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Nanoporous Superhydrophobic Coatings that Promote the Extended Release of Water-Labile Quorum Sensing Inhibitors and Enable Long-Term Modulation of Quorum Sensing in *Staphylococcus aureus*

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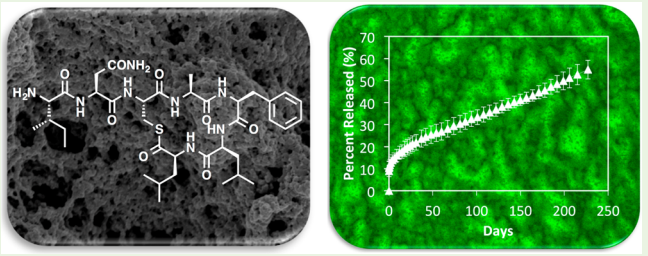
Supporting Information

ABSTRACT: Materials and coatings that inhibit bacterial colonization are of interest in a broad range of biomedical, environmental, and industrial applications. In view of the rapid increase in bacterial resistance to conventional antibiotics, the development of new strategies that target nonessential pathways in bacterial pathogens—and that thereby limit growth and reduce virulence through nonbiocidal means—has attracted considerable attention. Bacterial quorum sensing (QS) represents one such target, and is intimately connected to virulence in many human pathogens. Here, we demonstrate that the properties of nanoporous, polymer-based superhydrophobic coatings can be exploited to host and subsequently sustain the extended release of potent and water-labile peptide-based inhibitors of QS (QSIs) in *Staphylococcus aureus*. Our results demonstrate that these peptidic QSIs can be released into surrounding media for periods of at least 8 months, and that they strongly inhibit agr-based QS in *S. aureus* for at least 40 days. These results also suggest that these extremely nonwetting coatings can confer protection against the rapid hydrolysis of these water-labile peptides, thereby extending their useful lifetimes. Finally, we demonstrate that these peptide-loaded superhydrophobic coatings can strongly modulate the QS-controlled formation of biofilm in wild-type *S. aureus*. These nanoporous superhydrophobic films provide a new, useful, and nonbiocidal approach to the design of coatings that attenuate bacterial virulence. This approach has the potential to be general, and could prove suitable for the encapsulation, protection, and release of other classes of water-sensitive agents. We anticipate that the materials, strategies, and concepts reported here will enable new approaches to the long-term attenuation of QS and associated bacterial phenotypes in a range of basic research and applied contexts.



KEYWORDS: *surface coatings, polymer multilayers, superhydrophobic, controlled release, anti-virulence, quorum sensing*

INTRODUCTION

Thin films and coatings that prevent or reduce the occurrence of bacterial infections and biofouling are of potential utility in a host of industrial, commercial, and biomedical contexts. Many approaches to the development of antimicrobial or antifouling surfaces have focused on the design of materials that can release antibiotics or other biocidal agents to kill surface-associated or nearby planktonic (nonsurface-associated) bacteria.[1−5] Growing concerns related to evolved resistance and the decreased efficacy of conventional antibiotics, however, have motivated searches for new nonbiocidal strategies that could be used to prevent infection and fouling more effectively.[6−9] In this broad context, approaches based on modulation of quorum sensing (QS)—the small-molecule or peptide-based signaling system that governs many population-dependent behaviors in bacteria and fungi[10−12]—have emerged as attractive alternatives to conventional microbiocidal approaches because they can modulate and mitigate virulent behaviors without inducing cell death.[8,13,14]

Our group[15−20] and others[21−26] have reported synthetic compounds that act as potent inhibitors of QS in bacteria and demonstrated recently that these "quorum sensing inhibitors" (QSIs) and other nonbactericidal agents can be imbedded into or immobilized onto a range of materials and surfaces.[27−39] Those studies provide guiding principles useful for the development of anti-QS-based approaches to preventing bacterial virulence and fouling. However, many challenges remain with respect to encapsulating, conferring appropriate chemical protection, and controlling the release of these QSIs in practical contexts. The work reported here takes a step toward addressing several of these challenges through the use of novel nonwetting surface coatings to promote the extended, long-term release of macrocyclic peptide-based QSIs that modulate virulence in *Staphylococcus aureus*, a notorious Gram-

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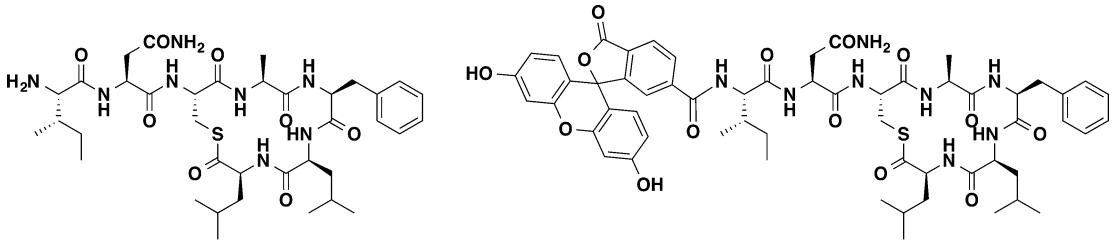

Figure 1. Structures of the macrocyclic peptides used in this study. Peptide **1** is a potent QSI in *S. aureus*; peptide $\mathbf{1}_{FL}$ is a fluorescein-labeled analog of peptide **1**.

positive human pathogen. Our approach exploits the unique physicochemical properties of nanoporous and "superhydrophobic" polymer-based coatings as matrices for the encapsulation and chemical protection of water-soluble and water-labile peptide structures.

We recently reported that thin films of rapidly dissolving water-soluble polymers containing peptidic QSIs can inhibit QS and substantially reduce QS-controlled toxin production in *S. aureus*.[29] In that study, we used carboxymethylcellulose as a model water-soluble polymer and cyclic peptide **1** (Figure 1)—a potent synthetic QSI that can inhibit the agr-type QS circuits in all four specificity groups of *S. aureus* at subnanomolar levels[16−18]—to design thin polymer coatings that can release or "dump" QSI into surrounding media rapidly on exposure to aqueous environments (e.g., over several minutes). That work also demonstrated that peptide **1** released from film-coated surfaces could strongly modulate QS in group-III *S. aureus*[29] and provided potential means of inhibiting the production of toxic shock syndrome toxin-1 (TSST-1), a causative agent of staphylococcal toxic shock syndrome, in this bacterium.

We note that while the rapid release of QSIs would be useful for certain potential applications (e.g., anti-infective wound dressings, on tampons, or other disposables used or replaced over short time periods),[29] materials that release or deliver QSIs over a broad range of time scales and conditions will be necessary to develop anti-QS-based strategies for preventing bacterial fouling or virulence in other contexts. These challenges have been addressed, in part, through the covalent attachment,[30,33,35,36] physical adsorption,[31] or encapsulation[27−29,32,37−39] of QSIs or other nonbactericidal agents on surfaces,[27−33,35,36,38] within degradable coatings,[27,28,34] or as the payloads of nanoparticle formulations.[37,39] The study reported here was motivated by the potential utility of surface coatings that could inhibit QS for long periods (i.e., from weeks to years), and thus prevent or reduce bacterial virulence and fouling on interventional devices and implants (e.g., indwelling catheters, etc.) or on other objects of commercial or industrial importance that have long service lives or residence times.

To design coatings that could sustain the release of peptidic QSIs for prolonged time periods, we investigated thin polymer-based "multilayers" fabricated by the covalent layer-by-layer assembly of branched polyethylenimine (PEI) and the amine-reactive polymer poly(4,4-vinyldimethylazlactone) (PVDMA).[40,41] We selected this system for several reasons: (i) PEI/PVDMA multilayers are chemically stable and physically durable as a result of covalent cross-links formed between polymer chains during assembly;[42,43] (ii) these films can be fabricated readily on a variety of topologically complex substrates, including woven and nonwoven fiber mats and the inner surfaces of tubes;[43−46] (iii) the presence of residual azlactone functionality on and within the films provides a convenient reactive handle[41,47] for the introduction of secondary functionality that can be used to tune bulk and interfacial properties;[40,43,48−50] and (iv) PEI/PVDMA multilayers can be fabricated to have nanoporous morphologies that, when combined with strategies for postfabrication functionalization noted above, can be used to design coatings that are nonwetting to aqueous fluids and exhibit robust superhydrophobicity[49−51] (superhydrophobicity is defined here as having an advancing water contact angle (θ) greater than 150° and a water droplet "roll-off" angle of less than 10°).[52,53]

We recently reported that the unique physicochemical properties of superhydrophobic PEI/PVDMA multilayers could be exploited to host and subsequently promote the extended release of water-soluble small-molecule agents for a period of ∼1 year upon immersion in aqueous environments.[51] This superhydrophobicity-based approach is novel compared to other methods for the long-term release of small molecules, with prolonged release made possible through a mechanism that involves the slow and gradual displacement of air trapped in and around these porous and extremely nonwetting materials by surrounding liquid media (shown schematically in Figure 2).[51,54−56] The model compounds and active agents used in our past study were of low molecular weight and relatively hydrophobic, and could thus be loaded into superhydrophobic coatings using a variety of organic solvents.[51] In this current study, we sought to determine (i) whether the properties of these superhydrophobic coatings could be exploited to load and sustain the release of substantially higher molecular weight and water-soluble peptides, and (ii) whether these nonwetting materials could protect, and thus also prolong the useful active lifetimes, of water-labile peptides upon long-term immersion and storage in aqueous environments. Here, we demonstrate that superhydrophobic PEI/PVDMA coatings can be loaded with water-soluble and water-sensitive peptide **1**, and a related fluorescent model QSI, without negatively affecting the nonwetting properties of the polymer matrix. We further demonstrate that these peptide-loaded superhydrophobic materials can be used to protect and sustain the long-term (at least 8-month long) release of these QSIs and report coatings that can modulate QS and the QS-controlled formation of *S. aureus* biofilms in in vitro environments for prolonged periods without any effect on cell growth. This approach for the controlled loading and release of water-soluble, hydrolytically unstable agents is likely general, and may also prove useful for the encapsulation, physical and chemical protection, and release of other classes of bioactive molecules and macromolecular agents.

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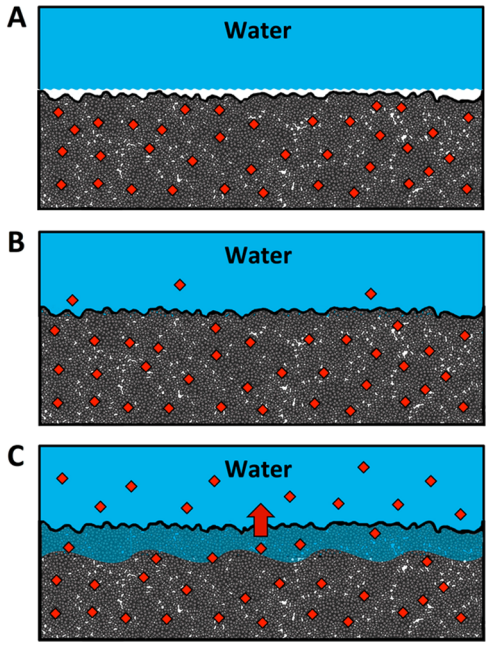

Figure 2. Schematic illustrations showing a nanoporous superhydrophobic PEI/PVDMA multilayer coating (gray) loaded with a model water-soluble agent (red) upon immersion in water (blue). (A) Upon initial immersion, the films are surrounded by a layer of trapped air and contain smaller pockets of air (white) trapped within the pores of the film; the presence of these pockets of air limits the contact of water with the surface and the interior of the film. (B) Eventual wetting of the surface of the film results in more intimate contact with water and the release of the water-soluble agent imbedded near the surface of the coating. (C) Gradual penetration of water into the interior of the coating results in the gradual release of the agent imbedded in the interior of the coating.

METHODS AND MATERIALS

Materials. Branched poly(ethylenimine) (PEI, MW ~ 25 000), acetone, dimethyl sulfoxide (DMSO), dichloromethane, tetrahydrofuran (THF), 2,2′azoisobutyronitrile (AIBN), 5-(6)-carboxyfluorescein, and *n*-decylamine were purchased from Sigma-Aldrich (Milwaukee, WI). Peptide **1** was synthesized and purified as described previously.[16] 2-Vinyl-4,4-dimethylazlactone (VDMA) was generously provided by Dr. Steven M. Heilmann (3M Corporation, Minneapolis, MN). Poly(2-vinyl-4,4-dimethylazlactone) (PVDMA, MW ~ 20 700; PDI = 2.4) was synthesized by free radical polymerization of VDMA in the presence of 7 wt % intentionally-added cyclic azlactone-functionalized oligomers using a previously reported method.[49]

Biological Reagents and Strain Information. All biological reagents were purchased from Sigma-Aldrich and used according to enclosed instructions. *S. aureus* strains AH1677[57] and RN6390B[58] were grown in Brain-Heart Infusion (BHI) medium. *S. aureus* AH1677 is a methicillin-resistant group-I strain harboring a P3-*gfp* reporter plasmid. Activation of the P3 promoter via the agr system in this strain leads to GFP production. *S. aureus* RN6390B is a wild-type group-I strain. Bacterial cultures were grown in a standard laboratory incubator at 37 °C with shaking (200 rpm) unless noted otherwise. A Biotek Synergy 2 microplate reader running Gen5 software was used to measure absorbance and fluorescence of biological cultures.

Instrumentation and Methods. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using a Shimadzu system equipped with an SLC-10Avp controller, an LC-10AT pump, a FCV-ALvp solvent mixer, and a SPC-10MAvp UV/vis diode array detector. An analytical Phenomenex Gemini C18 column (5 μm, 4.6 × 250 mm^2, 110 Å) was used for analytical RP-HPLC work. A semipreparative Phenomenex Gemini C18 column (5 μm, 10 × 250 mm^2, 110 Å) was used for preparative RP-HPLC work. Contact angle measurements were made using a Dataphysics OCA 15 Plus instrument with an automatic liquid dispenser at ambient temperature. Advancing and receding contact angles were measured using 5 μL droplets of deionized water (18 MΩ). Fluorescence microscopy images were acquired using an Olympus IX70 microscope and analyzed using the Metavue version V7.7.8.0 software package (Molecular Devices, LLC). Scanning electron micrographs were acquired using a LEO 1530 scanning electron microscope (SEM) at an accelerating voltage of 3 kV. SEM samples were coated with a thin layer of gold using a gold sputterer operating at 45 mA under a vacuum pressure of 50 mTorr for 40 s prior to imaging. All statistical comparisons were made using Student's *t* test.

Synthesis of Fluorescently Labeled Peptide $\mathbf{1}_{FL}$. Linear peptides were synthesized on Dawson Dbz AM resin (0.42 mmol/g) using standard Fmoc-based solid-phase synthesis protocols[59,60] with an additional 2-h HBTU coupling of 5-(6)-carboxyfluorescein to the *N*-terminus. Cleavage from the resin was performed using the Dawson protocol to afford C-terminal peptide-Nbz.[61] The linear peptide was purified by RP-HPLC and subsequently cyclized using our previously reported methods.[16] The purity of the resulting fluorescein-labeled cyclic peptide was assessed by analytical RP-HPLC (>99%) and its identity was confirmed by exact mass measurement (calculated for $C_{58}H_{69}N_8O_{14}S^+$ = 1133.4648; observed = 1133.4645).

Fabrication and Functionalization of Superhydrophobic Coatings. Prior to film fabrication, glass slides were cut to appropriate sizes, cleaned by sonication in a methanol/water solution, rinsed with acetone, and then dried using filtered compressed air. Superhydrophobic PEI/PVDMA films were fabricated using a layer-by-layer protocol.[49−51] Briefly: (1) Glass substrates were immersed in an acetone solution of PEI (20 mM with respect to the molecular weight of the polymer repeat unit) for 20 s, (2) substrates were removed and immersed in an initial acetone bath for 20 s, followed by a second acetone bath for 20 s, (3) substrates were submerged in an acetone solution of PVDMA (20 mM with respect to the molecular weight of the polymer repeat unit) for 20 s, and (4) substrates were rinsed in the same manner as described in step 2. This cycle was repeated until 100 PEI/PVDMA layers (or "bilayers") were deposited. The volumes of the polymer dipping solutions and rinse baths were maintained by the addition of acetone as needed to replace solvent evaporation. Solutions of polymer were replaced with fresh solutions after every 25 dipping cycles; rinse baths were replaced with fresh acetone after every 20 dipping cycles. After fabrication, film-coated substrates were placed directly into a decylamine solution (30 mM) in THF at room temperature overnight. After this functionalization step, substrates were rinsed with THF and dried under a stream of filtered, compressed air.

Loading and Release of Peptides. Substrates coated with superhydrophobic coatings fabricated as described above (1 cm × 1 cm) were prepared for loading and release experiments by first using a razor blade to remove the entire coating from one side of the coated glass substrates (the layer-by-layer dipping procedure described above results in fabrication of a coating on both sides of a substrate; all experiments in this study were performed using substrates for which the coating on one side was physically removed prior to peptide loading). For solvent-assisted loading, a 40-μL aliquot of dichloromethane was added directly to the surface of the film by pipet, followed by a droplet of DMSO containing either peptide $\mathbf{1}_{FL}$ (2 μL; 1 mM; for experiments to characterize loading and release) or peptide **1** (10 μL; 1 mM; for biological experiments involving bacteria). These solvent-treated samples were then allowed to air-dry overnight and then broken in half to yield two samples 0.5 cm × 1.0 cm in size. For release experiments designed to characterize peptide release profiles, the short edges of the film-loaded substrates (0.5 cm × 1.0 cm) were attached using Five-Minute Epoxy resin (ITW Devcon, Danvers, MA) to the lids of 96-well microtiter plates in a manner that allowed them to be suspended simultaneously in PBS buffer (200 μL; pH 7.4) contained in the wells of the plates when the lid was attached. The film-coated substrates were removed periodically, the concentration of peptide $\mathbf{1}_{FL}$ in solution was measured using fluorometry, and the film-

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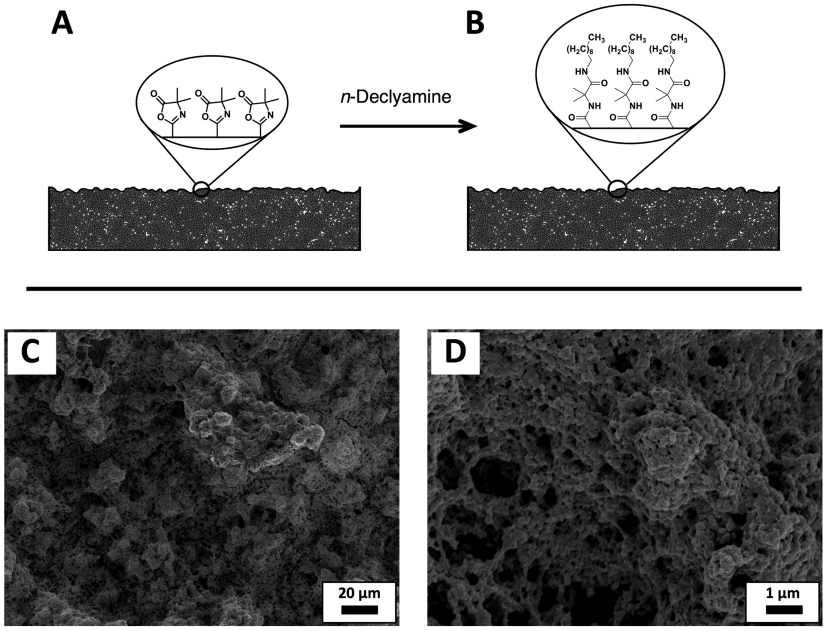

Figure 3. (A, B) Schematic showing the functionalization of (A) amine-reactive azlactone groups in micro/nanoporous PEI/PVDMA multilayer coatings by (B) postfabrication treatment with *n*-decylamine. (C, D) Low- and high-magnification SEM images of decylamine-treated multilayers showing micro- and nanoscale porosity and other topographic features.

coated substrates were placed in fresh buffer. Each experiment was performed in triplicate.

Incubation of Peptide-Loaded Films in the Presence of Bacteria. Release experiments performed in the presence of bacteria were conducted in a similar manner to that described above, with the exception that the film-coated substrates attached to the lids of 96-well microtiter plates were suspended in wells containing (i) growth medium and bacteria or (ii) wells containing PBS (no growth medium or bacteria). Cultures of bacteria used in these experiments were prepared using specific strain-dependent procedures described in the sections below. For these experiments, samples were incubated in wells containing media and cells for 24 h and then moved to a plate containing PBS buffer for 30 s (to remove any attached bacteria and media components), moved to a new plate containing PBS buffer for another rinse, and then placed into wells containing PBS (without bacteria or nutrients). After a predetermined period of time (usually between 1−6 days after the previous exposure to bacteria), the substrates were removed from PBS and again suspended in wells containing growth medium and bacteria for 24 h. This cycle of 24 h exposure to bacteria and incubation in PBS buffer was repeated multiple times over the course of these experiments (see main text). Samples of bacterial cultures resulting from these experiments were characterized using fluorescence reporter assays or crystal violet biofilm assays as described below.

GFP Reporter Gene Assay. An overnight culture of *S. aureus* AH1677 was diluted 1:50 with fresh BHI medium and 200 μL of that diluted culture was added to the wells of a black 96-well microtiter plate. Bacteria were incubated in the presence of film-coated substrates for 24 h (see procedure described above for additional details), and the fluorescence (excitation, 500 nm; emission, 540 nm) and optical density (OD_{600}) of each well (in the absence of substrate) were then characterized using a plate reader.

Crystal Violet Biofilm Assay. An overnight culture of *S. aureus* RN6390B was diluted 1:100 with fresh BHI medium augmented with 1% glucose, and 200 μL of that diluted culture was added to the wells of a clear 96-well microtiter plate. Bacteria were incubated in the presence of film-coated substrates for 24 h at 37 °C under static conditions (see procedure described above for additional details). Amounts of biofilm formation in each well were quantified using a modified crystal violet assay.[62,63] Briefly: OD_{600} values were measured for each well prior to decanting the liquid culture. The wells were then washed gently with 250 μL of PBS three times. The 96-well plate was then incubated at 55 °C for 1 h to fix the biofilm bacteria, and 200 μL of crystal violet solution (0.1%) was added to the wells and incubated for 5 min. Each well was then washed twice with 200 μL of water and treated with 30% acetic acid for 15 min with slight agitation. The absorbance of each well at 595 nm was then characterized using a plate reader.

RESULTS AND DISCUSSION

Fabrication and Characterization of QSI-Loaded Coatings. For all of the studies described below, we used nanoporous PEI/PVDMA multilayers ∼80 μm thick fabricated by reactive layer-by-layer assembly and functionalized by treatment with *n*-decylamine to impart superhydrophobicity (Figure 3A, B; see Materials and Methods for details of film fabrication and characterization).[49−51] Characterization of these coatings revealed them to have micro- and nanoscale pores and other topographic features similar to those reported in past studies (by SEM, Figure 3C, D) and average advancing water contact angles (θ) of 155 ± 1°.[49−51] We selected peptide **1** (Figure 1) as a model QSI for loading into these coatings because this peptide has been demonstrated to be one of the most potent inhibitors of agr-type QS in *S. aureus* reported to date.[16] The loading of peptide **1** into our multilayer films, however, provided an initial challenge. Our past study demonstrated that certain types of small-molecule water-soluble agents could be loaded into superhydrophobic multilayers (which are extremely nonwetting to aqueous solutions) when dissolved in organic solvents, such as acetone, that readily wet and penetrate into these porous materials.[51] While this approach to loading is useful with many types of small-molecule agents, peptide **1** is insoluble in all common solvents other than water, DMSO, and acetonitrile/water mixtures. Aqueous solutions of peptide **1** applied to the surfaces of these coatings beaded up immediately and rolled off of the coatings, and solutions of peptide **1** in DMSO or water/DMSO mixtures

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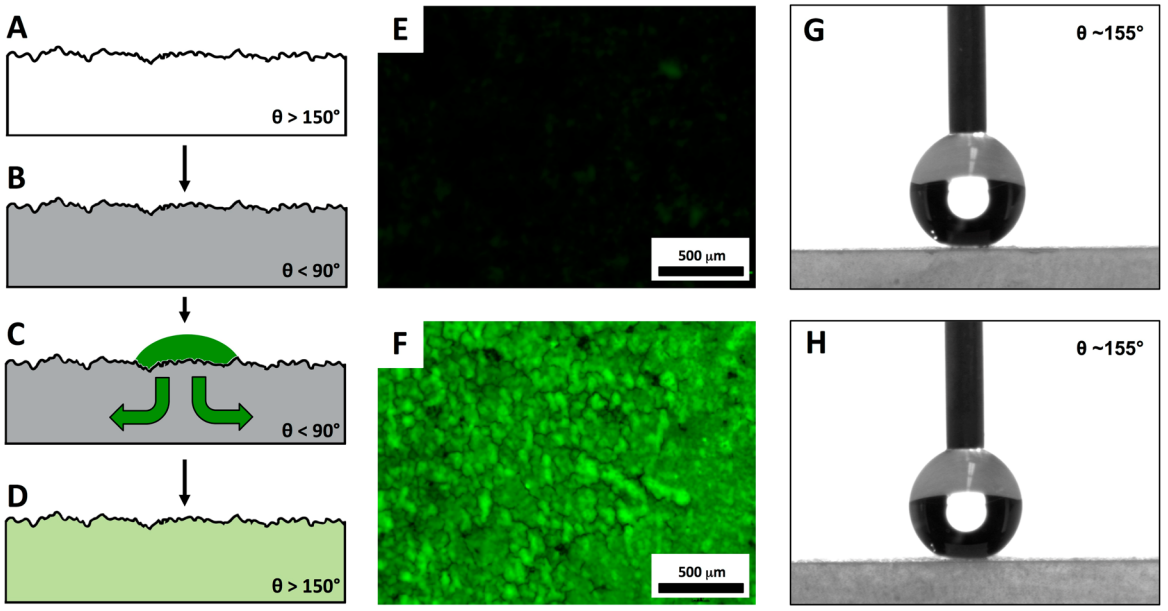

Figure 4. (A–D) Schematic illustrations showing the organic solvent-assisted approach used to load water-soluble peptides **1** and $\mathbf{1}_{FL}$ into superhydrophobic PEI/PVDMA multilayers. (A) Films as-fabricated (white) are superhydrophobic and resist wetting by water or DMSO (θ > 150°). (B, C) Temporary wetting by dichloromethane yields films (gray) that are readily wet by DMSO (θ < 90°). (C) Addition of a droplet of DMSO containing a known concentration of peptide (dark green) results in the rapid penetration and spreading of the solution into the bulk of the film. (D) Removal of solvents by evaporation yields films impregnated with peptide (light green) that are superhydrophobic and resist wetting by water to extents similar to unloaded films in A (θ > 150°). (E, F) Representative fluorescence microscopy images showing (E) a superhydrophobic film prior to the loading of peptide $\mathbf{1}_{FL}$ and (F) a superhydrophobic film after the loading of peptide $\mathbf{1}_{FL}$ using the solvent-assisted approach shown in A–D. (G, H) Images showing the advancing contact angles of water droplets placed on superhydrophobic multilayers (G) prior to ($\theta \sim 155 \pm 1°$) and (H) after ($\theta \sim 155 \pm 1°$) the loading of peptide.

failed to wet or penetrate the surfaces of these coatings in a manner sufficient to permit high loading.

To develop an approach that would enable peptide **1** to be loaded into these superhydrophobic coatings uniformly, we adapted a two-step solvent-assisted approach developed previously for the deposition and patterning of aqueous solutions of proteins on superhydrophobic PEI/PVDMA films.[64] We performed a series of experiments in which solutions of peptide in DMSO were brought in contact with superhydrophobic films that were temporarily infused with a volatile organic solvent (dichloromethane, DCM; Figure 4A, B). To facilitate characterization of loading (and the subsequent release) of peptide in experiments described below, these studies were performed using peptide $\mathbf{1}_{FL}$, an analog of peptide **1** covalently labeled with the fluorophore fluorescein (Figure 1). The addition of droplets of DMSO containing peptide $\mathbf{1}_{FL}$ (2 μL, 1.0 mM; 2.0 nmol) to these DCM-saturated films resulted in the immediate spreading and penetration of the DMSO solution into the coating (over an area of 1 cm^2 in these proof-of-concept experiments; Figure 4C, D). Subsequent evaporation of both solvents yielded films containing peptide $\mathbf{1}_{FL}$. Characterization of peptide-treated surfaces by fluorescence microscopy revealed green fluorescence distributed over the entire film-coated substrate, suggesting that solutions of DMSO were able to wick into and spread uniformly within the solvent-treated multilayers (Figure 4F; a representative image of a film prior to peptide loading is shown in Figure 4E for comparison).

Our past study on the use of this solvent-assisted approach to pattern and impregnate superhydrophobic PEI/PVDMA films with proteins (bovine serum albumin, 66.5 kDa) revealed large changes in the wetting properties of protein-treated coatings—specifically, those films became highly hydrophilic ($\theta \sim 0°$) after treatment with protein, resulting in surfaces that were rapidly wet and infiltrated by water when immersed in aqueous solutions.[64] In contrast, the contact angles of films treated with peptide $\mathbf{1}_{FL}$ here remained essentially unchanged (values of θ before and after peptide loading were ~155 ± 1°; Figure 4G, H). This result suggests that the peptide is not located or presented at the film/air interface and that the bulk of the loaded peptide resides in the interior of these porous coatings. We conclude that solvent-assisted loading provides a convenient and practical approach to loading water-soluble peptides into superhydrophobic substrates in ways that do not compromise the underlying nonwetting behavior of the porous polymer matrix. We also note that, relative to our past immersion-based approach to the loading of small molecules into these materials,[51] the approach used here permits precise and known quantities of peptide (or combinations or more than one peptide) to be loaded and varied over a broad range simply by control over the composition, volume, and/or concentration of the droplets used to treat the surface, or by subjecting the surfaces to multiple different cycles of solvent-assisted loading.

Superhydrophobic Coatings Promote the Long-Term Release of Peptidic QSIs. Objects coated with peptide-loaded superhydrophobic films remained surrounded by a visible layer or "jacket" of trapped air when they were submerged in PBS buffer at 37 °C (as shown schematically in Figure 2A; consistent with the results of our past studies and other superhydrophobic surfaces in the Cassie–Baxter state),[51,54−56,65] and released peptide $\mathbf{1}_{FL}$ into solution over a period of at least 8 months. Figure 5A shows a representative release profile for film-coated glass slides loaded with 1.0 nmol

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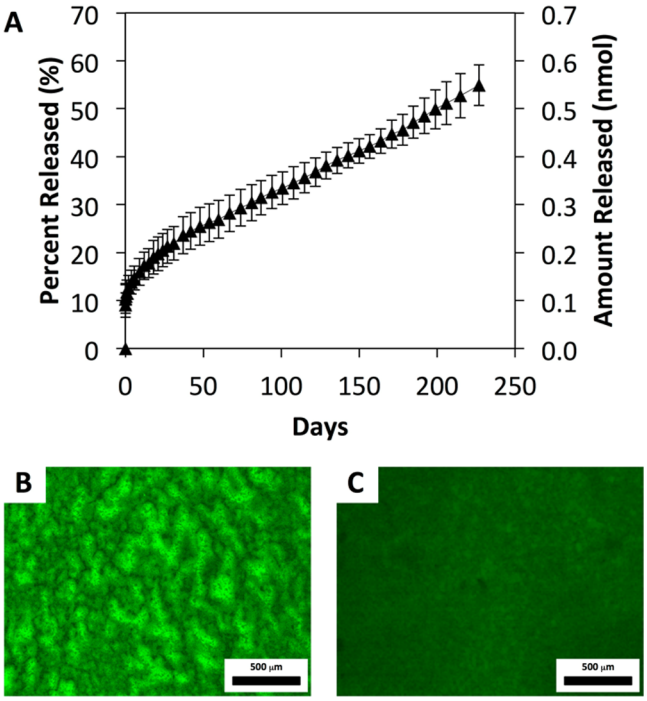

Figure 5. (A) Plot showing the release of peptide $\mathbf{1}_{FL}$ from substrates coated with peptide-loaded superhydrophobic PEI/PVDMA multilayers containing 1.0 nmol of peptide as a function of time incubated in PBS buffer (see text for details). Results are shown as the total amount of peptide released over time and as a percentage of the total amount of peptide loaded. (B, C) Representative fluorescence microscopy images showing (B) a superhydrophobic film loaded with peptide $\mathbf{1}_{FL}$ prior to incubation and (C) a superhydrophobic film loaded with peptide $\mathbf{1}_{FL}$ after incubation for 240 days.

of peptide $\mathbf{1}_{FL}$. Inspection of these data reveals ~15% of the loaded peptide to be released over the first several days of incubation, followed by the linear release of an additional ~40% over the remainder of the 240-day experiment. Fluorescence microscopy images of films acquired during this experiment revealed a substantial decrease in total fluorescence after 240 days (Figure 5C), but that the peptide was not completely released over this time period, consistent with the release profile shown in Figure 5A (a representative image of a loaded film prior to incubation is shown for comparison in Figure 5B).

Characterization of release profiles beyond 240 days in this experiment was complicated by the onset of partial delamination of the films from their underlying substrates, which was associated with the frequent handling of the substrates during these release experiments. However, on the basis of the results shown in Figure 5A, we estimate these films to have the potential to sustain the release of remaining imbedded peptide for up to an additional 9 months (~17 months for complete release of peptide $\mathbf{1}_{FL}$). Films loaded with higher amounts of peptide $\mathbf{1}_{FL}$ (2 nmol) exhibited release profiles with salient features that were similar to those shown in Figure 5A (e.g., an initial burst release, followed by an extended period of relatively linear release for at least 365 days prior to the onset of film delamination; Figure S1). We note, however, that the magnitude of the burst release was higher for those films (~30%; Figure S1), suggesting that, at higher loadings, more peptide may reside near the surfaces of the films (or, alternatively, that the peptide could begin to influence the surface or bulk wetting properties of these materials in ways that allow water to penetrate more rapidly; see discussion below). Additional physicochemical characterization will be required to characterize relationships between peptide loading and peptide release profiles more completely, and to understand the ways in which these parameters could be exploited to tune the rates and extents of peptide release under various conditions.

The release profile shown in Figure 5A is, in general, consistent with the release profiles reported in past studies for the long-term release of water-soluble small-molecules from superhydrophobic PEI/PVDMA films.[51] It is also consistent with a proposed release mechanism that involves the gradual and slow displacement of pockets of air trapped around and within the bulk of these superhydrophobic coatings by the surrounding aqueous media (as noted above and shown schematically in Figure 2).[51,54−56] The higher rate of release that occurs over the first several days is similar to "burst release" profiles observed for many other polymer-based controlled release systems,[66] and likely occurs as a result of the partial wetting of some surface features upon initial immersion, followed by the subsequent faster release of small amounts of peptide residing near the surface of the film. The observation that release slows down substantially after this initial period (and is maintained at a steady rate of ~1.6 pmol/day thereafter) suggests that water does not penetrate rapidly into the bulk of these porous materials, even after an initial breach or partial wetting of the surface of the coatings. We believe that the relatively unique "bulk" or "internal" superhydrophobicity of these porous coatings[50,51,64] plays a critical role in this context. Many conventional superhydrophobic coatings, for example, are nonporous and allow water to wet their surfaces (and thus allow water to penetrate and infiltrate their underlying substrates) rapidly once their outermost low energy surface barriers are breached. In contrast, the internal features of porous superhydrophobic materials can confer "bulk" superhydrophobicity that can prevent the rapid ingress of water into the bulk of these materials (and, in the work presented here, slow the subsequent release of peptide residing in the bulk of the material) even after surface superhydrophobicity is compromised.[51,54−56] Porous materials exhibiting such "bulk" superhydrophobicity thus appear particularly well-suited for controlled release applications in which long-term release is desired.[51,55,67]

In addition to providing means to promote extended release profiles, the ability of these porous superhydrophobic materials to halt, limit, or substantially slow the ingress of water also has potential practical implications with respect to increasing the stability and long-term storage/release of active agents that can be hydrolyzed or otherwise degraded by contact with water. Peptide **1** and peptide $\mathbf{1}_{FL}$, for example, have macrocyclic structures specifically designed to interact with AgrC-receptor proteins in *S. aureus*,[16,17] but these cyclic structures are maintained by water-labile thioester bonds. Thioester bonds of this type typically hydrolyze (with pH-dependent half-lives of approximately 4−72 h) in physiologically relevant media to yield linear peptides that are biologically inactive.[68] The hydrolytically degradable nature of these bonds may ultimately be important for native AIP-type ligands in the context of regulating QS signaling in bacteria,[69] but they are a liability with respect to the storage and maintenance of reservoirs of active peptide in wet environments (that is, if water were to penetrate a thin film containing these peptides quickly, the peptides would be rapidly converted to an inactive form—in addition to

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increasing the likelihood of more rapid release from the water-logged polymer matrix). Accordingly, a "bulk" superhydrophobic matrix that prevents or slows the entry of water could also help prevent or limit contact with water until the point at which the peptide is released into surrounding media (thus providing a means to protect the peptide from degradation and increasing the likelihood that the peptide is released in its cyclic, QS-active form for the duration of the extended period over which it is to be released). The results of bacterial assays using peptide **1**-loaded films described below provide general support for this view.

QSI-Loaded Coatings Modulate QS and Biofilm Formation in *S. aureus* for Prolonged Periods. To determine whether the peptide released from these materials is released in a QS-active form—and whether this approach could be used to design coatings that prevent or attenuate virulence factor production and biofilm formation in clinically relevant contexts—we conducted a series of experiments in which peptide-loaded superhydrophobic films were incubated directly in bacteria-containing media. We used two assays to characterize the biological activity of the released peptides: (i) a quantitative fluorescence-based assay using a group-I *S. aureus* reporter strain that produces GFP under QS control,[57] and (ii) a biofilm production assay using a wild-type group-I *S. aureus* strain.[58] We selected group-I *S. aureus* for these experiments because it is frequently linked to invasive disease and is considered one of the more clinically relevant groups among the four *S. aureus* agr classes.[69]

For all of the experiments described below, we used superhydrophobic PEI/PVDMA films loaded with peptide **1** (Figure 1), rather than fluorescein-labeled peptide $\mathbf{1}_{FL}$, because peptide $\mathbf{1}_{FL}$ contains a fluorophore that could mask characterization of activity in the GFP reporter assay. As noted above, peptide **1** is also one of the most active inhibitors of agr-based QS reported in *S. aureus* (e.g., IC_{50} = 485 pM in group-I *S. aureus*).[16,17] The lack of a fluorescent label, however, prevented us from directly characterizing the release of peptide **1** from peptide-loaded films. We therefore used films loaded with 5.0 nmol of peptide **1**, an amount five times higher than that used in the experiments described above using peptide $\mathbf{1}_{FL}$, to increase the likelihood that the amount of peptide released into solution over any 24 h period would be sufficient to yield concentrations above its IC_{50}. All long-term bacterial assays were conducted using a continuous "challenge-and-hold" approach, in which peptide-loaded films were repeatedly (i) "challenged" by incubation in fresh bacteria-containing media for a 24-h period and then removed, rinsed, and (ii) "held" by incubation in buffer free of bacteria or nutrients prior to a subsequent challenge with bacteria (see Materials and Methods for additional details). This approach permitted us to characterize the biological activity of released peptide and challenge peptide-loaded films directly over multiple 24 h periods during the extended incubation and continuous release of peptide.

Using the *S. aureus* GFP reporter strain assay to monitor peptide activity, we observed the in situ release of peptide **1** to limit the production of GFP (and thereby agr-type QS) to less than 10% (relative to control coatings without loaded peptide) through the first 28 days of these experiments (representative results are shown in Figure 6A; see Materials and Methods for details of assay protocol). Optical density measurements performed as part of these GFP assays revealed that these reductions in fluorescence did not arise from bacterial cell death

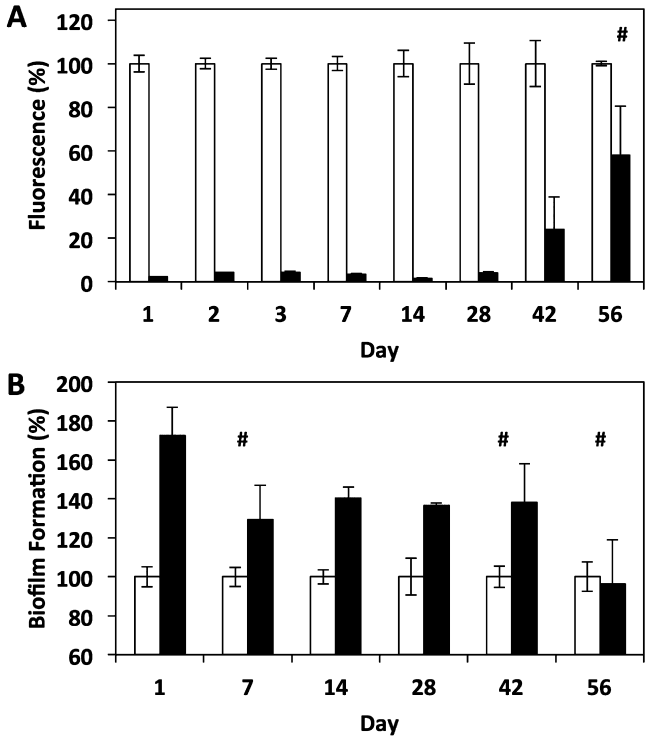

Figure 6. Plots of (A) fluorescence versus time and (B) biofilm formation versus time, normalized to controls, for the (A) *S. aureus* GFP reporter strain and (B) wild-type *S. aureus* incubated with peptide-loaded coatings during extended challenge-and-hold experiments (see text). Black bars show results for experiments using peptide-loaded films; white bars show results for control experiments using nonpeptide-loaded films. All experiments were performed in replicates of four; # indicates lack of significance ($p > 0.05$).

(Figure S3). Characterization of samples at later time points (e.g., days 42 and 56) revealed that GFP production began to increase but was still lower than that observed for untreated controls (all values from days 1 through 42 were statistically significant ($p < 0.05$)). This experiment was discontinued after day 56 because visual inspection revealed signs of film delamination and cracking at these longer time points. This behavior was similar to that observed during experiments to characterize release profiles (discussed above) but occurred much earlier in these experiments, presumably due to the additional repetitive washing and media changes required by these bacterial assays. We attribute the decrease in inhibition of GFP production at 42 and 56 days to result, at least in part (see discussion below), from these physical changes. Support for this view is provided by the results of otherwise identical experiments in which film delamination was not observed; production of GFP remained low for up to 56 days in those experiments (Figure S2A).

Dilution of released peptide **1** solutions into PBS (by 10–1000 fold) and the subsequent characterization of these solutions in our GFP reporter assay revealed a clear dose-dependent activity trend (see Figure S4), with peptide solution activity decreasing gradually over time. For example, 10-fold dilutions of released peptide solution still showed almost complete inhibition of GFP production after 7 days, whereas 100-fold dilutions showed a marked drop in activity by day 2. These results, when combined, suggest (i) that solvent-assisted loading and physical contact with these superhydrophobic coatings does not significantly limit the ability of peptide **1** to

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inhibit AgrC-I activity in *S. aureus*, and (ii) that this approach can be used to sustain the release of peptide in amounts sufficient to strongly inhibit this receptor, and thus also inhibit agr-type QS, over a period of ∼2 months.

Perhaps more importantly, these results suggest that encapsulation of peptide **1** in a superhydrophobic coating can confer some level of protection against hydrolysis during long-term storage in aqueous media. Specifically, peptide **1** remains active when it is released at later time points, even after having been immersed in an aqueous environment for prolonged periods. Without the protection of the coating, the thioester bond in the loaded peptide would have been hydrolyzed and rendered inactive if it had been solvated by water during the entirety of these prolonged release periods ($t_{1/2} \approx 4-72$ h; see discussion above). The fact that levels of inhibition were reduced at longer times in some experiments (e.g., Figure 6A; attributed, at least in part, to changes in film morphology as described above) could also hint that this form of protection may not be perfect. It is possible, for example, that reductions in inhibition observed at days 42 and 56 could also result from reductions in the amount of active, unhydrolyzed peptide that is released at those time points. We note here that these two effects could also be related (that is, film delamination, if it occurs, could enable more rapid entry of water; this would, in turn, result in more rapid hydrolysis of the peptide). More extensive analytical studies will be required to characterize the percent hydrolysis of peptides contained within or released from these films as a function of incubation time and water penetration. Nevertheless, in the context of this current study, we conclude that these superhydrophobic coatings enable the release of bioactive peptide at amounts above IC_{50} at time points well beyond what would be expected if water were to penetrate the films and hydrate or dissolve the peptide rapidly.

Finally, we characterized the ability of these peptide **1**-loaded films to modulate biofilm formation, an important virulence phenotype that is also at least partially under the control of agr-type QS in *S. aureus*.[69,70] Unlike many other known bacterial QS systems, inhibition of agr-type QS in *S. aureus* promotes biofilm formation rather than biofilm inhibition.[71,72] Indeed, *S. aureus* uses QS to disperse from biofilms and upregulate toxin production at high cell densities. The former phenotype appears to be more relevant under chronic infection conditions, whereas the latter may play a more significant role in acute infections.[73] Their interplay, however, is quite complex during bacterial colonization and both QSIs and QS agonists are of interest for therapeutic control in different stages and types of infections.[69,74] Accordingly, we reasoned that films containing peptide **1**, a QSI, should promote biofilm formation in *S. aureus* when the peptide was released.

We characterized the ability of peptide **1**-loaded superhydrophobic films to stimulate biofilm growth in wild-type group-I *S. aureus* using a standard static biofilm assay that quantifies biofilm growth via crystal violet staining (see Materials and Methods for assay details).[62,63] We tested samples of peptide **1** released from films at specific 24-h time points over several weeks using the same "challenge-and-hold" approach used in the GFP reporter strain experiments above. We observed a significant increase in biofilm formation over at least 42 days of peptide release, consistent with our findings in the reporter gene assay (Figure 6B; see Figure S2B for the results of additional experiments). Over this time period, the amount of increased biofilm growth at each time point was similar and ∼30−40% higher than the levels promoted by samples from control wells containing films that did not contain peptide **1** (differences at 7 and 42 days were statistically insignificant ($p > 0.1$); see Figure 6B).

Taken together, the results of these biofilm growth assays and the GFP reporter assays directly measuring AgrC-I activity indicate that these peptide **1**-loaded superhydrophobic coatings release active peptide at levels sufficient to inhibit agr-type QS in group-I *S. aureus* over at least 4−5 weeks. The results reported here are likely influenced, at least in part, by the assay format used for these proof of concept studies. It is likely that these release and inhibition periods could be extended or modulated in future studies by manipulating peptide loading levels or other variables such as the thickness, porosity, or degree of chemical functionalization of the coatings. We note again that stimulating biofilm growth, as evaluated here, may or may not be a desirable biomedical outcome (e.g., depending on infection type, etc.).[74] Such stimulation would, of course, be interesting for research purposes, however, as many fundamental questions remain about the interplay between QS and biofilm formation in *S. aureus* and its role in disease. In view of our past studies demonstrating the ability of peptide **1** to strongly inhibit toxin production in *S. aureus* (e.g., production of hemolysin and TSST-1),[16] we anticipate that these peptide **1**-loaded coatings will be capable of promoting the prolonged inhibition of toxin production, a phenotype of clear importance for the abatement of acute infections. Studies to evaluate the potential utility of these materials in this context are currently ongoing.

SUMMARY AND CONCLUSIONS

Materials that promote the long-term release of bioactive QSIs could attenuate bacterial virulence and biofilm formation in many important applications. Herein, we have demonstrated that polymer-based superhydrophobic coatings that are nanoporous and exhibit "bulk" or internal superhydrophobicity can be used to host and subsequently sustain the release of water-soluble, peptidic QSIs for at least 8 months. The release of peptide **1** from these films strongly inhibited agr-based QS in group-I *S. aureus* for at least 40 days without any effect on cell growth. These results, when combined, suggest that these superhydrophobic coatings can also confer protection against the rapid hydrolysis of these water-labile peptides, and thus expand the range of times over which these peptides can be made available in their bioactive forms. This approach has the potential to be general, and could prove useful for the encapsulation, physical and chemical protection, and release of other classes of water-sensitive agents. Finally, we demonstrated that these superhydrophobic coatings can strongly modulate the QS-controlled formation of biofilm in wild-type *S. aureus*. We conclude that these nanoporous superhydrophobic films provide a new and useful nonbiocidal approach to the design of coatings that attenuate bacterial virulence. We anticipate that the materials, loading strategies, and new concepts reported here will enable new approaches to the long-term attenuation of QS and associated phenotypes in a range of basic research and applied contexts.

ASSOCIATED CONTENT

Supporting Information

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Additional release profiles and the results of additional bacterial assays (PDF)

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Notes

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REFERENCES

(1) Wu, P.; Grainger, D. W. Drug/device combinations for local drug therapies and infection prophylaxis. *Biomaterials* **2006**, *27*, 2450−2467.
(2) Hetrick, E. M.; Schoenfisch, M. H. Reducing implant-related infections: active release strategies. *Chem. Soc. Rev.* **2006**, *35*, 780−789.
(3) Zilberman, M.; Elsner, J. J. Antibiotic-eluting medical devices for various applications. *J. Controlled Release* **2008**, *130*, 202−215.
(4) Lichter, J. A.; Van Vliet, K. J.; Rubner, M. F. Design of Antibacterial Surfaces and Interfaces: Polyelectrolyte Multilayers as a Multifunctional Platform. *Macromolecules* **2009**, *42*, 8573−8586.
(5) Banerjee, I.; Pangule, R. C.; Kane, R. S. Antifouling Coatings: Recent Developments in the Design of Surfaces That Prevent Fouling by Proteins, Bacteria, and Marine Organisms. *Adv. Mater.* **2011**, *23*, 690−718.
(6) Clatworthy, A. E.; Pierson, E.; Hung, D. T. Targeting virulence: a new paradigm for antimicrobial therapy. *Nat. Chem. Biol.* **2007**, *3*, 541−548.
(7) Cegelski, L.; Marshall, G. R.; Eldridge, G. R.; Hultgren, S. J. The biology and future prospects of antivirulence therapies. *Nat. Rev. Microbiol.* **2008**, *6*, 17−27.
(8) Rasko, D. A.; Sperandio, V. Anti-virulence strategies to combat bacteria-mediated disease. *Nat. Rev. Drug Discovery* **2010**, *9*, 117−128.
(9) Allen, R. C.; Popat, R.; Diggle, S. P.; Brown, S. P. Targeting virulence: can we make evolution-proof drugs? *Nat. Rev. Microbiol.* **2014**, *12*, 300−308.
(10) Fuqua, C.; Parsek, M. R.; Greenberg, E. P. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **2001**, *35*, 439−468.
(11) Camilli, A.; Bassler, B. L. Bacterial small-molecule signaling pathways. *Science* **2006**, *311*, 1113−1116.
(12) Rutherford, S. T.; Bassler, B. L. Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harbor Perspect. Med.* **2012**, *2*, a012427.
(13) Njoroge, J.; Sperandio, V. Jamming bacterial communication: New approaches for the treatment of infectious diseases. *EMBO Mol. Med.* **2009**, *1*, 201−210.
(14) Gerdt, J. P.; Blackwell, H. E. Competition studies confirm two major barriers that can preclude the spread of resistance to quorum-sensing inhibitors in bacteria. *ACS Chem. Biol.* **2014**, *9*, 2291−2299.
(15) Welsh, M. A.; Eibergen, N. R.; Moore, J. D.; Blackwell, H. E. Small Molecule Disruption of Quorum Sensing Cross-Regulation in *Pseudomonas aeruginosa* Causes Major and Unexpected Alterations to Virulence Phenotypes. *J. Am. Chem. Soc.* **2015**, *137*, 1510−1519.
(16) Tal-Gan, Y.; Stacy, D. M.; Foegen, M. K.; Koenig, D. W.; Blackwell, H. E. Highly Potent Inhibitors of Quorum Sensing in *Staphylococcus aureus* Revealed Through a Systematic Synthetic Study of the Group-III Autoinducing Peptide. *J. Am. Chem. Soc.* **2013**, *135*, 7869−7882.
(17) Tal-Gan, Y.; Ivancic, M.; Cornilescu, G.; Cornilescu, C. C.; Blackwell, H. E. Structural Characterization of Native Autoinducing Peptides and Abiotic Analogs Reveals Key Features Essential for Activation and Inhibition of an AgrC Quorum Sensing Receptor in *Staphylococcus aureus*. *J. Am. Chem. Soc.* **2013**, *135*, 18436−18444.
(18) Tal-Gan, Y.; Stacy, D. M.; Blackwell, H. E. N-Methyl and peptoid scans of an autoinducing peptide reveal new structural features required for inhibition and activation of AgrC quorum sensing receptors in *Staphylococcus aureus*. *Chem. Commun.* **2014**, *50*, 3000−3003.
(19) Stacy, D. M.; Welsh, M. A.; Rather, P. N.; Blackwell, H. E. Attenuation of quorum sensing in the pathogen *Acinetobacter baumannii* using non-native N-Acyl homoserine lactones. *ACS Chem. Biol.* **2012**, *7*, 1719−1728.
(20) Palmer, A. G.; Streng, E.; Blackwell, H. E. Attenuation of virulence in pathogenic bacteria using synthetic quorum-sensing modulators under native conditions on plant hosts. *ACS Chem. Biol.* **2011**, *6*, 1348−1356.
(21) Galloway, W. R. J. D.; Hodgkinson, J. T.; Bowden, S. D.; Welch, M.; Spring, D. R. Quorum Sensing in Gram-Negative Bacteria: Small-Molecule Modulation of AHL and Al-2 Quorum Sensing Pathways. *Chem. Rev.* **2011**, *111*, 28−67.
(22) Galloway, W. R.; Hodgkinson, J. T.; Bowden, S.; Welch, M.; Spring, D. R. Applications of small molecule activators and inhibitors of quorum sensing in Gram-negative bacteria. *Trends Microbiol.* **2012**, *20*, 449−458.
(23) Muh, U.; Schuster, M.; Heim, R.; Singh, A.; Olson, E. R.; Greenberg, E. P. Novel *Pseudomonas aeruginosa* quorum-sensing inhibitors identified in an ultra-high-throughput screen. *Antimicrob. Agents Chemother.* **2006**, *50*, 3674−3679.
(24) Amara, N.; Mashiach, R.; Amar, D.; Krief, P.; Spieser, S. A. H.; Bottomley, M. J.; Aharoni, A.; Meijler, M. M. Covalent Inhibition of Bacterial Quorum Sensing. *J. Am. Chem. Soc.* **2009**, *131*, 10610−10619.
(25) Amara, N.; Krom, B. P.; Kaufmann, G. F.; Meijler, M. M. Macromolecular Inhibition of Quorum Sensing: Enzymes, Antibodies, and Beyond. *Chem. Rev.* **2011**, *111*, 195−208.
(26) O'Loughlin, C. T.; Miller, L. C.; Siryaporn, A.; Drescher, K.; Semmelhack, M. F.; Bassler, B. L. A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 17981−17986.
(27) Breitbach, A. S.; Broderick, A. H.; Jewell, C. M.; Gunasekaran, S.; Lin, Q.; Lynn, D. M.; Blackwell, H. E. Surface-mediated release of a synthetic small-molecule modulator of bacterial quorum sensing: Gradual release enhances activity. *Chem. Commun.* **2011**, *47*, 370−372.
(28) Broderick, A. H.; Breitbach, A. S.; Frei, R.; Blackwell, H. E.; Lynn, D. M. Surface-Mediated Release of a Small-Molecule Modulator of Bacterial Biofilm Formation: A Non-Bactericidal Approach to Inhibiting Biofilm Formation in *Pseudomonas aeruginosa*. *Adv. Healthcare Mater.* **2013**, *2*, 993−1000.

DOI: 10.1021/acsbiomaterials.5b00313
ACS Biomater. Sci. Eng. 2015, 1, 1039−1049

(29) Broderick, A. H.; Stacy, D. M.; Tal-Gan, Y.; Kratochvil, M. J.; Blackwell, H. E.; Lynn, D. M. Surface Coatings that Promote Rapid Release of Peptide-Based AgrC Inhibitors for Attenuation of Quorum Sensing in *Staphylococcus aureus*. *Adv. Healthcare Mater.* **2014**, *3*, 97−105.
(30) Hume, E. B. H.; Baveja, J.; Muir, B. W.; Schubert, T. L.; Kumar, N.; Kjelleberg, S.; Griesser, H. J.; Thissen, H.; Read, R.; Poole-Warren, L. A.; Schindhelm, K.; Willcox, M. D. P. The control of *Staphylococcus epidermidis* biofilm formation and in vivo infection rates by covalently bound furanones. *Biomaterials* **2004**, *25*, 5023−5030.
(31) Baveja, J. K.; Wilcox, M. D. P.; Hume, E. B. H.; Kumar, N.; Odell, R.; Poole-Warren, L. A. Furanones as potential anti-bacterial coatings on biomaterials. *Biomaterials* **2004**, *25*, 5003−5012.
(32) Melander, C.; Moeller, P. D. R.; Ballard, T. E.; Richards, J. J.; Huigens, R. W., III; Cavanagh, J. Evaluation of dihydrooroidin as an antifouling additive in marine paint. *Int. Biodeterior. Biodegrad.* **2009**, *63*, 529−532.
(33) Ho, K. K. K.; Cole, N.; Chen, R.; Willcox, M. D. P.; Rice, S. A.; Kumar, N. Characterisation and in vitro activities of surface attached dihydropyrrol-2-ones against Gram-negative and Gram-positive bacteria. *Biofouling* **2010**, *26*, 913−921.
(34) Nowatzki, P. J.; Koepsel, R. R.; Stoodley, P.; Min, K.; Harper, A.; Murata, H.; Donfack, J.; Hortelano, E. R.; Ehrlich, G. D.; Russell, A. J. Salicylic acid-releasing polyurethane acrylate polymers as anti-biofilm urological catheter coatings. *Acta Biomater.* **2012**, *8*, 1869−1880.
(35) Gomes, J.; Grunau, A.; Lawrence, A. K.; Eberl, L.; Gademann, K. Bioinspired, releasable quorum sensing modulators. *Chem. Commun.* **2013**, *49*, 155−157.
(36) Ho, K. K. K.; Chen, R.; Willcox, M. D. P.; Rice, S. A.; Cole, N.; Iskander, G.; Kumar, N. Quorum sensing inhibitory activities of surface immobilized antibacterial dihydropyrrolones via click chemistry. *Biomaterials* **2014**, *35*, 2336−2345.
(37) Nafee, N.; Husari, A.; Maurer, C. K.; Lu, C.; de Rossi, C.; Steinbach, A.; Hartmann, R. W.; Lehr, C.-M.; Schneider, M. Antibiotic-free nanotherapeutics: Ultra-small, mucus-penetrating solid lipid nanoparticles enhance the pulmonary delivery and anti-virulence efficacy of novel quorum sensing inhibitors. *J. Controlled Release* **2014**, *192*, 131−140.
(38) Shenderovich, J.; Feldman, M.; Kirmayer, D.; Al-Quntar, A.; Steinberg, D.; Lavy, E.; Friedman, M. Local sustained-release delivery systems of the antibiofilm agent thiazolidinedione-8 for prevention of catheter-associated urinary tract infections. *Int. J. Pharm.* **2015**, *485*, 164−170.
(39) Lu, H. D.; Spiegel, A. C.; Hurley, A.; Perez, L. J.; Maisel, K.; Ensign, L. M.; Hanes, J.; Bassler, B. L.; Semmelhack, M. F.; Prud'homme, R. K. Modulating *Vibrio cholerae* Quorum-Sensing-Controlled Communication Using Autoinducer-Loaded Nanoparticles. *Nano Lett.* **2015**, *15*, 2235−2241.
(40) Buck, M. E.; Zhang, J.; Lynn, D. M. Layer-by-layer assembly of reactive ultrathin films mediated by click-type reactions of poly(2-alkenyl azlactone)s. *Adv. Mater.* **2007**, *19*, 3951.
(41) Buck, M. E.; Lynn, D. M. Azlactone-functionalized polymers as reactive platforms for the design of advanced materials: Progress in the last ten years. *Polym. Chem.* **2012**, *3*, 66−80.
(42) Broderick, A. H.; Carter, M. C. D.; Lockett, M. R.; Smith, L. M.; Lynn, D. M. Fabrication of Oligonucleotide and Protein Arrays on Rigid and Flexible Substrates Coated with Reactive Polymer Multilayers. *ACS Appl. Mater. Interfaces* **2013**, *5*, 351−359.
(43) Manna, U.; Lynn, D. M. Synthetic Surfaces with Robust and Tunable Underwater Superoleophobicity. *Adv. Funct. Mater.* **2015**, *25*, 1672−1681.
(44) Buck, M. E.; Lynn, D. M. Functionalization of Fibers Using Azlactone-Containing Polymers: Layer-by-Layer Fabrication of Reactive Thin Films on the Surfaces of Hair and Cellulose-Based Materials. *ACS Appl. Mater. Interfaces* **2010**, *2*, 1421−1429.
(45) Broderick, A. H.; Manna, U.; Lynn, D. M. Covalent Layer-by-Layer Assembly of Water-Permeable and Water-Impermeable Polymer Multilayers on Highly Water-Soluble and Water-Sensitive Substrates. *Chem. Mater.* **2012**, *24*, 1786−1795.
(46) Manna, U.; Lynn, D. M. Fabrication of Liquid-Infused Surfaces Using Reactive Polymer Multilayers: Principles for Manipulating the Behaviors and Mobilities of Aqueous Fluids on Slippery Liquid Interfaces. *Adv. Mater.* **2015**, *27*, 3007−3012.
(47) Heilmann, S. M.; Rasmussen, J. K.; Krepski, L. R. Chemistry and technology of 2-alkenyl azlactones. *J. Polym. Sci., Part A: Polym. Chem.* **2001**, *39*, 3655−3677.
(48) Buck, M. E.; Breitbach, A. S.; Belgrade, S. K.; Blackwell, H. E.; Lynn, D. M. Chemical modification of reactive multilayered films fabricated from poly(2-alkenyl azlactone)s: design of surfaces that prevent or promote mammalian cell adhesion and bacterial biofilm growth. *Biomacromolecules* **2009**, *10*, 1564−1574.
(49) Buck, M. E.; Schwartz, S. C.; Lynn, D. M. Superhydrophobic Thin Films Fabricated by Reactive Layer-by-Layer Assembly of Azlactone-Functionalized Polymers. *Chem. Mater.* **2010**, *22*, 6319−6327.
(50) Manna, U.; Broderick, A. H.; Lynn, D. M. Chemical Patterning and Physical Refinement of Reactive Superhydrophobic Surfaces. *Adv. Mater.* **2012**, *24*, 4291.
(51) Manna, U.; Kratochvil, M. J.; Lynn, D. M. Superhydrophobic Polymer Multilayers that Promote the Extended, Long-Term Release of Embedded Water-Soluble Agents. *Adv. Mater.* **2013**, *25*, 6405−6409.
(52) Feng, L.; Li, S. H.; Li, Y. S.; Li, H. J.; Zhang, L. J.; Zhai, J.; Song, Y. L.; Liu, B. Q.; Jiang, L.; Zhu, D. B. Super-hydrophobic surfaces: From natural to artificial. *Adv. Mater.* **2002**, *14*, 1857−1860.
(53) Li, X. M.; Reinhoudt, D.; Crego-Calama, M. What do we need for a superhydrophobic surface? A review on the recent progress in the preparation of superhydrophobic surfaces. *Chem. Soc. Rev.* **2007**, *36*, 1350−1368.
(54) Yohe, S. T.; Colson, Y. L.; Grinstaff, M. W. Superhydrophobic Materials for Tunable Drug Release: Using Displacement of Air To Control Delivery Rates. *J. Am. Chem. Soc.* **2012**, *134*, 2016−2019.
(55) Yohe, S. T.; Herrera, V. L. M.; Colson, Y. L.; Grinstaff, M. W. 3D superhydrophobic electrospun meshes as reinforcement materials for sustained local drug delivery against colorectal cancer cells. *J. Controlled Release* **2012**, *162*, 92−101.
(56) Yohe, S. T.; Freedman, J. D.; Falde, E. J.; Colson, Y. L.; Grinstaff, M. W. A Mechanistic Study of Wetting Superhydrophobic Porous 3D Meshes. *Adv. Funct. Mater.* **2013**, *23*, 3628−3637.
(57) Kirchdoerfer, R. N.; Garner, A. L.; Flack, C. E.; Mee, J. M.; Horswill, A. R.; Janda, K. D.; Kaufmann, G. F.; Wilson, I. A. Structural Basis for Ligand Recognition and Discrimination of a Quorum-quenching Antibody. *J. Biol. Chem.* **2011**, *286*, 17351−17358.
(58) Novick, R. P.; Ross, H. F.; Projan, S. J.; Kornblum, J.; Kreiswirth, B.; Moghazeh, S. Synthesis of Staphylococcal Virulence Factors Is Controlled by a Regulatory Rna Molecule. *EMBO J.* **1993**, *12*, 3967−3975.
(59) Carpino, L. A.; Han, G. Y. 9-Fluorenylmethoxycarbonyl amino-protecting group. *J. Org. Chem.* **1972**, *37*, 3404−3409.
(60) Albericio, F.; Carpino, L. A. Coupling reagents and activation. *Methods Enzymol.* **1997**, *289*, 104−126.
(61) Blanco-Canosa, J. B.; Dawson, P. E. An efficient Fmoc-SPPS approach for the generation of thioester peptide precursors for use in native chemical ligation. *Angew. Chem., Int. Ed.* **2008**, *47*, 6851−6855.
(62) Christensen, G. D.; Simpson, W. A.; Younger, J. J.; Baddour, L. M.; Barrett, F. F.; Melton, D. M.; Beachey, E. H. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J. Clin. Microbiol.* **1985**, *22*, 996−1006.
(63) O'Toole,G. A.; Pratt, L. A.; Watnick, P. I.; Newman, D. K.; Weaver, V. B.; Kolter, R. Genetic approaches to study of biofilms. In *Biofilms*; Methods in Enzymology; Elsevier: Amsterdam, 1999; Vol. *310*, pp 91−109.
(64) Manna, U.; Lynn, D. M. Patterning and Impregnation of Superhydrophobic Surfaces Using Aqueous Solutions. *ACS Appl. Mater. Interfaces* **2013**, *5*, 7731−7736.
(65) Yan, Y. Y.; Gao, N.; Barthlott, W. Mimicking natural superhydrophobic surfaces and grasping the wetting process: A review

DOI: 10.1021/acsbiomaterials.5b00313
ACS Biomater. Sci. Eng. 2015, 1, 1039−1049

on recent progress in preparing superhydrophobic surfaces. *Adv. Colloid Interface Sci.* **2011**, *169*, 80−105.
(66) Huang, X.; Brazel, C. S. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. *J. Controlled Release* **2001**, *73*, 121−136.
(67) Yohe, S. T.; Colson, Y. L.; Grinstaff, M. W. Superhydrophobic Materials for Tunable Drug Release: Using Displacement of Air to Control Delivery Rates. *J. Am. Chem. Soc.* **2012**, *134*, 2016.
(68) Wright, J. S.; Jin, R.; Novick, R. P. Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 1691−1696.
(69) Thoendel, M.; Kavanaugh, J. S.; Flack, C. E.; Horswill, A. R. Peptide Signaling in the Staphylococci. *Chem. Rev.* **2011**, *111*, 117−151.
(70) Boles, B. R.; Horswill, A. R. Staphylococcal biofilm disassembly. *Trends Microbiol.* **2011**, *19*, 449−455.
(71) Boles, B. R.; Horswill, A. R. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog.* **2008**, *4*, e1000052.
(72) Beenken, K. E.; Blevins, J. S.; Smeltzer, M. S. Mutation of sarA in *Staphylococcus aureus* limits biofilm formation. *Infect. Immun.* **2003**, *71*, 4206−4211.
(73) Otto, M. Quorum-sensing control in Staphylococci - A target for antimicrobial drug therapy? *FEMS Microbiol. Lett.* **2004**, *241*, 135−141.
(74) Khan, B. A.; Yeh, A. J.; Cheung, G. Y. C.; Otto, M. Investigational therapies targeting quorum-sensing for the treatment of *Staphylococcus aureus* infections. *Expert Opin. Invest. Drugs* **2015**, *24*, 689−704.

DOI: 10.1021/acsbiomaterials.5b00313
ACS Biomater. Sci. Eng. 2015, 1, 1039−1049