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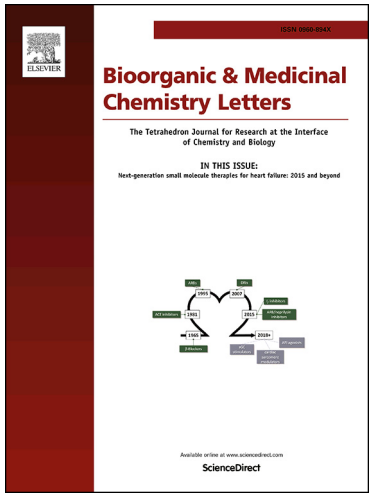

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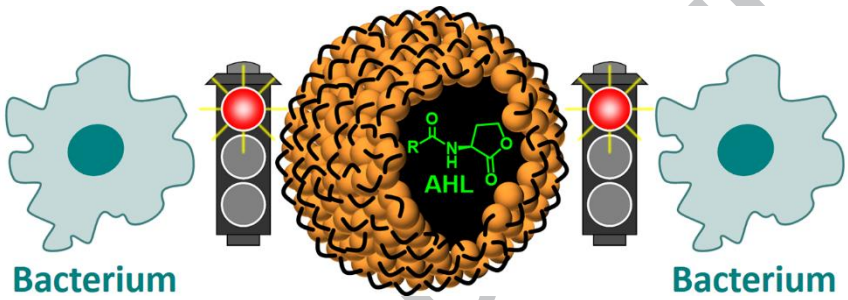

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Synthetic Nanoparticles for Selective Hydrolysis of Bacterial Autoinducers in Quorum Sensing

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ABSTRACT

N-acyl homoserine lactones (AHLs) are signal molecules used by a large number of gram-negative bacteria in quorum sensing and their hydrolysis is known to inhibit biofilm formation. Micellar imprinting of AHL-like templates with catalytic functional monomers yielded water-soluble nanoparticles with AHL-shaped active site and nearby catalytic groups. Either Lewis acidic zinc ions or nucleophilic pyridyl ligands could be introduced through this strategy, yielding artificial enzymes for the hydrolysis of AHLs in a substrate-selective fashion.

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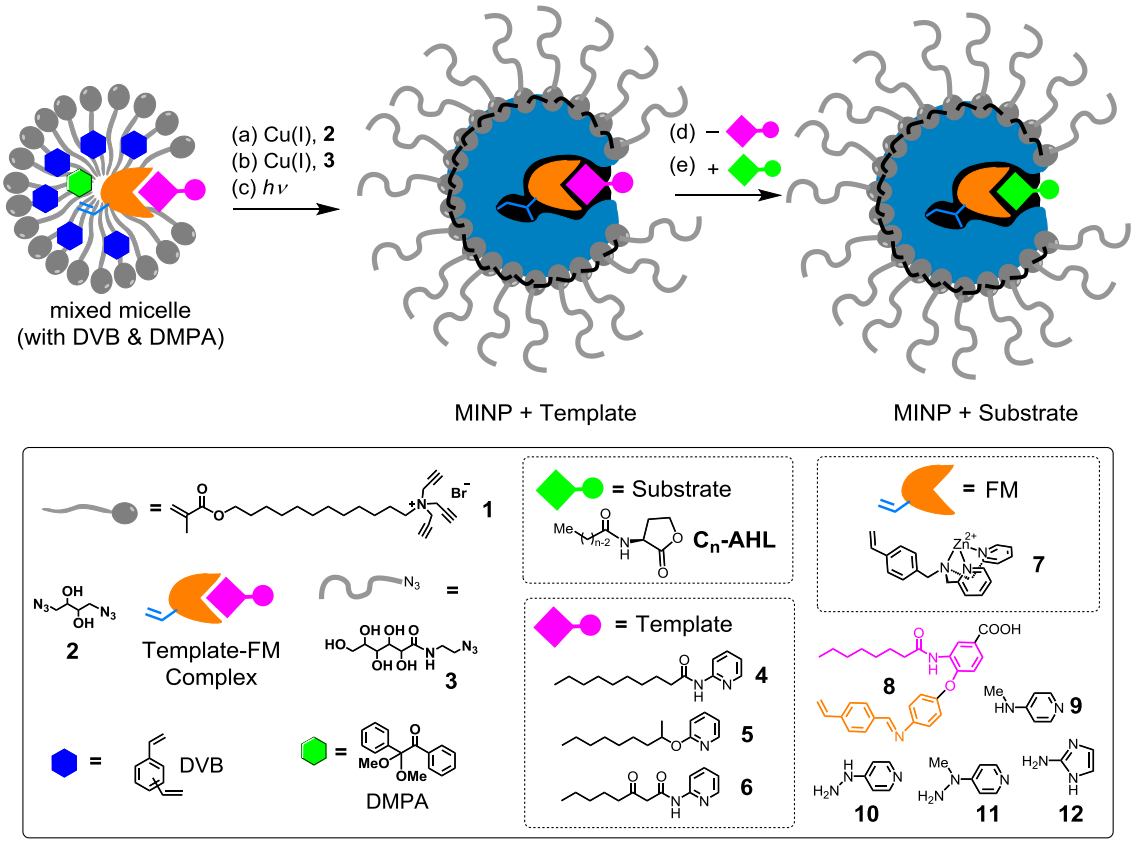


Despite their unicellular nature, bacteria can communicate with one another and recognize others' presence through quorum sensing (QS). The process happens as signal molecules (i.e., autoinducers) are secreted and accumulate within a bacterial community.[1] Because the secretion occurs more or less at a constant rate for an individual bacterium, the local concentration of the autoinducer correlates with the bacterial population in the immediate environment.[2] Once the autoinducer reaches a threshold concentration, QS-regulated genes are turned on, triggering group behavior including biofilm formation and enhanced virulence for pathogenic bacteria. Because bacteria can be 1000-times more resistant to antibiotics in a biofilm than individually, effective inhibition of QS is extremely important in fight against bacterial infection.[3]

N-acyl homoserine lactones (AHLs) are used by >70 strains of gram-negative bacteria in QS. Hydrolysis of AHLs deactivates the autoinducer[4] and is used by organisms including gram-positive bacteria, plants, and mammals to intervene bacterial QS.[2, 5] In this work, we report a method to equip conventional Lewis acidic and nucleophilic catalysts with molecular recognition features for the selective hydrolysis of AHLs. Our testing of two different strategies to construct AHL-shaped active sites and two different mechanisms to hydrolyze the bound AHL revealed that selective hydrolysis of the autoinducer was indeed feasible with readily synthesized synthetic materials, opening up new ways to inhibit biofilm formation.

Our strategy to convert conventional catalysts into "artificial AHL hydrolases" is through micellar imprinting developed in our laboratory in recent years.[6] Molecular imprinting[7] is a powerful technique to create guest-complementary binding sites[8] including those to bind AHLs to control bacterial communications.[9] Some notable challenges associated with traditional molecularly imprinted polymers (MIPs),[10] however, make it difficult to manipulate their active sites precisely for the intended catalysis. Our imprinted micelles, referred to as molecularly imprinted nanoparticles (MINPs), have been shown to produce binding sites with a high level of fidelity to the templates. They could, for example, distinguish leucine- and isoleucine-containing oligopeptides that only differ by the position of a single methyl group,[11] as well as carbohydrates that differ by the stereochemistry of a single hydroxyl.[12] Their solubility in water and selected organic solvents (e.g., DMF and DMSO) allow facile post-modification by chemical derivatization.[13]

Table 1. Hydrolysis of C_8-AHL by MINP catalysts in 10 mM HEPES buffer (pH 7.4) at 37 °C.[a]

entry	Amount of MINP	template	FM	Time (h)	yield (%)
1	0%	-	-	8	40 ± 1
2	5%	none	-	8	41 ± 3
3[b]	5%	**4**	**7**	8	50 ± 1
4	5%	**5**	**7**	8	45 ± 2
5	5%	**6**	**7**	8	48 ± 1

[a] The reactions were performed in duplicates and the yields were determined by reversed phase HPLC analysis. [AHL] = 50 μM. [MINP] = 2.5 μM. The catalyst was kept at 5 mol% in all cases for comparison. [b] Yields of hydrolysis were very similar for C_6, C_8, and C_{10}-AHLs.

Synthesis of the AHL-hydrolyzing MINPs is shown in Scheme 1, through double cross-linking of mixed micelles of surfactant **1** containing divinyl benzene (DVB) and DMPA (2,2-dimethoxy-2-phenylaceto-phenone, a photoinitiator).[6, 13-14] The surface-cross-linking was accomplished by the click reaction using diazide **2** and the core-cross-linking by free radical polymerization using DVB as the cross-linker and DMPA as the photoinitiator. During the core-cross-linking, the functional monomer (FM) was co-polymerized with the methacrylate tail of **1** and DVB to become covalently attached to the MINP core. Sugar-derived **3** was also clicked onto the surface of the cross-linked micelles for better water-solubility. The template was removed by repeated washing with organic solvents (methanol and acetone).

The most important design in the current work is the template and the FM that binds the template. When metal–ligand

complexation was used for the binding, we employed pyridyl ligands such as **4**–**6** as the templates and dipicolylamine(DPA)-based **7** as the FM. The templates resemble C_8-AHL by either having the same acyl chain or something similar in size and shape. We focused on the C8 derivative because the long acyl chain was expected to provide a significant hydrophobic driving force for the template to enter the micelle and for the substrate to enter the imprinted active site later on. The pyridyl mimics the 5-membered lactone ring of AHL but is a stronger ligand for Lewis acidic Zn. Molecular imprinting would then produce within the cross-linked micelle an AHL-binding pocket that has a nearby catalytic group (Lewis acidic zinc in this case) to facilitate the hydrolysis of the lactone. Zinc is known to promote the hydrolysis of acyl derivatives by either activating the metal-bound water or the carbonyl.[15] The substrate-shaped active site is meant to give selectivity in the hydrolysis. The design was partly inspired by naturally occurring AHL-degrading AiiA-like enzymes that use zinc for the hydrolysis.[16] Another motivation for the design was our recent success in using DPA-based FMs to construct highly selective artificial esterases for activated esters.[17] Because DPA binds zinc very strongly in water with a binding constant of $K_a \approx 10^7$ M^{-1},[18] these FMs easily retain the metal ion throughout the preparation and purification of MINPs.[17]

Detailed procedures for the synthesis and characterizations of MINPs have been reported previously[6, 13-14] and are included in the Supporting Information. The surface- and core-cross-linking were monitored by ^{1}H NMR spectroscopy. The surface-cross-linking has been verified by mass spectrometry (after the 1,2-diol in the cross-linked **2** was cleaved).[19] M.W. of the nanoparticles and their size (~5 nm with ligand **3**) were determined by dynamic light scattering (DSL). The DLS size has been confirmed by transmission electron microscopy (TEM).[20]

Table 1 shows the hydrolysis of AHLs under physiological conditions, at 37 °C in 10 mM HEPES buffer (pH 7.4). Yields of hydrolysis were determined by reversed-phase HPLC analysis following reported procedures.[21] Entry 1 shows that 40% of the lactone hydrolyzed into the carboxylic acid after 8 h in buffer. The yield of hydrolysis was essentially the same in the presence of 5 mol % nonimprinted nanoparticles (NINPs) prepared without any FM and template (entry 2). Thus, the backbone structure of the MINP was catalytically inactive. In the presence of the 5 mol % of the imprinted catalysts, the hydrolysis yield increased to 45–50% (entries 3–5). Thus, only a small increase was observed in the hydrolysis, regardless of the template used. For MINP prepared with template **4**, we also tried different AHLs (C6, C8, and C10), anticipating a more hydrophobic substrate would have a stronger driving force to enter the catalytic pocket. Little improvement, however, was observed (data not shown). Evidently, although these Zn-based MINPs could promote the hydrolysis of AHLs, their activity and selectivity were lacking.

Template **6** has two potential binding sites for zinc, the pyridyl nitrogen and the 1,3-dicarbonyl. We were hoping that the template would enable inclusion of two metal ions in the active site, with one used for binding and the other for hydrolyzing the AHL. To our disappointment, the double metal strategy did not help at all (entry 5). We also tried Cu instead of Zn as the catalytic metal but only saw a small improvement in the hydrolytic yields (data not shown).

Table 2. Hydrolysis of C_8-AHL by MINP catalysts in HEPES buffer (pH 7.4, 10 mM) at 37 °C.[a]

entry	Amount of MINP	Catalyst	time (h)	yield (%)
1	0%	-	8	40 ± 1
2	5%	NINP	8	41 ± 3
3	5%	MINP(**9**)	8	73 ± 2
4	5%	MINP(**9**)[b]	8	60 ± 3
5	5%	MINP(**9**)[c]	8	51 ± 4
6	5%	MINP(**10**)	8	74 ± 4
7	5%	MINP(**11**)	8	83 ± 4
8	5%	MINP(**12**)	8	60 ± 6

[a] The reactions were performed in duplicates and the yields were determined by reversed phase HPLC analysis. [AHL] = 50 μM. [MINP] = 2.5 μM. [b] 0.5 equiv. of DVB was used. [c] 0 equiv. of DVB was used

Zn has been used in the construction of catalytic MIMPs active and highly selective for *p*-nitrophenyl esters.[17] The much smaller rate acceleration observed in the AHL hydrolysis was disappointing. One possible reason for the low activity could be the pyridyl group, which might be a poor mimic of the lactone ring and might not have positioned the zinc at the optimal position for the catalysis. Another reason, we suspect, is that the activated esters are much easier substrates to hydrolyze and their catalysis might have a higher tolerance for imperfect catalyst design than the less active lactones.[22]

The benefit of synthetic catalysts is that they are not limited to biologically available ligands and functional groups. Since the Zn-based MINPs only accelerated the AHL hydrolysis by 10–20%, we explored another strategy to construct an AHL-hydrolyzing catalyst.[13b] Color-coded compound **8** has two main components: the magenta moiety resembles the substrate and the orange moiety is a placeholder ultimately converted into the catalytic functionality (Scheme 1).[23] To do the latter, we included a cleavable imine linkage in its structure. The design was based on our recent discovery that imines could be hydrolyzed cleanly within MINP to create a binding site mono-functionalized with an aldehyde inside.[24] Reductive amination of the aldehyde[25] is an efficient way of post-modification to introduce additional functions to the MINP.[24]

In the current example, **8** has a polymerizable vinyl group that enabled this imine to be covalently attached to the cross-linked micelle (Scheme 2). Treatment with 6N HCl at 90 °C for 2 h, identified in our previous work to hydrolyze imines in MINPs,[24-25] produced MINP(**8**)-CHO that contained an aldehyde group in the active site. Similar reductive amination as previously reported with **9**–**12** produced MINP(**9**)–MINP(**12**) with an AHL-shaped active site and a nucleophilic catalyst in close proximity. Compounds **9**–**11** allowed us to install a catalytic group similar to the powerful transacylating *N*,*N*-dimethylaminopyridine (DMAP)[26] in the active site and amine **12** installed an imidazole. The pyridyl or imidazoyl group can promote the hydrolysis through either nucleophilic catalysis or a general-based catalyzed mechanism.[27]

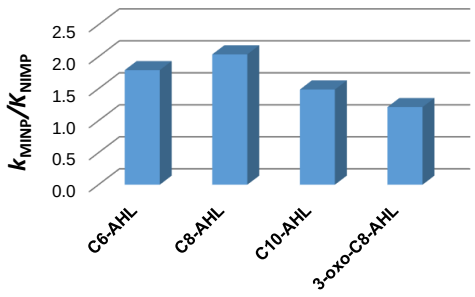
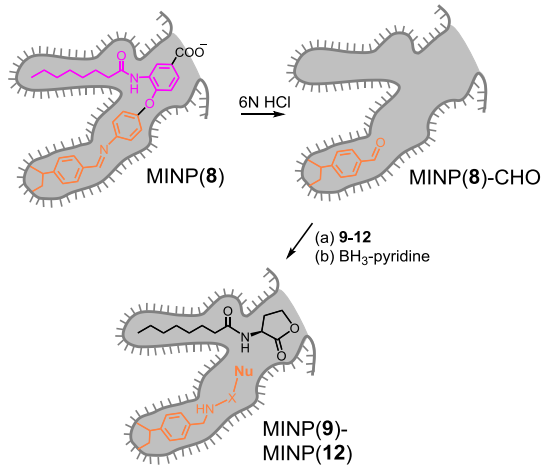


Figure 1. Rate acceleration for the hydrolysis of different AHL derivatives by MINP. [AHL] = 50 μM. [MINP] = 2.5 μM.

Table 2 summarizes the hydrolytic yields of C_8-AHL catalyzed by these nucleophilic catalysts. Encouragingly, the newly synthesized MINPs in general were more active than the Zn-based ones. Under the same reaction conditions, i.e., at 37 °C in 10 mM HEPES buffer, MINP(**9**) hydrolyzed 73% of the C_8-AHL in 8 h (entry 3). Our data also shows that rigidity of the active site was important. The highest amount of DVB solubilized by surfactant **1** is 1 equivalent.[6] When the amount of DVB in the core-cross-linking was reduced, for example, the yield decreased monotonously from 73% all the way to 51% (entries 3–5). This trend was more similar to what we have observed in the binding of MINPs[6] and was different from an earlier catalytic study that showed the best results with DVB/surfactant = 0.5.[17] The high level of DVB needed implied that collapse of the active site was particularly detrimental to the catalysis in the current system.

Among the nucleophilic catalysts, MINP(**11**) gave the best results and MINP(**12**) the worst, and MINP(**9**) and MINP(**10**) had similar activities (Table 2, entries 3 and 6–8). The dependence of the catalytic performance on the template is a good sign, suggesting that molecular imprinting has successfully transferred the information from the template to the imprinted active site. The poor performance of MINP(**12**) is understandable, because imidazole is a weaker nucleophile than DMAP. Another possibility is that the nucleophilic nitrogen and the lactone carbonyl carbon might be either misaligned or too far for efficient catalysis.

The better performance of MINP(**11**) over MINP(**10**) makes sense, as the electron-donating methyl in the former increases the nucleophilicity of the pyridyl nitrogen, consistent with our design of employing the pyridyl as a nucleophilic catalyst (or a general base). The better performance of MINP(**11**) over MINP(**9**) is interesting. Several possible factors might have contributed to the improvement: a stronger electron-donating ability of hydrazyl over amino, a closer distance between the pyridyl nitrogen and the lactone carbonyl, and higher flexibility in the pyridyl group in MINP(**11**) that could be helpful to the nucleophilic attack.



Scheme 2. Post-modification of the active site of MINP(**9**) with a nucleophilic catalytic group.

We also studied the selectivity of MINP(**11**), our best catalyst, for AHLs with varying chain lengths (C_6–C_{10}) and oxidation state (3-oxo-C_8) of the acyl chain. Figure 1 compares the k_{MINP}/k_{NINP} ratio for different AHLs. The ratios were obtained by dividing the hydrolytic yields of the MINP-catalyzed reactions with those of the NINP-catalyzed ones (Table S1), thus eliminating the influence of inherent reactivity—note that C_6-AHL and 3-oxo-C_8-AHL had a higher background reactivity due to their better solubility in water. Our data clearly shows the largest rate acceleration for C_8-AHL among the analogues, confirming that the micellar imprinting and post-modification yielded catalysts with predetermined selectivity.

Selective hydrolysis of AHLs is considered a particularly sustainable therapeutic approach to combat bacterial infections because it does not kill the bacteria but places a limited selective pressure for the survival of bacteria.[28] Catalytic antibodies are the only known "synthetic" catalysts that displayed modest hydrolytic activity for AHLs under biologically relevant conditions.[21, 29] Our MINPs are much easier to produce than antibodies and can tolerate high temperature and organic solvents.[13a] The clear structure–activity correlation observed in MINP(**9**)–MINP(**12**) suggests that the catalytic activities could be improved rationally through better designs of the active site. With many possible ways to fine-tune the structures of MINPs, further improvement should be possible, enabling new strategies to fight bacterial resistance.

Acknowledgments

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Supplementary Material

Synthesis and characterization of materials, experimental procedures for AHL hydrolysis, and NMR spectra for key compounds.

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