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Highly branched poly(N-isopropyl acrylamide) functionalized with an inducer molecule suppresses quorum sensing in Chromobacterium violaceum

Homoserine lactone rings were attached to a highly branched polymer chain end and used to influence the quorum sensing of Chromobacterium violaceum. This functional macromolecule prevented quorum sensing amongst the bacteria acting as an efficient auto-inducer.

As featured in:

See Joanna Shepherd et al., Chem. Commun., 2019, 55, 9765.
Highly branched poly(N-isopropyl acrylamide) functionalized with an inducer molecule suppresses quorum sensing in Chromobacterium violaceum†

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Bacterial quorum sensing has been implicated in a number of pathogenic bacterial processes, such as biofilm formation, making it a crucial target for developing materials with a novel antibiotic mode of action. This paper describes poly(N-isopropyl acrylamide) that has been covalently linked, at multiple chain ends, to homoserine lactone to give a highly branched polymer functionalized with a key messenger molecule implicated in QS. This novel functional material has shown promising anti-QS activity in a Chromobacterium violaceum assay.

Quorum sensing (QS) intercellular communication allows individual bacteria to modify their behaviour collectively in response to the local population density through small molecules termed autoinducers (AI). QS has been implicated in bacterial gene regulatory networks for regulating and coordinating several bacterial behaviours including biofilm formation, virulence expression and antibiotic resistance. Genes responsible for AI production are also included among the QS-regulated genes leading to an autoinductive signalling feedback loop and the modulation of pathogenic factors. A number of different classes of compounds have been identified as acting as inducers in QS. N-Acyl homoserine lactones (AHL) are major signalling molecules synthesised by LuxI homologs that induces LuxR homologs in Gram-negative bacteria. Examples include as N-butanoyl-homoserine lactone and N-(3-oxododecanoyl)-homoserine lactone produced by pathogenic Pseudomonas aeruginosa. Around 10% of downstream genes, including key virulence genes, in the genome of P. aeruginosa are regulated by these two AHL mediated QS systems both directly and indirectly. In Chromobacterium violaceum the production of violacein, a purple pigment with antibacterial activity, is regulated by the cviI/cviR (luxI/luxR homolog, respectively) QS system through N-hexanoyl-L-homoserine lactone. The C. violaceum CV026 strain, a cviI mutant, only produces violacein in presence of exogenic AHLs. This indicative feature makes CV026 a convenient biosensor for detecting exogenic AHL molecules or investigating AHL-based QS inhibitors when supplying exogenic AHLs. Due to the regulatory roles of QS in bacterial virulence, the disruption of QS offers a novel way in which to potentially treat bacterial infections. It is particularly attractive since QS is not required for survival of bacteria, so anti-QS therapies are suggested to be unlikely to lead to bacteria developing antimicrobial resistance, although this is hotly contested. As such, there have been considerable research efforts directed at disrupting the separate stages of the bacterial QS process. Approaches include halting the action of the LuxI region responsible for the synthesis of the AHL molecules, sequestering the inducers once they have been released into the intrabacterial matrix, and blocking the LuxR receptor region through the synthesis of small molecule antagonists. Each of these lines of enquiry has showed promising results, but an antimicrobial treatment based on disrupting QS remains elusive. Functionalized polymers have recently emerged as a potential tool in disrupting QS, with examples reported of the use of linear polymers to sequester AI molecules through covalent and non-covalent interactions. Also, recently Piletska and coworkers have developed molecular imprinted polymers for the passive sequestration of AHL molecules and others have used polymer films to release AHL based agonists or probe furanosyl borate ester (known as autoinducer-2 [AI-2]). Other systems known in the literature include studies of polymers functionalized with a diol on the back bone to competitively bind boronic acid for AI-2 production. To our knowledge, the direct functionalization of polymers with AIs has not been investigated, but this would seem to offer an alternative mechanism for disrupting QS.

We have recently demonstrated that highly branched (HB) polymer architectures have an advantage over linear polymer systems with regards to biological binding. HB-poly(N-isopropylacrylamide)
Laboratory experimental assays for quorum sensing through to distribution with high dispersity. However, after post-functionalization the self-condensing vinyl polymerization produced a bimodal material that contained the homoserine lactone ring with a N-hydroxysuccinimides as shown in Fig. 1. This produced a material that contained the homoserine lactone ring with a short alkyl linker onto the PNIPAM repeat units.

Full polymer synthesis and characterization is shown in the ESI†. As seen in previous studies of highly branched polymers the self-condensing vinyl polymerization produced a bimodal distribution with high dispersity. However, after post-functionalization the low molar mass portion is removed (alongside any unreacted residual AHL) via ultra-filtration leaving only the high molar mass fraction of the polymer. Conjugation of chain ends with HL led to a substantial increase in the average molar masses. This was supported by diffusion ordered spectroscopy (DOSY) $^1$H NMR in chloroform and the hydrodynamic radii of the polymers was shown to have increased by more than 3 nm following functionalization and purification (see ESI† for full details).

Analysis of the HB-PNIPAM, HB-PNIPAM-COOH and HB-PNIPAM-HL polymers, using differential scanning calorimetry, showed that the properties of the LCST had substantially changed due to the modification of the chain end group (see ESI†). This indicated that at temperatures, which span laboratory experimental assays for quorum sensing through to body temperature (around 32 °C for skin temperature), both polymers will be desolvated and exist in a globular state. However, we have recently shown that several different functional end segments of this class of polymer can remain solvated in a core–shell morphology in which core is desolvated but the shell is solvated before binding. In this core–shell structure or the fully desolvated state the chain ends of the polymer remain exposed to the solvent, thus ensuring that the HL groups are likely available for binding.

Initial tests using the CV026 biosensor assay indicated HB-PNIPAM-HL was able to block AHL signalling in CV026 bacteria. CV026 was co-incubated with both HB-PNIPAM-HL and AHL. After 48 hours co-incubation, AI signalling was indicated from the increase in intensity at 590 nm (Fig. 2).

In the absence of polymer there was an increase in signalling with increasing concentrations of AHL as expected (Fig. 2a). The presence of HB-PNIPAM-HL blocked this signalling in a concentration dependent manner (Fig. 2a and b). It is important to note the lack of signalling observed is not due to any bactericidal effects of the polymer. CV026 incubated with HB-PNIPAM-HL for 48 h remained viable. The live cell counts were comparable to bacteria incubated in LB broth. This was tested via Live/Dead staining, which showed no difference in viable cell numbers between experimental groups (Fig. 2c and d).

As discussed, previous studies investigating the use of polymeric systems as anti-QS agents have focused on the polymer's ability to sequester AI molecules. Therefore, it was important to examine the capacity of control polymers, not functionalised with AHL, to sequester AHL. As a control experiment HB-PNIPAM, with carboxylic acid end groups was used in place of HB-PNIPAM-HL and this had no effect on violacein production at any concentration (Fig. 3). These data indicated that HB-PNIPAM-HL is not acting as a sequestering agent. Next, we sought to confirm this hypothesis by assessing the impact of HB-PNIPAM-HL on the transcription levels of several CviR-target genes in the presence...
and absence of HB-PNIPAM-HL. As can be seen in Fig. 3B, co-incubation of CV026 with AHL led to a significant increase in the expression of vioA, vioC and vioD, compared with the untreated control. Strikingly, this increase was dramatically and significantly ($p < 0.01$) reduced in cells co-treated with AHL and HB-PNIPAM-HL, but not with the control polymer (HB-PNIPAM).

Violacein production in C. violaceum is promoted by a LuxR-type regulator, CviR. After binding to its native ligand, C6-HSL, CviR-AHL complex will be stabilized and form into a dimer to bind to DNA and to interact with RNA polymerase for target gene expression. A study has shown that lengthening of the acyl tail of AHLs enhances the antagonism of C. violaceum quorum sensing.22 Crystal structure data from this work suggests that antagonist binding increases the distance of DNA-binding domains between two subunits of dimeric CviR, resulting in a predominantly open and competent structure when binding to native ligand.22 The data showed that the highly branched polymer (HB-PNIPAM-HL) inhibited violacein production in C. violaceum and it may also have a strong antagonistic effect on CviR activity.

During auto-induction AHLs cross the cell membrane and bind to LuxR-type proteins in the cytoplasm. The Lux-R-AHL complex then induces transcription of quorum sensing genes. Thus, implicit in the data reported here is the realisation that the HB-PNIPAM-HL is predicted to enter the cytoplasm. The precise mechanism of how this would occur is not available but it is interesting to note that DOSY NMR data of the polymer in water indicates the hydrodynamic radii is $\approx 17$ nm (the size distribution is broad with an interquartile range from 14 to 22 nm – see ESI†) and this seems to be too large for channel mediated transport. More work is required on this aspect but we have previously shown that similar branched amphipathic polymers are also capable of crossing the membranes in eukaryotic cells.23 Also the HB-PNIPAM-HL molecule chain ends are indicated to interact with the LuxR receptors despite the structural difference in the PNIPAM chain compared to the alkyl linker in the small molecule AI.

In conclusion, the first highly branched polymer functionalized with quorum sensing auto inducers has been synthesized. This novel functional material has been shown to act as an anti-quorum sensing compound. Examining the gene expression of bacteria in the presence of polymers showed that the polymer scaffold interrupts the dimerization of LuxR homologs crucial for downstream signalling preventing AHL from inducing QS.

We gratefully acknowledge funding from the MRC/DBT MR/N501888/2. We also thank Dr Jonathan Shaw (The University of Sheffield) for the C. violaceum CV026 bacteria and David...
Pownall at the University of Bradford for assistance developing polymer synthetic protocols (Wellcome Trust 099800/C/12/Z).

Conflicts of interest

There are no conflicts to declare.

Notes and references


