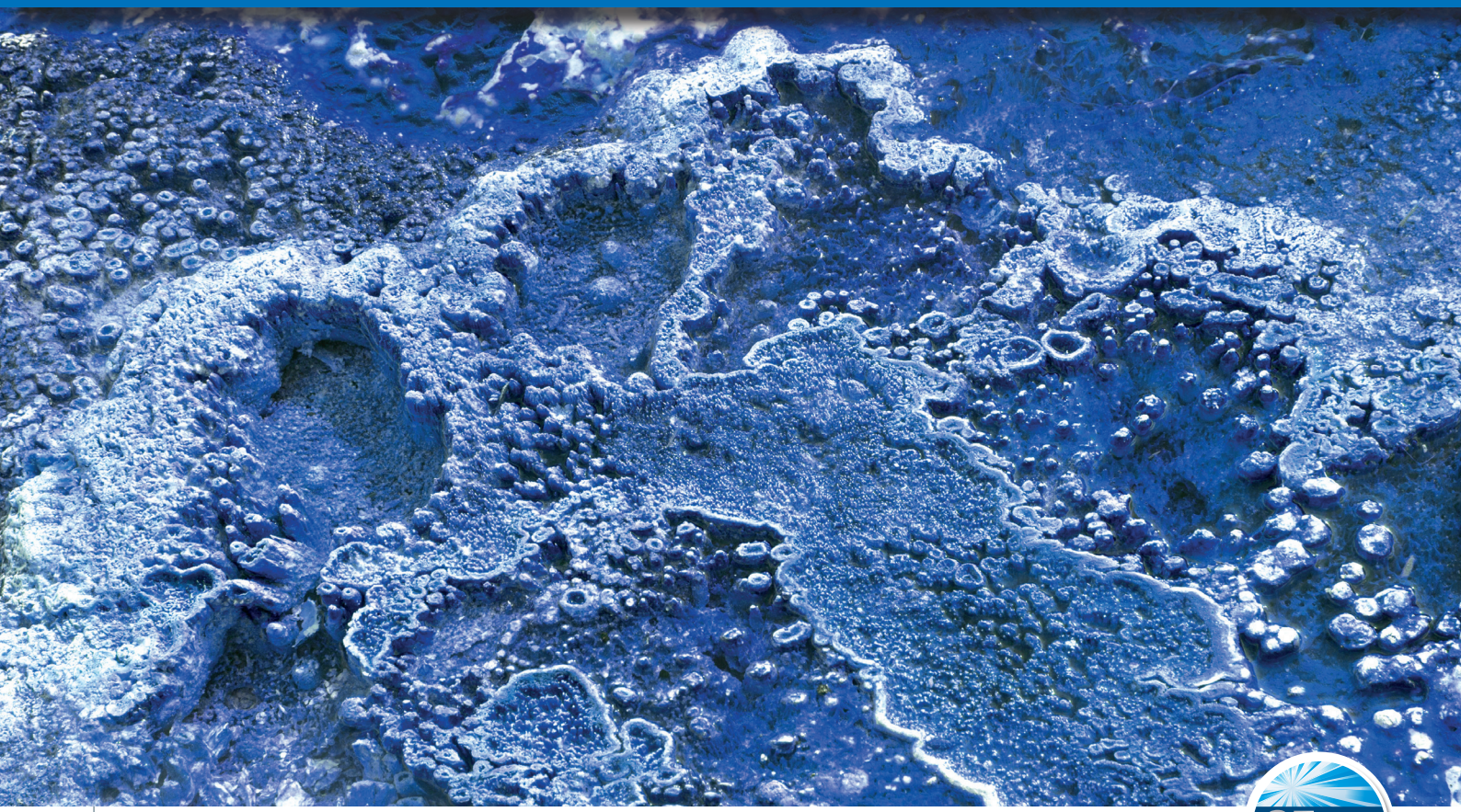

Re-discovering Bacterial Biofilm Heterogeneity with MALDI Imaging Mass Spectrometry

Biofilms are multicellular microbial communities where cells stick to each other and often adhere to a surface. The study of biofilms is important given that most infections in the human body are caused by biofilms originated from one, or a combination of bacterial pathogens

Keywords: MALDI-TOF, imaging, rapifleX, bacteria

Introduction

Biofilms exhibit tissue-like heterogeneity due to no t only the different bacterial subpopulations, but also the spatially-defined differentiation of cells that supports the overall survival of the biofilm [1]. During the process of infection in particular, concentration and distribution of nutrient metals fluctuates duet the efforts by the host to sequester essential minerals to limit pathogenicity (known as nutritional immunity). Identification of a particular bacterial sub-population within a biofilm has been

previously achieved by RNA FISH (fluorescence in situ hybridization) and reporter gene fusion techniques. The biggest limitation of these methods however, is that they require prior knowledge of the molecule of interest. Furthermore, the number of targets that can be assessed at a single time is also restricted.

P. aeruginosa is a biofilm-forming bacterium associated with pulmonary infections, particularly in patients with cystic fibrosis (CF) *P. aeruginosa* infects the respiratory tracts of 60-70% of CF patients by the age of 20 [2]. MALDI imaging mass spectrometry has proven a powerful technology to detect analytes in tissue while preserving their spatial distribution, and thus comprises a unique approach to study bacterial biofilm heterogeneity. Here the user was interested in studying biofilm architecture (i.e. Determination of subpopulations, as well as metal/ nutrient/protein gradients) in *P. aeruginosa* by imaging protein expression in 1) biofilms grown in vitro, and 2) biofilms developed in CF human lung. In addition, the effect of calprotectin, a key player in nutritional immunity, on biologically-relevant protein profiles was examined.

***P. aeruginosa* Biofilms**

Pre-wash

Post-wash

Experimental

Samples/Sample preparation

Bacterial biofilms were grown over 5–6 days, in a Drip Flow Biofilm Reactor (DFR) (BioSurface Technologies) using glass microscope slides (VWR, USA; #48300-025) as growth surfaces. Below is a diagram of the experimental workflow, from biofilm culture, to IMS data acquisition (Figure 1). Biofilm was embedded in 25% OCT and sectioned at 12 µm thickness using cryostat (cryostar Thermo NX70). Sections were mounted onto ITO slides, and washed with 70%, 90%, and 95% EtOH. Each wash was 30 sec. matrix (DHB (15 mg/mL) and CHCA (5 mg/mL) in 9:1 ACN: H_2O with 0.2% TFA) was applied using a robotic sprayer (HTX TM-Sprayer, HTXimaging) before MALDI analysis.

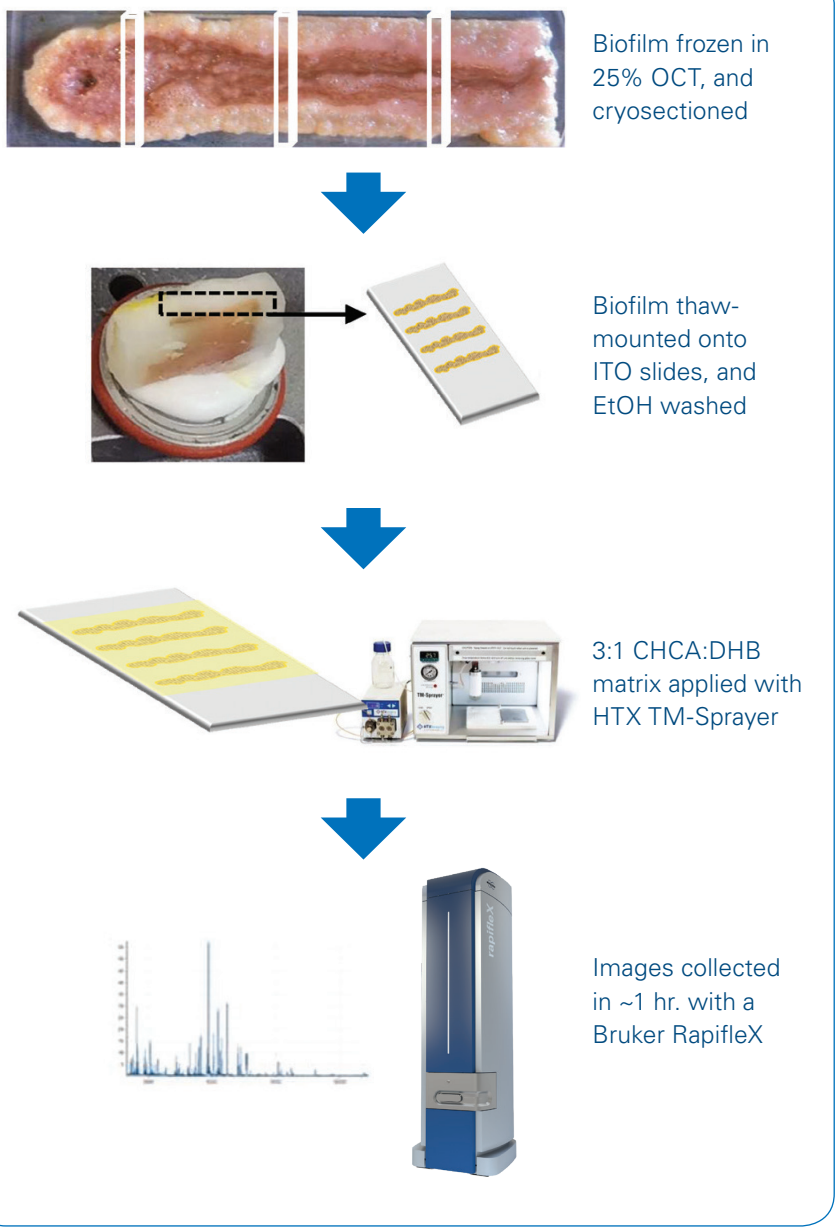

Figure 1. Summarized experimental work-flow for IMS of bacterial biofilms. See tables for further technical details and parameter optimization.

CF lungs sections (10 µm thickness, Leica CM3050S Cryostat) was sequentially washed with 70% EtOH (30 s), 100% EtOH (30 s), 6:2:1 EtOH / chloroform / acetic acid (2 min), 100% EtOH (30 s), H_2O (30 s), and EtOH 100% (30 s), followed by matrix coating (DHA (15 mg/mL) in ff9:1 ACN: H_2O with 0.2% TFA) and MALDI analysis.

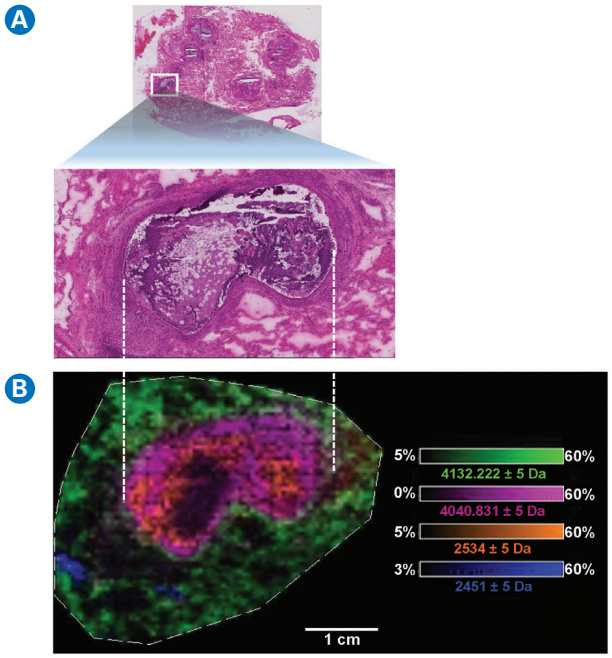

Figure 2. **A** *H&E histology of CF human lung (right lower lobe). The inflamed airspace is shown enlarged (inset, gram stained). Optical images obtained at x 20 magnification using a Leica SCN400 Brightfield Slide Scanner.* **B** *CF human lung at 50 µM using the RapifleX MALDI Tissuetyper, on linear positive ion mode. Overlay: m/z 4,132.222 (green), m/z 4,048.831 (pink), m/z 2,534 (orange), and m/z 2,451 (blue). 100 shots/pixel in 20 µm steps, 4537 positions. Scale bar 1 cm.*

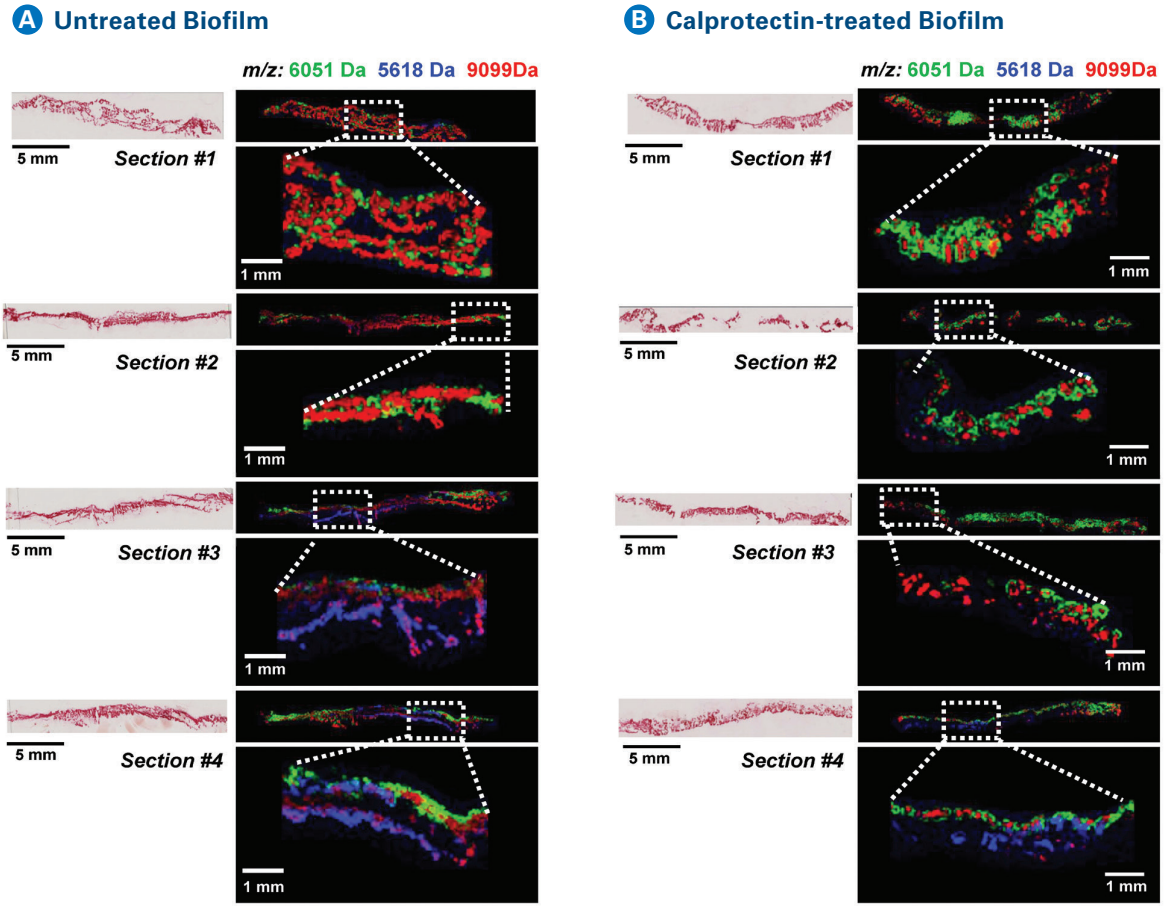

Figure 3. Four different cross-sections of Pseudomonas aeruginosa strain PA14 biofilms, untreated **A** *or exposed to calprotectin in the media* **B**. *Gram-Safranin stained images (post-analysis) are presented on the left, and MALDI IMS signals with differential biofilm localization are shown on the right. Overlay: m/z 6051 (green), m/z 5618 (blue), m/z 9099 (red). MALDI images collected at 50 µm, 66,571 pixels.*

MALDI imaging

All MALDI imaging of biofilms were acquired on a Bruker rapifleX TOF/TOF system in linear positive ion mode. Biofilm samples were performed with 50µm pixel size. 50 laser shots were collected in random-walk mode at each pixel in single beam mode. CF lung samples were operated at 50 µm pixel size with 500 shots per pixel, in 50 shot increments. All data analysis was performed using FlexImaging.

Results

Figure 2A shows an H&E stain of CF human lung tissue presenting significant inflammation. Gram-staining further revealed that bacteria with features consistent with *P. aeruginosa* and *S. aureus* co-colonized the infected airspace in the lung. A series of m/z protein ions were observed to localize in the inflamed airspace (Figure 2B). Calprotectin was also found to co-localize in these areas [3] from a CF patient.

Figure 3A shows MALDI IMS of four different cross-sections of a control *P. aeruginosa* biofilm. MALDI images highlight the heterogeneity of m/z spatial distributions along, and within, the biofilm. Analysis of *P. aeruginosa* biofilms challenged with the host protein calprotectin (an antimicrobial protein that chelates essential nutrient metals) revealed metal-sensitive bacterial subpopulations within the biomass (Figure 3B).

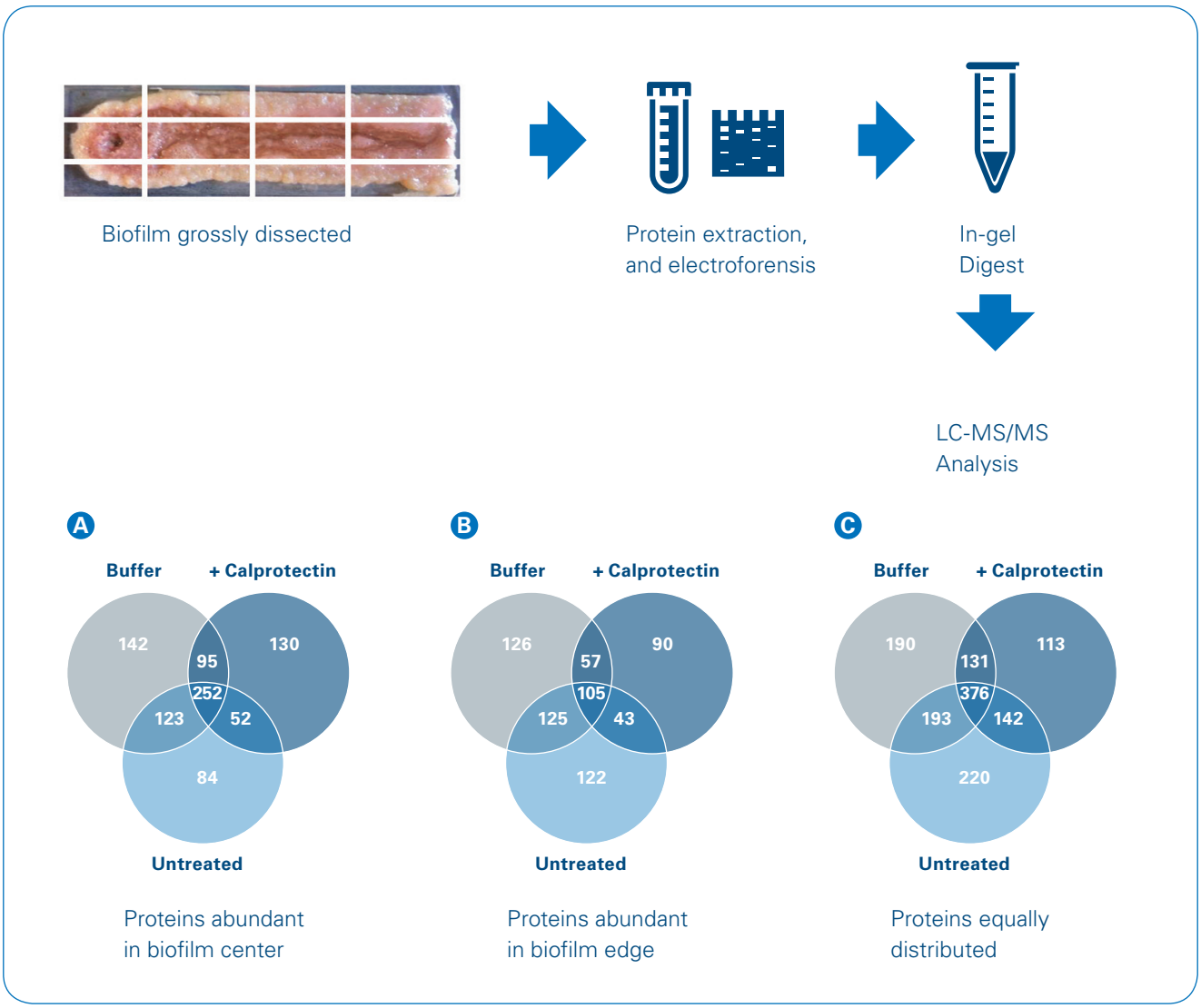

Figure 4. Summarized workflow for proteomics analysis of PA14 biofilms. Central channel (A) or nutrient deplete (edge) regions (B) were untreated / treated with buffer / or exposed to calprotectin, dissected and lysed (80% ACN, 5% formic acid, 400 µL bacterial protein extraction reagent) for protein extraction. Protein samples were separated in a 10% Novex Bis-Tris gel at 200 V, 5 min. Stained gel bands were excised and subjected to in-gel reduction, alkylation and tryptic digestion. Peptides were sequenced on a Thermo LTQ MS and the resulting spectra were searched against the Uniprot PA14 database using Sequest. Data were compiled using Scaffold version 4.4.3 (A) displays the number of proteins abundant in the biofilm center, (B) in biofilm edges, and (C) proteins that were equally distributed through the biofilm.

Bottom-up proteomics analysis of grossly-dissected biofilms allowed for the identification of hundreds of proteins related to metabolism, stress, protein synthesis, and other biological processes. MALDI IMS signals identified using bottom-up proteomics (Figure 4) revealed distinct protein profiles in the center compared to the edge of the biofilm, as predicted by the presence of nutrient gradients (i.e. proximal vs. distal to nutrient influx).

These protein profiles were also found responsive to calprotectin exposure (Figure 4A-C). The application of MALDI IMS to the study of structural microbiology significantly furthers our understanding of how microbial communities respond to the host-imposed nutrient limitations during the process of infection.

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Conclusions

- MALDI Imaging provides a powerful tool for the study of protein profiles (i.e. identification and spatial localization) and metal homeostasis within a bacterial biofilm, granting us the opportunity to re-discover biofilm architecture and heterogeneity, which seems tightly correlated with patterns of nutrient(s) availability.
- The information and data collected by these technologies has high clinical implications given the frequent occurrence of bacterial co-infections in human disease.

Learn More

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Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0)421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com