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Bacterial Patterning Controlled by Light Exposure

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Patterning of multiple bacterial strains in one system is achieved by employing a single photo-activated antibiotic. Varying the light-exposure time results in zones with mixed and single populations.

Controlling the growth of bacteria on surfaces[1–4] is important for the investigation of bacterial interactions.[5,6] The study of microbial interplay has helped to gain knowledge on how bacteria communicate[7] and defend themselves,[8] which is essential information for the advancement of novel strategies for antimicrobial therapy.[8–10] Additionally, the controlled growth of bacteria on surfaces has found applications in microrobotics[11] and is crucial for the development of sensing and diagnostic devices.[12] In particular, patterning of bacteria can be a useful tool in microbiology to study interactions between microorganisms, like competition, horizontal gene transfer and quorum sensing.[13,14] Several methods have been employed to control cellular deposition on surfaces, mostly relying on microdevices and microfabrication techniques.[15–17] While proven extremely useful, these methods all require highly specialized equipment and facilities.

A different approach exploits the extraordinary spatiotemporal resolution, offered by light-irradiation, to control the growth of cells on surfaces,[18,19] for example by the use of photo-activated antibiotics to control bacterial patterning.[20,21] In a recent report, we have used photo-activated antibiotics that can be orthogonally addressed to control the growth of different bacterial strains in one system,[22] which is an important feature for studying bacterial interactions.[6] Though this showed to work well, the preparation of multiple photoprotected antibiotics can be laborious. Therefore, we aimed at controlling the growth of different bacterial strains by employing only a single photo-activated antibiotic. To accomplish this goal we envisioned the exploitation of the intrinsic difference in antibiotic-susceptibility of different bacterial strains. By varying the light exposure time, it is possible to precisely create surface areas with various antibiotic concentrations and gradients. Doing so, the growth of multiple bacterial strains can be controlled (Fig.1).

Here, we report on the controlled and combined growth of *Escherichia Coli* CS1562[23] (*E. coli*) and *Micrococcus Luteus* ATCC 9341 (*M. luteus*) using only a single photo-activated antibiotic **1** (Fig. 2). This photo-activated antibiotic was designed to be stable under ambient conditions in the laboratory and showed to have a superior quantum yield compared to previously-reported compounds.[22] Changes in colony morphology were investigated to prove the usefulness of this approach for studying bacterial interactions. We envision that this method might be extended to controlling the growth of additional strains and observing bacterial interactions using more advanced technologies, as it would allow for studying genomic and metabolic variations upon interstrain and intrastrain interactions.

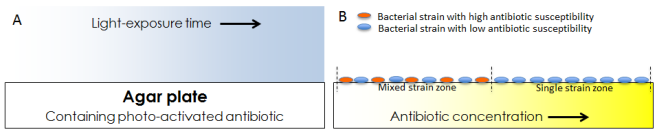

Fig. 1 Schematic overview of the presented approach for bacterial patterning of mixed bacterial populations. A) An agar plate containing a photo-activated antibiotic can be exposed to light for varying amount of times, creating zones with different antibiotic concentrations. B) Short-exposure times will generate small amounts of antibiotic and these zones will contain mixed populations of bacterial strains with high and low susceptibility. Long-exposure times result in a high antibiotic concentration and these zones will only contain bacterial strains with low susceptibility.

The design of the photo-activated antibiotic **1**, which consists of a broad-spectrum fluoroquinolone antibiotic and a coumarin-based photoprotecting group,[24,25] is depicted in Fig. 2. The carboxylate group of the fluoroquinolone is an essential part of the quinolone's pharmacophore and is known to bind to DNA gyrase, and therefore is crucial for its antimicrobial activity.[26] Caging of this particular acid group results in a dramatic decrease in activity. A methoxycoumarin derivative was chosen as a photocleavable group,[27] because it can

only be cleaved with UV-light and is stable to visible light exposure, which ensures easy handling in the lab.

The UV-Vis absorption spectrum of compound **1** shows an absorption maximum around 323 nm (Fig. 3A). Exposure of a solution of compound **1** to UV-light results in uncaging, which can be observed by a distinct decrease in absorption at λ_{max}. Fig. 3B shows that exposing compound **1** to UV-light results in a rapid decrease in absorption at λ_{max}, implying the release of fluoroquinolone. However, exposure to a powerful visible light source (150 W Thor Labs OSL1-EC Fiber Illuminator, see Fig. S1 for lamp emission spectrum) does not result in any substantial change in absorption, proving the stability of compound **1** under ambient conditions.

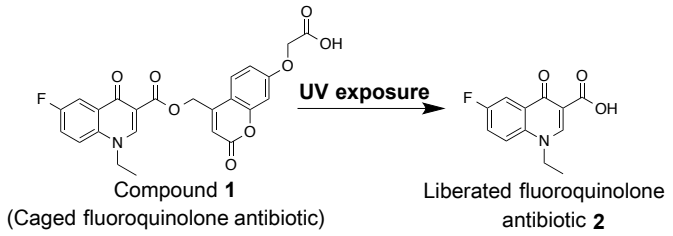

Fig. 2 The molecular structure of compound **1** and the fluoroquinolone antibiotic **2** liberated after exposure of **1** to UV-light (312 nm).

Using ferrioxalate actinometry[28] (for details, see SI) the quantum yield for the uncaging of compound **1** was determined to be ~0.128. This is substantially larger as compared to a visible-light-sensitive, photo-activated fluoroquinolone, which was reported earlier.[22] A relatively high quantum-yield is an important prerequisite for developing an easy-to-handle biological tool, because it allows for short light-exposure times for activating the antibiotic.

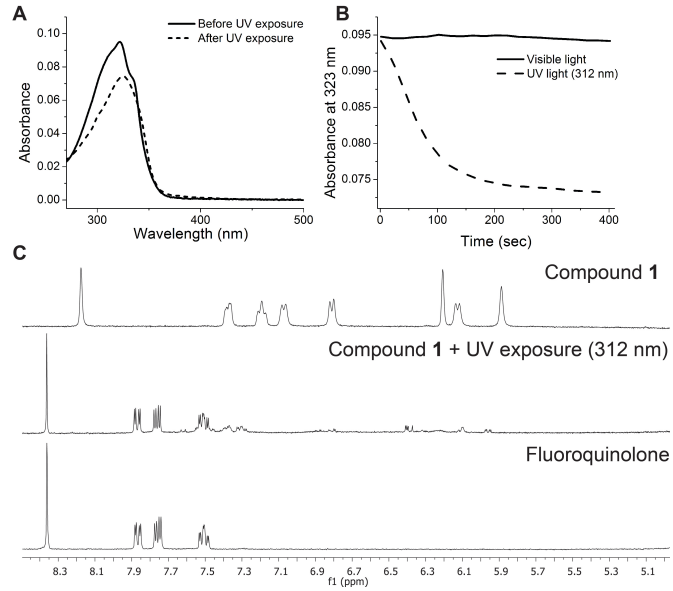

Fig. 3 The uncaging process of compound **1**, resulting in the liberation of a fluoroquinolone. A) The UV-Vis absorption spectra of compound **1** in water (5 μM) before and after exposure to UV-light. B) Temporal evolution of the absorbance at 323 nm (=λ_{max}) of a 5 μM solution of compound **1** in water, when exposed to visible light and when exposed to UV-light. C) ^{1}H NMR spectra of compound **1** (10 mM, D_2O), compound **1** after UV-light exposure for 60 min and the fluoroquinolone **2**.

To confirm the liberation of fluoroquinolone from compound **1,** ^{1}H NMR spectroscopy studies were performed (Fig. 3C). A distinct change in chemical shifts was observed when a 10 mM solution of compound **1** in D_2O was exposed to UV-light for 60 min. The chemical shifts of the UV-exposed sample exactly overlap with the chemical shifts of an original fluoroquinolone sample, proving the release of fluoroquinolone from compound **1**. The aromatic signals from the coumarin part of the molecule have disappeared, probably due to photodegradation of the cage group.

Bacterial strains differ in their susceptibility to antibiotics. We hypothesized that these inherent differences might be exploited to develop a method for patterning of multiple bacterial strains using a photo-activated antibiotic. Using light to control the antibiotic concentration in different zones of the agar plate would allow for bacterial patterning with high precision.

By exposing regions of an agar plate, containing compound **1**, to UV-light, for various amounts of time, different concentrations of liberated fluoroquinolone are obtained in the respective regions. An agar plate containing compound **1** (22 μM) was divided in 25 zones and, by using a mask and a hand-held UV-illuminator (Spectroline ENB-280C/FE UV lamp, 312 nm), the zones were exposed to UV-light for various periods of time (Fig. 4A). Subsequently, the plates were inoculated with *E.coli* or *M.luteus* and incubated overnight. The resulting agar-plates (Fig. 4B and C) show that an UV exposure-time of 10 seconds liberates sufficient antibiotic to inhibit *E.coli* growth. However, UV exposure-times up to 40 seconds did not result in any growth-inhibition of *M.luteus*. This large difference in UV-exposure time allows for the creation of regions with combined bacterial growth and regions with a single bacterial strain. Therefore, we set out to exploit these properties for bacterial patterning and show the usefulness of this approach for studying basic bacterial interactions.

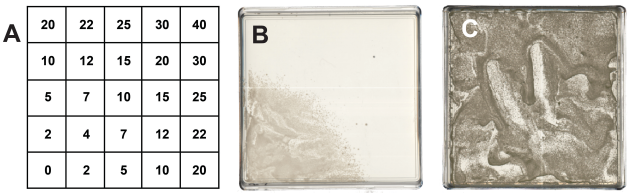

Fig. 4 Bacterial growth in regions on agar-plates containing compound **1**, which were exposed to UV-light for different amounts of time. A) Different regions on an agar-plate containing 22 μM of compound **1**, with respective UV-light exposure times in sec. B) *E.coli* growth at the different regions as described in A. *E.coli* growth is inhibited at UV-exposure times of 10 sec and higher. C) *M.luteus* growth at the different regions of the agar-plate as described in A. *M.luteus* growth was not inhibited up to UV-exposure times of 40 sec.

First, a mask consisting of five black squares was placed on top of an agar-plate containing 22 μM of compound **1**. Next, the agar-plate was exposed to UV-light for 20 sec and the plate was inoculated with *E.coli* and incubated overnight. Bacterial growth could only be observed in the five squares (Fig. 5A). A similar experiment was conducted, but now the agar-plate was inoculated with a mixture of *E.coli* and *M.luteus*. After incubation overnight, bacterial growth could be observed on the entire plate. As expected, colonies of *M.luteus* were growing on the entire plate, but *E.coli* colonies were only present at the five squares (Fig. 5B). This experiment shows how the

presented method can be employed for creating bacterial patterns of mixed populations.

To examine the interaction between the two different bacterial strains, a change in colony morphology was studied using phase-contrast microscopy. In the regions where *E.coli* was grown individually, a confluent layer of bacteria could be observed, and single colonies were not distinguishable (Fig. 5C). The parts of the plate where *M.luteus* was grown as a single strain contained large colonies (Fig. 5D). Interestingly, the regions where *E.coli* and *M.luteus* were grown together, consisted of a large confluent layer, just as in the *E.coli* region, but now it was interrupted with small colonies of *M.luteus* (Fig. 4E). The *M.luteus* colonies were substantially smaller as compared to the zones were this bacterial strain was grown by itself. This can be attributed to the interaction between the two bacterial strains: when two bacteria are cultured together at a high density they will have to compete for nutritional resources and habitable space.[29,30]

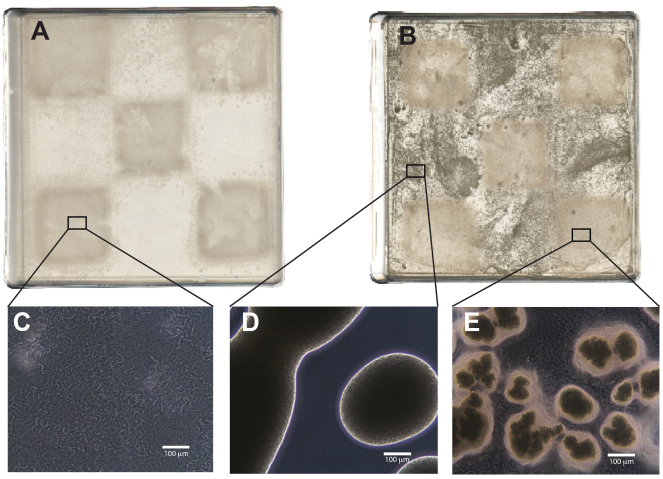

Fig. 5 Bacterial patterning. A) *E.coli* is grown in square-shaped patterns by exposing a mask-covered agar-plate, containing 22 µM of compound **1**, to UV-light for 20 sec. B) Square-shaped patterns of *M.luteus* and patterns of mixed *M.luteus* and *E.coli* colonies. C) Close-up of *E.coli* colonies. D) Close-up of *M.luteus* colonies. E) Close-up of mixed colonies *of E.coli* and *M.luteus*.

These 'proof-of-principle' experiments show the usefulness of employing only a single photo-activated antibiotic to create patterns of different bacterial strains and allowing to study how colony growth is affected. Altering light-exposure times allows for patterning of bacterial strains in a single system without perturbation to the studied microbial population, with just one photo-activatable antibiotic. This is a significant improvement compared to our previous reported system where multiple photo-activatable antibiotics were needed to obtain the same result.[22] In the next step, more sophisticated technology, such as genome sequencing or bioanalyte detection, would enable a more advanced study of bacterial interplay. We foresee that photocaging of antibiotics with activity against other bacteria, would allow for patterning of a range of microbes by simply altering the light-exposure time.

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