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Spatial patterning of engineered biofilms

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14. ABSTRACT
Bacteria living in biofilms can have wide-ranging impacts on society, ranging from threatening human health to purifying drinking water. Biofilms are aggregates of bacterial cells surrounded by a self-produced, spatially-structured extracellular matrix. During biofilm
growth, bacteria develop enhanced resistance to both antibacterial treatments and mechanical stressors, making them difficult to eradicate but robust enough for usage in beneficial environmental applications. In order to develop new anti-biofilm treatments and to efficiently deploy beneficial biofilms, a reproducible, engineerable biofilm model system is urgently needed. In this project, we used our home-built 3D bacteria printer in combination with engineered bacteria to create model biofilms containing spatially-patterned gradients of biofilm components. The printed biofilm bacteria were tested for their emergent biological properties, including the ability to resist antibacterial treatments. These model biofilms will be crucial for future development of anti-biofilm strategies and therapeutics, as well as for the production of beneficial biofilms for use in variable, challenging environments.

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Project Abstract

Bacteria living in biofilms can have wide-ranging impacts on society, ranging from threatening human health to purifying drinking water. Biofilms are aggregates of bacterial cells surrounded by a self-produced, spatially-structured extracellular matrix. During biofilm growth, bacteria develop enhanced resistance to both antibacterial treatments and mechanical stressors, making them difficult to eradicate but robust enough for usage in beneficial environmental applications. In order to develop new anti-biofilm treatments and to efficiently deploy beneficial biofilms, a reproducible, engineerable biofilm model system is urgently needed. In this project, we used our home-built 3D bacteria printer in combination with engineered bacteria to create model biofilms containing spatially-patterned gradients of biofilm components. The printed biofilm bacteria were tested for their emergent biological properties, including the ability to resist antibacterial treatments. These model biofilms will be crucial for future development of anti-biofilm strategies and therapeutics, as well as for the production of beneficial biofilms for use in variable, challenging environments.

Research background

Biofilms are three dimensional networks of bacteria embedded in a self-produced extracellular matrix composed of proteins (curli), polysaccharides (cellulose), lipids and extracellular DNA. Bacteria in biofilms are resilient to harsh chemical treatments and extreme environmental conditions. The robustness of bacterial biofilms and their mechanical and biological endurance have made them attractive to be used for beneficial environmental applications such as waste water processing, extraction of heavy metals and drinking water treatment plants. Despite of the extensive research on bacterial biofilms, the factors that govern their biological and mechanical toughness remain undetermined. This question forms the basis of our research project. In this project, we aim to identify the design principles of *Escherichia coli* bacterial biofilms (**Figure 1A**) that direct their subsequent emergent properties with the aid of 3D printing technology (**Figure 1B**).

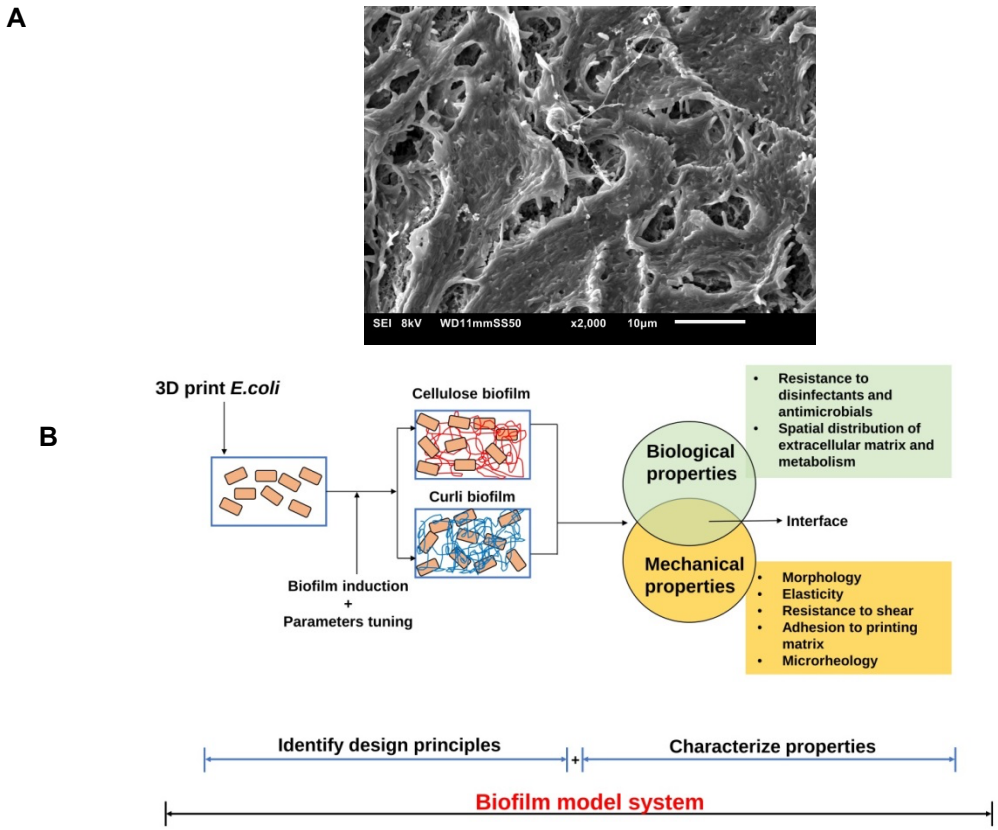

Figure 1: (A) Scanning electron micrograph of an *E. coli* biofilm on LB agar (5 d) grown at room temperature; (B) Schematic workflow of the study involving the construction of a biofilm model system with 3D printing technology

Briefly, genetically engineered *E. coli* expressing either curli or cellulose are made into bacterial inks (bioink) with sodium alginate solution. Bioinks are then 3D printed with our home-built DIY 3D printer into specific designs and shapes on a $CaCl_2$ containing agar surface. This results in formation of stable 3D printed-hydrogels containing bacteria and alginate (**Figure 2A**). Such 3D printed structures are then incubated at specific temperatures and time periods to allow biofilm formation by *E.coli*. After mature biofilms are formed, they are probed for their emergent properties. Tuning of parameters (such as bacterial density, curli density, cellulose density, nutrient/oxygen availability, rugosity, etc.) are done during the process of 3D printing specifically by varying the components of the bioinks, printing set-ups and incubation conditions (temperatures and media composition). Tuning of parameters is designed in such a way that the 3D printed-biofilms can mimic the structure-function relationships of natural bacterial biofilms over time. Thus, our project is expected to identify the design principles of biofilms and lead to construction of a biofilm model system (**Figure 2B**) that could be used for

practical applications such as testing potential anti-biofilm treatments, evaluating the adequacy of mathematical models of biofilms, etc.

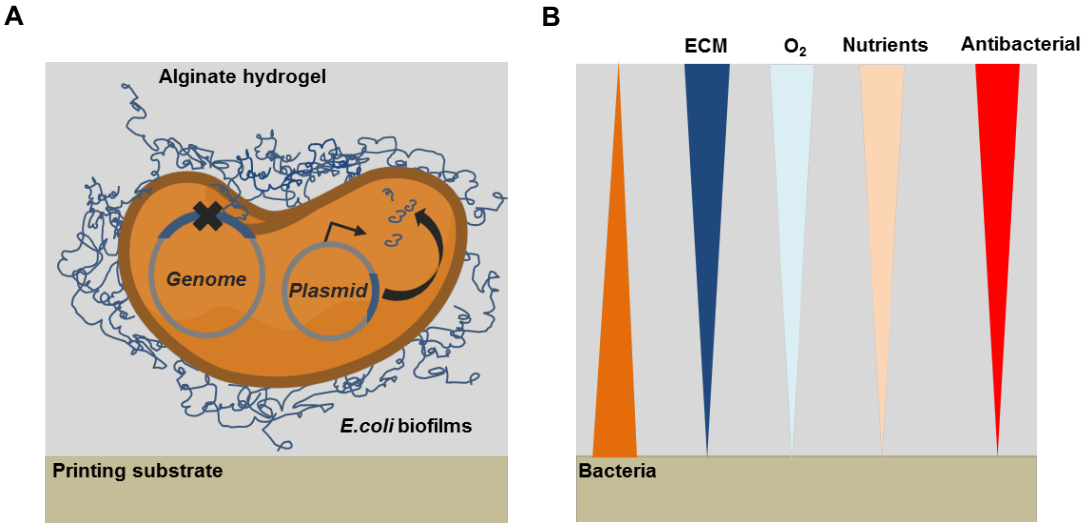

Figure 2: (A) 3D printed *E.coli* biofilm. Genetically engineered *E.coli* expressing tuneable curli or cellulose proteins (blue) are embedded in an alginate hydrogel of specified geometry and design over a printing substrate containing the nutrient medium for bacterial growth; **(B) Reproducible biofilm model system obtained by 3D printing.** This model will simulate the structure-function relationships of a natural biofilm over time. Gradients of different components (such as extracellular matrix (ECM) composed of curli and/or cellulose, oxygen and nutrient levels, bacterial density, antibacterial distribution) are achieved with our 3D printing set-ups.

Results and discussion

1. Optimization of 3D printing of *E.coli* biofilms

We use our customized DIY 3D bio printer for printing *E.coli* on $CaCl_2$ containing LB agar surfaces using printer-specific G-code commands for printing various shapes and designs (**Figure 3**). After 3D printing, bacteria are incubated at either room temperature or 37ºC for 5d to allow biofilm induction and subsequent studies.

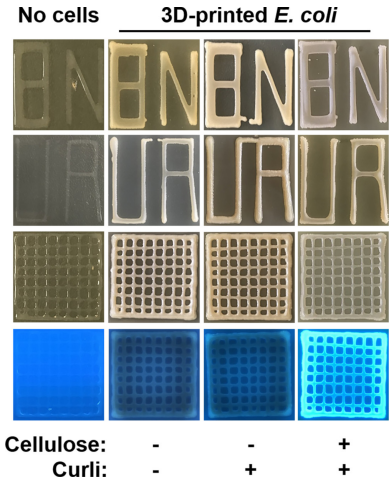

Figure 3: 3D printability of 7 day *E. coli* biofilms expressing cellulose and/or curli. Different types of possible patterning of 3D-printed biofilms (top three rows) and their fluorescence under UV in a calcofluor assay (bottom row). Calcofluor fluorescence under UV (wavelength: 312 nm) indicates cellulose production in the 3D-printed biofilms.

The 3D printed-*E.coli* are assessed for biofilm formation with citrate treatment assay in which the added citrate solution dissolves the alginate hydrogel leaving behind the biofilm structures. In this assay, biofilm-forming *E.coli* show resistance to citrate treatment whereas biofilm-negative *E.coli* are citrate sensitive. An example of this is shown in **Figure 4**.

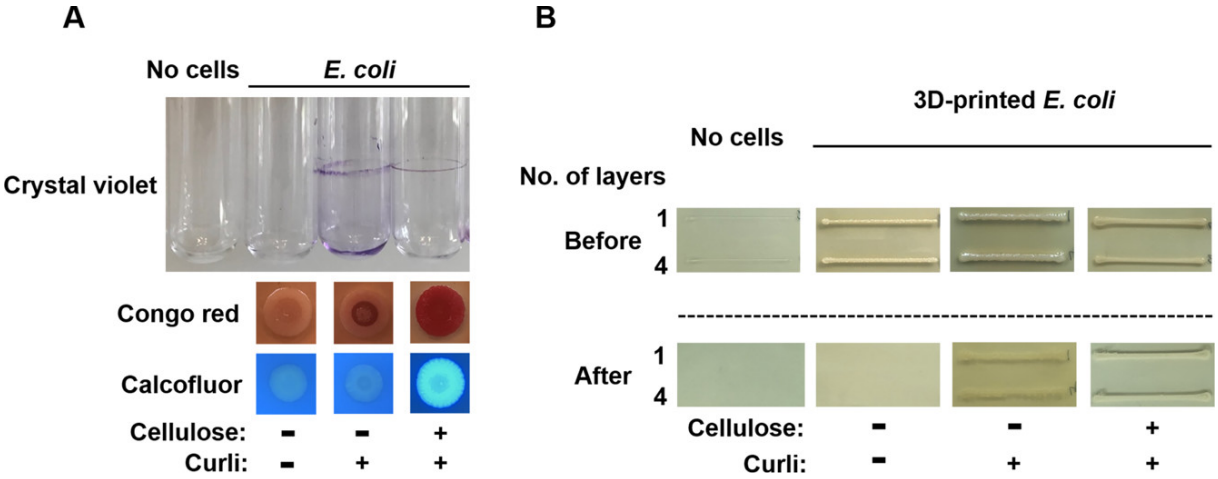

Figure 4: Biofilm formation by *E. coli* strains in this study. (A) Crystal violet (top), Congo red (middle), and calcofluor assays (bottom) for visualization of total biofilm and to identify the biofilm-matrix components. The crystal violet assay detects the total biofilm formation in liquid culture, whereas the Congo red assay detects the presence of cellulose and/or curli, and the calcofluor assay detects the presence of cellulose in colony biofilms (hydrogel culture) and (B) resistance of 3D-printed biofilms (one- or four-layered prints) to citrate treatment. Images on the top depict 3D-printed biofilms before citrate treatment, and the images on the bottom depict 3D-printed biofilms after citrate treatment. All biofilm samples were grown at room temperature for 7 days before these experiments were carried out.

2. Profile of oxygen level across the 3D printed biofilms

In a natural biofilm, oxygen levels typically vary across the biofilm structure with saturating levels of O_2 in the surface to absolutely anaerobic conditions (Zero O_2 levels) at the bottom of the biofilm where the metabolic activity of the bacteria is absolutely limited. We measure the O_2 levels across varying depths in our 3D printed-biofilms using an O_2 microsensor **(Figure 5)**.

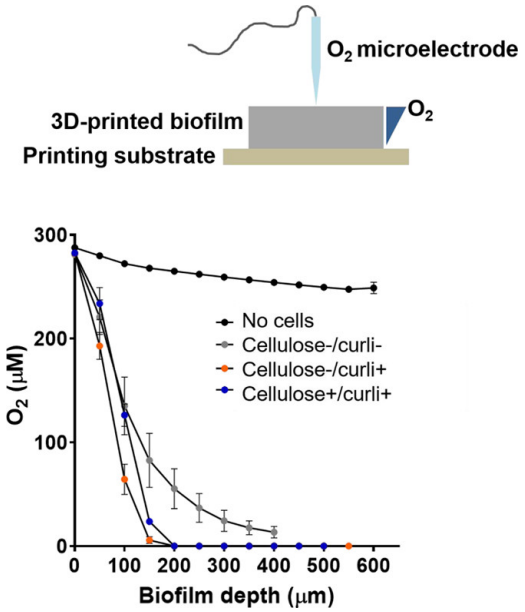

Figure 5: Oxygen profiles of four-layered 3D-printed biofilms revealing the presence of zero-O2 zones in the bottom layers. An oxygen microelectrode was used to profile the oxygen concentration at different depths in 3D-printed *E. coli*. The deepest O2 measurement coincides with the bottommost point of the 3D print at the interface with the supportive media such that the thickness of 3D prints can be compared with this method.

3. Measuring the emergent biological properties of 3D printed-biofilms

As a step forward, the biological properties (resistance to disinfectants) of 3D printed-biofilms were measured using Colony Forming Units (CFUs) measurement assays. Briefly, bacteria in different set-ups are 3D printed and allowed to form mature biofilms. After maturation, the biofilms are exposed to different concentrations of EtOH (ethanol) or Virkon S and assessed for viability based on CFUs (**Figure 6**).

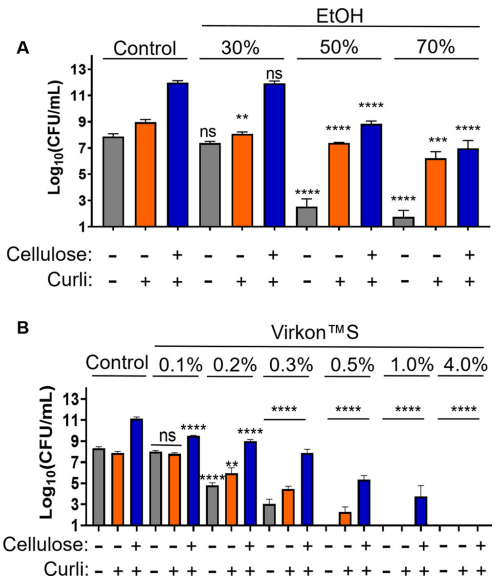

Figure 6: Emergent disinfectant resistance of 7 day-old 3D-printed biofilms to a 10 min exposure to varying concentrations of (A) ethanol or (B) Virkon S. Gray bars depict cellulose–/curli–, orange bars depict cellulose–/curli+, and blue bars depict cellulose+/curli+ 3D-printed *E. coli*. The control condition indicates treatment with sterile saline (0.9% (w/v) sodium chloride). Ns, not significant; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Statistical significance was assessed by comparing the disinfectant samples with their respective control sample using Student's *t*-test ($p < 0.05$; statistically significant).

4. Physical stability of 3D-printed biofilms

3D-printed biofilms could be used in various beneficial applications including bioremediation, wastewater treatment, or probiotic coatings on medical devices and surfaces to prevent colonization by pathogenic bacteria. In order to be employed in such applications, reversible adhesion of 3D-printed biofilms to different surfaces and physical stability are important aspects. We tested these parameters by removing fully grown (7 days old) 3D-printed *E. coli* (cellulose+/curli+) biofilms from agar and attaching them to fresh surfaces composed of bacterial cellulose, glass, or polystyrene. The 3D-printed biofilms displayed reversible attachment to fresh bacterial cellulose as well as glass and polystyrene surfaces (**Figure 7**).

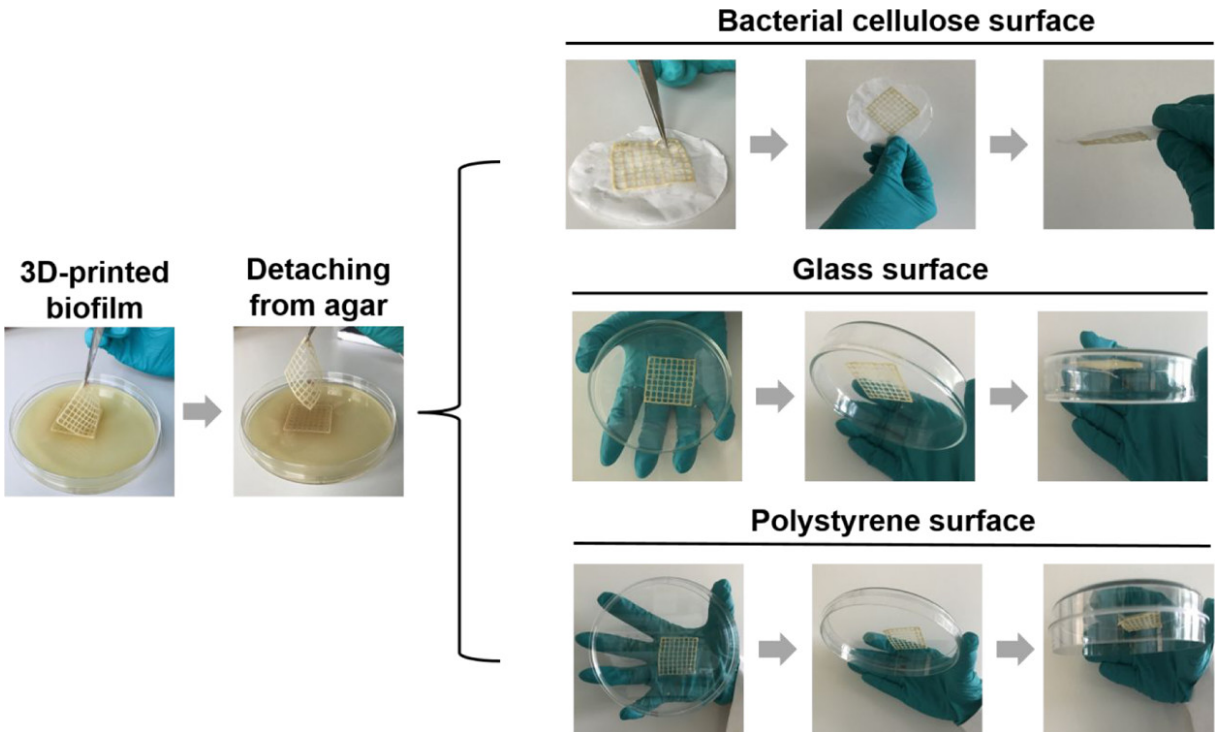

Figure 7: Adhesion of 3D-printed *E. coli* cellulose+/curli+ biofilms to bacterial cellulose, glass, and polystyrene surfaces after detachment from agar.

Conclusions and future perspectives

With our optimized 3D printing approach, we could effectively 3D print *E. coli* and induce them genetically to produce stable spatially patterned biofilms. Further, we are able to tune the design principles of biofilms including cell density, extracellular matrix components density (curli and cellulose), oxygen availability, etc. With our constructed 3D printed-biofilms, the emergent properties (for instance resistance to disinfectants) and oxygen distribution can be effectively gauged. Parameters like tolerance to ethanol treatments, presence of anaerobic zones in the 3D printed-biofilms closely match to that of natural *E.coli* biofilms. Future plans include further tuning the design principles and assessing other emergent biological properties (resistance to other disinfectants, antibiotics and distribution of metabolism), and mechanical properties (viscoelasticity). Overall, the results of this project will lead to construction of a reproducible biofilm model system that could be used as platform for testing new anti-microbials or to assessing the efficacy of theoretical models.

Publication outcomes from this funding

1. Balasubramanian S, Yu K, Cadenas DV, Aubin-Tam ME, Meyer AS. (2021) Emergent biological endurance depends on extracellular matrix composition of 3D-printed Escherichia coli biofilms. ACS Syn. Bio. 10, 2997-3008.
2. Balasubramanian S, Yu K, Meyer AS, Karana E, Aubin-Tam ME. (2021) Bioprinting of regenerative photosynthetic living materials. Adv. Funct. Mater. 2011162.
3. Balasubramanian S., Aubin-Tam ME., and Meyer AS. (2019) 3D printing for the fabrication of biofilm-based functional living materials. ACS Syn. Bio. 8, 1564-1567.
4. Spiesz EM., Yu K., Lehner BAE., Schmieden DT., Aubin-Tam ME., and Meyer AS. (2019) Three-dimensional patterning of engineered biofilms with a do-it-yourself bioprinter. J. Vis. Exp., 147, e59477.