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Modulation of Quorum Sensing in a Gram Positive Pathogen by Linear Imprinted Copolymers with anti-Infective Properties

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Abstract: Here we describe the development, characterization and biological testing of a new type of linear molecularly imprinted polymer (LMIP) designed to act as anti-infective by blocking the quorum sensing (QS) mechanism and so preventing virulence of the pathogen *Streptococcus pneumoniae*. The LMIP is prepared (polymerized) in presence of a template molecule, but unlike in traditional molecular imprinting approaches no cross-linker is used. This results in soluble low molecular weight polymer, which can act as drug agent in vitro and in vivo. The LMIP was characterized by mass spectrometry in order to determine its monomer composition. The fragments identified were then aligned along the peptide template by computer modeling, predicting the possible monomer sequence of the LMIP. These findings provide proof of principle that LMIP can be used to block QS, setting the stage for studies on a novel drug-discovery platform and class of materials to target Gram positive pathogens.

Antibiotic resistance is one of the top threats to global health.[1] Thus, there is an urgent need to develop antimicrobial agents that are effective and less prone to microbial resistance. Interfering with bacterial communication systems is considered to be an effective strategy to develop novel anti-infective agents.[1] Bacteria communicate with each other to coordinate their behavior by production and detection of signalling molecules through a mechanism called quorum sensing (QS).[2] A wide range of phenotypes are modulated by QS systems including growth, biofilm formation and virulence expression.[3,4] As QS systems are absent in mammalians, this minimises the risks of host-toxicity of therapies which target microbial QS.[5] The QS system of Gram negative bacteria use diffusible small molecules (like homoserine lactones), Gram positives utilize exported, processed peptides.[2] Conventional methods to manipulate QS systems include the use of lactonases and amidases (to degrade the homoserine lactone) or removal of the signalling molecule using affinity ligands like peptides and antibodies.[6–8] While a degree of success in modulation of QS by these two methods has been reported in in vitro and ex vivo models, more work is apparently needed to improve the stability of therapeutic agents based on biomolecules. [9,10] Alternatives include synthetic cross-linked adsorbents like molecularly imprinted (and non-imprinted) polymers which are not susceptible to biodegradation. These have been used to successfully remove homoserine lactones from solution and prevent QS.[11,12] Linear random co-polymers have also been tested, but less successfully due to lower affinity towards the signalling molecule.[13] Unfortunately cross-linked polymers have limitations as drug candidates since it is impossible to elucidate their structure due to presence of large bulk of randomly cross-linked material. Affinity ligands made with linear molecularly imprinted polymers (LMIP)[14] can overcome some of these limitations as they are smaller in size and can conceivably be sequenced. These are ideal attributes which should allow for their use as drug candidates or drug-discovery tools.

The recently characterized TprA receptor and its signalling peptide PhrA[15] was chosen as QS target for two reasons. First, it is widespread across bacilli and streptococci thus relevant to many human pathogens.[16] Second, we present data that demonstrate that the TprA/PhrA system is a major virulence determinant. It is important for pneumococcal growth on galactose and mannose as well as on mucin (**Figure S1**), the primary carbon sources for pneumococcus *in vivo*.[17,18] The PhrA peptide expression is induced both by galactose and mannose (**Table S1**). Independent deletion mutation of TprA and PhrA both abrogates pneumococcal virulence in a mouse model of pneumonia and septicemia (**Figure S2**), as well as in a chinchilla model of otitis (**Figure S3**). Therefore, it was envisaged that by interfering with the binding of PhrA with TprA would block the phenotypic manifestations of the TprA/PhrA QS system. In order to prepare a modulator of this system, its inducer (signalling) peptide (PhrA10) was to create the LMIP. PhrA10 is the active signal peptide with capability to induce PhrA expression,[15] the C-terminal end of this peptide with the sequence SNGLDVGKAD was used as template for LMIP preparation. Unlike binary functional monomer/cross-linker compositions commonly used for production of MIPs, a mixture of different monomers with various functionalities was used to prepare the LMIP and so avoid formation of homopolymers (which would be incapable of *selective* binding). It is hypothesized that in order to obtain good affinity and selectivity, the sequence of monomers along the polymer backbone should be directed pre-polymerization by self-assembly around the template. Two monomers capable of establishing ionic interactions were used, acrylic acid and *N*-(3-aminopropyl)methacrylamide. Acrylamide was used as it can establish hydrogen bonds; *N-tert*-butylacrylamide to target hydrophobic areas on the template. To perform solid-phase

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imprinting, PhrA10 was first immobilized at the surface of glass beads, the solid-phase (SP) according to an established protocol,[19] see SI. These were then placed in contact with the aqueous monomer mixture and polymerization initiated.

After polymerization and affinity purification, 9 mg of polymer were obtained, equivalent to 0.15 mg per gram of SP used. As determined by mass-spectrometry the molecular weight of the polymer was on average 1006 Da (**Figure S4 b**). The following identifiable fragments were observed in the SIR mode: *m/z* 73 (acrylic acid, M+1), *m/z* 214 (triplet of acrylamide, 3M+1), *m/z* 270 (one molecule of *N*-tert-butylacrylamide and doublet of acrylamide); *m/z* 593 (three acrylamide and three molecules of butyl acrylamide). The MRM fragmentation of both *m/z* 740 and the largest ion (*m/z* 1006) generated *m/z* 381.4 daughter fragment might be a triplet of butyl acrylamide moieties, also observed in the spectrum generated using the SIR mode (Figure S4). There were occurring losses of 28 Da associated with *m/z* 381.4, 739.6 and 1005.9 ions. Another recurrent pattern was the loss of a neutral fragment (358 Da), observed during the fragmentation of *m/z* 739.6 and *m/z* 1005.9 that could correspond to two moieties of acrylamide and three moieties of acrylic acid. Accordingly the polymer consisted mainly of sequences containing three acrylamide, three butyl acrylamide and two or three acrylic acid blocks. LIPs might not be necessarily chemically monodisperse, even if fractionated by affinity. There might be variations in polymer composition between molecules. However MS analysis was performed with dominant peak hence this should be representative of most of the polymer molecules present in the sample.

Surflex-Dock and Sybyl Leapfrog molecular modeling techniques were applied to predict the positions of the LMIP fragments along the PhrA10 sequence [20] (**Figure 1**). It was found that acrylic acid trimer showed relatively high and equal affinity for either of two arrangements around the asparagine residue, forming either a four point interaction (hydrogen bond donation to the C and N residue carbonyls, and accepting from the N amine and side chain amide) or five point interaction (hydrogen bond donation to the carbonyls of G and the N side chain, and acceptance from the N amide and amines of the G and N backbone), in which the two interactions with the G residue are relatively weaker. The strongest interaction found by Leapfrog screening was that of a three-point interaction between the acrylamide trimer with carbonyl and amine of the leucine residue and the carboxylate of the aspartic acid residue. Butyl acrylamide trimer also showed three points of interaction with PhrA10, all hydrogen donations going from the fragment amides to the backbone carbonyls of the V, G and K residues. The LMIP structure is therefore predicted to be X-AA-AA-AA-X-Ac-Ac-Ac-X-BA-BA-BA-X, were AA is acrylic acid, Ac acrylamide, BA butyl acrylamide and X either a join between the adjacent subunits or another fragment of the polymer, with the total mass of X being approximately 195 Da (**Figure S4**). This approach is similar in nature to fragment-based drug discovery with added advantage that the sequence of appropriate fragments is guided by a self-assembly process taking place during molecular imprinting.[21] The affinity of synthesized LMIP to PhrA10 peptide was studied by surface plasmon resonance (SPR) (**Figure 2**). Results indicate PhrA10 binds LIMP in a dose-dependent and sequence-specific manner. The dissociation constant (K_D) was estimated to be 8.16 µM (**Figure 2A**), which correlated well with the bioactive concentration range. Conversely, the K_D of the interaction between LMIP and a scrambled control peptide with the same amino acids (DAKGVDLGNS) and in similar concentration range was much higher >0.13 mM though practically no binding was observed (**Figure 2B**).

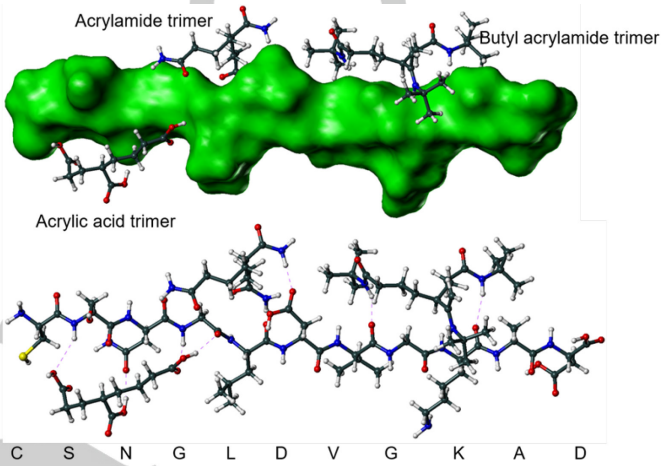


Figure 1. Molecular complex between LMIP fragments and C-terminal end of PhrA peptide (SNGLDVGKAD) as predicted by computational modeling. N-terminal C added for SH coupling onto the solid-phase.

An SPR assay was then performed to assess if the LMIP could affect the binding of PhrA10 to TprA receptor. For this recombinant TprA was immobilized on a SPR chip then PhrA10 (ranging from 0.2 to 50 µM) injected in order to establish the minimal concentration of PhrA10 necessary for binding to occur to TprA, which was 25 µM. PhrA10 at 25 µM (**Figure 2C**) was then co-injected on the TprA receptor with LMIP at a concentration of 100 nM as used in the microbiological inhibition assays (see SI). LMIP could significantly alter the binding behavior of PhrA10 to TprA with 84% reduction in binding response and greatly reducing the dissociation rate of the complex, resulting in irreversible binding, possibly due to the binding of PhrA10/LMIP complex to the TprA receptor site. No measurable interaction was observed upon injection of 100 nM LMIP onto immobilized TprA in absence of PhrA10. Having demonstrated the specific binding and consequent blocking of TprA receptor by the PhrA10/LMIP, LMIP was then tested to determine if it would attenuate the induction of β-galactosidase activity driven by the phr*A* promoter (P*phrA*), and decrease pneumococcal growth on galactose, for which the TprA/*Phr*A system is required.[15] The inhibitory role of LMIP on P*phrA* driven β-galactosidase activity in the presence of synthetic PhrA10 was evaluated as PhrA is known to activate the transcriptional expression of PhrA on galactose. Addition of PhrA10 resulted in approximately 5.7-fold increase in the activity

relative to a control that was not induced with the peptide (**Figure 3A)**. In contrast, in the presence of LMIP the β-galactosidase activity was still higher than the control (no PhrA10) but was significantly lower, by 1.8-fold, compared to induction by PhrA10 alone ($p<0.001$), demonstrating the efficacy of LIMP in inhibiting promoter activation by PhrA10. These results were also confirmed by qRT-PCR, see SI.

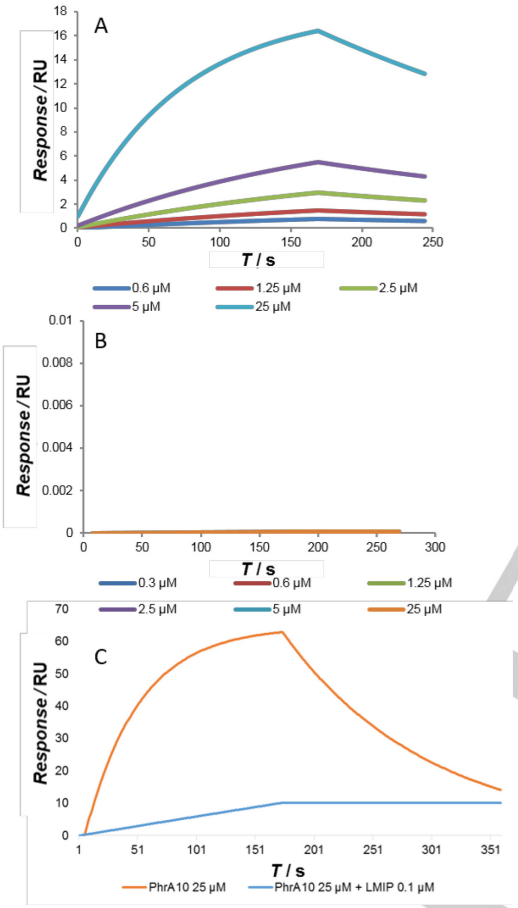
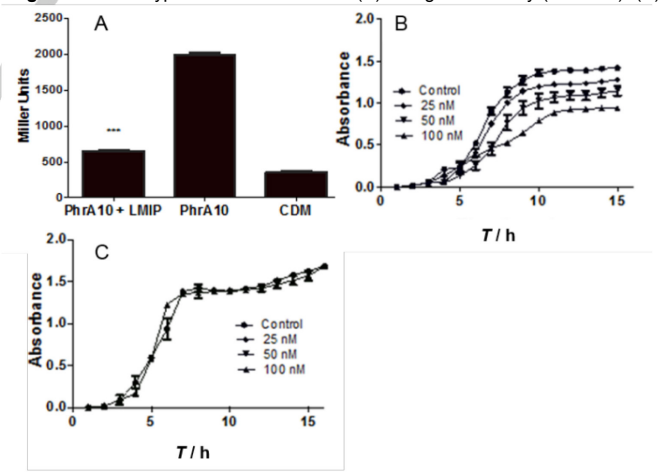


Figure 2. SPR sensorgrams. (A) Injections of PhrA10 onto immobilized LMIP, (B) injections of scrambled PhrA10 onto LMIP. (C) PhrA10 at 25 µM injected onto immobilized TprA (curve PhrA10) and PhrA10 (at 25 µM) injected alongside LMIP onto immobilized TprA.

To demonstrate the growth inhibitory effect of LMIP, the wild-type strain was grown in CDM supplemented with galactose, in the presence of varying concentration of LMIP (25-100 nM) (**Figure 3B)**. Growth yield was significantly lower at all the concentrations tested and growth rate was significantly decreased at 100 nM LMIP compared to the control in absence of LMIP (**Figure 3B** and **S1**). Consistent with the patterns of TprA expression (**Table S1**), LMIP had no influence on growth on glucose (**Figure 3C**), demonstrating a lack of toxicity at these concentrations. TprA was also found to regulate neuraminidase (**Figure S5**) so its activity was assessed in pneumococcal cultures grown on galactose supplemented or in absence of 100 nM LMIP. Neuraminidase is responsible for cleaving complex host sugars, playing an important role in pneumococcal colonization and invasiveness.[22–24] There was a significant decrease in neuraminidase activity (26.7 ±1.5 U) in the presence of LMIP compared to the control that did not contain LMIP (53.3±3.0 U) ($p<0.001$), indicating that LMIP by blocking TprA abrogates neuraminidase activity, hence virulence. LMIP impact on virulence was further confirmed by *in vivo* evaluation against lethal microbial challenge in a mouse model of pneumococcal pneumonia that progresses to bacteremia (**Figure S2)**. Three groups were infected intranasally either with 7.5×10^5 pneumococci/mouse in 50 µL PBS (control), or with 1.4×10^6/mouse suspended in the same volume containing 100 nM LMIP solution in PBS. The third group was infected with 1.4×10^6/mouse, in the presence of 100 nM LMIP targeting the scrambled 10 aa peptide (LMIPscramble). After 24 h results show that despite receiving a higher infective dose the cohort that received the inoculum together with PhrA10 LMIP had significantly lower blood counts (Log10 0.61±0.28 CFU mL^{-1}, n=15) than the cohort that had received the dose in PBS (Log10 2.96±0.54 CFU mL^{-1}, n=13) ($p<0.01$). There was no difference between the number of pneumococci in control and LMIPscramble groups (Log10 2.49±1.04 CFU mL^{-1}, n=5) ($p>0.05$). This shows that LMIP prevents the translocation of pneumococci from lungs to blood. Consistent with reduction in bacterial blood count, the cohort that received LMIP survived significantly longer (65 h±15.1, n=13) than control (37 h±8.4, n=15) ($p<0.0001$), whereas there was no difference between LMIPscramble group (44 h±4.6, n=5) and the control ($p>0.05$).

Figure 3. Phenotypic evaluation of LMIP (A) and growth assay (B and C). (A)



LMIP inhibits β-galactosidase activity driven from the promoter of phrA (P*phr*A) in Δ*phr*A::phrA, a strain deficient in endogenous production of PhrA. P*phr*A was induced with 10 nM synthetic PhrA10 peptide or with PhrA10 plus 100 nM LMIP, and galactosidase activity without PhrA in CDM supplemented with galactose is also shown. In growth assays, 25-100 nM LMIP attenuates S. pneumoniae growth in medium supplemented with galactose (B) but not with glucose (C). Control cultures did not receive LMIP. Each experiment repeated at least in triplicate, ** $p<0.01$, **** $p<0.0001$.

In conclusion, we found that LMIP interfere with the QS signals selectively, and curtail the phenotypic manifestation of this system in in vitro and in vivo assays. These results indicate that LMIP can be developed as an effective anti-infective against pathogenic Gram positive bacteria. In the future, we plan to use this innovative technology in a more challenging clinical situation, and to determine optimal dose and dosing regimen. Also, we will assess the performance of synthetic ligands against the same target but prepared by chemical synthesis. The placement of functional groups along the synthetic ligand will mimic that of the LMIP, thus using this approach for drug discovery.

Experimental Section

Synthesis of LMIP by solid-phase imprinting: In 100 mL deionised and degassed water the following monomers were dissolved: 24 mg acrylamide (3.3×10^{-4} mol), 33 mg *N*-tert-butylacrylamide (2.60×10^{-4} mol) and 2.2 µL acrylic acid (2.24×10^{-5} mol, d = 1.051 g mL^{-1}), 5.5 mg *N*-(3-aminopropyl)methacrylamide hydrochloride (3.1×10^{-5} mol). Then 60 g of PhrA10 or scrambled peptide-derivatised glass beads (see SI for coupling protocol) were placed in a 100 mL glass bottle and then 50 mL of the monomer solution added under N_2 atmosphere. Polymerization was initiated with 0.5 mL ammonium persulphate solution (60 mg mL^{-1}) containing 54 mg mL^{-1}sodium bisulfite and carried out at RT for 15 h. Afterwards, beads and solution were decanted onto a fritted SPE cartridge washed with 5×40 mL DI water at RT, followed by 4×40 mL DI at 70 °C to elute the LMIP. See SI for additional methods.

Keywords: Host-guest systems • Supramolecular chemistry • Receptors • Transcriptional regulation • Quorum sensing

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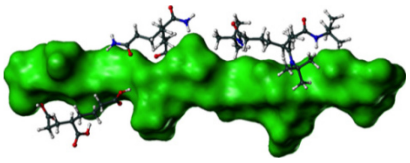

Linear molecularly imprinted polymers prepared against a signalling peptide of the pathogen *Streptococcus pneumoniae* can block its quorum sensing mechanism and abrogate its virulence. Obtained results indicate that this approach has the potential to be used for the development of effective anti-infective agents against pathogenic Gram positive bacteria.

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