Electronic Supplementary Material (ESI) for Chemical Communications.

Boosting the inactivation of bacterial biofilms by photodynamic targeting of matrix structures with Thioflavin T.

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Materials and methods.

Thioflavin T (ThT) was purchased from Sigma-Aldrich and recrystallized twice from water. For each experiment, fresh solutions were prepared by dissolving a small amount of the purified powder in PBS pH 7.4, 0.01 M, (Sigma-Aldrich). ThT concentration was determined by absorbance using an extinction coefficient of 36,000 $M^{-1} \cdot cm^{-1}$ at 412 nm.[1] Gentamicin and Ampicillin (PanReac AppliChem) were dissolved at 1 $mg \cdot mL^{-1}$ aliquots and stored at -80 °C. Prior to use, the stock solution was appropriately diluted in PBS to obtain the desired concentration.

Bacterial culture conditions.

The bacterial strains used in the study are *S. aureus* CECT 240 and *E. coli* DH10β. Overnight cultures in 10 mL Luria Bertani (LB) medium (Fisher BioReagents) were obtained from single colonies of each bacteria. For biofilm formation, 2 µl of the overnight culture was added to 198 µl LB in a 96-well plate and incubated statically at 37 °C for 24 or 48 h.[2] For planktonic studies, 100 µl of the overnight culture was added to 10 mL of fresh medium and grown at 37 °C and 250 rpm until the suspension reached the appropriate optical density at 600 nm (OD_{600}), see below.

Spectroscopic measurements.

Absorption and fluorescence spectra were recorded on a Cary 50 UV-vis spectrophotometer (Varian) and a Fluoromax-4 (Horiba) respectively, using a 1 cm pathlength cuvette.

For fluorescence measurements, biofilms were gently washed with PBS to remove planktonic and loosely attached cells and disrupted by repeated pipetting. Bacterial cells in the suspension were counted using a Neubauer chamber (Marienfeld) and 2 mL solutions containing $3 \cdot 10^5$ $cell \cdot mL^{-1}$ were prepared. ThT was added to the biofilm suspensions to a final concentration of 5 µM and incubated for 30 min in the dark before measuring its fluorescence (λ_{ex} = 440 nm, λ_{obs} = 455 – 700 nm). Data were analyzed and represented using GraphPad Prism 9.1.1.

Fluorescence microscopy.

Confocal fluorescence images were acquired on a commercial Confocal multispectral TCS SP5 system (Leica Microsystems). 48 h-old biofilms were washed 3x PBS and incubated with 5 µM ThT and 1 µM PI for 15 min in the dark. Biofilms were imaged using a 20x/0.75 HCX PL APO CS objective (Leica Microsystems, Ex 458 nm, Em 470 – 550 nm and Ex 514 nm, Em 550 – 700 nm for ThT and PI, respectively). To examine the entire thickness of the biofilm, stacks of 0.88 µm optical sections were collected. For visualization of the PDI effect, biofilms were irradiated with a blue light-emitting diode (LED) light (λ_{max} = 448 nm, 24 $J \cdot cm^{-2}$). Raw images were analyzed with open-source Fiji (imagej.net/Fiji). Biofilm thickness and PI fluorescence increase were determined with COMSTAT2[3,4] ImageJ plugin and by z-stacks projections, respectively, from at least 3 independent replicates using 776 x 776 μm^2 areas

from 3-5 sets of stacks selected randomly for each biofilm sample. Data in Figure 1 shows biofilm thickness and PI intensity normalized to the values from untreated biofilms and error bars represent the standard deviation. Statistical analysis was performed using GraphPad 9.1.1, *P* values less than 0.05 were considered statistically significant. The average thickness of untreated biofilms was 12±2 µm and 18±2 µm for 24 h-old *S. aureus* and *E. coli* biofilms, respectively, and 18±3 µm and 22±2 µm for 48 h-old cultures.

Widefield fluorescence images were captured using a commercial DMI 3000B inverted microscope (Leica Microsystems) equipped with an ORCA Flash 4.0 LT CMOS camera (Hamamatsu), a SOLA Light Engine (Lumencor), a 20x/0.40 HCX PL FLUOTAR objective (Leica Microsystems) and Leica filter cubes L5 (Ex 480/40, Em 527/30) for ThT, and TX2 (Ex 560/40, Em 645/75) for PI. To visualize both bacterial cells and matrix components, biofilms were suspended in PBS, washed three times with PBS and spread on a glass coverslip[5] coated with 0.1% poly-L-lysine solution (Sigma-Aldrich) for ten minutes. Coverslips were washed with PBS and immobilized biofilms were stained with 5 µM ThT in PBS.

Photodynamic studies with ThT alone and combined with antimicrobials.

For planktonic assays, bacteria were allowed to grow to OD_{600} = 0.2, harvested by centrifugation and washed 3x PBS. Bacteria were incubated with 2.5 – 5 µM ThT in PBS for 30 min in the dark and 200 µL of the suspensions were irradiated in 96-well plates using a blue LED (λ_{max} = 448 nm, 20 mW·cm^2). At different time points, 50 µL were collected for colony-forming units (CFU) quantification (see below).

Biofilms were allowed to grow statically for 24 or 48 h, gently washed with PBS to remove planktonic cells and incubated with 5 µM ThT for 30 min in the dark. Biofilms were illuminated with the blue LED at 20 mW·cm^{-2} for different time periods. For experiments combining PDI and antimicrobials, biofilms were primarily treated with ThT and blue light as described, followed by additional 24 h incubation with the corresponding antimicrobial in LB media and at 37 °C. Controls of PDI or antimicrobials alone were also carried out. The effect of the combined treatment was evaluated by applying the method described by Valeriote and Lin,[6] and defined as synergistic: [A + B] < [A] x [B] / 100, where [A] and [B] are the bacteria viability for treatments A (only ThT), B (only antimicrobials), and [A + B] for the combination of treatments A + B (ThT + antimicrobials).

For CFU quantification of planktonic or biofilm cultures, bacteria were 10-fold serially diluted and 10 µL of each dilution was streaked on LB-agar plates. After overnight incubation at 37 °C, the number of CFU·mL^{-1} was counted. Light and dark control assays were carried out for all experiments. In the light controls, the bacterial suspensions without ThT were exposed to the highest light dose applied. In the dark controls, suspensions containing ThT remained in the dark for the duration of the experiments. Data correspond to the mean ± SD from at least 3 independent triplicates.

References.

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Supporting figures

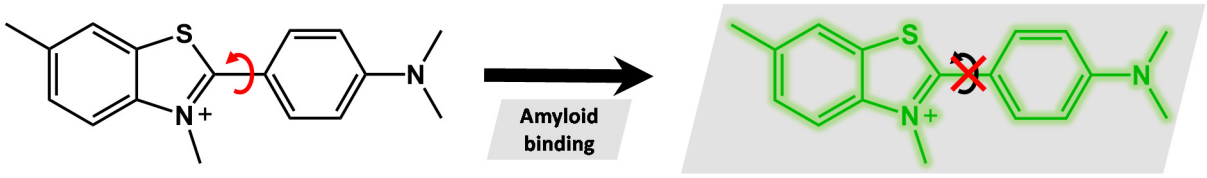

Figure S1. Chemical structure of ThT showing the rotation around the C-C bond connecting the benzothiazole and *N,N*-dimethylaniline moieties (red arrow) that enables non-radiative relaxation of the excited states in solution. Upon binding to amyloids, the rotation is blocked and fluorescence is activated.

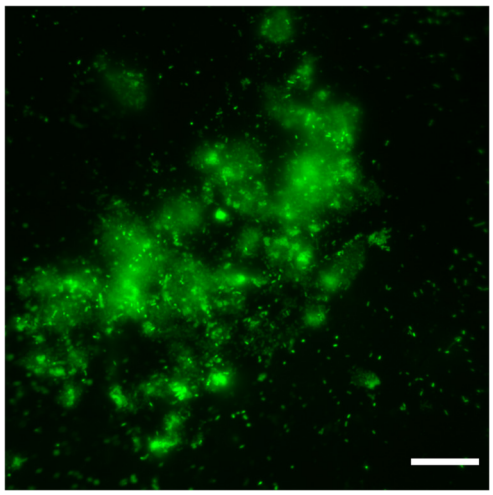

Figure S2. Fluorescence microscopy image of *E. coli* biofilm harvested from the growth medium and spread on a glass coverslip showing the activation of ThT fluorescence emission upon binding to bacterial cells and matrix components. Scale bar is 25 µm.

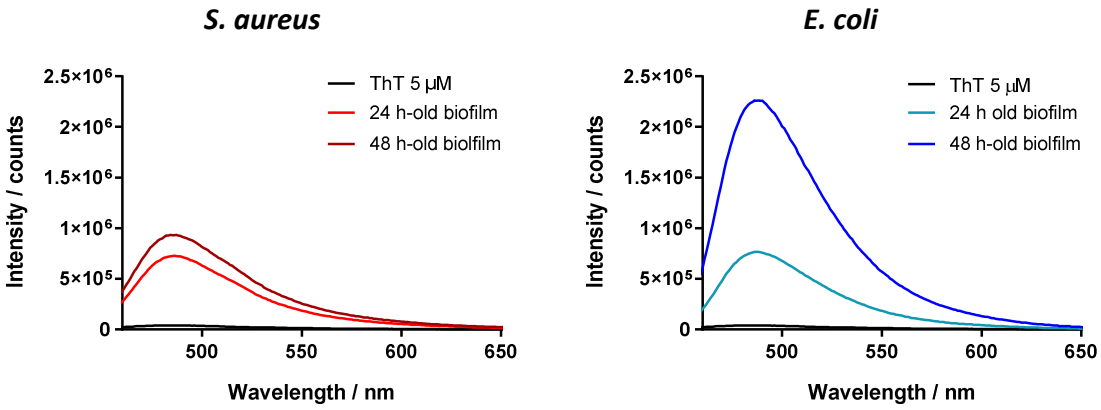

Figure S3. Activation of the fluorescent properties of 5 µM ThT in the presence of 24-h and 48-h old biofilm suspensions of *S. aureus* and *E. coli* containing $3 \cdot 10^5$ bacteria·mL^{-1}. In 24 h old cultures, the fluorescence enhancement was comparable in both biofilms with respect to free ThT in solution. However, it further increased in 48 h-old *E. coli* cultures (3-fold) as compared to *S. aureus* (1.3-fold), revealing superior ThT binding and activation capacity towards EPS components in *E. coli* biofilms.

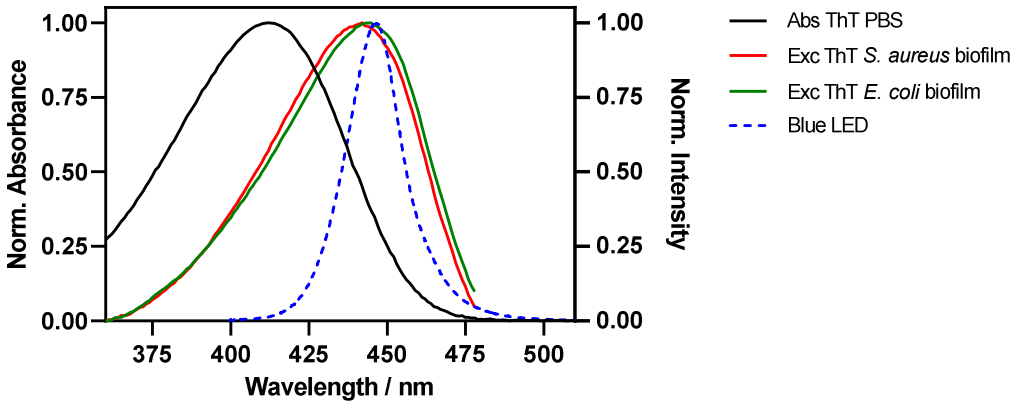

Figure S4. Normalized absorbance of free ThT in PBS (black) and its red-shifted excitation spectra in the presence of *S. aureus* (red) and *E. coli* (green) 48 h-old biofilms (λ_{obs} = 500 nm). The emission spectrum of the blue LED used for PDI experiments (blue dashed line) is centered at 448 nm, maximizing the excitation of bound-ThT.

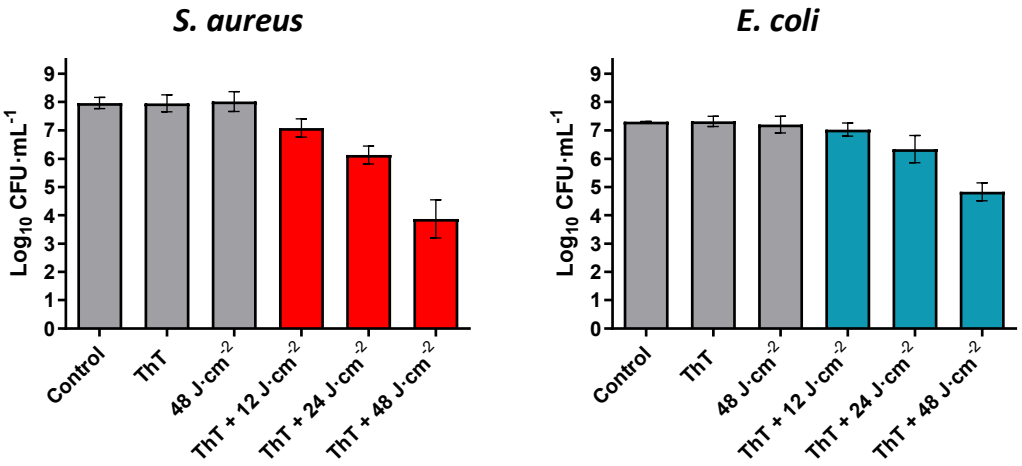

Figure S5. Photodynamic effect of 2.5 µM ThT against planktonic *S. aureus* and *E. coli,* reducing the number of CFU·mL^{-1} by >4 $\log_{10}$ and >2 $\log_{10}$, respectively. Increasing ThT concentration to 5 µM led to >7-$\log_{10}$ CFU·mL^{-1} reduction of *S. aureus* at light doses higher than 12 J·cm^{-2}, and 24 J·cm^{-2} for *E. coli* (not shown).