Model Biofilms to Evaluate Antimicrobial Treatments and Structure-Function Relationships

Kirsten Parratt[1*], Joy Dunkers[1], Heidi Leonard[1], Charles Camp Jr.[1], and Nancy J. Lin[1]

[1.] Biosystems and Biomaterials Division, NIST, Gaithersburg MD, USA.
* Corresponding author: kirsten.parratt@nist.gov

Motivation: There is great need for methods to sterilize healthcare-associated surfaces to prevent the spread of infection, and this need is especially great since the recent discovery that biofilms can persist in dried form after cleaning [1, 2]. While antimicrobial agents are most commonly tested against planktonic microbes, many microbes in nature are actually found in an adherent state that offers additional protection against removal. Biofilms form on surfaces when microbes strongly adhere and secrete extracellular matrix components (DNA, proteins, polysaccharides, etc.) onto their surroundings. This matrix protects the biofilms against removal by physical or chemical means and is partly why biofilms require much higher antimicrobial doses to eradicate compared to planktonic cells [2, 3]. Biofilm removal involves the additional challenges of altered microbial phenotypes and viscoelastic behavior, which can impact antimicrobial treatment efficacy [4]. Many of these challenges are particularly important considerations for ultraviolet (UV) light-based treatment options as microbial cells within the biofilms will experience different light exposures as a result of scattering and absorption by the matrix components and overlapping cells. The goal of this project is to develop and characterize a model biofilm system in terms of composition, structure, functional behavior, and repeatability. By varying the number of cells at the initial adhesion event, diverse structures were obtained. It was our hypothesis that these structures would result in different functional behaviors, which might relate to our ability to eradicate them.

Experimental procedures: *Streptococcus mutans* (*S. mutans*, an oral pathogen and model biofilm-forming bacterial species) were inoculated into Todd Hewitt Broth (THB) and cultured overnight in a 37 ^{o}C incubator with 5 % (by volume) CO_2. Biofilm-forming media (BFM) was prepared from 25 % (by volume) THB supplemented with 5 mg/mL Yeast Extract, 10 % (by volume) 300 mmol/L sucrose, and 65 % (by volume) distilled water. Optical density (OD) was measured at 600 nm using a spectrophotometer, and cells were diluted to an OD of 0.1 in BFM. From this stock concentration, cells were serially diluted in BFM to achieve the desired inoculation concentrations (with an OD ranging from 0.1 to 0.0001). Biofilms were inoculated on tissue-culture treated polystyrene plates and cultured in the incubator for 48 h with a media exchange at 24 h. To measure total biomass, 0.1 % (by mass) Crystal Violet dye was incubated with biofilms, rinsed, and destained in 75 % (by volume) ethanol and 5 % (by volume) acetic acid. Crystal Violet absorbance was read at 570 nm. To measure DNA content, Syto9 was diluted in phosphate buffered saline (PBS) to 1 µmol/L and incubated with biofilms for up to 3 h. Syto9 fluorescence was read at an excitation of 485 nm and an emission of 500 nm. To measure metabolic activity, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was used. The 48 h biofilms were incubated with fresh BFM for 2 h to stimulate metabolic activity. After 1 h incubation at 37 ^{o}C with 1 mg/ml MTT in PBS, biofilms were destained with dimethyl sulfoxide, and MTT absorbance was read at 540 nm. For antibiotic challenge experiments, 10 mg/mL erythromycin was dissolved in ethanol and serially diluted from 100 µg/mL to 1 ng/mL in BFM. Biofilms were incubated in erythromycin-BFM for 2 h as an antibiotic challenge. Control biofilms received no antibiotics. Biofilms were either analyzed immediately or exchanged with fresh BFM, incubated for 24 h

to recover, and then analyzed for metabolic activity. To measure acid generation, biofilms exposed to antibiotics were incubated with fresh BFM for up to 8 h (either immediately or after incubation with fresh BFM for 24 h), and the pH of the supernatant was measured over time. For Scanning Electron Microscopy, biofilms were cultured on glass coverslips and fixed in 4 % (by volume) formaldehyde.

Main results: *S. mutans* biofilms were found to form diverse structures ranging from large individual colonies to continuous biofilm by controlling the initial inoculation concentration. These structures were visible as early as 6 h after inoculation and persisted for > 80 h of culture. For the 48 h biofilms in this study, biomass was relatively constant across the inoculation concentrations; however, greater metabolic activity was seen in biofilms with a higher starting OD. Localized measurements of biomass suggested that substantial well edge effects may exist for biomass measurements, with these effects being a function of plate size and initial OD. However, large well effects and OD differences were not seen for DNA measurements. Antibiotic challenge experiments showed that the normalized metabolic activity (relative to the no antibiotic control) for biofilms treated with lower antibiotic concentrations was relatively constant regardless of OD. This was true when biofilms were evaluated immediately after antibiotic exposure and after a 24 h recovery in fresh media. However, there were differences between the two timepoints for higher antibiotic concentrations, showing greater antibiotic efficacy in the recovered biofilms. The acid generation capability of the biofilms was also measured after antibiotic challenge. Unlike the metabolic measurements, an effect of OD was present. While all biofilms were unable to generate significant reductions in pH when tested immediately after the antibiotic challenge, lower OD biofilms that were allowed to recover for 24 h were able to generate acid.

Conclusions: In this work, an approach to produce model biofilms with varying properties was demonstrated. *S. mutans* biofilm models were developed using inoculation concentration as an independent variable to control biofilm structure. Biofilm composition, structure, and function were characterized. While biofilms from different initial ODs demonstrated noticeably different structures, not all compositional and functional measurements showed differences. The results highlight the complexities of biofilms and the need for additional biofilm studies, particularly as related to treatment, removal, and prevention of biofilms. Ongoing work includes efforts to classify biofilm images as a function of OD using machine learning, to evaluate biofilm formation as a function of OD for other microbial species, and to understand how metabolic activity relates to acid generation in *S. mutans*. We propose that model biofilms developed with the simple approach described herein could be used to evaluate and challenge antimicrobial methods across biofilms with a range of extracellular matrices and physical structures, properties that are likely to impact UV light-based treatment of biofilms.

[1] J. Otter et. al., Journal of Hospital Infection **89** (2015), p. 16 -27.
[2] P Stewart, International Journal of Medical Microbiology **292** (2002), p. 107 – 113.
[3] K Smith, I Hunter, Journal of Medical Microbiology **57** (2008), p. 966 - 973.
[4] T Garrett, M Bhakoo, Z Zhang, Progress in Natural Science **18** (2008), p. 1049 – 1056.
[5] KP and HL acknowledge funding from the NIST National Research Council Post-Doctoral Research Associateship Program.