

Application of molecularly imprinted polymer nanoparticles for degradation of the bacterial autoinducer N-hexanoylhomoserine lactone

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A novel bacterial quorum quenching system is presented. For the first time the degradation of N-L-hexanoyl homoserine lactone (C6-AHL), a Gram-negative quorum sensing autoinducer, has been enhanced using molecularly imprinted nanoparticles (MIP NPs) which were prepared using transition state analogue of the γ -lactone ring hydrolysis as template.

Recently, the effectiveness of antibiotics has been declining dramatically, and since the introduction of fluoroquinolones in the eighties the development of new antibiotics has slowed considerably.¹ According to the World Health Organisation, research into the development of novel treatments against bacterial infections is currently a priority.² The main reason is the incredible capability of bacteria for mutations and, therefore, building resistance against antibiotics.³ Bacterial biofilm (BF), which is a bio-layer composed of a polymeric matrix mainly formed of exopolysaccharides and proteins, is one of the main sources of protection and resistance.⁴ It protects bacteria against a wide variety of threats, such as antibodies, macrophages and antibiotics.^{5,6} Some bacterial BFs are even resistant to detergents and disinfectants.⁷ Besides its defensive capability, the BF works as a nutrient reservoir, bacterial cells source (persister cells) or ideal media to exchange genetic material and information (e.g. signal molecules or secondary metabolites).⁸ The BF formation and maturation, as well as other bacterial phenotypes such as motility, bioluminescence, aggregation or virulence, are triggered by the process referred to as quorum sensing (QS).^{9,10} QS is the mechanism by which bacteria (and other micro-organisms) coordinate gene expression. For the purpose of sensing their population density, bacteria release small signal molecules

known as autoinducers (AI) into their environment. By sensing the AI concentration, the microorganisms are able to act synchronously. For many pathogenic Gram-negative bacteria, such as *Staphylococcus aureus*, *Vibrio cholera* or *Pseudomonas aeruginosa*, various N-acyl homoserine lactones (AHL) are employed as AIs, activating or deactivating different behaviours.^{11,12} The typical structure of an AHL includes a γ -lactone ring, a hydrocarbon acyl chain variable in the number of carbons and the presence or absence of a carbonyl or hydroxyl group on the third carbon (Fig. 1a). The acyl chain is never shorter than four carbons and determines the lipophilic character of AHLs, which increases in proportion to the number of carbon atoms. This hydrophobic nature allows them to remain in the vicinity of the bacterial cells after release.

Among the most popular tools used to fight bacteria are antibiotics, QS inhibitors and bacteriophages.^{13,14} Unfortunately, these approaches are all likely to trigger the development of resistance due to their direct interactions with bacteria. Recently a new type of linear molecularly imprinted polymer (LMIP) capable of blocking the quorum sensing (QS) mechanism of *Streptococcus pneumoniae* and abrogating its virulence was reported.¹⁵ LMIP was shown to interfere with QS signals selectively *in vivo* and *in vitro* and could potentially be developed into a new anti-infective agent against pathogenic Gram-positive bacteria.

The development of QS phenotypes could impart a huge advantage in the very competitive arena of bacterial communities, which is the reason why many organisms have developed anti-QS mechanisms, commonly named as quorum quenching (QQ) mechanisms.^{16–18} One of the most successful mechanisms used by bacteria is the secretion of hydrolytic enzyme AHL-lactonase, which is used to silence the QS of other bacterial species by the hydrolysis of AHL.

To the best of our knowledge, this is the first work showing QQ systems based on catalytic molecularly imprinted polymer nanoparticles (MIP NPs). The only previously reported approach using MIP microparticles was based on adsorption of the bacterial autoinducers by a polymer matrix, thus inhibiting the

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QS pathways.^{19,20} The effectiveness of this approach was limited by the capacity of the polymer to adsorb the signal molecules. Once this limit is reached, the adsorbent may instead act as a source, compounding the problem it was designed to tackle. To avoid this important drawback, our aim was the development of a system capable of deactivation of AHL by a catalytic mechanism.

The first MIPs with catalytic activity were developed by Mosbach and his group in 1989 using a transition state analogue (TSA) as the template to catalyse the hydrolysis of p-nitrophenyl acetate.²¹ Since then, the use of a TSA as template was shown to be a successful strategy in the synthesis of MIPs able to mimic the activity of the enzyme carboxypeptidase A.²² This approach has been applied focusing on the development of micro- and nanogels, which showed higher solubility and efficiency.^{23,24} In our work, we present a new approach to the synthesis of catalytic MIP nanogels using a TSA-template covalently linked to a solid phase adapted from the protocol developed and optimised by Canfarotta *et al.*²⁵ The solid phase synthesis approach facilitates the preparation and purification of MIP NPs and, potentially, re-use of the template which was synthesised mimicking the shape and positions of the functional groups of the target molecule. The sulfone ring used in the template mimics the transition state for hydrolysis of N-hexanoyl homoserine lactone and addition of the primary amine group allows template immobilization on the solid phase surface (Fig. 1).

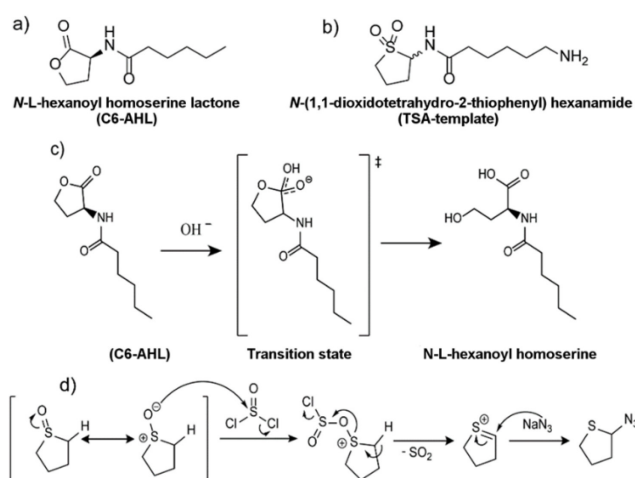


Fig. 1. Chemical structures of: the bacterial autoinducer, N-L-hexanoyl homoserine lactone (C6-AHL) (a), N-(1,1-dioxidotetrahydro-2-thiophenyl) hexanamide (b), mechanism of the hydrolysis reaction of C6-AHL (c) and the reaction mechanism of the Pummerer rearrangement, using 1-oxido-tetrahydrothiophene and thionyl chloride to obtain the α-functionalised tetrahydrothiophene (d).

The TSA-template was synthesised in a few steps. First, tetrahydrothiophene functionalised at the α-carbon with an azide group was synthesised by the Pummerer reaction from the corresponding sulfoxide to obtain 2-azidotetrahydrothiophene (Fig. 1d). Subsequently, the sulphide was oxidised to the sulfone using magnesium monoperoxyphthalate hexahydrate (MMPP) obtaining the part

of the template that mimics the transition state analogue. The N-Boc-protected acyl chain was coupled by Staudinger ligation to prepare the TSA-template which was deprotected before immobilisation to the solid phase (see ESI section for detailed protocols).

The stabilisation of the transition state by the active site in the polymer is the key to the catalytic activity of MIP NPs. In order to orientate the functional monomers in the correct position, the template must be as similar as possible to the transition state in size, shape and functionalities. Using ACD/Labs software with three-dimensional modelling the transition state analogue was optimised and the close similarity between the template and actual TSA is shown in Fig. 2.

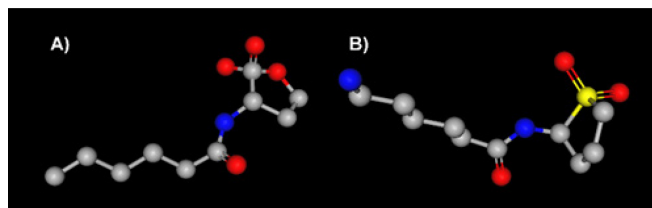


Fig. 2. The 3D structures of C6-AHL (a) and its purported transition state analogue modified with amino group for immobilisation ("TSA-template") (b).

The TSA-template was immobilised on glass beads by coupling its primary amine group with the epoxy group of the solid phase. MIP NPs were prepared using solid phase synthesis approach. The UV polymerisation reaction was initiated using *N,N'*-diethyldithiocarbamic acid benzyl ester as "living" photoiniferter which provides more control over the size of the particles produced.²⁶ A combination of two cross-linkers (trimethylolpropane trimethacrylate and ethylene glycol dimethacrylate, 30% w/w) was used to obtain a high degree of cross-linking. Three inexpensive and commercially available functional monomers were chosen for their ability to produce strong noncovalent bonds with the template molecule: methacrylic acid (MAA), itaconic acid (IA) and 2-(dimethylamino)ethylmethacrylate (DEAEM). After polymerisation, the reaction mixture was removed, and NPs retained by the solid phase were washed using cold acetonitrile (+4 °C) several times to remove unreacted monomers and low affinity MIPs. Subsequently, hot acetonitrile (60 °C) was used to disrupt the non-covalent interaction between template and MIP NPs, resulting in the elution of MIPs free of template. Consequently, the high affinity NPs collected did not require any further purification (Supplementary Information, Fig. 1S).

The size distribution of the polymeric NPs, measured in acetonitrile using dynamic light scattering (DLS), demonstrated a low polydispersity index (PDI), therefore, confirming the homogeneity of the samples. The correlation curves showed that the MIP NPs had a low tendency to form aggregates. The size of all obtained nanoparticles was between 175 nm to 213 nm, indicating the reproducibility of the synthesis method (Table 1). The TEM images confirmed the uniform size of the particles. The size of the MIP NPs in their dry state was in the range of 50–80 nm (ESI section, Fig. 2S). As expected, these values were lower than values obtained by DLS, which could be

explained by swelling of low cross-linked polymer nanoparticles in acetonitrile during DLS measurement.

The ability of the MIP NPs to catalyse the hydrolysis of C6-AHL was evaluated by measuring the decrease in the concentration of the autoinducer over time using an LC-MS set-up (Waters, UK). MIP NPs synthesized using different functional monomers were compared. It was found that the highest hydrolytic activity was demonstrated by MAA-based MIP NPs. The concentration of TSA-NPs catalyst that showed the best performance in water was found to be equal to 1 mg mL⁻¹.

Table 1. The size of nanoparticles obtained using DLS.

Monomer	MAA	DEAEM	IA
TSA-NPs			
Diameter, nm	204±7	175±4	198±4
PDI	0.2±0.01	0.22±0.01	0.24±0.02
Control-NPs			
Diameter, nm	193±6	213±14	212±7
PDI	0.35±0.01	0.41±0.03	0.38±0.01

The degradation of C6-AHL (initial concentration 500 ng mL⁻¹) using 1 mg mL⁻¹ of TSA-NPs or non-specific NPs was evaluated and compared with the natural degradation of C6-AHL at 25 °C in water. It was found that no degradation was observed in the first 30 min in samples incubated with non-specific NPs and without NPs. It was found that the concentration of C6-AHL had decreased after 2 h incubation in the NPs-containing samples. The highest catalytic activity was demonstrated by MAA-based TSA-NPs that produced a reduction of 41% of C6-AHL after only 2 h. At the same time, a reduction of only about 17% was detected in samples containing the non-specific (control) NPs and absolutely no natural degradation was observed in the samples without any NPs. After 20 h, the concentration of C6-AHL had reduced by 57%, 31% and 16% for MAA-based TSA-NPs, control NPs and without NPs, respectively (Fig. 3).

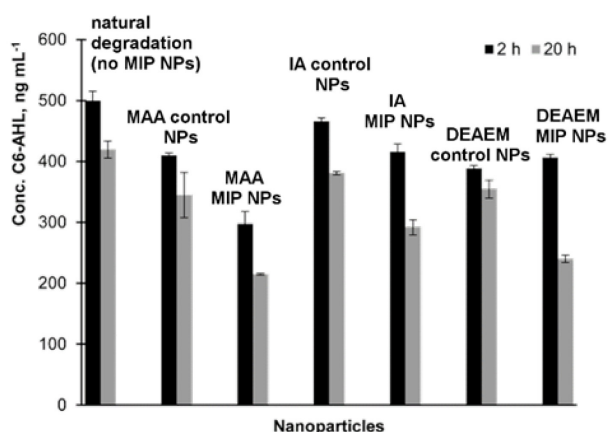


Fig. 3. Graph of the degradation of 500 ng mL⁻¹ of C6-AHL after 2 h and 20 h using different catalytic and control NPs and

without NPs (natural degradation). The experiment was repeated three times.

The obtained results clearly suggest that the activity of MAA-based MIP NPs was due to molecular recognition within the imprinted cavity of the TSA NPs stabilising the transition state, resulting in hydrolytic cleavage of the signal molecule. It is possible that a decrease in pH due to the formation of the acidic hydrolysis product could account for the reduced rate of hydrolysis that was observed after 20 h, since hydrolysis of the lactone ring is very sensitive to changes in pH. It is known that a decrease in pH similarly slows the hydrolytic activity of the natural lactonase which becomes inactive at pH < 5.27.

A kinetic analysis of the NPs was performed using MAA-based MIP NPs in water solution with different concentrations of C6-AHL at 25 °C. The data presented in Figure 4 shows of the variation of reaction velocity with decreasing substrate concentration using the TSA-imprinted NPs synthesised with MAA. A linear behaviour is observed as expected in a catalysed reaction over the saturation concentration of the enzyme (TSA-NPs in this case).

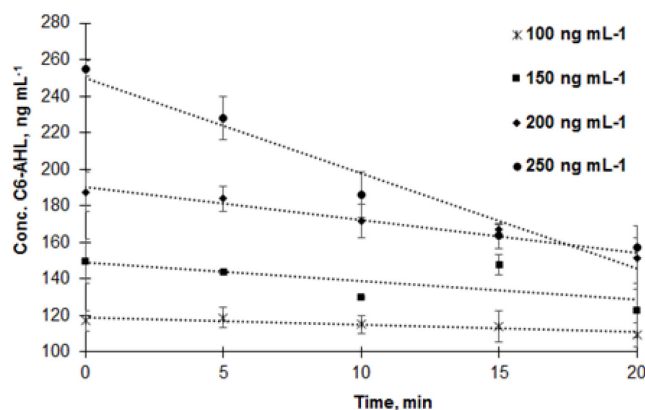


Fig. 4. Initial reaction velocity (v_i) determination for the hydrolysis of C6-AHL performed in the presence of 1 mg mL⁻¹ of MAA-based MIP NPs in water at 25 °C. The data for each concentration of substrate was fitted to a linear regression. The experiment was repeated three times.

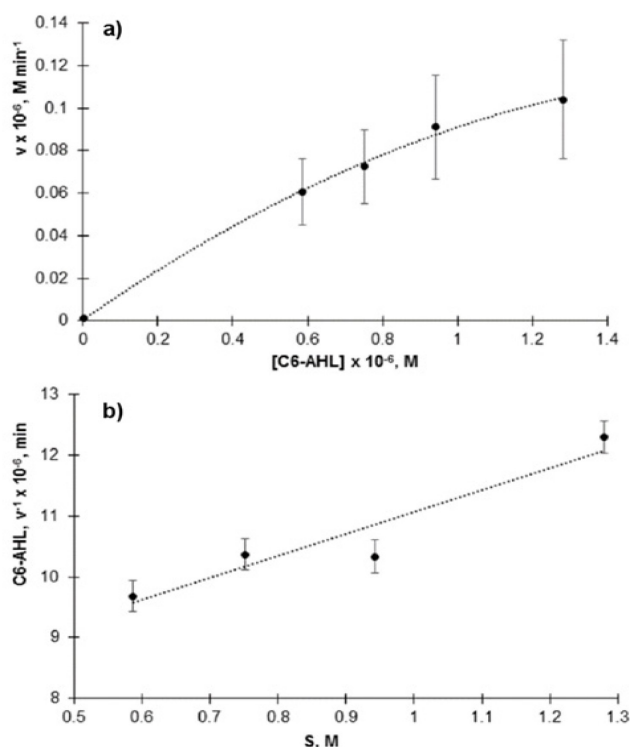
The kinetic values for tested materials were obtained using the OriginPro 2016 software with reaction rate and concentration fitted to a non-linear Michaelis-Menten saturation curve, are the following: $V_{max} = (26 \pm 5) \times 10^{-8}$ M min⁻¹; $K_m = (1.9 \pm 0.5)$ M; $K_{cat} = V_{max}/[catalyst] = 26$ min⁻¹ (Figure 5a).

According to the literature, a Hanes-Woolf plot can be used to corroborate the homogeneity of the activity of a catalyst.^{28, 29} A high variety of kinetic parameters bestow a plot of $[S] \cdot v^{-1}$ against $[S]$ with a concave shape towards the abscissa axis.^{24, 30} However, when the same data fits a linear regression, as shown in Figure 6b, the system corresponds to a single-site saturation model, where the variation in catalytic activities is low.

The catalytic efficiency (K_{cat}/K_m) of the MIP NPs was considerably lower than that reported for the natural lactonase enzyme from the Gram-positive bacteria *Bacillus* sp: 0.23 M⁻¹ s⁻¹.

1 and $9.31 \text{ mM}^{-1} \text{ s}^{-1}$, respectively.²⁷ Taking into account that AHL signal molecules are found in the pico-molar concentration range in the supernatant of bacterial cultures,³¹ it is feasible that the developed NPs could be used to control bacterial QS processes triggered by C6-AHL.

Fig. 5. Plot of the initial reaction rate (v_i) against the



concentration of substrate (C6-AHL), which fits a Michaelis-Menten non-linear equation (a); and plot of $[S] \cdot v^{-1}$ against $[S]$, which fits a Hanes–Woolf linear regression, supporting the proof of enzyme-like behaviour in this reaction (b). Each experiment was repeated three times.

The first case of catalytically-active nanoparticles based on molecular imprinting, which mimics the activity of the natural lactonase, is presented. It is important to highlight that the synthetic protocol of the TSAs synthesis could be used as a blueprint for the synthesis of the TSAs corresponding to any of the other signal molecules by changing the acyl chain used in the Staudinger ligation. The MIP NPs prepared for TSAs of the signal molecules of the different Gram-negative bacteria would provide new tools to control biofilms and other quorum sensing phenotypes. Comparing these results with those achieved using the non-specific NPs, we can conclude that this is a promising new technology that will help to fight bacteria without the risk of developing resistance.

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There are no conflicts of interests to declare.