Surface-attached molecules control *Staphylococcus aureus* quorum sensing and biofilm development

Minyoung Kevin Kim, Aishan Zhao, Ashley Wang, Zachary Z. Brown, Tom W. Muir, Howard A. Stone and Bonnie L. Bassler

Bacteria use a process called quorum sensing to communicate and orchestrate collective behaviours, including virulence factor secretion and biofilm formation. Quorum sensing relies on the production, release, accumulation and population-wide detection of signal molecules called autoinducers. Here, we develop concepts to coat surfaces with quorum-sensing-manipulation molecules as a method to control collective behaviours. We probe this strategy using *Staphylococcus aureus*. Pro- and anti-quorum-sensing molecules can be covalently attached to surfaces using click chemistry, where they retain their abilities to influence bacterial behaviours. We investigate key features of the compounds, linkers and surfaces necessary to appropriately position molecules to interact with cognate receptors and the ability of modified surfaces to resist long-term storage, repeated infections, host plasma components and flow-generated stresses. Our studies highlight how this surface approach can be used to make colonization-resistant materials against *S. aureus* and other pathogens and how the approach can be adapted to promote beneficial behaviours of bacteria on surfaces.
Detection of AIP launches the autoinduction positive feedback loop that increases AIP production, resulting in amplification of the quorum-sensing response. There are four S. aureus Agr allelic variants (I to IV), which make four AIPs differing only in a few amino-acid residues. AIPs activate quorum sensing in the S. aureus cells that produce them, and they generally inhibit quorum sensing in heterologous S. aureus cells possessing different AIP variants. In the S. aureus agr-I strain that we study here, AIP-I is the native autoinducer. TrAIP-II, a truncated AIP-II with the exocyclic tail replaced by an acetyl group, is a universal inhibitor for all four agr quorum-sensing systems. TrAIP-II competes with AIP-I, the reporter strain encoding transcription factor SarA, and agrP3 is activated by AgrA → P in response to quorum sensing and AIP accumulation. A constitutively expressed sarAP1 drives expression of the gene encoding transcription factor SarA, and agrP3 is activated by SarAP1. The plasmid also harbours the Agr-activated mkate2 reporter. In response to exogenously provided agonists and antagonists. AIP-I, the reporter strain GFPMut2 is activated and red fluorescence is observed. This quorum-sensing system is found worldwide in nosocomial infections. We use AIP-I as an autoinducer agonist and we use TrAIP-II as a competitive antagonist.

**Results**

To follow the quorum-sensing status of S. aureus cells growing on unmodified and chemically modified surfaces, we used confocal microscopy and an S. aureus reporter strain that produces the fluorescent protein mKate2 in response to exogenous addition of AIP-I. The reporter strain is a ΔagrBDCA strain harbouring a multicopy plasmid carrying agrCA driven by the native agrP2 promoter. The plasmid also harbours the Agr-activated agrP3 promoter fused to mkate2. Therefore, in response to exogenously provided AIP-I, the reporter strain fluoresces red. This quorum-sensing response can be repressed by the administration of TrAIP-II (Fig. 1a). A constitutively expressed sarAP1-gfpmut2 gene was introduced onto the chromosome to enable normalization of quorum-sensing responses. We validated the reliability of our reporter strain before examining the effects of surface-attached quorum-sensing modulators, noting some heterogeneity in expression level (Supplementary Fig. 1 and accompanying text).

To demonstrate that the S. aureus reporter strain faithfully reports on Agr quorum sensing, we incubated it in the absence and presence of 100 nM AIP-I (that is, the agonist).

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**Figure 1** A strategy to quantify the Agr quorum-sensing responses of S. aureus to exogenously supplied agonists and antagonists. a. The S. aureus reporter strain used in this study. b. Fluorescence images of the S. aureus reporter strain from after 3 h incubation with buffer or buffer containing 100 nM AIP-I. Left panels (i) and (vi): constitutive sarAP1-gfpmut2 reporter. Middle panels (ii) and (v): quorum-sensing-controlled agrP3-mKate2 reporter. In S. aureus, sarAP1 is expressed constitutively and drives expression of the gene encoding transcription factor SarA, and agrP3 is activated by AgrA → P in response to quorum sensing and AIP accumulation. Right panels (iii) and (vi): merged images from the left and middle panels. Top panels (i), (ii) and (iii): buffer. Bottom panels (iv), (v) and (vi): 100 nM AIP-I. c. Fluorescence images of the S. aureus reporter strain following 3 h incubation with TrAIP-II or combinations of AIP-I and TrAIP-II. Left panels (i), (iv) and (vii): constitutive sarAP1-gfpmut2 reporter. Middle panels (ii), (v) and (viii): quorum-sensing-controlled agrP3-mKate2 reporter. Right panels (iii), (vi) and (ix): merged images from the left and middle panels. Top panels (i), (ii) and (iii): 2.5 μM TrAIP-II + 100 nM AIP-I. Middle panels (iv), (v) and (vi): 250 nM TrAIP-II + 100 nM AIP-I. Bottom panels (vii), (viii) and (ix): 2.5 μM TrAIP-II + 100 nM AIP-I. In b and c, images are based on n = 3 independent replicates. Scale bars, 20 μm.
was produced irrespective of the presence of AIP-I, but mKate2 was produced only when AIP-I was supplied to the cells (Fig. 1b). When the reporter strain was simultaneously provided with 100 nM AIP-I and 2.5 μM TrAIP-II (that is, the antagonist), little mKate2 production occurred, indicating inhibition of Agr quorum sensing (Fig. 1c(iii)). Reducing the TrAIP-II antagonist concentration by tenfold caused a corresponding ninefold decrease in quorum-sensing inhibition (Fig. 1c(v)). Addition of TrAIP-II alone elicited no mKate2 production (Fig. 1c(viii)).

**Surface-attached AIP-I activates quorum sensing.** Our overarching goal was to investigate whether we can positively and negatively manipulate *S. aureus* Agr quorum sensing using surface-attached compounds. To achieve this goal, we used copper-catalysed azide-alkyne cyclo-addition click chemistry to attach active compounds to surfaces33. We chose polyethylene glycol (PEG) polymers decorated with azide groups as the surface linkers because PEG is flexible, hydrophilic and non-bulky, and we used glass as a model surface material.

To make the AIP-I amenable to surface attachment, we synthesized AIP-I containing an alkyne at the N terminus (Supplementary Figs 2a and 3a and Methods). We call this compound ‘Alkyne-AIP-I’ (Fig. 2a). Before attaching the Alkyne-AIP-I to the surface linkers, we carried out the click reaction between the Alkyne-AIP-I and a surface-free version of the PEG₃₃₀-azide linker to test if the AIP-I derivatives retained their activation capability following the click reaction. The reaction between the alkyne and azide produces a triazole ring linking AIP-I to the PEG₃₃₀ polymer. We call this compound ‘PEG₃₃₀-triazole-AIP-I’ (Fig. 2a(iii) and Supplementary Figs 2a and 3b). In solution, AIP-I, Alkyne-AIP-I and PEG₃₃₀-triazole-AIP-I activated Agr quorum sensing in the *S. aureus* reporter strain with similar efficacy, albeit with different potencies (see ‘Solution assay’, Fig. 2a(iii),b). The calculated EC₅₀ (effective half-maximal concentration for activation) values were 28 nM (±3) for AIP-I, 190 nM (±10) for Alkyne-AIP-I and 1.1 μM (±0.20) for PEG₃₃₀-triazole-AIP-I (Table 1). Addition of 2.5 μM TrAIP-II repressed the Agr quorum-sensing response to all three compounds by a comparable magnitude (Fig. 2b). We interpret these results to mean that, analogous to AIP-I, both Alkyne-AIP-I and PEG₃₃₀-triazole-AIP-I activate quorum sensing by targeting the AgrC-I receptor.

For the surface modification work, we used silanization and maleimide-thiol chemistry to decorate the surface with PEG₁₀₀₀₀ polymers carrying azide moieties at one end (Supplementary Fig. 4 and Methods). We name this unit the ‘Surface-PEG₁₀₀₀₀-azide’.

Calculations of the height of the PEG brush suggest that a PEG₁₀₀₀₀ polymer would have sufficient length to span the peptidoglycan layer and position the attached AIP-I to interact with the AgrC-I receptor located on the cell membrane. We could not, however, carry out our companion solution assay with the PEG₁₀₀₀₀ polymer attached to AIP-I due to limitations in the purification and characterization of soluble PEG₁₀₀₀₀ entities. In solution, PEG₁₀₀₀₀ polymers adopt a random-coiled configuration that does not mimic the extended conformation they possess when they are attached to surfaces with high grafting density34. We carried out the click reaction to covalently attach the Alkyne-AIP-I to the Surface-PEG₁₀₀₀₀-azide (Fig. 2a(iv)), generating ‘Surface-PEG₁₀₀₀₀-triazole-AIP-I’. To examine whether the PEG-triazole-AIP-I moiety remained functional when attached to the surface, we provided the *S. aureus* reporter strain to the Surface-PEG₁₀₀₀₀-triazole-AIP-I in microfluidic chambers. The Agr quorum-sensing response was induced (see ‘Surface assay’, Fig. 2a(iv),c). Compared to *T* = 0 h, when cells were in the quorum-sensing-off mode, the response was activated over time and reached an average of 25-fold activation at *T* = 6 h (Fig. 2c,d, Supplementary Fig. 5a and Supplementary Video 1). These results suggest that the surface-attached AIP-I is indeed recognized as an autoinducer by the cognate membrane-bound AgrC-I receptor. To confirm our interpretation of surface-tethered AIP-I eliciting the Agr quorum-sensing response in *S. aureus*, 2.5 μM TrAIP-II antagonist was provided in solution to the *S. aureus* cells residing on the AIP-I-coated surface. The AIP quorum-sensing response was repressed (Fig. 2c and Supplementary Fig. 5b(ii)).

To reinforce the above results, we show that *S. aureus* did not activate a quorum-sensing response when introduced onto the identical surface lacking the triazole-AIP-I decoration (Surface-PEG₄₀₀₀⁰ₐzide) or that had not undergone the click reaction (Surface-PEG₄₀₀₀⁻triazole) (Fig. 2c and Supplementary Fig. 5b(i,iii)). Furthermore, the reporter strain that was presented with a surface coated with the identical PEG₄₀₀₀₀ polymer attached, via a triazole ring, to a ring-opened version of AIP-I did not elicit a response (compound 6 in Supplementary Fig. 2a, Fig. 2c and Supplementary Fig. 5b(iv); called Surface-PEG₄₀₀₀⁻triazole-Linear-AIP-I). Identical results were obtained using the Surface-PEG₄₀₀₀⁻triazole-AIP-I in which the thioester ring was opened via treatment with 10 mM cysteine (Supplementary Fig. 5b(v)). Consistent with these results, an *S. aureus* reporter strain lacking AgrC-I did not respond to the surface-attached AIP-I (Supplementary Fig. 5b(vi)). Thus, surface-attached AIP-I specifically and reversibly binds to AgrC-I, indicating that it functions as an autoinducer.

**Crucial features of surface-attached quorum-sensing molecules.** We investigated the requirements for the surface-attached AIP-I to bind AgrC-I and activate Agr quorum sensing in *S. aureus* by changing particular features of the tethered molecules. Presumably, AgrC-I diffuses freely in the membrane35. In *S. aureus*, the plasma membrane is covered by a 15–30 nm peptidoglycan layer with 4– to 5-nm-diameter pores36. Peptidoglycan is considered to be an elastic mesh network that can expand, contract and tolerate transport of globular molecules of up to 100 kDa (ref. 36). First, we examined how altering the length of the polymer attaching the AIP-I to the surface influenced the *S. aureus* Agr quorum-sensing response. We attached AIP-I with two different surface linkers: Surface-PEG₁₀₀₀₀⁻azide and Surface-PEG₄₀₀₀⁻azide. Using atomic force microscopy, the heights of the linkers were measured to be 26.5 nm (±2.5) and 1.9 nm (±0.2), respectively. The calculated Flory radius of PEG₁₀₀₀₀ in solution is ~10 nm (ref. 34), indicating that the surface-attached PEG₁₀₀₀₀ exists in an extended configuration due to local crowding. This configuration suggests that the terminal functional groups are exposed and thus could properly position attached AIP-I molecules to interact with AgrC-I receptors. Indeed, we confirmed that the azide moieties on both polymers were amenable to the click reaction using alkyne-functionalized dyes (Surface-PEG₁₀₀₀₀⁻azide, Supplementary Fig. 4c; Surface-PEG₄₀₀₀⁻azide, Supplementary Fig. 6a). Finally, we attached the Alkyne-AIP-I to both surfaces, and found that the Surface-PEG₁₀₀₀₀⁻triazole-AIP-I activated the *S. aureus* Agr quorum-sensing response, whereas the Surface-PEG₄₀₀₀⁻triazole-AIP-I did not (Supplementary Fig. 6b,c). We presume that the latter result is due to a geometrical restriction related to the PEG₁₀₀₀₀ length and this shorter linker does not appropriately position the AIP-I to access AgrC-I receptors on the plasma membrane. As a control, we show that the *S. aureus* reporter strain could activate Agr quorum sensing on the Surface-PEG₄₀₀₀⁻triazole-AIP-I if it was also provided with 50 nM AIP-I in solution (Supplementary Fig. 6b,c(iii)).

To examine how the surface coverage density of PEG₁₀₀₀₀⁻triazole-AIP-I affects the activation of AgrC-I-directed quorum sensing, we measured the number of reacted azides in a unit area on the Surface-PEG₁₀₀₀₀⁻azide following the click reaction. To do this, we clicked a fluorescent dye harbouring an alkyne to the Surface-PEG₁₀₀₀₀⁻azide (Supplementary Fig. 4c(iii)). The intensity of fluorescence from the surface is directly proportional to the number of reacted azide moieties. We obtained an average intensity for each dye molecule.
which enabled us to calculate the coverage density of reacted azides by dividing the total integrated intensity in a unit surface area by the average single-molecule intensity (see Methods). We calculate the coverage density of the clicked azide to be $2.1 \times 10^4 \mu m^{-2} (\pm 0.11)$. We assume that the Alkyne-dye and the Alkyne-AIP-I have identical reactivity in the click reaction \(^{33}\), thus rendering the same surface coverage density, which is sufficient to stimulate the \(S.\) \textit{aureus} Agr quorum-sensing response (Supplementary Fig. 6e). We reduced the surface coverage density of active AIP-I by mixing the Alkyne-AIP-I and the Alkyne-Linear-AIP-I (that is, the inactive counterpart) at different ratios before attachment to the surface. We found that Agr quorum-sensing output, which is defined as the quorum-sensing-controlled reporter output divided by the output from the constitutive reporter, in response to AIP-I (circles), Alkyne-AIP-I (squares), PEG\(_{330}\)-triazole-AIP-I (triangles), AIP-I + 2.5 \(\mu\)M TrAIP-II (asterisks), Alkyne-AIP-I + 2.5 \(\mu\)M TrAIP-II (diamonds) and PEG\(_{330}\)-triazole-AIP-I + 2.5 \(\mu\)M TrAIP-II (inverted triangles). Data points indicate means and error bars denote standard deviations from triplicate experiments. Normalized Agr quorum-sensing output from the \(S.\) \textit{aureus} reporter strain measured as a function of time in the presence of Surface-PEG\(_{10000}\)-triazole-AIP-I (asterisks), Surface-PEG\(_{10000}\)-triazole-AIP-I + 2.5 \(\mu\)M TrAIP-II in solution (diamonds), Surface-PEG\(_{10000}\) (squares), Surface-PEG\(_{10000}\)-azide (circles) and Surface-PEG\(_{10000}\)-triazole-Linear-AIP-I (triangles). Supplementary Fig. 5 provides details of the chemical procedures in each panel. Normalized quorum-sensing outputs were measured as means from 1,000 to 4,000 individual cells in each experiment. Data points indicate means and error bars denote standard deviations from triplicate experiments. An analysis of variance (ANOVA) test with Tukey–Kramer post hoc analysis was used to assess the statistical significance between the means for the AIP-I-coated surface and the control surfaces using the \(T=6\) data (\(P<0.0001\) for all pairwise comparisons). Representative merged fluorescence images of the \(S.\) \textit{aureus} reporter strain on the Surface-PEG\(_{10000}\)-triazole-AIP-I at \(T=0\), 2, 4 and 6 h. Images are based on \(n=3\) independent experiments. One representative image for each condition was chosen from ~50 images acquired from different regions of each surface. Scale bar, 20 \(\mu\)m. a.u., arbitrary units.

**Table 1 | EC\(_{50}\) values (from \(n=3\) experiments) for AIP-I and its derivatives and IC\(_{50}\) values (from \(n=4\) experiments) for TrAIP-II and its derivatives when AIP-I is present at 100 nM (that is, EC\(_{95}\)).**

| EC\(_{50}\) (nM) | AIP-I | Alkyne-AIP-I | PEG\(_{330}\)-triazole-AIP-I |
|----------------|-------|--------------|-----------------|
|                 | 28 (±3) | 190 (±40) | 1,100 (±200) |
| IC\(_{50}\) (\(\mu\)M) | TrAIP-II | Alkyne-TrAIP-II | PEG\(_{330}\)-triazole-TrAIP-II |
|                 | 1.5 (±0.5) | 0.21 (±0.05) | 3.1 (±1.5) |

EC\(_{50}\) and IC\(_{50}\) are the effective half-maximal concentrations for activation and inhibition, respectively.

Supplementary Fig. 6d), which enabled us to calculate the coverage density of reacted azides by dividing the total integrated intensity in a unit surface area by the average single-molecule intensity (see Methods). We calculate the coverage density of the clicked azide to be $2.1 \times 10^4 \mu m^{-2} (\pm 0.11)$. We assume that the Alkyne-dye and the Alkyne-AIP-I have identical reactivity in the click reaction \(^{33}\), thus rendering the same surface coverage density, which is sufficient to stimulate the \(S.\) \textit{aureus} Agr quorum-sensing response (Supplementary Fig. 6e). We reduced the surface coverage density of active AIP-I by mixing the Alkyne-AIP-I and the Alkyne-Linear-AIP-I (that is, the inactive counterpart) at different ratios before attachment to the surface. We found that Agr quorum-sensing output, which is defined as the quorum-sensing-controlled reporter output divided by the output from the constitutive reporter, in response to AIP-I (circles), Alkyne-AIP-I (squares), PEG\(_{330}\)-triazole-AIP-I (triangles), AIP-I + 2.5 \(\mu\)M TrAIP-II (asterisks), Alkyne-AIP-I + 2.5 \(\mu\)M TrAIP-II (diamonds) and PEG\(_{330}\)-triazole-AIP-I + 2.5 \(\mu\)M TrAIP-II (inverted triangles). Data points indicate means and error bars denote standard deviations from triplicate experiments. An analysis of variance (ANOVA) test with Tukey–Kramer post hoc analysis was used to assess the statistical significance between the means for the AIP-I-coated surface and the control surfaces using the \(T=6\) data (\(P<0.0001\) for all pairwise comparisons). Representative merged fluorescence images of the \(S.\) \textit{aureus} reporter strain on the Surface-PEG\(_{10000}\)-triazole-AIP-I at \(T=0\), 2, 4 and 6 h. Images are based on \(n=3\) independent experiments. One representative image for each condition was chosen from ~50 images acquired from different regions of each surface. Scale bar, 20 \(\mu\)m. a.u., arbitrary units.

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| EC\(_{50}\) (nM) | AIP-I | Alkyne-AIP-I | PEG\(_{330}\)-triazole-AIP-I |
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| IC\(_{50}\) (\(\mu\)M) | TrAIP-II | Alkyne-TrAIP-II | PEG\(_{330}\)-triazole-TrAIP-II |
|                 | 1.5 (±0.5) | 0.21 (±0.05) | 3.1 (±1.5) |

EC\(_{50}\) and IC\(_{50}\) are the effective half-maximal concentrations for activation and inhibition, respectively.
sensing activation depends on the surface-attached AIP-I coverage density (Supplementary Fig. 6e). At a coverage density below $2.1 \times 10^2 \mu m^{-2}$, the surface-attached AIP-I did not elicit the quorum-sensing response in S. aureus, and above a coverage density of $1.6 \times 10^3 \mu m^{-2}$, the response was saturated (Supplementary Fig. 6e). Together, our results show that the length of the PEG polymer and the coverage density of the surface-bound AIP-I molecules are key factors for AgrC-I-directed quorum-sensing activation.

**Quorum sensing is inhibited by surface-attached TrAIP-II.**

We investigated whether a quorum-sensing antagonist, when immobilized on a surface, could interfere with Agr quorum-sensing signal transduction. Using the above strategy, we synthesized a clickable quorum-sensing antagonist, the Alkyne-TrAIP-II (Supplementary Figs 2b and 3c), and attached it to the PEG$_{330}$-azide in solution and to the Surface-PEG$_{10000}$-azide (Fig. 3a), yielding PEG$_{330}$-triazole-TrAIP-II and Surface-PEG$_{10000}$-triazole-TrAIP-II, respectively. We characterized the products as outlined above for AIP-I (Supplementary Fig. 3d). In solution, in the absence of AIP-I, the PEG$_{330}$-triazole-TrAIP-II elicited no Agr quorum-sensing activation (Fig. 3b). When AIP-I was supplied at 0.1 $\mu M$ (that is, at its EC$_{95}$), all of the TrAIP-II derivatives (that is, Alkyne-TrAIP-II and PEG$_{330}$-triazole-TrAIP-II) showed dose-dependent inhibition of Agr quorum sensing (Fig. 3b). The Alkyne-TrAIP-II was the most potent of the compounds (Table 1). We presume that modification of the inhibitor with the alkyne at the N-terminus endows the inhibitor with enhanced accessibility to the receptor and/or tighter binding.
We next examined quorum-sensing inhibition by surface-bound TrAIP-II. Introduction of the S. aureus reporter strain to the Surface-PEG$_{10000}$-azide in the presence of AIP-I in solution at its EC$_{50}$ (30 nM) resulted in activation of Agr quorum sensing (Fig. 3c and Supplementary Fig. 7). However, if, in the presence of 30 nM AIP-I, the strain was presented to the Surface-PEG$_{10000}$-triazole-TrAIP-II, quorum-sensing inhibition occurred (Fig. 3c,d and Supplementary Video 2), albeit with modest activation occurring at $T = 6$ h (Supplementary Fig. 7(i)). As controls, we increased the AIP-I concentration to 1 μM in solution and found that this was sufficient to outperform the surface-bound TrAIP-II, causing strong activation of Agr quorum sensing over the 6 h period of the experiment (Fig. 3c and Supplementary Fig. 7(ii)). This result indicates that inhibition by surface-bound TrAIP-II is competitive, as it is in solution. Furthermore, Agr quorum-sensing inhibition did not significantly occur when S. aureus cells were added to the Surface-PEG$_{10000}$-triazole-Linear-TrAIP-II (Fig. 3c and Supplementary Fig. 7(iv)), demonstrating the requirement for specific structural elements in the antagonist.

**Wild-type S. aureus responds to surface-attached quorum-sensing molecules.** We investigated whether surface-attached pro- and anti-quorum-sensing molecules could control S. aureus behaviours in the more natural and clinically relevant context of S. aureus strains that are capable of producing AIP-I (ref. 37), unlike the reporter strain used above. For this analysis, we introduced the fluorescent quorum-sensing reporter genes into wild-type S. aureus agr-I (RN6390b) and S. aureus MRSA agr-I. In the presence of the Surface-PEG$_{10000}$-azide, S. aureus agr-I activated Agr quorum sensing in response to the accumulation of endogenously produced AIP-I (Fig. 4a,b(i) and Supplementary Video 3). The Surface-PEG$_{10000}$-triazole-AIP-I caused an earlier and higher-magnitude induction than did the Surface-PEG$_{10000}$-azide, showing that S. aureus agr-I responded to both the surface-tethered AIP-I and to its endogenously produced AIP-I (Fig. 4a,b(ii) and Supplementary Video 3). The attached wild-type cells clearly responded to the surface-attached AIP-I (Fig. 4b(ii)). This event elicited the positive autoinduction feedback loop, causing the surface-adhered S. aureus cells to induce the production and release of endogenous, soluble, AIP-I autoinducer. That autoinducer, in turn, activated the Agr quorum-sensing response in unattached S. aureus cells residing in solution above the surface, as shown by their red fluorescence emission (Supplementary Fig. 8a(ii)). Thus, attached cells, if they have the capacity to make autoinducer, can rapidly propagate the signal to neighbouring, non-surface-adhered cells. The Surface-PEG$_{10000}$-triazole-TrAIP-II repressed the Agr quorum-sensing response of the surface-adhered S. aureus cells over the 6 h period of the experiment (Fig. 4a,b(iii) and Supplementary Video 3). Furthermore, the surface-attached TrAIP-II also repressed the quorum-sensing response of the neighbouring, non-surface-adhered S. aureus cells (Supplementary Fig. 8a(iii)). Presumably, surface-attached TrAIP-II, by repressing quorum sensing in the surface-adhered S. aureus cells, decreased their endogenous production of AIP-I, retarding the autoinduction feedback loop, which, in turn, delayed signal propagation beyond the surface. Consistent with this interpretation, exogenous provision of 1 μM AIP-I in solution relieved inhibition from the Surface-PEG$_{10000}$-triazole-TrAIP-II (Fig. 4a,b(iv) and Supplementary Video 3), confirming, as above, that the Surface-PEG$_{10000}$-triazole-TrAIP-II functions competitively. Analogous results were obtained using the clinical pathogen S. aureus MRSA (Supplementary Fig. 8b), which highlights the generality of our results and approach for regulating quorum sensing by surface modification.

Next, we explored how the modified surfaces influence S. aureus biofilm colonization dynamics. We introduced a constitutively expressed mKO fluorescent reporter into the wild-type S. aureus agr-I strain and grew the strain on the Surface-PEG$_{10000}$-azide, the Surface-PEG$_{10000}$-triazole-AIP-I and the Surface-PEG$_{10000}$-triazole-TrAIP-II (Fig. 4c, d); all surfaces had comparable initial cell attachment (Supplementary Fig. 8c(i)). Consistent with our above quorum-sensing transcriptional reporter results and with the known role for Agr quorum sensing in triggering biofilm dispersal in S. aureus, when grown on the Surface-PEG$_{10000}$-triazole-AIP-I, the S. aureus agr-I strain induced biofilm dispersal, which resulted in an ~80% reduction in biofilm coverage compared to when the strain was grown on the Surface-PEG$_{10000}$-azide. When the S. aureus agr-I strain was grown on the Surface-PEG$_{10000}$-triazole-TrAIP-II, repression of Agr quorum sensing increased biofilm coverage approximately twofold compared to when the strain was grown on the Surface-PEG$_{10000}$-azide. Analogous results were obtained using the wild-type S. aureus agr-II (that is, subgroup II: RN6607) strain grown on the Surface-PEG$_{10000}$-azide, the Surface-PEG$_{10000}$-triazole-AIP-I and the Surface-PEG$_{10000}$-triazole-TrAIP-II (Supplementary Fig. 8c(ii)). Specifically, in the S. aureus agr-II strain, Agr quorum sensing was activated by the surface-attached AIP-II, which results in biofilm dispersal, and quorum sensing was inhibited by the Surface-PEG$_{10000}$-triazole-TrAIP-II, which results in increased biofilm coverage of the surface.

**Applications of surface-attached quorum-sensing molecules.** The analyses presented here focused on glass surfaces. However, other surfaces are relevant in industry and medicine, for example metals and plastics. To expand our results, we chemically modified gold and polydimethylsiloxane (PDMS) surfaces with the dye-triazole-PFG moieties (Supplementary Fig. 8d). Given the effectiveness and specificity of the click reaction, our preliminary results with the dye molecules suggest that AIP-I or TrAIP-II can be attached to these two surfaces. These results suggest that the logic used here is general and could be broadened to other materials as well as other molecules of interest.

Having a reasonable shelf lifetime will also be crucial for chemically coated surfaces in medical and industrial applications. We examined the long-term stability of the Surface-PEG$_{10000}$-triazole-dye and the Surface-PEG$_{10000}$-triazole-AIP-I as a proxy for modified surface longevity. Irrespective of the presence or absence of S. aureus cells, the intensity of the surface-attached dye did not change for 15 h (Fig. 5a). In storage, in the absence of cells, the intensity did not change for 40 days (Supplementary Fig. 8e(i)). Furthermore, the response of S. aureus cells to AIP-I-attached surfaces that had been stored for 40 days was identical to that of freshly generated surfaces (Supplementary Fig. 8e(ii)). We interpret these findings to mean that the surface-tethered entities do not detach over time and the components remain stable for long time periods. These results are especially important in the context of materials such as submerged prosthetics, as our results suggest that concerns about cytotoxicity due to leaching or instability may not be highly relevant to the strategy we have developed.

To further explore issues pertaining to medical applications of modified surfaces, we performed the quorum-sensing analysis in the presence of human blood plasma because S. aureus frequently causes bacteraemia. Having blood plasma present throughout the experiment did not alter the ability of the AIP-I-coated surface to stimulate S. aureus quorum sensing (Supplementary Fig. 8f). We also investigated the long-term effectiveness of surface-attached quorum-sensing molecules to repeated ‘infection’. We introduced the S. aureus reporter strain to the Surface-PEG$_{10000}$-triazole-AIP-I (Fig. 5b(i)) and, after 3 h, we removed the S. aureus cells by repeatedly introducing low shear ($\text{shear} = 0.03$) and air bubbles$^{58}$. We subsequently added a second dose of S. aureus reporter cells to the surface (Fig. 5b(ii)). The two S. aureus reporter strains were labelled with different constitutively fluorescent colours so we could monitor each of them. Cells
from both the first and second inoculations colonized the same region (Fig. 5b(iii)), and both responded to the modified surface, activating Agr quorum sensing to a comparable magnitude (Fig. 5b(iv)). Thus, the surface-attached molecules remained effective following mechanical shear. This feature is especially important in the biomedical arena, where long-term activity of surface-attached molecules will be essential to combat repeated infection.

Finally, we demonstrate the potential to generate multifunctional surfaces by simultaneously attaching an equal mixture of red and green dyes to the same surface (Fig. 5c). As noted, we estimate that there are 2.1 × 10⁴ µm⁻² sites on the surface available for attachment to biomolecules (Supplementary Fig. 6e). However, an AIP-I surface coverage density of 1.6 × 10⁴ µm⁻² is sufficient to fully induce *S. aureus* Agr quorum sensing (Supplementary Fig. 6e). Thus, ~25% of the sites (or 0.5 × 10⁴ µm⁻²) are not required to achieve the maximal quorum-sensing response. These sites could conceivably be used to attach a different biomolecule, for example, one with orthogonal activity. Thus, our preliminary results suggest surprising versatility in this approach: quorum-sensing molecules conceivably be used to attach a different biomolecule, for example, one with orthogonal activity. Thus, our preliminary results suggest surprising versatility in this approach: quorum-sensing molecules could be simultaneously or sequentially attached in combination
with other biomolecules, such as antimicrobial agents, enzymes or perhaps quorum-sensing compounds that target other bacteria.

**Discussion**

We have discovered and documented that surface-attached molecules can be used successfully to control bacterial quorum sensing, which has potential implications for the development of anti-biofouling or anti-colonization materials in industry and medicine, respectively. As our test case, we have considered a crucial medical example: *S. aureus* Agr quorum sensing. In *S. aureus*, Agr quorum-sensing activation leads to the production of a battery of virulence factors that are responsible for invasion and dissemination in host tissues. Agr quorum sensing in *S. aureus* also activates the biofilm disassembly process. Thus, precisely manipulating Agr quorum sensing using synthetic strategies that rely on soluble or surface-bound compounds will require tuning.

**Figure 5 | Features of autoinducer-attached surfaces.**

(a) Stability of surface-attached molecules. Mean surface fluorescence intensities were measured from the Surface-PEG10000-triazole-dye over time in the presence and absence of *S. aureus* cells. Alexa Fluor 555 alkyne dye (Thermo Fisher) was attached to the surface using the click reaction. Data points indicate means and error bars denote standard deviations from *n* = 4 experiments.

(b) Stability of surface-attached AIP-I and its activity following repeated introduction of *S. aureus* cells. (i) Relevant genotypes and fluorescent reporters used for the multiple inoculations (mtur2 denotes mturquoise2). (ii) Representative merged fluorescence images of the *S. aureus* reporter strains on the Surface-PEG10000-triazole-AIP-I. At *T* = 0 h, the *S. aureus* reporter strain (quorum-sensing-off, blue; quorum-sensing-on, purple) was introduced. At *T* = 3 h, *S. aureus* cells were washed off the surface and the second reporter strain (quorum-sensing-off, green; quorum-sensing-on, yellow) was introduced to the same surface. Scale bar, 20 μm. (iii) Merged fluorescence images of the area colonized by both strains. The constitutive fluorescent colours from the two *S. aureus* reporter strains (blue, *T* = 3 h; green, post-wash, *T* = 3 h) were artificially aligned. The light blue area with the white circles shows that cells from both the first and second inoculations colonized the same region. Scale bar, 20 μm. In (ii) and (iii), images are based on *n* = 3 independent experiments.

(c) Mixtures of dye molecules containing alkyne moieties can be simultaneously attached to the Surface-PEG10000-azide. Dyes: Alexa Fluor 488 alkyne (green) and Alexa Fluor 594 alkyne (red). Three-dimensional renderings of the surface that underwent the click reaction with a mixture of the two dyes are shown. Images are based on *n* = 4 independent experiments. Scale bars, 10 μm.
functions. Such strains would not be vulnerable to the strategies of extreme courses of antibiotics and/or that have impaired immune strains primarily occur in patients that have been subjected to formation and virulence factor production. Thus, surfaces harbouring E. faecalis could provide simpler cases for deployment. For example, the Gram-negative bacteria can be explored perhaps by exploiting because they do not possess an outer membrane. Strategies for we have developed is primarily useful for Gram-positive bacteria because of the urgent medical need for its control. We have explored variations in surface coatings to target S. aureus Agr quorum sensing for different applications. First, antagonist-coated surfaces could be used in scenarios such as acute infections, for example, staphylococcal scaled skin syndrome and toxic shock syndrome, where it is essential to halt the production of exo-toxins. TrAP-II is a global antagonist that represses quorum sensing in all four S. aureus Agr subgroups. In the current work, we have demonstrated that surface-attached TrAP-II represses Agr quorum sensing in strains of Agr subgroups I and II. Presumably, subgroup III and IV strains would be repressed similarly, although we did not test them in this study. In the opposite vein, autoinducer-coated surfaces could have merit in scenarios including chronic infection, for example, pneumonia or medical device-related infections, where S. aureus biofilms are the major issue. Indeed, S. aureus cells residing in surface-bound biofilms are more resistant to antibiotics and host immune defences than are their planktonic counterparts. AIP-I-coated surfaces, by triggering biofilm dispersal and transitioning the S. aureus cells to the planktonic lifestyle, could render surfaces resistant to biofilm colonization and, furthermore, render the dispersing cells more susceptible to antibiotics and to host immune defences. In these contexts, it could be ideal to attach a global quorum-sensing agonist to the surface, if one existed. Such a strategy could disperse biofilms composed of any Agr subgroup. With these ideas in mind, our research raises the exciting, but now plausible possibility that surfaces decorated with quorum-sensing-modulating molecules can be assessed for anti-infective properties in animal models harbouring in-dwelling devices. We note that instances of Agr-deficient virulent S. aureus strains have been reported. Infections by Agr-defective strains primarily occur in patients that have been subjected to extreme courses of antibiotics and/or that have impaired immune functions. Such strains would not be vulnerable to the strategies proposed here. Our strategy for coating surfaces with pro- or anti-quorum-sensing molecules and using them to influence bacterial behaviours is not limited to the model bacterium S. aureus. Indeed, other bacteria could provide simpler cases for deployment. For example, the Gram-positive bacterium Enterococcus faecalis causes life-threatening urinary tract infections, bacteremia, endocarditis and meningitis in humans. The pathogenicity of E. faecalis relies on the Fsr quorum-sensing system, which is homologous to the S. aureus Agr quorum-sensing system. However, importantly, in the case of E. faecalis, activation of Fsr quorum sensing promotes both biofilm formation and virulence factor production. Thus, surfaces harbouring Fsr quorum-sensing antagonists (for example, ZI4015) could have a dual benefit in preventing biofilms and reducing exo-toxin production. Such dual benefits can also be imagined for other bacteria such as Listeria monocytogenes and Streptococcus pyogenes, as both pathogens possess Agr-type quorum-sensing systems that activate biofilm formation and virulence factor expression at high cell density. Moreover, the beneficial bacterium Lactobacillus plantarum, which is important in the dairy and fermented food industries, also has an Agr-type quorum-sensing system called Lam that could be manipulated using surfaces in applications in food production.

In summary, we have shown that surface-attached molecules can be used to manipulate a particular bacterial signalling system. Our strategy has the potential to be expanded to other systems with known ligands and with accessible cognate receptors. The strategy we have developed is primarily useful for Gram-positive bacteria because they do not possess an outer membrane. Strategies for Gram-negative bacteria can be explored perhaps by exploiting surfaces coated with quorum-sensing-manipulation compounds together with molecules that form pores in the outer membrane, such as holins, endolysins or bacteriocins. Similarly, surface-attached quorum-sensing molecules could be examined with other orthogonal approaches that exploit the surface release of active compounds.

### Methods

#### Bacterial strains and plasmids.

The strains and plasmids used are listed in Supplementary Table 1. S. aureus strains RN4220, RN9011, RN69309, RN6911 and RN6607 and plasmids pC1111 and pRN7062 were gifts from the group of R. Novick (New York University). S. aureus strains MK121 (ref. 18) (RN63909 carrying pMK1; AgrP3-gfpmut2, sarAP1-mkate2) and S. aureus MRSA strain MK131 (ref. 18) (BAA1680 carrying pMK1; pRN7025) were used. DNA polymerase, dNTPs, T7 promoters and restriction enzymes were purchased from New England Biolabs (NEB). DNA extraction and purification kits were acquired from Qiagen. DNA oligonucleotides were purchased from Integrated DNA Technologies. Sequences of plasmids were verified by Genewiz.

Plasmids carrying constitutively expressed fluorescent fusions were constructed by replacing the mKate2 gene from pMK104 (sarAP1-mkate2) with genes encoding different fluorescent proteins (gfpmut2, mTurquois2 and mko). To make these plasmids, the gfp mutant gene was amplified by PCR from pMK021 (ref. 18) using primers MKF013/MKR013, the mTurq mutant gene was amplified by PCR from (ref. 18) (BAA1680 carrying pMK021) using primers MKF019/MKR010, and the mko gene was amplified by PCR from pCN005 (ref. 52) using primers MKF010/MKR014. The amplified genes were used to replace mKate2 by overlap extension PCR cloning. These plasmids are called pMK102 (sarAP1-gfpmut2), pMK101 (sarAP1-mTurquois2) and pMK103 (sarAP1-mko).

We integrated the constitutively expressed reporter fusions onto the S. aureus chromosome. To do this, we used a site-specific integration suicide vector, pCI111, carrying a cadmium resistance cassette and the SaPI-1 attS sequence that integrates into the S. aureus chromosomal attachment site (attC) of pathogenicity island 1 (SaPI-1). This plasmid is integrated in single copy and maintained stably. We digested pCI111 using restriction enzymes NarI/SphI. The sarAP1-gfpmut2 gene was amplified by PCR from pMK012 using primers MKF031/MKR031, followed by digestion with NarI/Sph1 and ligations into digested pCI111. This plasmid is called pMK302 (sarAP1-gfpmut2 in the suicide vector). We used the same procedure for other fluorescent genes: pMK303 (sarAP1-mTurq2 in the suicide vector) and pMK233 (sarAP1-mko in the suicide vector). The plasmids were introduced into Escherichia coli DH5a using chemical transformation (NEB) followed by selection with ampicillin. The plasmids were purified from E. coli, introduced by electroporation into S. aureus strain RN9011, which expresses the SaPI-1 integrase, and colonies containing the fusions integrated onto the chromosome were selected with cadmium. Subsequently, the chromosomal integrants were transduced into S. aureus strain RN6911 using standard phage transduction techniques with phage 80a. These strains are called MK232 (sarAP1-gfpmut2 in the genome), MK231 (sarAP1-mTurq2 in the genome) and MK233 (sarAP1-mko in the genome).

A plasmid carrying a transcriptional fusions to monitor S. aureus Agr quorum-sensing activity was constructed by replacing the lacZ gene from pCI7062 (ref. 54) (AgrP3-lacZ) with the mKate2 gene. pRN7062 also harbours the genes encoding the Agr quorum-sensing detection compounds agrCA under their native agrP2 promoter but driven in the opposite direction. To make this plasmid, the lacZ gene was removed from pRN7062 by digestion with EcoRI/NarI. The digested mKate2 gene was ligated into digested pRN7062. This plasmid is called pMK305 (agrP2-agrCA, agrP3-mKate2). This construct was first introduced into E. coli, purified, and subsequently introduced into S. aureus strain RN4220 using selection with erythromycin. Subsequently, using plage transduction, the plasmid was introduced into S. aureus strains MK232, MK231 and MK233. The resultant strains are called MK242 (sarAP1-gfpmut2 in the genome and pMK305), MK241 (sarAP1-mTurq2 in the genome and pMK301) and MK243 (sarAP1-mko in the genome and pMK301). To construct the S. aureus ΔagrBΔca strain harbouring agrP3-mKate2 on a plasmid, the vector pMK004 (ref. 18) (AgrP3-mKate2) was introduced into S. aureus strain MK232 (sarAP1-gfpmut2 in the genome of S. aureus ΔagrBΔca), leading to strain MK245. Introduction of these plasmids into S. aureus strains does not alter growth or quorum-sensing phenotypes.

We constructed control strains to study the heterogeneity of the Agr quorum-sensing response. The first control strain has the agrP2-agrCA and agrP3-mKate2 genes inserted into the genome of RN9011 and harbour sarAfp1-gfpmut2 on a plasmid. To make this strain, we amplified the agrP2-agrCA and agrP3-mKate2 genes from pMK051 using primers MKF032/MKR032, and inserted this fragment into the suicide vector pC1111 by overlap extension PCR cloning. This plasmid is called pMK104 (agrP2-agrCA, agrP3-mKate2 in the suicide vector). The gene was introduced into the S. aureus strain RN9011 chromosome by conjugation. We call this strain MK264 (agrP2-agrCA, agrP3-mKate2 in the genome). Vector pMK012 (sarAP1-gfpmut2) was introduced into MK264, leading to strain MK265 (agrP2-agrCA, agrP3-mKate2 in the genome and pMK012). The second control strain was constructed by introducing pMK014 (sarAP1-mKate2) into strain MK232, leading to
MK242 (surAPl-fgfmut2) on the genome and pMK014). The third control strain has the surAPl-fgfmut2 insertion into the genome of RN6911 and the surAPl-fgfmut2 insertion into the suicide vector pC1111 by overlap extension PCR cloning. This plasmid is called pMK060 (apg2-apgCA in the suicide vector). The gene was integrated into the S. aureus strain RN6911 chromosome as described above. We call this strain MK260 (apg2-apgCA in the genome). Vector pMK004 (ref. 18) (apg3-mkat2e) was introduced into MK260, leading to strain MK261 (apg2-apgCA in the genome and pMK004). Finally, a constitutively expressed mKO fluorescent reporter (surAPl-mko in pMK013) was introduced into wild-type S. aureus agr-I (strain RN6390b) and wild type S. aureus agr-II (strain RN6607) to measure the number of cells in biofilms on surfaces.

Growth conditions. S. aureus RN6911 derivatives were grown overnight at 37 °C with shaking in tryptic soy broth (TSB, Difco) with 10 mg ml⁻¹ tetracycline and 10 μg ml⁻¹ erythromycin to maintain plasmids, back-diluted 1:200 and regrown for 3 h (to an optical density at 600 nm (OD₆₀₀) of ~0.05–0.1). S. aureus MK121, MK131, MK125 and MK126 were grown overnight at 37 °C with shaking in TSB with 10 μg ml⁻¹ erythromycin, back-diluted 1:200 and regrown for 3 h (to OD₆₀₀ = 0.05–0.1).

Synthesis of AIP-I, AIP-II and TraIP-II derivatives. AIP-I, AIP-II and TraIP-II derivatives were synthesized using a combined solid-phase/solution-phase approach. Linear peptide α-thioester precursors were generated using Fmoc-solid-phase peptide synthesis using a hydrazine linker system. The peptides were then cyclized in solution to install the thiolactone macrocycle. Synthetic details are provided in the Supplementary Methods.

Fluorescence reporter assay. Transcription from fluorescence reporter genes was measured in S. aureus strain MK242. Overnight cultures were diluted 1:200 into fresh TSB with 10 μg ml⁻¹ tetracycline and 10 μg ml⁻¹ erythromycin, regrown, and 90 μl of each of these cultures was distributed into wells of 96-well plates (MatTek), followed by addition of 10 μl of AIP-I and/or TraIP-II and/or derivatives.

Subsequently, 50 μl mineral oil was added (Sigma) to prevent evaporation. Using a Synergy 2 plate reader (Biotek), GFPmut2 and mKate2 levels were measured at 485/508 nm and 588/633 nm, respectively. Measurements were conducted at 15 min intervals at 37 °C with shaking. This assay is called ‘Solution assay’ in the main text.

Surface fabrication. Surface-PEG₁₀₀₀₀-azide, Surface-PEG₄₀₀₀-azide, Surface-PEG₂₄₄ and Surface-PEG₄₄₄ were fabricated as follows (Supplementary Fig. 4a). Glass slides (56 × 60 mm², Ted Pella) were heated for 2 h in 2% sodium peroxodisulfate (Sigma) at 50 °C and washed twice with Millipore water. The washed surfaces were submerged in piranha etch solution (3:1 H₂SO₄:H₂O₂, Fisher Scientific) for 1 h at 50 °C and washed twice with Millipore water. The washed surfaces were bonded to microfluidic chambers for 30 min to remove the planktonic cells. After this step, chambers were washed with acetone and subsequently washed with 100% blood plasma solution (Biological Specialty Corp), 100% blood plasma solution equipped with a liquid nitrogen-cooled MCT/A detector (Supplementary Fig. 4b). Before taking the spectra of the Surface-PEG₁₀₀₀₀-azide, the unmodified glass surface was used to provide the background spectrum. Background-subtracted measurements were taken only after the sample chamber was purged sufficiently with dry nitrogen to reduce the levels of carbon dioxide and water vapour.

To characterize the Surface-PEG₁₀₀₀₀-azide, Alexa Fluor 555 dye functionalized with an alkylamine moiety (Thermo Fisher) was used. The Surface-PEG₁₀₀₀₀ was used as the negative control. The surfaces were treated with click solution containing the Alexa Fluor 555 alkyl amine. After washing away the unreacted dyes, the surfaces were imaged by confocal microscopy (Supplementary Fig. 4c). Surface-PEG₄₀₀₀-azide (Supplementary Fig. 6a), gold-based Surface-PEG₄₀₀₀-azide and PDMS-based Surface-PEG₁₀₀₀₀-azide (Supplementary Fig. 8c) were characterized using an identical procedure.

To investigate long-term surface stability, the Surface-PEG₁₀₀₀₀-triazole-AIP-I and Surface-PEG₁₀₀₀₀-triazole-dye were stored at 4 °C for 40 days in a wet container covered with aluminum foil to prevent desiccation and exposure to light.

Surface characterization. The modified surfaces were characterized using a Fourier transform infrared (FTIR) spectrometer, fluorophore labelling or atomic force microscopy (AFM) as follows. Two-point absorbance spectra for the Surface-PEG₁₀₀₀₀-azide were obtained as averaged signals by scanning the sample 128 times at 4 cm⁻¹ resolution using a Thermo Nicolet Nexus 670 FTIR spectrometer (Thermo Electron Corp) equipped with a liquid nitrogen-cooled MCT/A detector (Supplementary Fig. 4b). Before taking the spectra the Surface-PEG₁₀₀₀₀-azide, the unmodified glass surface was used to provide the background spectrum. Background-subtracted measurements were taken only after the sample chamber was purged sufficiently with dry nitrogen to reduce the levels of carbon dioxide and water vapour.

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Quorum-sensing gene expression analyses on surfaces. The chemically modified surfaces were bonded to microfluidic chambers (400 μm × 100 μm × 2 cm) using an epoxy glue (Fishier Scientific). The assembled chambers were inoculated with the S. aureus agr-I reporter strain cultures and cells were able to settle onto surfaces for 10 min, after which sterile M63 medium containing 10 μg ml⁻¹ erythromycin, 0.5% glucose, 0.5% casamino acid, 5 mM NaCl, 50 mM Tris, and 0.1 mM CaCl₂, was flowed steadily into the devices for 30 min to remove planktonic cells. After this step, chambers were placed on the microscope and the fluorescent reporter output was measured. In experiments containing autoinducer or antagonist in solution, molecules were added in the wash medium. In experiments containing human blood plasma (Biological Specialty Corp), Human 100% blood plasma solution was initially flowed into the chambers containing the chemically modified surfaces for 10 min. Subsequently, S. aureus agr-I reporter cells were seeded for 10 min and then 20 or 30% blood plasma diluted in the above medium was flowed into the chambers for 30 min to remove the planktonic cells. After this step, chambers were placed on the microscope and the fluorescent reporter output was measured.

Biofilm analyses on surfaces. Chambers containing modified surfaces were seeded with wild-type S. aureus strains harbouring constitutively expressed mko on a plasmid and the cells were able to settle onto the surfaces for 10 min, after which sterile M63 medium containing both a <1000 cells per mm² on the edge of the image or if they were smaller than 0.3% of the average cell size, suggesting they were of low focus. In the area of an individual cell, both the constitutive GFPmut2 fluorescence and the quorum-sensing controlled mKate2 fluorescence were measured, subtracted from background signals, and summed. The normalized quorum-sensing output was calculated as the ratio of the quorum-sensing fluorescence of the Mko cells divided by the constitutively expressed GFPmut2 intensity in individual cells. In each experiment,
images of many regions on the surfaces were taken to include 1,000 to 4,000 individual cells. Each plate was performed using independent cultures and independent surfaces at room temperature. Identical procedures were performed for the strains harbouring different constitutive fluorescent proteins such as mTurquoise2 and mKO (Fig. 5b). Custom code was used to count the cells in the biofilms. Each image was segmented in the z-plane and assessed independently (Fig. 4d and Supplementary Fig. 8c).

Quantitation of surface coverage density and single-molecule microscopy. The coverage density of ‘clicked’ azides on the Surface-PEG$_{1000}$-azide was quantified based on flophore labelling. We assume that the Alkyne-dye, the Alkyne-AlIP and the Alkyne-Tr mAIP II have identical reactivity in the click reaction, rendering the same surface coverage density. Thus, surface coverage density can be quantified using flophore labelling as a proxy for surface-attached quorum-sensing compounds. In the flophore labelling assay, the surface-attached azides underwent the click reaction with 100 μM Alexa Fluor 555 alkyne flophore. After completion, the total intensity of flophores from the surface was measured (at 10% laser power), the background was subtracted, and the difference was divided by the average single-molecule intensity. The average single-molecule intensity was obtained by measuring the intensity of single Alexa Fluor 555 molecules as follows. We treated the Surface-PEG$_{1000}$-azide with 0.1–10 nM Alexa Fluor 555 alkyne flophore and this low concentration of Alkyne-dye produced individually discernable fluorescent spots (20–300 in 5 × 5 μm) (Supplementary Fig. 6d(i)). The individual fluorescent spots were subjected to photobleaching. They displayed stepwise decreases in intensity. Background subtracted intensities of a few thousand spots across five independent dye-linked surfaces were measured and analysed using our custom in-house software. Specifically, the obtained images were smoothed and filtered to produce a zero-based image in which bright fluorescent spots were located with pixel-level accuracy by a peak-finding algorithm. With the high spatial resolution of the microscope (100 nm per pixel), the intensity of each spot was measured from an 8 × 8 pixel$^2$ region centred at each of these peaks. The integrated fluorescence emission within each region was measured over consecutive frames while exposing the sample to high-intensity illumination (at 100% laser power with 500 μs exposure time) as the photobleaching process. The photobleaching intensity trace revealed the number of fluorophores present in that spot and the size of these intensity steps yielded the intensity of emission from the fluorophores in the spot. Most fluorescent spots decreased in one step following bleaching (Supplementary Fig. 6d(ii)). A small number of spots showed stepwise decreases in fluorescence intensity (Supplementary Fig. 6d(iii)). By fitting the bleaching step sizes for over 3,600 spots with a normal distribution (Supplementary Fig. 6d(iii)), we were able to arrive at the average single-molecule intensity (7,000 a.u.), which was further verified by counting fluorescent spots from the surface. Unlike the reaction of the surface with the low concentration of Alkyne-dye, the surface intensity from the typical surface that had reacted with 100 μM Alkyne-dye was above the maximum detection limit of the camera when exposed to 100% laser power. Thus, we used a two-step calibration process in which we measured the total intensity of uniform surfaces that had reacted with 1, 5 and 10 nM Alkyne-dye at 100% laser power and compared them to that obtained with 100% laser power, yielding an average ratio of 90. For the final surface reaction at 10% laser power with 500 μs exposure time, we multiplied the integrated intensity by 49 and divided that by the average single-molecule intensity (7,000 a.u.), finally giving a surface coverage density.

Data availability. All data sets that support the findings of this study are available in the present manuscript in either the main text or the Supplementary Information.
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**Author contributions**

M.K.K., H.A.S. and B.L.B. conceived the idea. M.K.K., A.Z., T.W.M., H.A.S. and B.L.B. designed the experiments. M.K.K. and A.Z. performed the majority of the experiments. A.W. helped with the solution assay. M.K.K., A.Z., A.W. and Z.Z.B. contributed new reagents/analytic tools. M.K.K., A.Z., T.W.M., H.A.S. and B.L.B. analysed the data. M.K.K., A.Z., H.A.S. and B.L.B. wrote the manuscript.

**Additional information**

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Correspondence and requests for materials should be addressed to H.A.S. and B.L.B.

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**Competing interests**

The authors declare no competing financial interests.