Published in final edited form as:

Chem Commun (Camb). 2011 January 7; 47(1): 370–372. doi:10.1039/c0cc02316g.

Surface-mediated release of a synthetic small-molecule modulator of bacterial quorum sensing: Gradual release enhances activity

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Abstract

We demonstrate an approach to the surface-mediated release of a synthetic *N*-acylated L-homoserine lactone (AHL) modulator of bacterial quorum sensing (QS). AHL released gradually from thin films of poly(lactide-co-glycolide) (PLG) is shown to activate QS in the model symbiont *Vibrio fischeri* at levels that exceed those promoted by direct solution-based administration.

Many types of bacteria communicate to assess their local population densities in a phenomenon known as “quorum sensing” (QS).^{1–3} This signaling system is based on the intercellular exchange of small-molecule signals (or autoinducers). Several of the most notorious human pathogens, including *Pseudomonas aeruginosa* and *Staphylococcus aureus*, use QS to organize into structured impermeable communities called “biofilms” and activate virulence pathways at high cell densities that are the basis for acute and chronic infections.⁴ As a result, QS presents an important and relevant target for the development of new anti-virulence strategies.^{5,6} Such an approach to the prevention and/or treatment of infection is also particularly attractive in the longer term, as it could provide a potential means to avoid evolved-resistance mechanisms that plague most currently used antibiotics.

Gram-negative bacteria have the best-understood QS systems, which are largely based on *N*-acylated L-homoserine lactone (AHL) autoinducers and their associated LuxI-type synthase enzymes and LuxR-type receptors.^{3,7} AHL:LuxR-type receptor binding is essential for the QS system to activate, and thus represents a central target for the interception of QS networks.^{8,9} As part of a broader program aimed at elucidating the chemical mechanisms of QS in bacteria, we have recently identified a series of non-native AHLs capable of either strongly inhibiting or activating LuxR-type receptors in a range of bacterial pathogens and symbionts.^{10–12} These compounds are readily synthesized and provide pools of potent antagonists and agonists of QS that can be used to dissect and understand fundamental mechanisms of bacterial communication. In addition, these AHL-derived antagonists serve as lead scaffolds for the development of new anti-virulence agents.¹³

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[‡]Electronic Supplementary Information (ESI) available: Full details for film fabrication, compound quantification, and bacteriological assays. See DOI: 10.1039/b000000x/

One challenge to the application of these new QS inhibitors as anti-virulence agents lies in developing methods for the administration of these compounds in ways that can be tailored in a range of different therapeutic contexts (e.g., systemic vs. localized delivery, rapid vs. sustained release, and addressing issues associated with the stability of these molecules in physiological media). Bacterial colonization and the formation of biofilms on the surfaces of indwelling medical devices, for example, represent two primary points of entry for bacteria into the body.^{14,15} Approaches aimed at inhibiting or attenuating QS in bacteria locally (i.e., at or near the surfaces of these objects) presents challenges that differ substantially from strategies based on the systemic delivery of these molecules (via injection, etc.). The work reported here takes a first step toward addressing several of these challenges through the design of thin polymer films that provide time-dependent control over the surface-mediated release of a non-native, AHL-derived QS modulator. We demonstrate that this materials-based approach can be used to control and activate a QS phenotype in a model bacterial system, and that this surface-mediated approach to delivery has several potential advantages relative to methods for the direct (i.e., solution-based) administration of AHLs used to disrupt QS pathways in several past studies.⁸ Although many different approaches have been reported for the design of materials that control the release of antibiotics and other bacteriocidal agents,^{16,17} materials-based approaches to the release of agents that intercept and disrupt bacterial communication directly have not, to our knowledge, been reported.

We selected the synthetic QS modulator *N*-(3-nitro phenylacetanoyl)-L-homoserine lactone (AHL **1**; Fig. 1) for use in our initial studies for several reasons: (i) past work in our group has shown that this non-native AHL is a potent modulator of QS in bacteria, most notably as an inhibitor in the pathogen *P. aeruginosa* and as a “super-activator” in the bioluminescent symbiont *Vibrio fischeri*,^{10–12} and (ii) the nitrophenyl substituent on this molecule provides a convenient method for monitoring time-dependent concentrations of AHL in solution (i.e., by UV absorbance). We selected poly(lactide-co-glycolide) (PLG) as a matrix for the encapsulation and surface-mediated release of AHL **1**. PLG is both biocompatible and biodegradable, and it has a well-documented history of use in drug delivery and other biomedical applications.^{16,18–20} This polymer is also commercially available in a broad range of copolymer compositions and molecular weights that can be used to influence and control the rates of release of encapsulated compounds.^{18,21} Finally, and of particular relevance to the work reported here, the microenvironments within matrices of PLG have been demonstrated to be acidic (owing, in part, to the presence of carboxylic acid groups that arise from backbone ester hydrolysis).^{22–24} In this respect, PLG can serve to stabilize the structures of molecules that are base-sensitive or that may otherwise hydrolyze or degrade upon prolonged exposure to aqueous media.²⁴ We note, in this context, that AHLs contain a hydrolyzable lactone moiety (Fig. 1), and that past studies by our group and others have demonstrated that the time-dependent hydrolysis of these lactone groups (in both native and non-native AHLs; half-lives from ~12 to 48 hours) in aqueous media leads to ring-opened products that are essentially inactive as QS modulators.^{10,25,26} The use of PLG as a matrix for the release of AHLs could therefore lead to materials that both stabilize *and* prolong the release of these agents, and thereby lead to surfaces and coatings that modulate QS more effectively than the direct administration of these compounds in solution.

To explore the feasibility of this approach, we performed a series of experiments to characterize the encapsulation and release of AHL **1** from thin solvent-cast films of PLG fabricated directly in the wells of 96-well microtiter plates (see Supp. Info. for full details of solvent-casting methods and the characterization of AHL-loaded films). This approach resulted in uniform and transparent thin films of polymer with loadings of AHL **1** that could be controlled reproducibly. To characterize the release of AHL **1** from these films under physiologically relevant conditions, we incubated AHL-loaded films in an M9-type aqueous buffer (pH = 7.5) at 37 °C. The concentration of AHL released into solution was monitored

over time by characterizing the UV absorbance of the buffer solution at 267 nm (the absorbance maximum for AHL 1).

Fig. 2 shows representative release profiles of two films fabricated to have initial loadings of either 9 or 36 μg of AHL 1. These results demonstrate that AHL 1 is released relatively rapidly (~80% of the encapsulated compound is released over a period of 4.5 d) and that the amount of AHL released can be controlled by the amount of the compound incorporated into the film during fabrication. We comment in this context that, in general, the release of small molecules from PLG can be made to occur over a broad range of times (e.g., from hours or days to several weeks or months) by manipulation of polymer structure and other factors (film thickness, method of fabrication, etc.).^{16,18–20} The relatively rapid release profiles shown in Fig. 1 suggest that release occurs by a mechanism (e.g., diffusion of AHL 1 from a water-swollen film) that does not require substantial polymer degradation or physical film erosion. Although it should be possible to design films that provide for more extended release, the timescales of the release profiles shown in Fig. 1 are relevant in the context of certain potential applications (e.g., in the context of preventing biofilm growth on short-term indwelling devices) and were suitable for all subsequent biologically oriented *in vitro* studies described below.

We next performed a series of cell-based experiments to characterize the functional activity of AHL 1 released from the polymer films described above. We used *V. fischeri* as a model for these experiments because this organism uses QS to control bioluminescence at high population densities and thus provides a straightforward means of characterizing changes in QS. We selected a *V. fischeri* mutant strain (ES114; $\Delta\text{-luxI}$) that lacks a functional AHL synthase (as a result, the exogenous addition of an appropriate agonist is required to activate QS). As mentioned above, AHL 1 behaves as a highly potent QS agonist in this strain, with an EC_{50} value of ~0.2 μM (10-fold more potent than *V. fischeri*'s native autoinducer).¹⁰ Cell-based experiments were performed by collecting aliquots of AHL 1 released from our polymer films during release experiments similar to those described above, diluting these samples in series into separate 96-well plates, and then adding suspensions of *V. fischeri*. For these cell-based experiments, films containing AHL 1 were incubated in LBS growth medium, a medium that is similar to M9 buffer but also contains additional nutrients and salts required to support *V. fischeri* bacterial growth (see Supp. Info. for full details of assay protocol).

After an appropriate period of growth, the bioluminescence of *V. fischeri* in each well was measured, normalized to cell density, and plotted as percent of the authentic AHL 1 positive control vs. concentration to generate sigmoidal dose curves and determine EC_{50} values for released AHL 1 at each time point. We note here that the absorbance spectrum of LBS media at 267 nm prevented direct characterization of concentrations of the AHL released from the films in these cell-based experiments. As a result, we used concentrations of released AHL measured using otherwise identical films incubated in M9 media (as described above) to generate the dose-response curves shown in Fig. 3.

Fig. 3A shows representative dose curves and corresponding EC_{50} values for *V. fischeri* using polymer films fabricated to have an initial loading of 36 μg of AHL 1 (i.e., identical to the films shown in Fig. 2, closed squares). Inspection of these data reveals that the AHL released from these films retains its biological activity as a QS agonist at each time point sampled over the ~4 day course of this experiment. Further inspection also reveals the dose curves to shift to higher concentrations as a function of incubation time [shown in Fig. 3A and summarized in Fig. 3B (black bars)]. This shift to higher concentrations over time was also observed for control solutions of authentic AHL 1 (36 μg /well; see Fig. 3B (white bars) for a comparison) incubated under otherwise identical conditions (we note, however, that

these two shifts in EC₅₀ values occur to two different extents; we return to this observation again in the discussion below).

As described above, both naturally occurring and synthetic AHLs undergo time-dependent hydrolysis in physiologically relevant media to yield ring-opened structures that are QS-inactive (e.g., Fig. 1). On the basis of this knowledge, we interpret the time-dependent shifts in EC₅₀ values observed in Fig. 3B to arise, at least in part, from the partial and time-dependent hydrolysis of AHL **1** during the course of these experiments. Such hydrolysis would, over time, result in a decrease in the amount of active compound present in solution and lead to apparent EC₅₀ values that increase over time (as observed in Fig. 3A). Support for this view is provided by the results of additional solution-based experiments that confirm that the hydrolysis of AHL **1** leads to a product that is indeed QS-inactive in *V. fischeri* (see Supp. Info.).

Finally, a comparison of the data in Fig. 3B reveals that the time-dependent shift to higher EC₅₀ values occurs more rapidly for solutions of AHL **1** (white bars) than it does for experiments using compound that was released gradually from the polymer films (black bars). These results demonstrate that the gradual, surface-mediated release of AHL **1** yields solutions of agonist that are more active for longer periods of time (compared to the activity of an equivalent amount of AHL incubated in solution) and hint that the polymer used to fabricate these films could play a protective role in these experiments. Additional characterization will be required to understand the origin of the increased activity observed in these controlled release experiments more completely. However, our current results are consistent with the broader view that the acidic microenvironments of water-swollen PLG matrices can stabilize the structures of base-sensitive drugs²⁴ and thereby prolong the effectiveness of certain active agents in ways that extend beyond control over rates of release.

In summary, we have reported a polymer-based approach to the surface-mediated release of AHL-derived modulators of bacterial QS. Our results demonstrate that this approach can be used to provide control over the release of a synthetic AHL and that this compound is released in a form that is biologically active and able to modulate (turn on) QS in the marine symbiont *V. fischeri*, a bacterial model used widely for fundamental studies of QS. Our results also show that this polymer-based approach can be used to prolong the biological activities of AHLs relative to one-time treatments with equivalent amounts of AHL in solution. To the best of our knowledge, this study presents the first demonstration of the controlled release of a non-native, AHL-derived QS modulator from a polymer matrix. The results of this study, when combined, could thus provide a basis for the design of surfaces and coatings that intercept or disrupt bacterial communication in ways that are important in a broad range of fundamental and applied contexts. Experiments to extend this general approach to the design of surfaces that prevent the formation of *P. aeruginosa* biofilms are underway and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support to H.E.B. was provided by the NIH (AI063326), the Greater Milwaukee Foundation, the Burroughs Welcome Fund, Johnson & Johnson, and the Alfred P. Sloan Foundation. Support to D.M.L. was provided by the Alfred P. Sloan Foundation, the University of Wisconsin, and the UW Vilas Trust. A.S.B. was funded in part by an NIH Chemistry Biology Interface Training Grant (NIGMS T32 GM008505). A.H.B. is a NSF Graduate Research Fellow.

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Fig. 1.
Chemical structures of AHL **1** (264.23 g/mol) and its hydrolysis product.

Fig. 2. Plot of release vs. time for two PLG thin films containing AHL **1** (with initial loadings of either 9 µg or 36 µg) incubated in M9 buffer (pH 7.5) at 37 °C. Dashed lines indicate the initial loading of AHL **1** in each film. Each data point represents the average for 4 replicate wells; error bars are STE.

Fig. 3.

(A) Dose-response curves for aliquots of AHL 1 released from PLG films incubated in buffer (LBS; pH 7.5, 37 °C) at selected time points and added to *V. fischeri* (ES114) (see text). Initial film loading of AHL 1 = 36 μg . QS activation measured via luminescence output per well. Concentration of AHL 1 determined from replicate wells of PLG:AHL 1 films in M9 buffer (see Fig. 2). Each data point is an average of 4 replicate wells; error bar is STE. (B) Comparison of the change in EC_{50} value for AHL 1 vs. time for AHL 1 released from PLG films (loading of 1 = 36 μg) or direct treatment with a bolus of 36 μg AHL 1 (see text). Error bars are 95% CI.