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Bioinspired, releasable quorum sensing modulators†

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We demonstrate the synthesis and immobilization of natural product hybrids featuring an acyl-homoserine lactone and a nitrodopamine onto biocompatible TiO_2 surfaces through an operationally simple dip-and-rinse procedure. The resulting immobilized hybrids were shown to be powerful quorum sensing (QS) activators in *Pseudomonas* strains acting by slow release from the surface.

Nosocomial infections constitute a major health care concern worldwide and in particular bacterial biofilm formation on indwelling medical devices poses a significant challenge. Direct systemic treatment with antibiotics remains difficult both due to tissue encapsulation, leading to an up to 1000-fold decrease in antibiotic efficacy,[1,2] and due to the rise of resistant bacterial strains.[1–5] Several approaches aimed at inhibiting bacterial biofilm formation *via* the generation of antimicrobial or antifouling surfaces have been documented[6–10] that could, however, give rise to resistance upon long term exposure. Another approach to prevent biofilm formation involves the inhibition of quorum sensing (QS),[11–15] a cell-to-cell signalling system that is used by numerous bacteria to control expression of certain genes in a cell-density dependent manner. Chemically, QS is based on the exchange of diffusible small-molecule signals (often also referred to as autoinducers (AIs)),[16] such as, for example, *N*-acyl-L-homoserine lactones (AHLs), between cells of many gram-negative bacteria.[17–19] Interfering with QS with small molecules[20–24] therefore represents a fundamentally different approach for the prevention and eradication of bacterial biofilms.[25–27] Blackwell and coworkers recently reported the controlled release of non-native QS modulators from thin PLG films.[28] However, covalently bound QS modulators on metal oxide surfaces have, to the best of our knowledge, not yet been reported. In this communication, we report the immobilization of QS activators on TiO_2 beads and their successful biological evaluation in *Pseudomonas* strains.

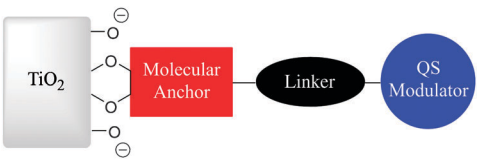

Fig. 1 Conceptual design of a QS modulator attached to a TiO_2 surface using a molecular anchor connected by a linker.

A first challenge consisted in finding suitable surface-active hybrids that can still be recognized by the bacteria as QS modulators. As anchor groups, we hoped to exploit catechols that have been shown to bind strongly to several metal oxides and can thus be used as binding groups for generating self-assembled monolayers using an operationally simple dip-and-rinse procedure.[29] Therefore, we envisaged the synthesis of hybrids as shown in Fig. 1. These compounds would consist of a bioactive moiety, which should interfere with QS (blue in Fig. 1), a catechol anchor able to bind to surfaces (red), and a suitable linker to combine these two fragments. AHL was chosen as the bioactive moiety as it is frequently used for cell-to-cell communication by various gram-negative bacteria. Dopamine, nitro-dopamine and the anachelin chromophore[7,8] were selected as possible anchors to investigate the recognition impact of the anchor group in the hybrids. These two fragments were

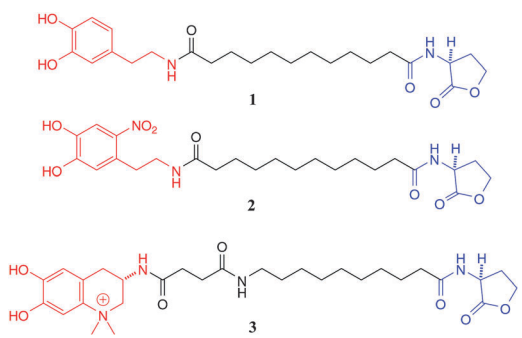

Fig. 2 Natural product hybrids as QS modulators with different anchors.

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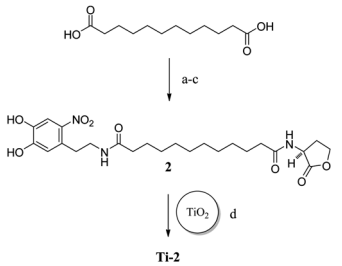

[a] (a) NSC, pyridine, MeCN, rt, 7 h, quant.; (b) nitrodopamine, Et_3N, THF/DMSO, rt, 20 h; (c) HSL, Et_3N, THF/DMSO, rt, 20 h, 72 % (over two steps); (d) 0.1 M MOPS/0.6 M NaCl/0.6 M K_2SO_4, 4 h, 50 °C NSC = *N,N'*-disuccinimidyl carbonate; rt = room temperature; THF = tetrahydrofuran; DMSO = dimethyl sulfoxide; HSL = L-homoserine lactone .

Scheme 1 Synthesis of compound **2** and immobilization on TiO_2 beads **Ti-2**.

connected by alkyl linkers of various lengths and preliminary screening showed that the hybrid featuring a C_{12} linker (Fig. 2) induced QS in the AHL biosensor strain *C. violaceum* CV026.[30] Other linker lengths did not lead to QS induction, even at high concentrations. The influence of the catechol anchor group on activity was studied next, and no significant difference between compounds **1–3** was observed. We therefore chose the nitro-dopamine derivative **2** for further studies, due to the ease of accessibility, strong binding properties towards metal oxides, and stability towards oxidation.[29c] The synthesis of hybrid **2** is shown in Scheme 1 and was achieved in 3 steps from commercial dodecanedioic acid in 72% yield.

After having established that hybrid **2** is able to interfere with QS in a liquid based assay (see ESI†), we wanted to evaluate its activity after immobilization on surfaces. We decided to use titanium dioxide as this metal oxide is widely used for orthopedic and dental implants due to its biocompatibility. For ease of handling and visualization, we decided to use TiO_2 beads with an average diameter of 1 μm. Therefore, a modified dip-and-rinse procedure[29] was applied: the beads were incubated in a dilute solution of **2** in high salt buffer (0.1 M MOPS/0.6 M NaCl/0.6 M K_2SO_4) for 4 hours at 50 °C to result in the functionalized beads **Ti-2**. In a control experiment, no hydrolysis products of **2** could be detected after 4 hours at 50 °C by UPLC-MS. After evaluating several bacterial AHL reporter strains, we observed that hybrid **2** and **Ti-2** activated the GFP-based biosensor *P. putida* F117 (pAS-C8) similarly to *N*-octanoyl-L-homoserine lactone (C_8-AHL), for which it was shown to be most sensitive.[31] GFP fluorescence is induced in this strain only when AHL molecules with C_6 to C_{12} acyl side chains are provided externally. The functionalized beads **Ti-2** induced a similar fluorescence response as the natural agonist in this assay.

We then evaluated if the immobilized hybrid **2** on TiO_2 beads could be removed by sequential washing operations (Fig. S1, ESI†) thus potentially impacting the activity. Interestingly, washing the beads up to ten times did not alter the biological activity of **Ti-2** as judged by measured GFP levels in the pAS-C8 reporter strain. These results clearly demonstrate that the immobilized hybrid **Ti-2** is a potent QS inducer which retains activity even upon repeated rinsing.

Next, we wanted to address the mode of action of the functionalized beads **Ti-2**. In particular, we were interested to determine if QS is induced exclusively at the surface of the beads or in the entire bacterial population. Therefore, the

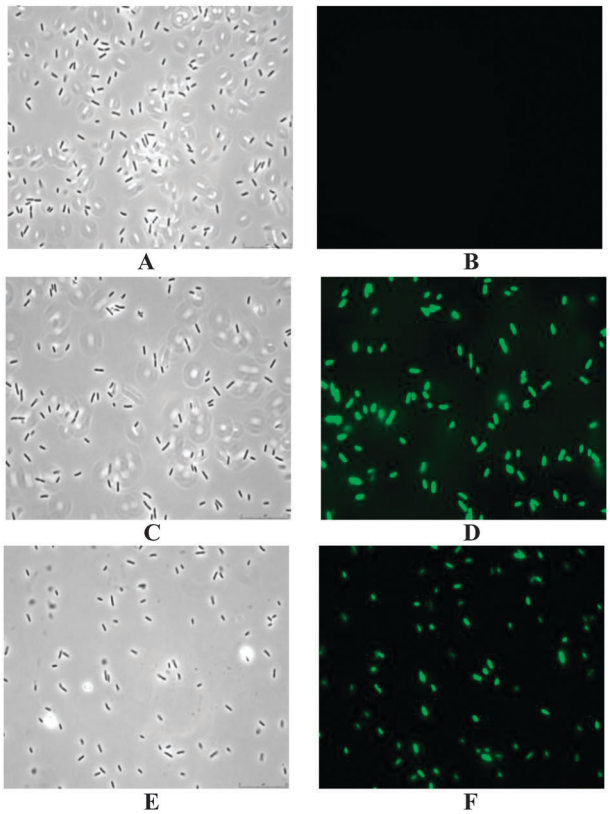

Fig. 3 Phase contrast (A) and GFP fluorescence (B) of the sensor as a negative control. Phase contrast (C) and GFP fluorescence (D) of the sensor incubated with compound **2** as a positive control. Phase contrast (E) and GFP fluorescence (F) of the sensor incubated with the functionalized beads **Ti-2**.

functionalized beads **Ti-2** were incubated with the *P. putida* F117 (pAS-C8) biosensor strain for 2 hours and the experiments were monitored by fluorescence microscopy (Fig. 3). In the absence of an inducer, no fluorescence could be observed in the negative control (Fig. 3A and B). By contrast, in the presence of compound **2**, which served as a positive control, the reporter strain produced GFP as shown in Fig. 3C and D. Similarly to compound **2**, the functionalized beads **Ti-2** (visible in the phase contrast image Fig. 3E) induced the biosensor (Fig. 3F), suggesting that hybrids are released from the surface.

Several mechanisms can be formulated to explain these observations. On one hand, induction of QS by contact of a bacterium with the functionalized bead **Ti-2** and concomitant capture of the QS modulator can be hypothesized ('the billiards hypothesis'). As an alternative, more feasible hypothesis, slow release of QS modulators from the surface and subsequent induction of QS in the reporter strains might also be operative. In order to address this question, we performed dialysis assays using cellulose ester membranes with 3.5–5 kDa molecular weight cut off. In this assay the beads are retained in dialysis bags but released AHL mimics can diffuse into the surrounding medium and induce the biosensor. The functionalized beads **Ti-2** were dialyzed overnight in buffer solution, and the supernatant was then incubated with the sensor for 3 hours at 30 °C (see ESI†). Fluorescence was observed in the reporter strain, which supports the mechanism that AHL mimics are released from the beads. In order to test the kinetics of release, samples were taken

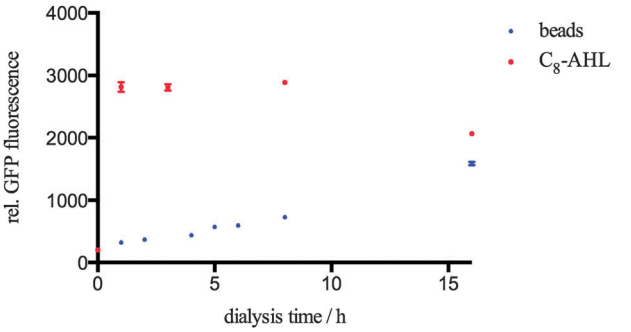

Fig. 4 Comparison of activity of dialyzed **Ti-2** (0.6 mg mL^{-1}) and dialyzed C8-AHL (0.3 μM). Data are reported as mean ± SEM, $N = 9$.

after different incubation times and the activity of functionalized beads **Ti-2** was compared to the natural agonist, both positioned inside the dialysis tubes. The results (Fig. 4) demonstrate that the positive control using dialyzed compound C_8-AHL reaches its maximum activity after a short period of time as expected. The activity decreased after overnight incubation, which can be explained by the fact that the lactone in AHL is known to be hydrolyzed non-enzymatically under physiological conditions.[32] The functionalized beads **Ti-2** release the hybrids much slower and an increase of activity is observed even after overnight incubation. The release appears to be linear over the measured experiment time. However, the mechanism of release and relative rates for the hydrolysis of compounds in solution *vs.* on the surface remain unknown. Quantitative measurements were performed showing hybrid concentrations increasing from 4 to 270 nM after 1 hour and 16 hours incubation time, respectively.

In conclusion, we report the preparation of hybrid compound **2** combining a nitrodopamine anchor and an acylated homoserine lactone as the bioactive moiety tethered *via* a C_{12} linker. These hybrids are recognized by the AHL biosensor *P. putida* F117 (pAS-C8). Functionalized TiO_2 beads **Ti-2** were obtained by an operationally simple dip-and-rinse procedure and the resulting beads **Ti-2** are capable of inducing QS. The activity of the **Ti-2** beads was retained even after extensive washing with water. The bacteria do not accumulate on the beads and the hybrids are slowly released as suggested by dialysis assays. This hybrid system expands the catechol surface modification platform[7,9,29,33] and this approach might provide a straightforward method for the preparation of coated surfaces in medical devices able to interrupt the QS signalling pathway in a wide range of different bacterial strains. Such surfaces could find an application in various bacteria related to medicine (*e.g.*, implants), agriculture, and material sciences.

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