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Multivalent alteration of quorum sensing in *Staphylococcus aureus*†‡

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Virulence in *Staphylococcus aureus* is strongly and positively correlated with local cell density. Here we present an effective approach to modulate this group behaviour using multivalent peptide–polymer conjugates. Our results show that by attaching multiple AIP-4′ units to macromolecular scaffolds, the *agr* QS response in *S. aureus* was affected strongly, while displaying a clear multivalency effect.

Staphylococcus aureus is a Gram-positive human pathogen that can cause problems ranging from minor skin infections to life-threatening health conditions.[1] Pathogenesis is caused mainly by the secretion of a wide array of toxins and tissue-degrading enzymes.[2] One of the primary mechanisms for controlling the secretion of these virulence factors is a quorum sensing (QS) system called the accessory gene regulator (agr).[3,4] The agr system monitors the extracellular concentration of unique autoinducing peptides (AIPs) that are produced and secreted by *S. aureus*. Each bacterium secretes AIPs that then accumulate extracellularly. Once these ligands reach a certain threshold concentration they bind to the transmembrane domain receptor AgrC, the agr QS system. Taking into account that coordinated virulence factor synthesis in *S. aureus* is largely under the control of the agr QS system, significant efforts have been made to identify effective inhibitors of AgrC.[5,6]

As mentioned above, QS responses are only initiated upon reaching a certain threshold concentration of the appropriate receptor ligand. Therefore, exploiting multivalency effects may be a promising strategy to achieve a marked increase in "effective ligand/inhibitor concentration". There are two main types of multivalency in biomolecular recognition: the first is dependent on multiple binding events, and the second relies on high local concentration.[7] While it is not known how far apart different AgrC proteins are located on the cell membrane, even in the case that only one AgrC receptor is bound by the AIP–polymer, this first event will increase the likelihood that a nearby AIP moiety will be bound upon release of the first unit, thereby effectively raising the affinity of AgrC for the whole polymer construct. Nature frequently uses multivalency to achieve strong binding in situations where univalent receptor–ligand interactions are weak.[8] One good example of such a phenomenon, for instance, is the recognition of carbohydrate ligands by bacterial and mammalian lectins. Recently, Janda and coworkers described multivalent dendrimers based on the quorum sensing molecule AI-2.[9] Here, we present the first example of quorum sensing attenuation based on and strengthened by multivalent polymeric constructs in *S. aureus*.

Multivalent ligand-induced binding of signaling molecules can result in antagonist- or agonist-like effects, thereby forming multivalent inhibitors or effectors, respectively. Multivalent inhibitors were shown before to effectively block receptor function. On the other hand, multivalent effectors were also demonstrated to activate various cellular processes.[10] Moreover, the use of polymeric scaffolds for synthetic multivalent ligands has been explored previously. For instance, polymers that were generated by ring opening metathesis polymerization (ROMP) were used to investigate cell–surface interactions, multivalent binding of histidine-tagged proteins and DNA binding analysis.[11–13] This synthetic method not only allows control over the degree of polymerization and over the display and density of ligands,[14,15] it is also a straightforward procedure that allows the assembly of polymer constructs in a single step.[16]

In order to assess potential multivalent interactions with the AgrC receptor, we prepared constructs based on non-native AIP-4 (Fig. SI-3, ESI;‡ we replaced methionine with norleucine, yielding a more stable isosteric analog (AIP-4′) that is identical in activity to the native AIP-4 EC50 33). Next, a modular approach to synthetic copolymers functionalized with multiple AIP-4′ units was designed (Fig. 1). As the N-terminal end would be the most convenient site of attachment to the polymer, an N-terminal modified AIP-4′ was synthesized to assess the effect on its activity. For a monomeric norbornene label only a minor change in the EC_{50} value was observed (Fig. SI-4, ESI‡). Consequently, several AIP-4′ containing polymers, differing in polymer backbone structure and in the average number of AIP-4′ units per

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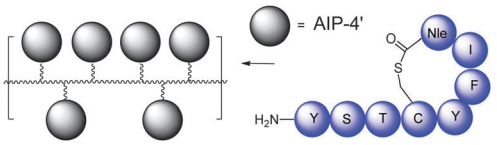
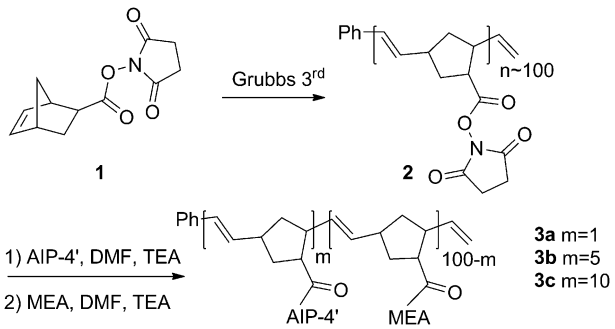


Fig. 1 Polymeric quorum sensing constructs based on AIP-4.

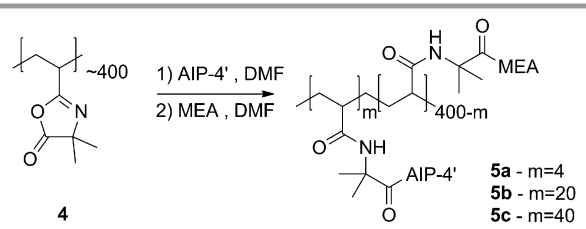
polymer chain were prepared. To control the latter, a "graft-to" approach was adopted, linking the appropriate amount of AIP-4′ peptide moieties to *N*-hydroxysuccinimide (NHS) active sites in the polymer backbone. Any unreacted active groups on the polymer were saturated with 3,6,9-trioxadecylamine (MEA) to increase solubility and minimize undesired subsequent side reactions. Thus, polymer **2** was synthesized by ROMP of a NHS- activated norbornene carboxylate monomer (**1**)[17] using a Grubbs 3rd generation catalyst.[18] The degree of polymerization (DP) of polymer **2** was determined by triple-detector GPC to be around 100. Consequently, three different polymers, containing 1, 5 or 10% AIP-4′ peptide (*i.e.* 1, 5 and 10 average units of AIP-4′ per polymer) were prepared using the NHS strategy. The disappearance of free peptide was monitored by LC-MS. Once all monomeric peptides had reacted, a reaction with excess of MEA was carried out to afford polymers **3a–c** as presented in Scheme 1.

In order to also analyze higher molecular weight polymers, a second type of polymer (**5**) was prepared by attaching 1, 5 or 10% AIP-4′ peptide to poly(2-vinyl-4,4-dimethylazlactone) (DP ~ 400) (**4**)[19,20] by azlactone ring opening, followed by reaction with excess MEA (Scheme 2).

Due to the higher degree of polymerization and concomitant monomer content, it was possible to achieve a much higher number of AIP-4′ moieties in polymers **5a–c** than in polymers **3a–c**. Notably, for concentrations up to 10 μM none of the studied polymers showed growth inhibitory effects on *S. aureus*. In order to determine whether the polymers could attenuate activation of the agr QS system, a modified strain of *S. aureus* class-4 (RN9371), containing a plasmid-carried P3 promoter fused to a β-lactamase reporter gene,[4] was grown in the presence of AIP-4′ and varying concentrations of polymers **3** or **5** (both polymers without AIP-4′ did not show either activation or



Scheme 1 Synthesis of polymers **3a–c**.



Scheme 2 Synthesis of polymers **5a–c**.

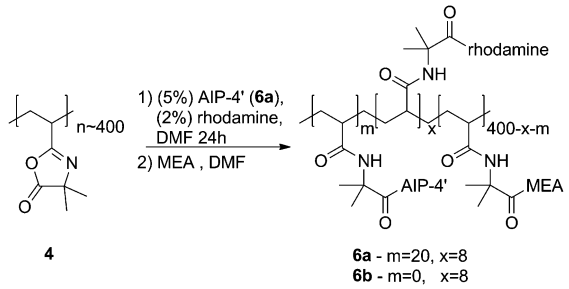
Table 1 Activation and inhibition by **3**, **5** and free AIP-4′

Compound	AIP-4′ units per compound	EC_{50} [nM]	IC_{50} [nM]
AIP-4′	1	33 ± 13	
3a	1	149 ± 26	
3b	5	52 ± 20	
3c	10	35 ± 4	
5a	4	22 ± 13	
5b	20		145 ± 59
5c	40		89 ± 41

EC_{50}/IC_{50} values for AIP-4′, **3** and **5**. All results were determined through β-lactamase assays by incubating *S. aureus* strain RN9371 with AIP-4′ containing compounds. Control experiments with buffer alone or polymers without AIP-4′ showed no activation above background.

inhibition of β-lactamase activity). In order to assess whether the increase in AIP-4′ loading per polymer results in a true multivalency effect, we plotted β-lactamase activity *versus* effective AIP-4′ molarity – and not polymer concentration (Fig. SI-5 and SI-6, ESI‡ and Table 1). Polymer **3a**, the short polymer that contains on average 1 unit of AIP-4′ peptide, acts as an agonist with an EC_{50} of 149 ± 26 nM. In comparison with the activity of free AIP-4′ it appears that the polymeric structure somewhat interferes with the affinity of AIP-4′ for its receptor, even though this effect is not very prominent. Interestingly, as loading of AIP-4′ on the polymer increases, the EC_{50} values of polymers **3b** and **3c** decrease. This suggests that the loss of affinity caused by the polymer construct was compensated for by a positive effect providing the first evidence that ligand multivalency can affect QS responses. In the longer polymers (**5a–c**), the average number of AIP-4′ units per chain is 4, 20 and 40 respectively. Polymer **5a**, loaded with 1% AIP-4′, activates the QS system to a similar degree as free AIP-4′ (EC_{50} of 33 ± 13 nM – reported EC_{50} values for native AIP-4 are 13–20 nM[5,21,22]). At higher loadings, however, we observed a remarkable switch from agonist to antagonist behavior. This change, also observed at very high concentrations (>5 μM) of free AIP-4′, could be due to either significantly higher local AIP concentrations and/or higher affinity of the peptide in the vicinity of the AgrC receptor, as would be predicted for multivalent systems. Addition of the AIP-4′ rich polymers **5b** and **5c** to *S. aureus* resulted in strong inhibition of its QS system with IC_{50} values of 145 ± 59 nM and 89 ± 41 nM respectively. While not unprecedented,[23] inhibition of QS by high concentrations of autoinducers is poorly understood. One possible explanation could be that a very high autoinducer concentration could signal to the bacteria that their population density is too high and coordinated group behaviour such as increased virulence factor expression would not add to their benefit at this point or might even harm the long-term survival of the population. We currently use the multivalent high-load polymer (**5c**) as a tool to further unravel this phenomenon.

To continue the analysis of multivalent binding of the polymers to the AgrC receptors, fluorescent probes were prepared. Thus, fluorescent polymers **6a** and **6b**, based on polymer **5b** conjugated to rhodamine-6G, were designed and synthesized (Scheme 3). These fluorescently labeled polymers could be utilized for direct receptor binding studies. Polymer **6a** contains on average 20 AIP-4′ units, while polymer **6b** is void of any AIP-4′ (and most likely void of any QS activity, although its fluorescence prevented proper determination of its activity). In order to quantify binding of polymer to the receptor, bacteria (OD_{600} = 0.7) were incubated in the presence of polymers **6a** and **6b**, and the fluorescence of both the supernatant and pellet were



Scheme 3 Synthesis of polymers **6a** and **6b**.

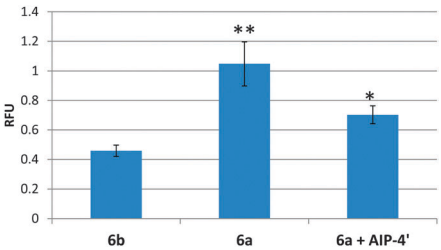


Fig. 2 Fluorimetric binding assay. *S. aureus* cells were incubated for 30 min at 37 °C in the presence of 0.5 μM polymer **6b**, 0.5 μM polymer **6a**, or 0.5 μM polymer **6a** + 0.5 μM AIP-4′. Cells were then washed with TRIS buffer and fluorescence of both pellet and supernatant was measured (λ_{exc} 490 nm, λ_{em} 520 nm), and their ratios are shown. ** and * denote statistically significant differences between **6b** and **6a** ($p < 0.02$) and between **6a** and **6a** + AIP-4′ ($p < 0.05$), respectively.

measured. As shown in Fig. 2, polymer **6a** shows significantly higher affinity to the cells compared to polymer **6b**. Moreover, in a competition assay between polymer **6a** and free AIP-4′ fluorescence levels are comparable to those with polymer **6b**. This suggests that the multivalency effect described in Table 1 is very likely due to an increase in the affinity of the multivalent ligands to the receptor. The competition assay also indicates that the general affinity of polymer **6a** to AgrC is lower than that of AIP-4′ (Fig. 2).

To detect and localize the polymers in live cells, bacteria (O.D.600-0.5) were incubated for 15 min at 37 °C in the presence of polymers **6a** and **6b**. Images were acquired immediately after 2 washes with TRIS buffer. Polymer **6a** (containing AIP-4′) was found attached to the bacteria (Fig. 3b). In contrast, none of the cells was labeled with polymer **6b** (Fig. 3d). Following these results, the inhibitory effects of AIP-4′ containing polymers **5b** and **5c** on QS activation and their effect on hemolytic activity of *S. aureus* was probed.[5] Accordingly, *S. aureus*

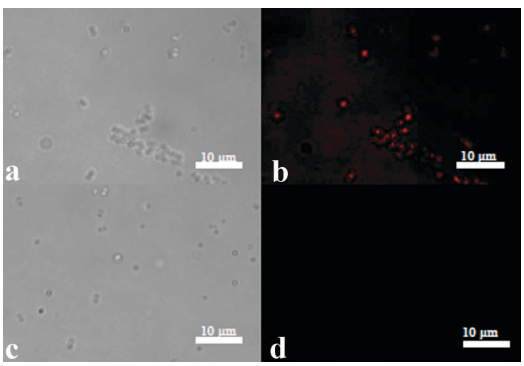


Fig. 3 Imaging of polymers **6a**, **b** in living cells. *S. aureus* cultures were incubated in the presence of 1 μM polymer **6a** (a, b), or in the presence of 1 μM polymer **6b** (c, d). Left panels, bright field; right panels, rhodamine channel. The scale bar represents 10 μm.

wild-type group IV strain, RN8242, was incubated in the presence of different concentrations of free AIP-4′ and polymers **5b** and **5c**. The hemolytic activity was measured following the release of heme from sheep red blood cells (RBCs). We verified that free AIP-4′, as expected, acts as activator at low concentrations (13 nM and beyond, Fig. SI-6, ESI‡) and as inhibitor at high concentrations (>5 μM). However, polymers **5b** and **5c** showed some inhibition at both low and high concentrations, while polymers devoid of AIP-4′ did not. The inhibitory pattern of **5b** and **5c** was quite unexpected and is currently further investigated. It should be noted that while the agr system attenuates hemolytic activity of *S. aureus*, it is not the sole or even primary regulator of hemolysis, and as such full inhibition is not expected upon agr inhibition.

This is the first description of a multivalent polymeric inhibitor of QS in *S. aureus*, using its autoinducing peptide as the active entitiy on two different types of polymers. This system may be a launching pad for creating new types of inhibitors by using agonists or antagonists as the recognition elements. In addition, it could deepen our understanding of multimeric receptor–ligand interactions in QS and aid in studying the importance of bacterial QS receptor topology on bacterial cell surfaces.

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