Evaluating the Activity of an Anti-biofilm Agent via Imaging

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***Abstract*—The majority of human infections are considered to be due to microbes growing in complex communities called biofilms. Biofilms are often highly resistant to common antibiotics as compared to their planktonic (suspended) counterparts, yet many strategies to evaluate antimicrobials focus primarily on planktonic cells. To address the need for test methods to assess antimicrobial activity in biofilms, we previously developed an experimental framework to evaluate three cell states (planktonic, biofilm-forming and biofilm). As a demonstration of this framework, a quaternary pyridinium salt (QPS-1: 4-acetyl-1-hexadecylpyridin-1-ium iodide) was shown to be efficacious in killing planktonic and biofilm cells, as well as preventing biofilm formation of oral pathogen, *Streptococcus mutans* (*S. mutans*). Here, we present laser scanning confocal microscopy (LSCM) as an orthogonal approach to evaluate effects of QPS-1 on *S. mutans* biofilms. Qualitatively, QPS-1 disrupted the cell membranes, confirming the bioassay results and demonstrating QPS-1 as a promising anti-biofilm agent. Moreover, our results show LSCM is a useful platform for assessment of antimicrobials through visualization of morphological features and changes in biofilm cell membrane integrity. There remains a need for quantitative imaging of biofilms and their complex chemical, biological, and structural features to further elucidate effects of antimicrobials on biofilms.**

***Index Terms*—Anti-biofilm agent, antimicrobial efficacy, laser scanning confocal microscopy, microbial biofilm imaging, *Streptococcus mutans*.**

I. Introduction

Microbial biofilms are inherently complex, three-dimensional (3D) structures that possess a protective coating (polymeric matrix) that increases resistance to the host immune response and diffusion of drugs into the matrix [1,2]. These communities of microbes are both structurally and metabolically diverse, making them much more challenging to study than free-floating (planktonic) cells [3]. Although advanced imaging techniques have been widely applied to probe biofilm properties [4,5], many of these approaches can be time consuming [6], destructive [7] and result in data that are difficult to quantify [8]. Herein, we describe how traditional measurements (bioassays) are combined with imaging methods to provide complementary insights into biofilm response to an antimicrobial compound. Further, we discuss some existing challenges associated with imaging of biofilms.

II. Materials & Methods

Cell Culture: *S. mutans* UA159 biofilms were grown in Todd Hewitt Broth (THB) with 5 mg ml^{-1} yeast extract diluted to 25 % (by volume) in water with 30 mmol l^{-1} sucrose. After 24 h incubation, spent medium was replaced with fresh medium containing a bactericidal concentration of QPS-1 (50 µg ml^{-1}) or controls of 0 µg ml^{-1} QPS-1, 0.5 % dimethyl sulfoxide (DMSO), 400 µg ml^{-1} erythromycin (ERY), or 4 % ethanol (EtOH), then further incubated for 2 h. For planktonic

cultures, *S. mutans* were grown for 16 h in THB, diluted to an optical density at 600 nm (OD_{600}) of 0.001, then incubated for 2 h with 0 μg ml^{-1} QPS-1, 12.5 μg ml^{-1} QPS-1, 0.125 % DMSO, 100 μg ml^{-1} ERY, or 1 % EtOH.

Biofilm Imaging: After 2 h incubation, biofilm medium was removed and replaced with 0.85 % (by mass) NaCl containing 5 μmol l^{-1} SYTO 9 and 30 μmol l^{-1} propidium iodide (PI). After 20 min at room temperature, dye solution was removed and replaced with 0.85 % NaCl and samples were immediately imaged using a LSCM (Leica TCS SP5 II, 40X water immersion objective with 0.8 numerical aperture, 63X water immersion objective with 0.9 numerical aperture). At least six images were acquired per sample (Z-stack voxel: 96 nm x 96 nm x 294 nm, 1 Airy unit, line average 1, 200 Hz, xy/xz voxel: 379 nm x 379 nm x 0 nm, 1 Airy unit, line average 1, 100 Hz) on red (excitation 543 nm, emission 697 nm to 735 nm) and green (excitation 476 nm, emission 500 nm to 530 nm) channels, and experiments were repeated four times.

Planktonic Imaging: After 2 h incubation, 100 μl of cells were centrifuged (1.5 r.c.f., 10 min), resuspended in 0.85 % NaCl containing 5 μmol l^{-1} SYTO 9 and 30 μmol l^{-1} PI, and stored at room temperature for 15 min. Aliquots of 4 μl were placed between two glass coverslips for imaging using epifluorescence microscopy (Zeiss Axiovert S100 TV, 20X dry objective with 0.4 numerical aperture). At least three images were collected on two samples per condition, and the experiment was repeated twice. Planktonic samples were also plated on THB agar and incubated for 48 h to check for viable cells.

Data Analysis: Fluorescence image data were viewed in ImageJ (Bio-formats package) to visualize cell morphological features and collective cell membrane integrity for the planktonic and biofilm sample conditions [9]. Due to the complexity of the biofilm physical structure, the image data set was collected for qualitative comparison to the bioassay results from prior experiments.

III. Results & Conclusions

In this work, imaging methods were applied as an orthogonal approach to investigate antimicrobial effects of QPS-1 on planktonic and biofilm cell states of oral pathogen *S. mutans*. Imaging results were consistent with previous bioassay findings, with biofilm cells exposed to a lethal QPS-1 concentration of 50 μg ml^{-1} for 2 h consistently stained red (compromised membranes), demonstrating QPS-1 as a promising positive control material for anti-biofilm assays.

The data also indicated that imaging results must be interpreted with care. Planktonic imaging data, combined with results from agar plating, revealed that imaging alone can be misleading, as cells that appear green can imply viability in cases where agar plating reveals cell growth is actually inhibited (nonviable). These results demonstrate the need to select an appropriate method and measurand for a given property of interest, based on knowledge of mechanisms of action, if available. Further, the use of multiple methods, such as traditional measurement methods (bioassays) combined with orthogonal approaches (such as imaging), can provide additional confidence in the experimental results, particularly in situations where mechanism of action is unknown.

Transitioning to quantitative biofilm imaging is challenging due to the complex, heterogeneous, dynamic properties of biofilms. Biofilms are 3D and typically require advanced imaging systems, such as confocal microscopy, to capture their features. Further, a sufficient number of images must be collected to adequately sample the biofilm, a process that was too time consuming for the live biofilm imaging performed herein. Finally, the images must be processed, and relevant biofilm characteristics must be quantified. Though challenging, quantitative imaging of biofilms is needed to advance the field, as spatial information is crucial to understanding biofilms and their response to perturbations such as antimicrobials.

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