (Whatman filter paper nº 1) and re-dissolved in DMF (~20-30 mL). This procedure was performed three times. The copolymers were dried under vacuum in a desiccator at room temperature for 4 days and kept at room temperature until use.

2.3 Linear Polymers characterization

2.3.1 Nuclear magnetic resonance (NMR) spectroscopy

The NMR spectra were obtained using a JEOL ECX-400 NMR spectrometer (Jeol, Welwyn Garden City, UK). The NMR solvents, $CDCl_3$ and CD_3OD, were obtained from Cambridge Isotopes Limited (UK). Twenty mg of copolymer were solubilized in NMR solvent. Itaconic acid polymers were solubilized in methanol-d_4 (CD_3OD) and methacrylic acid polymers were solubilized in chloroform-d ($CDCl_3$). All the polymers were analyzed by ^1H NMR (400MHz). The resulting NMR spectra were analyzed with JOEL Delta^{TM} data processing software.

2.3.2 Gel permeation chromatography

Gel permeation chromatography (GPC) was performed on a Polymer Labs GPC 50 Plus system (Agilent, Stockport, UK) fitted with a differential refractive index detector. Separations were performed on a pair of PLgel Mixed-D columns (300 × 7.8 mm, 5 µm bead size, (Agilent, Stockport, UK) fitted with a matching guard column (50 × 7.8 mm). The mobile phase was DMF with 0.1 % LiBr (w/v) at a flow rate of 1 mL min^{-1}. Column calibration was achieved using poly[methyl methacrylate] standards (1.96 – 790 kDa, (Agile, Stockport, UK)). Samples were prepared at 1–5 mg mL^{-1} in the mobile

phase and injected (100 µL) onto the column. Molecular weight and polydispersity indices were calculated using Polymer Labs Cirrus 3.0 Software (Agilent, Stockport, UK).

2.3.3 Binding capacity

Polymer's binding capacity towards the lactones 3-oxo-C_6-AHL, C_4-HSL and C_6-HSL was evaluated by HLPC-MS as described previously[26] with slight modifications. A stock solution of each AHL (1 mg mL^{-1}) was prepared in acetonitrile. Several dilutions were prepared in water: 0.1, 0.5, 1.0, 2.5, 5, 10, 20, 50, and 100 µg mL^{-1}, in order to build a calibration curve. Ten mg of each copolymer were suspended in 1 mL of each AHL solution (25 µg mL^{-1}) and incubated overnight with rotation of 200 rpm, at room temperature. After incubation, samples were centrifuged at 10,000 rpm for 20 minutes, and the supernatants were collected and filtered through 0.45 µm pore nylon filter. For the quantification of AHLs a Waters 2975 HPLC system equipped with a Luna C18 (2) column (150 x 3 mm, 3 µm, Phenomenex) was used. Elution was achieved by a gradient of methanol (0–70 %, v/v) acidified with formic acid (0.1 %) at a flow rate of 0.2 mL^{-1} min. A fragment of AHL with a m/z of 102 was detected by mass-spectrophotometer Micromass Quatro Micro (Waters, UK) equipped with an ESI interface in positive ion mode.[27] Mass Spectrometry parameters were: desolvation gas - 850 L h^{-1}, cone gas- 50 L h^{-1}, capillary- 4.5 kV, cone-25 V, CE- 20, source temperature- +120 ºC, desolvation temperature- +350 ºC, collision energy- 25 V, multiplier- 650 V [26]. The concentration of free AHL was determined and the amount of AHL adsorbed was calculated using a subtraction from the original concentration.

2.4 Immobilization of copolymers

Copolymers $pMAA_{25}$-*co*-$pMMA_{75}$ and pIA_{25}-*co*-$pMMA_{75}$ were solubilized in dry methanol (1 mg mL^{-1}). One hundred milliliters of solubilized copolymers were dispensed in a flat-bottom, polystyrene 12-well microplate. The solvent was allowed to evaporate overnight at room temperature.[28]

2.5 Bacterial strains and growth conditions with copolymers

The wild strain *Vibrio fischeri* ATCC 7744 and the environmental isolate of *Aeromonas hydrophila* strain IR13 were used as model organisms. *Vibrio fischeri* and *A. hydrophila* were grown overnight at 180 rpm, 25 ºC and 30 ºC, in Luria-broth (LB) and in Nutrient Broth nº 2 supplemented with 2% NaCl, respectively. Copolymers were added to 250 mL Erlenmeyer flasks (10 mg mL^{-1}), and sterilized by irradiation with UV light for 20 minutes. Fifty milliliters of culture medium and an aliquot of an overnight bacteria culture (500 µL) were added. All cultures were incubated at the bacteria optimum growth temperature with agitation at 180 rpm. Copolymer-free cultures and media supplemented with the respective copolymer were used as negative controls. At selected intervals, the optical density at 600 nm (OD_{600nm}) was measured using a UV mini-1240 UV-VIS Spectrophotometer (Shimadzu, Japan). At the same time, an aliquot was collected and diluted in PBS (10^{-1}-10^{-9}). The diluted aliquots were plated in Marine Broth Agar (MA) or Luria Broth Agar (LA), for *V. fischeri* or *A. hydrophila* and incubated at adequate temperature for 24 h, and the number of colonies forming units (CFU) was determined. All samples were analyzed in three independent experiments performed in triplicate.

2.6 Effect of copolymers on *Vibrio fischeri* bioluminescence

To evaluate the effect of copolymers on *Vibrio fischeri* ATCC 7744 bioluminescence, the culture was grown as described previously. *Vibrio fischeri*'s luminescence was measured using a luminometer (TD-20/20 Luminometer, Turner Designs, Inc., USA).

2.7 Biofilm formation in *Aeromonas hydrophila* strain IR13

Biofilm formation was analyzed in 12-well microplates coated with 0.1 mg mL-1 of solubilized polymers.[29,30] Microplates with polymers were then sterilized for 20 minutes under UV light radiation. Each well was inoculated with 1 mL of *A. hydrophila* culture diluted 100-times (stock solution ~0.9 O.D600nm). The microplates were then incubated for 27 hours without agitation at 30 ºC. Afterwards, the supernatant (planktonic cells) was collected and transferred to another 12-well sterilized microplate. Each well was gently rinsed three times with PBS. After adding 500 µL of 5% sterile resazurin solution (v:v) to each well, the microplates were incubated at cell's optimal growth temperature, as described in the literature.[31,32] Resazurin was solubilized in PBS and sterilized by filtration using 0.22 µm - syringe filter. After 1 hour for planktonic cells and 2 hours for biofilm cells, well contents were removed and transferred to another microplate. The absorbance of both, planktonic and sessile cells, were measured at 570 nm and 600 nm using a microplate reader (Multiskan Spectrum Microplate spectrophotometer, Thermo Scientific, UK). All the assays were performed in triplicate. *Aeromonas hydrophila* biofilm was determined by the ratio between planktonic (free cells) and biofilm (sessile) cells.

2.8 Cytotoxicity

The Vero cell line (ECACC 88020401, African Green Monkey Kidney cells, GMK clone) was grown and maintained according to.[33] 12-well microplates coated with copolymers (0.1 mg mL^{-1}) were sterilized by UV radiation for 20 minutes. Cytotoxicity evaluation was performed using Vero cell line (epithelial cells from African green monkey kidney). The cellular metabolic activity was assessed by resazurin (Alamar Blue) assay during 48 hours.[34] Vero cells were seeded into 12 well plates at a density of $1x10^5$ cells $well^{-1}$, and incubated for 24 h and 48 h at 37 ºC with 5% CO_2. After each time of incubation, the growth medium was aspirated and replaced with fresh medium supplemented with 10% of 0.1 mg mL^{-1} resazurin, for 2:30 h at 37 ºC. Afterwards, the well content was removed and transferred to another microplate and the absorbance at 570 nm and 600 nm was measured using a microplate reader (Multiskan Spectrum Microplate spectrophotometer, Thermo Scientific, UK). All the experiments were made in triplicate. Percentage of cytotoxicity for each copolymer was calculated as: (OD_{570}/OD_{600} sample - OD_{570}/OD_{600} medium) / (OD_{570}/OD_{600} control - OD_{570}/OD_{600} medium) x 100.[35] Vero cell morphology in the presence of copolymers was evaluated by inverted light microscopy. Images were acquired using a CKX41 Olympus inverse microscope with a digital color camera Olympus CAM-SC30 and a 20X objective (OLYMPUS, Tokyo, Japan). The image acquisition was obtained by the AnalySIS getIT software (Soft Imaging System, Munster, Germany).

2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism v.5 software (GraphPad Software Inc., San Diego California, USA). For cell viability one-way ANOVA

followed by Bonferroni's Multiple test with a statistical confidence coefficient of 0.95 was used; consequently p values <0.05 were considered significant.

3 Results and Discussion

Copolymerization synthesis

In a study performed previously by Piletska and co-workers, itaconic acid and methacrylic acid were already identified as monomers capable of interacting strongly with 3-oxo-C_6-HSL, C_4-HSL and C_6-HSL. Therefore, these two monomers were used here to synthesize two different types of linear polymers or copolymers. The linear polymers were produced using methyl methacrylate (MMA) as inert monomer, to build the backbone of the resulting materials and IA and MAA as functional monomers. Two different mixtures of copolymers (IA/MMA and MAA/MMA) were synthesized by free radical polymerization in a solvent mixture of 2-butanone: 2-methoxyethanol and AIBN as radical initiator. It is known that linear polymers prepared by free radical polymerization have several advantages compared to other polymerization methods (e.g. ionic chain polymerization), such as a relative insensitivity to monomer and media impurities (decreasing synthesis costs) and the possibility of using a broad range of monomers.[36] It was predicted that the chemical features of the monomers selected in this work for production of copolymers will produce material capable of binding to the target analytes through both hydrophobic and hydrophilic interactions.[37]

3.2 Characterization of polymers

The monomer composition of the polymers synthesized here was determined by NMR from the integral intensities of ^{1}H NMR signal of methyl peak (3.6 ppm) of methyl methacrylate (Table 2). The results showed that the functional monomer content of both polymers was close to the predicted one, with acceptable deviations. This means that the polymers production was properly controlled and reproducible.

When the polymers were analyzed by GPC analysis the results showed that *pMAA$_{25}$-co-pMMA$_{75}$* had a smaller molecular weight than pIA$_{25}$-co-pMMA$_{75}$ and a higher polydispersity index (Table 2). These could be a result of the different reactivity of IA and MAA monomers and could have an influence on the polymers performance.

The average binding capacity of the copolymers for AHLs was tested by batch binding experiments and the results are shown in Table 3. The *pMAA$_{25}$-co-pMMA$_{75}$* polymer showed higher binding capacity towards all assayed AHLs than the *pIA$_{25}$-co-pMMA$_{75}$* copolymer. ~~It was observed that t~~The polymers' binding to AHLs ~~was~~ seemed to be governed by a combination of hydrophobic, van der Waals and electrostatic interactions between the carboxylic groups of polymer and the lactone ring of AHLs. The copolymers binding capacities observed for all AHLs have underlined the potential of the material to be used as QQ agents and, therefore, both linear polymers were tested *in vitro* in order to investigate their ability to disrupt QS.

3.3 Effect of copolymers on QS-regulated phenotypes: *Vibrio fischeri* bioluminescence

Since the bioluminescence of *V. fischeri* is a QS-regulated phenotype,[3,9] it was used as a model to assess the efficiency of the copolymers to disrupt QS by their sequestration of corresponding AHLs.

The results reported in Figure 1A show that the greatest reduction (one logarithmic unit) was observed after 4 hours of incubation of bacteria (exponential phase) with *pIA$_{25}$-co-pMMA$_{75}$*. The *pMAA$_{25}$-co-pMMA$_{75}$* polymer also caused a decrease in bioluminescence (black squares, Figure 1B). The reduction on bioluminescence of *V. fischeri* caused by this polymer, although not as high as that seen for *pIA$_{25}$-co-pMMA$_{75}$*, was still significant. Interestingly, *pMAA$_{25}$-co-pMMA$_{75}$* showed a higher binding capacity for

AHLs than the *pIA$_{25}$-co-pMMA$_{75}$* polymer (Table 3), when experiments were performed in water. This demonstrates that testing conditions can influence the polymer's performance. It was also clear from the experiments that the reduction in *Vibrio* luminescence was not due to polymers toxicity, as the addition of *pMAA$_{25}$-co-pMMA$_{75}$* or *pIA$_{25}$-co-pMMA$_{75}$* did not have any effect on cell growth (Figure 2), suggesting that the linear polymers did interfere with QS by sequestration of lactones.

To demonstrate further that luminescence reduction was due to AHL sequestration by the polymers, signal molecule of *V. fischeri* (3-oxo-C6-HSL) was added to the culture. As expected, an increase in luminescence (open circles, Figure 1A) was noticed. Nevertheless, even after the addition of 3-oxo-C$_6$-HSL, *pIA$_{25}$-co-pMMA$_{75}$* was still able to reduce *V. fischeri* bioluminescence (open squares, Figure 1A). A similar trend was also observed after the addition of AHLs to the system containing *pMAA$_{25}$-co-pMMA$_{75}$*, (open squares, Figure 1B). In conclusions, both polymers demonstrated the reduction of *Vibrio*'s luminescence, when compared to the control. The same effect was observed by Piletska and colleagues [38] using cross-linked polymers based on the same functional monomers. It is possible to conclude that, similarly to the effect of the cross-linked polymers reported earlier, the linear polymers presented here had a direct impact on bacterial bioluminescence linked to strong interactions established between the functional monomers and AHLs.

3.4 Effect of copolymers on QS-regulated phenotypes: *Aeromonas hydrophila* biofilm formation

It is known that bacterial biofilm formation linked to QS via AHLs production strongly increases the survival and growth of the bacteria in the hostile environments.[2,39,40]

Biofilms contribute to the development of antibiotic resistance, playing a significant role in the virulence of several pathogenic bacteria.[41]

The effect of the linear polymers on *Aeromonas hydrophila* was evaluated using the strain IR13, with the polymers immobilized on wells of microplates as explained in the Methods section. It is known that biofilm formation in *A. hydrophila* is controlled by QS mediated by AHLs.[42] Similarly to *V. fischeri,* the presence of the polymers did not affect *A. hydrophila* growth (Figure 3). Nonetheless, it was found that the production of biofilm by *A. hydrophila* was significantly reduced in the presence of both polymers (Figure 4). To confirm that biofilm reduction resulted from sequestration of AHLs by the linear polymers, 100 μM of C_4-HSL (the signal molecule) was added to the cultures. The cultures with exogenous AHL recovered their ability to form biofilm, probably due to inability of the limited amount of copolymers attached to the wells to fully adsorb both, the endogenous and exogenous AHL. Nevertheless, this demonstrates that these polymeric materials were able to interfere with quorum sensing through sequestration of AHL, leading to reduced bacterial adhesion.[43]

3.5 Cytotoxicity evaluation

It was important to ensure that polymers capable to disrupting QS must not be toxic. Therefore, the cytotoxicity of the linear polymers was evaluated by resazurin method using the Vero cell line (Figure 5).

In the first 24 hours (Figure 5), cell viability was slightly affected ($p<0.05$) by both polymers. In fact, whereas in presence of $pMAA_{25}$-*co*-$pMMA_{75}$ cell viability was approximately 90%, in the presence of with pIA_{25}-*co*-$pMMA_{75}$, cell viability was reduced to 80 %. According to ISO 10993-5, materials producing percentages of cell viability above 80% are considered non-cytotoxic. Furthermore, after 48 hours of

contact with the polymers, Vero cells recovered nearly all their viability with values of 100% and almost 90% for *$pMAA_{25}$-co-$pMMA_{75}$ and pIA_{25}-co-$pMMA_{75}$* respectively (Figure 5). These results were confirmed by microscopy (Figure 6).

The effect of the polymers on the cell culture during the first 24 hours of incubation was probably due to the fact that the two polymers are acidic (with *pIA_{25}-co-$pMMA_{75}$* more acidic than *$pMAA_{25}$-co-$pMMA_{75}$*) and at the beginning of the experiments this might have lower the pH of the medium, slightly affecting the cell's growth. As the system matures, the pH is restored and the cell started to recover. In conclusion, the results demonstrate that the linear polymers were not cytotoxic, having potential for use on environmental and medical applications.

4 Conclusions

In this study, two non-toxic linear copolymers - *$pMAA_{25}$-co-$pMMA_{75}$* and *pIA_{25}-co-$pMMA_{75}$* - were synthesized by free radical polymerization. Their interference with QS-regulated phenotypes, such as the decrease of bioluminescence in *V. fischeri*, and the reduction of biofilms produced by *A. hydrophila*, confirmed the ability of the polymers to disrupt bacterial QS.

Phenotypes like bacterial virulence and resistance are regulated by QS. Therefore, the use of inert synthetic materials capable to interfere with QS is an exciting alternative to usually more toxic 'antiseptic' alternatives which are commonly used to prevent bacterial growth. When tested in model solutions (water) the two linear polymers developed in this study showed the ability to adsorb AHLs, with *$pMAA_{25}$-co-$pMMA_{75}$* demonstrating a higher binding capacity for AHLs than *$pMAA_{25}$-co-$pMMA_{75}$*. Nevertheless when the testing was performed in real conditions (in presence of medium), it was *pIA_{25}-co-$pMMA_{75}$* that showed a stronger interference with both *V.*

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Fischeri and *A. hydrophyla* QS, even though both polymers were capable or delaying and decreasing luminescence from *V. Fischeri* and significantly reduce biofilm formation by *A. hydrophyla*. ~~The linear~~In addition both polymers ~~developed in this study~~also did not interfere with bacterial grow and did not ~~did not show~~show cytotoxicity in the conditions tested, ~~being~~ which make them useful candidates for control of phenotypes associated to bacterial virulence. Moreover ~~T~~their solubility in organic solvents makes them excellent candidates for integration into the filters of water purification systems or as components of the paints which could be used for environmental and medical applications where the polymers could adsorb QS signal molecules delaying bacterial virulence, reducing bacterial resistance and enhancing the effect of commonly used antibacterial agents.

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Table 1. Composition of copolymers synthesis

Copolymer	Functional Monomers			Initiator
	IA	MAA	MMA	AIBN
pIA_{25}-co-$pMMA_{75}$	3.02 g; 0.023 mol	——	6.98 g; 0.069 mol	0.313 g
$pMAA_{25}$-co-$pMMA_{75}$	——	2.23 g; 0.026 mol	7.77 g; 0.078 mol	0.313 g

Table 2. Free-radical polymerisation of MAA/IA (M1) and MMA (M2). Characterization of copolymers determined by NMR and GPC

	Monomer feed (mol %)		**^{1}H NMR (mol %)**		**GPC**		
	M1	**M2**	**M1**	**M2**	**M_n (g/mol)**	**M_w (g/mol)**	**PDI**
$pMAA_{25}$-co-$pMMA_{75}$	25	75	28.28 ± 4.78	71.72 ± 4.78	11288.00	29634.50	2.63
pIA_{25}-co-$pMMA_{75}$	25	75	20.80 ± 8.03	79.20 ± 8.03	23729.00	42273.50	1.78

Table 3. Binding capacity of copolymers towards 3-oxo-C_6-HSL, C_6-HSL and C_4-HSL

	3-oxo-C_6-HSL	**C_6-HSL**	**C_4-HSL**
	(µg per mg of polymer)	**(µg per mg of polymer)**	**(µg per mg of polymer)**
$pMAA_{25}$-co-$pMMA_{75}$	0.95 ± 0.11	0.71 ± 0.11	0.66 ± 0.11
pIA_{25}-co-$pMMA_{75}$	0.28 ± 0.06	0.24 ± 0.12	0.13 ± 0.03

Figure 1 Bioluminescence (RLU) signal of *V. fischeri* in presence of pIA_{25}-*co*-$pMMA_{75}$ (A) and $pMAA_{25}$-*co*-$pMMA_{75}$ (B) copolymers. A supplement of 100 μM of AHL (3-oxo-C6-HSL) was add to each copolymer experiment. The data represent means (standard deviations) of three independent experiments performed in triplicate.

Figure 2 Bacterial growth (CFU/ml) of *V. fischeri* (A and B) in the presence of pIA25-co-pMMA75 and pMAA25-co-pMMA75 copolymers. The culture was supplemented with 100 μM of AHL for each copolymer.

Figure 3 Bacterial growth (CFU/ml) of *A. hydrophila* (A and B) in the presence of pIA_{25}-*co*-$pMMA_{75}$ and $pMAA_{25}$-*co*-$pMMA_{75}$ copolymer. The culture was supplemented with 100 μM of AHL for each copolymer.

Figure 4 Linear polymers effect on biofilm formation by A. hydrophila and A. hydrophila supplemented with 100 μM of AHL. One-way ANOVA, followed by a Bonferroni's multiple comparison test, was performed to determine statistical significance of copolymers against the control (***$p<0.001$) and the control supplemented with AHL (### $p<0.001$). Vertical bars represent standard deviation.

Figure 5 Cytotoxic effect of linear polymers evaluated by the viability of Vero cells, along 48 hours, determined by resazurin. One-way ANOVA was performed followed by a Bonferroni's multiple test ($p<0.05$). Data represents three independent experiments performed in triplicate.

Figure 6 Inverted microscopy of Vero cells exposed to copolymers after 24 and 48 hours. Images were taken under 200X magnification. Scale bar 100 μm.

Figure 1

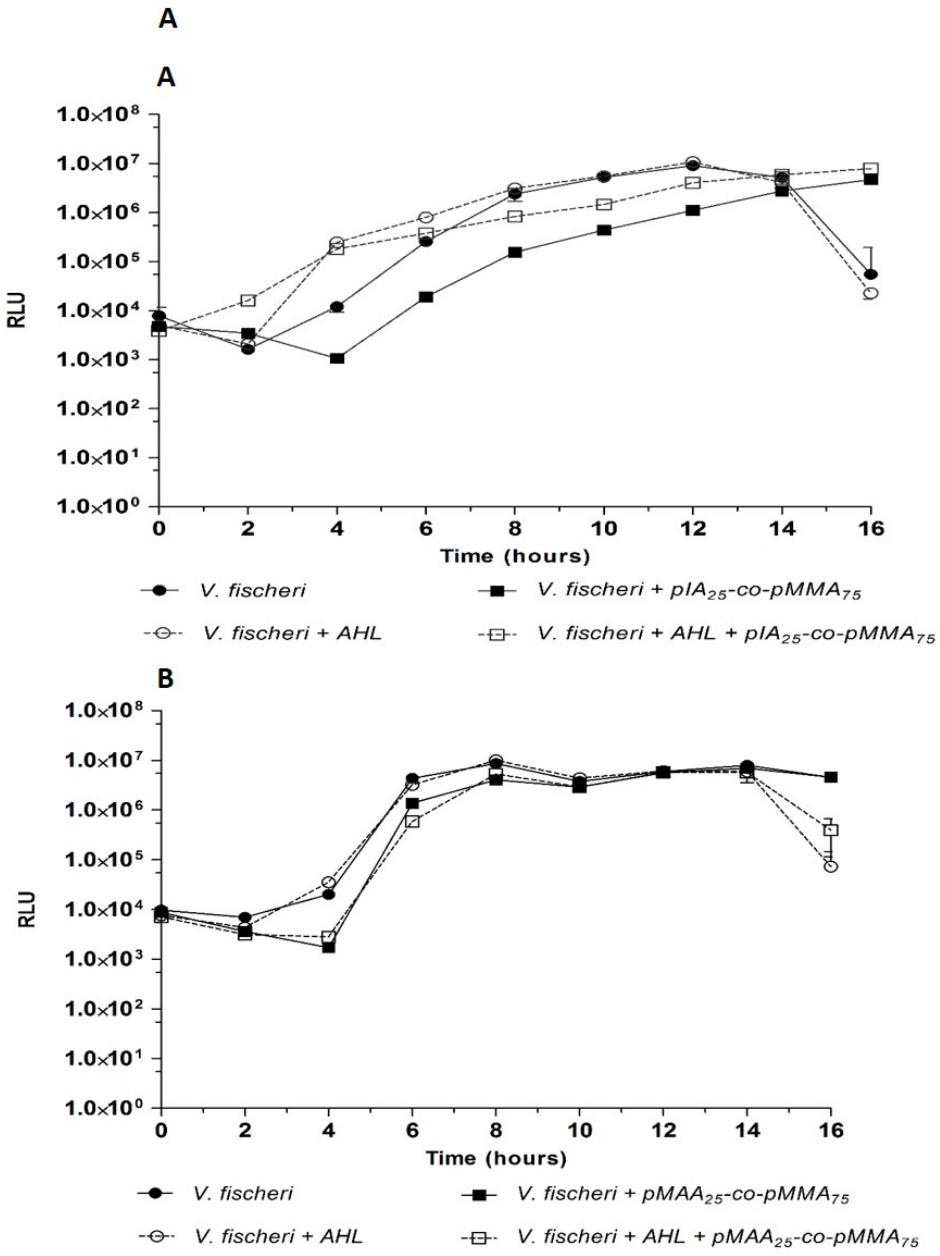

A
A
RLU
1.0×10⁸
1.0×10⁷
1.0×10⁶
1.0×10⁵
1.0×10⁴
1.0×10³
1.0×10²
1.0×10¹
1.0×10⁰
0
2
4
6
8
10
12
14
16
Time (hours)
V. fischeri
V. fischeri + pIA25-co-pMMA75
V. fischeri + AHL
V. fischeri + AHL + pIA25-co-pMMA75
B
RLU
1.0×10⁸
1.0×10⁷
1.0×10⁶
1.0×10⁵
1.0×10⁴
1.0×10³
1.0×10²
1.0×10¹
1.0×10⁰
0
2
4
6
8
10
12
14
16
Time (hours)
V. fischeri
V. fischeri + pMAA25-co-pMMA75
V. fischeri + AHL
V. fischeri + AHL + pMAA25-co-pMMA75

Figure 2

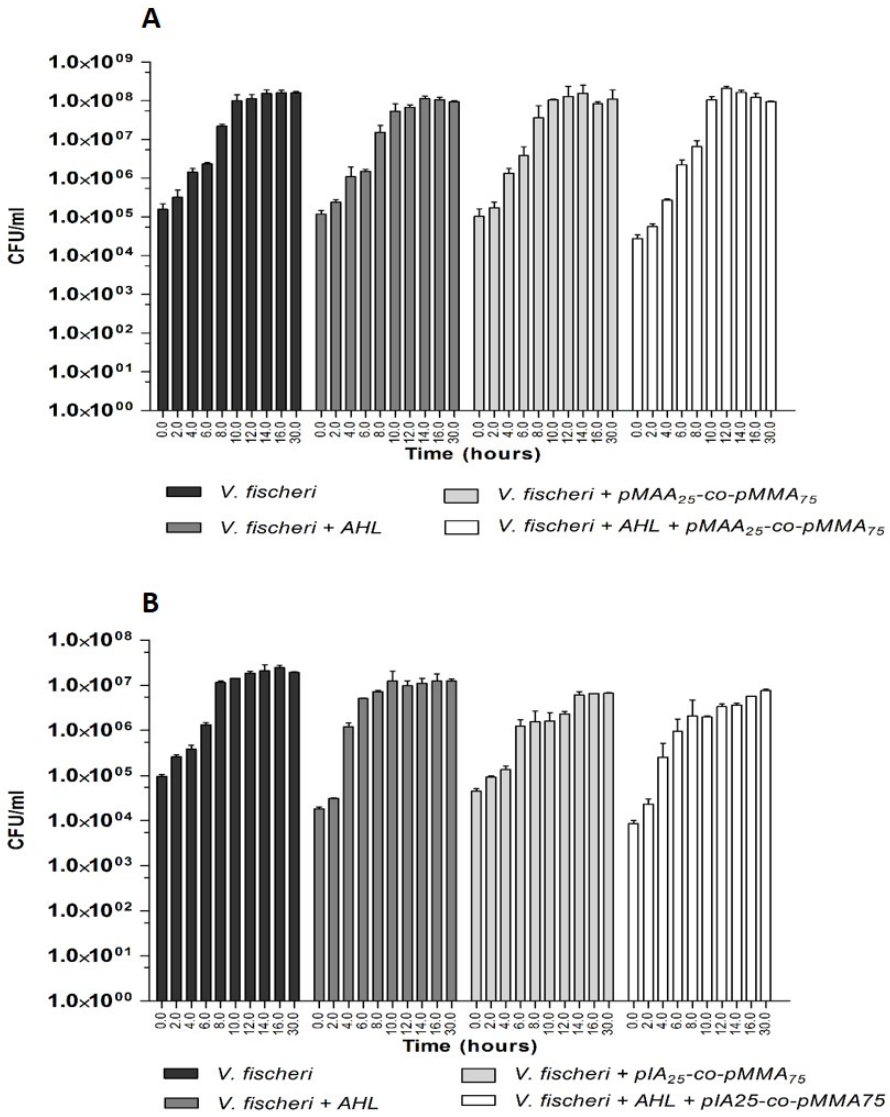
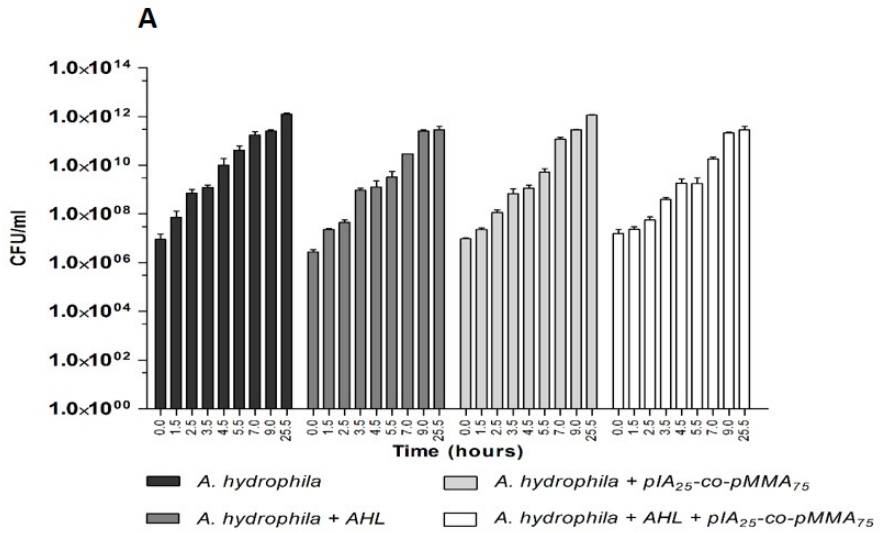

A
CFU/ml
1.0×10^09
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
1.0×10^02
1.0×10^01
1.0×10^00
0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 30.0
Time (hours)
V. fischeri
V. fischeri + AHL
V. fischeri + pMAA25-co-pMMA75
V. fischeri + AHL + pMAA25-co-pMMA75
B
CFU/ml
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
1.0×10^02
1.0×10^01
1.0×10^00
0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 30.0
Time (hours)
V. fischeri
V. fischeri + AHL
V. fischeri + pIA25-co-pMMA75
V. fischeri + AHL + pIA25-co-pMMA75

Figure 3



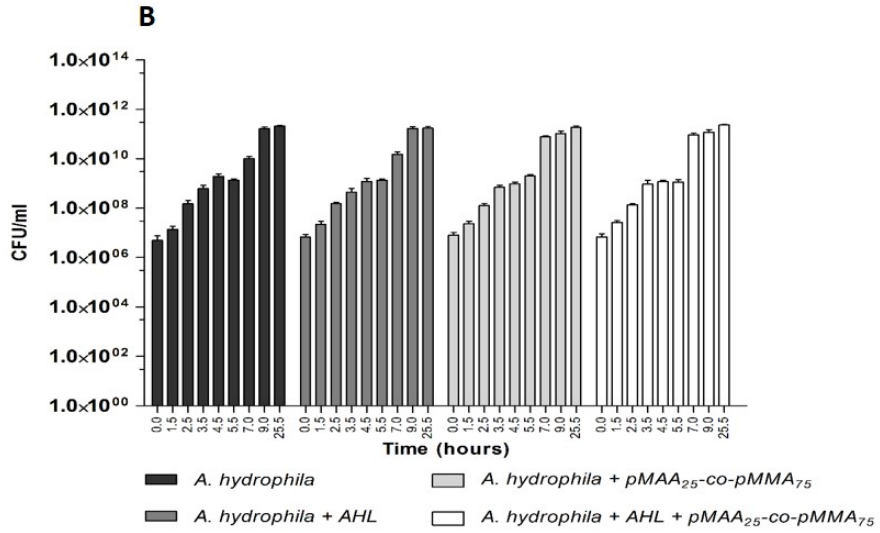


Figure 4

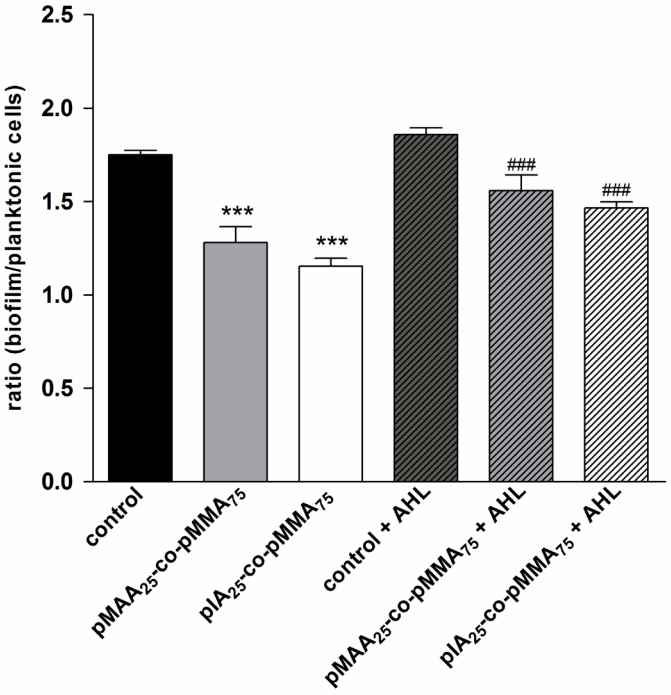

ratio (biofilm/planktonic cells)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
###
###
control
pMAA25-co-pMMA75
pIA25-co-pMMA75
control + AHL
pMAA25-co-pMMA75 + AHL
pIA25-co-pMMA75 + AHL

Figure 5

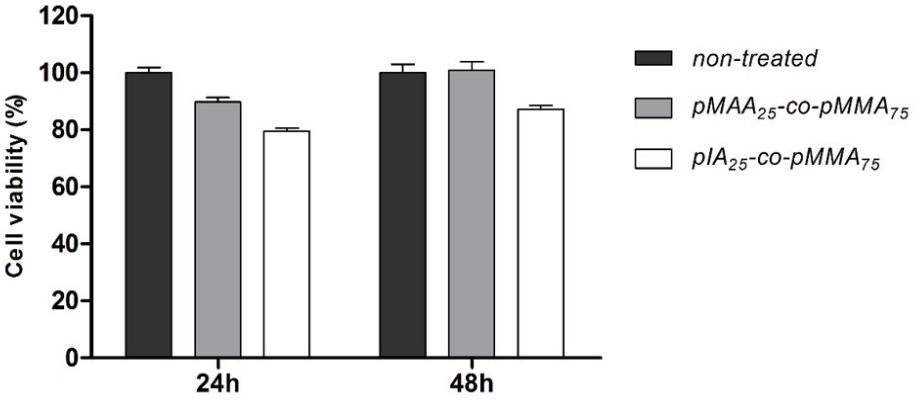
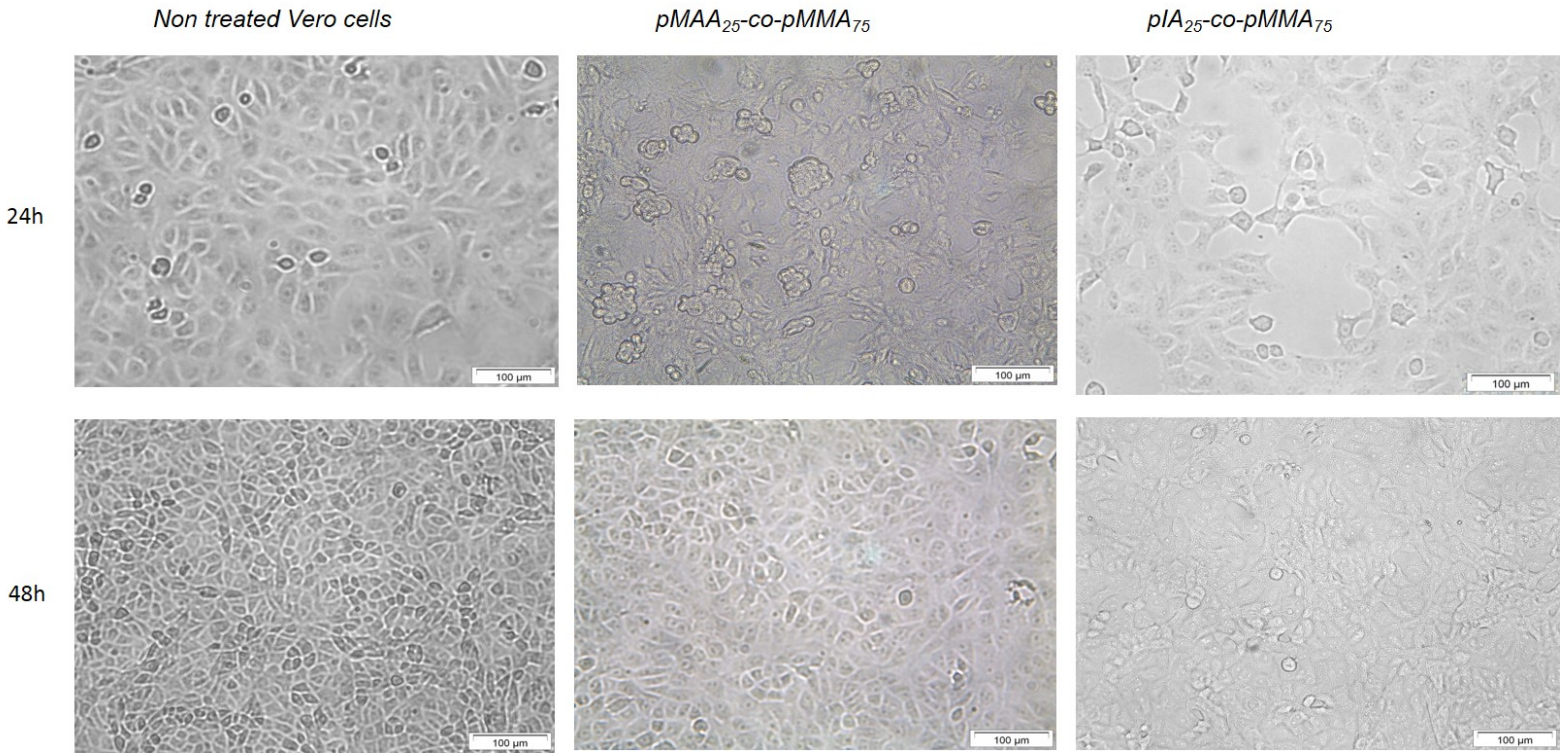

120
100
80
60
40
20
0
Cell viability (%)
24h
48h
non-treated
pMAA25-co-pMMA75
pIA25-co-pMMA75

Figure 6


Non treated Vero cells
pMAA25-co-pMMA75
pIA25-co-pMMA75
24h
48h
100 µm

Text and Graphic for the Table of Contents

The conventional treatment of bacterial infections is based on administration of antibiotics, which promotes bacterial resistance. We present the development of linear polymers able to disrupt Quorum Sensing as shown by reduction of bacterial bioluminescence and biofilm formation. The polymers could be used to control bacterial virulence without promoting resistance, thus constituting a safe alternative to the use of antibiotics.

KEYWORDS: Copolymers, Quorum Sensing, *Vibrio fischeri*, *Aeromonas hydrophila*