A New Effective Method for the Disruption of *Pseudomonas aeruginosa* Biofilms Using Adaptive Focused Acoustics™ (AFA)

Kenneth Flynn[1]
Mark Townley[2]
Gary Smejkal[1,3]
Hamid Khoja[3]
Srikanth Kakumanu[3]
Jim Laugharn[3]
Carl Beckett[3]
Vaughn Cooper[1]

Covaris®
the pre-analytical advantage™

Covaris, Inc. • 14 Gill Street, Unit H,
Woburn, Massachusetts 01801-1721 USA
Tel: +1 781-932-3959 • Fax: +1 781-932-8705
• Email: info@covarisinc.com • Web: www.covarisinc.com

1. Abstract

Proteomic analysis of the clinically and ecologically important microorganism *Pseudomonas aeruginosa* remains difficult due to its production of exopolysaccharides (EPS) that interfere with protein recovery and analysis. EPS anchors the cells to their substrate to protect the bacteria from physical or chemical host defenses and provide structure to the biofilm. The centrifugal removal of biofilms results in unacceptably low protein recovery and bias against biofilm-specific proteins. However, since the need to preserve post-translational protein modifications such as glycosylation prohibits the use of endoglycosidases to degrade interfering biofilms, new and improved pre-analytical methods are required to enable more reliable and comprehensive proteomic analyses.

Adaptive Focused Acoustics™ (AFA) effectively disrupted *P. aeruginosa* biofilms resulting in increased protein yields. Using Covaris Total Protein Reagent D, AFA yielded five times more total protein as determined by Bradford assay and confirmed by SDS PAGE, compared to negative controls. Scanning laser confocal microscopy using the fluorescent stain Calcofluor White M2R revealed extensive biofilms formed when bacteria were suspended in the lysis reagent. Counterstaining with SYTO-62 Red fluorescent cell permeable stain showed large numbers of intact cells encapsulated in the protective biofilm. Following AFA treatment, Calcofluor White M2R fluorescence showed the disruption of 99.6% of the biofilm. SYTO-62 fluorescence also decreased by 94.5% indicative of lysis of protected cells with only a few scattered fluorescent bodies remaining visible. Using AFA, the protein yields were nearly identical when the CHAPS concentration in the lysis reagent was lowered to 1% or 2%. Similar results were obtained when AFA was coupled to a preparative flow cell capable of processing 250 mL of liquid *Pseudomonas* culture.

2. Introduction

Pseudomonas aeruginosa is a monoflagellated gammaproteobacterium found in soil, water, and the normal human microflora. *Pseudomonads* are broadly resistant to antibiotics and are opportunistic pathogens of plants and animals. *P. aeruginosa* is the leading cause of mortality in cystic fibrosis[1]. *Pseudomonas* are also capable of growth on various hydrocarbons, including tar, oil, or jet fuel, and some species are used for bioremediation.

One of several extracellular polymeric substances secreted by *P. aeruginosa* is a repeating polymer of mannuronic and glucuronic acid referred to as alginate **(Figure 1)**. EPS biofilms anchor the cells to their substrate and protect the bacteria from host defenses such as macrophages and antibodies and impart antibiotic resistance[2].

Covaris AFA was investigated for the disruption of bacterial cells encapsulated in extensive biofilms. The need to preserve post-translational protein modifications such as glycosylation[3] and fucosylation [4,5] prohibits the use of endoglycosidases to degrade the interfering biofilms. Further, the dispersion of biofilms, rather than their removal by centrifugation, facilitates the isolation of biofilm- specific proteins[6,7].

FIGURE 1

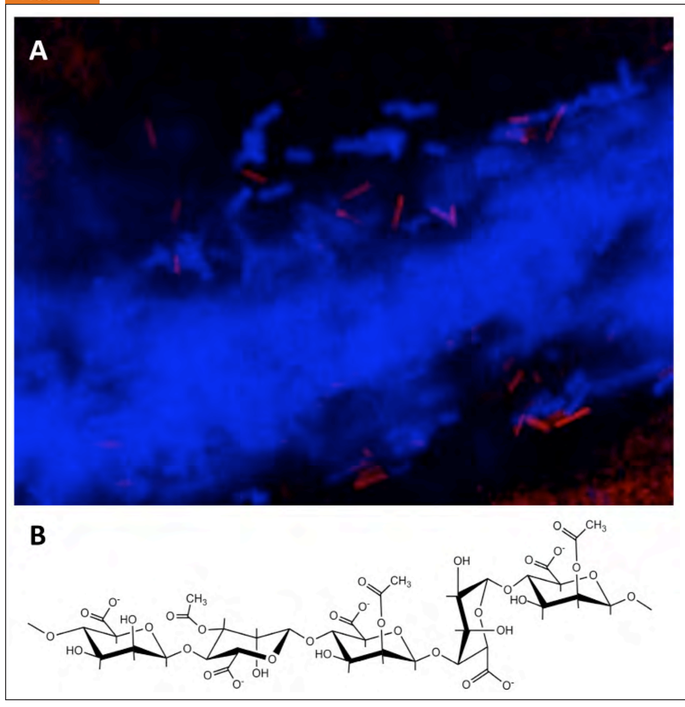

FIGURE 1.
(A) Dual fluorescence microscopy showing *P. aeruginosa* cells (red) and their biofilms (blue). Magnification 200X.
(B) Structure of possible arrangement of alginate subunits proposed by Franklin et al.[7]

3. Materials and Methods

Strains, media and culture conditions.

P. aeruginosa PA14 clones were grown in 5 mL tryptic soy broth for 6 hours at 37 °C. Multiple cultures were pooled in 10 mL volumes in tared 15 mL centrifuge tubes and incubated at 20 °C for 18 hours. Cells were recovered by centrifugation at 4,000 RCF for 30 minutes. Ten milliliter culture tubes yielded 195 ± 7 mg packed cells.

Covaris Adaptive Focused Acoustics™ (AFA)

Cell pellets were resuspended in 2 mL of Covaris Total Protein Reagent D supplemented with HALT Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim Germany). Other additives included 1%, 2%, or 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and reducing agents such as 5 mM tributylphosphine (TBP) or 20 mM tris (2-carboxyethyl) phosphine (TCEP). For each replicate, 1350 uL of cell suspension was transferred to a 15 x 19 mm glass vial with screw cap and the remaining 650 uL was reserved as negative control. Unless otherwise specified, the samples were processed at 18 °C in the Covaris E-220 at 275 W peak incidence power (PIP), 20% Duty Factor (DF), 200 cycles per burst (CPB) for 480 seconds.

AFA Treatment in a Covaris Flow Cell System

2.2 grams of pelleted biomass was resuspended in 250 mL TBS and processed in the Covaris SF200 Flow System at a flow rate of 25 mL/min. The cell suspension was recycled through the single cell flow system ten times and 1 mL samples were collected after each cycle. AFA conditions were 300 W PIP, 50% DF, and 200 CPB at 3 °C. Cell lysis was monitored by particle size analysis by dynamic laser light scattering using a Malvern Nano-S Instrument (Worcestershire, UK).

Protein analysis

Protein concentrations were estimated using the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA).

Laser scanning microscopy (LSM)

Cells were stained for nine minutes with 20 µM SYTO-62 red fluorescent dye (Invitrogen, Carlsbad, CA, USA). SYTO-62 binds to nuclei acids rendering it a good stain for visualizing intact cells, but does not discriminate between live or dead cells. Biofilms were counterstained for one minute with 135 µM Calcofluor White M2R as described by Cowan et al.[6]. Since Calcofluour stains polysaccharides, it was used to visualize EPS. LSM was performed using a Zeiss 510 META laser scanning confocal microscope (Zeiss, Oberkochen, Germany). Fluorescence was quantified using Image J open access Java based software (NIH, Bethesda, MD).

4. Results and Discussion

The precise mechanism by which AFA disrupts EPS biofilms is not known. It is likely that individual polymers are not sheared, but rather that non-covalently associated polymer networks are dispersed by AFA. **Figure 2** shows the nearly complete loss of Calcofluor White M2R staining following AFA treatment. Calcofluor White M2R fluorescence showed the disruption of 99.6% of the biofilm. SYTO-62 fluorescence also decreased by 94.5% indicating the lysis of encapsulated cells with only a few scattered fluorescent bodies remaining visible.

More total protein was recovered by AFA using the Covaris Total Protein Reagent D than with TBS or with 1% SDS in TBS, demonstrating the disruption of the EPS encapsulated cells. Further, AFA enabled CHAPS to be used effectively at lower than usual concentrations. **Figure 3** shows that as much total protein was recovered using only 1% CHAPS as with 2% or 4% CHAPS. Lowering the detergent concentration requirement during sample preparation significantly decreases the potential for interference with downstream mass spectrometry. Moreover, the inclusion of reducing agents such as TBP or TCEP did not significantly increase the total protein yield.

In the Covaris SF220 Flow System, 103 mg total protein was recovered by recycling the 250 mL cell suspension six times through the flow cell. This was increased to 111 mg after ten cycles, or 5.1% of the initial biomass. Particle size analysis showed 100% of the particles were too small to be intact cells following six cycles, despite the visualization of residual STYO Red fluorescence by LSM **(Figure 4)**. Lysates streaked onto agar plates failed to show growth after 24 hours.

5. Conclusion

AFA facilitates the proteomic analysis of recalcitrant microorganisms such as *P. aeruginosa*. AFA used in combination with optimized reagents effectively disrupted bacterial cells and associated biofilms in a non-contact isothermal process, resulting in higher protein yields while at the same time lowering the requirements for high detergency.

FIGURE 2

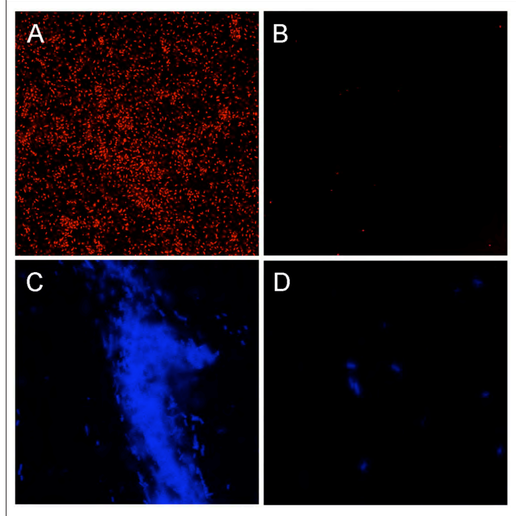

FIGURE 2. SYTO-62 red fluorescent staining of intact cells (A) before AFA and (B) after AFA. Mean fluorescence of identical areas was 11.02 and 0.04, respectively. Calcofluor White staining of (C) extensive biofilm before AFA and (D) fragments of biofilm after AFA. Mean fluorescence was 3.82 and 0.21, respectively. Magnification of 400X was used for A, B, and D. Magnification of 200X was used for C to show the extensive biofilm.

FIGURE 3

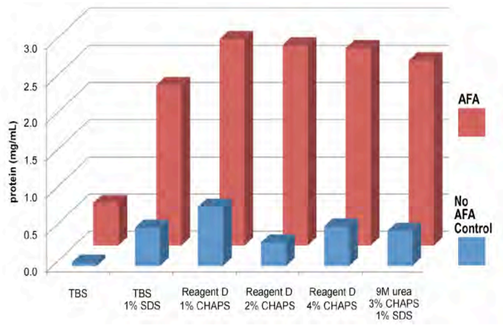

FIGURE 3. Optimized total protein recovery as a function of detergent type and concentration in TBS, Covaris Total Protein Re agent D supplemented with CHAPS, or 9M urea, 3% CHAPS, 1% SDS. AFA (red) extracted similar amounts of proteins at the lowest CHAPS concentration (1%) than at higher CHAPS concentrations. The addition of SDS did not increase protein yields. Negative controls (blue) are proteins extracted from cells resuspended in buffer without AFA treatment.

FIGURE 4

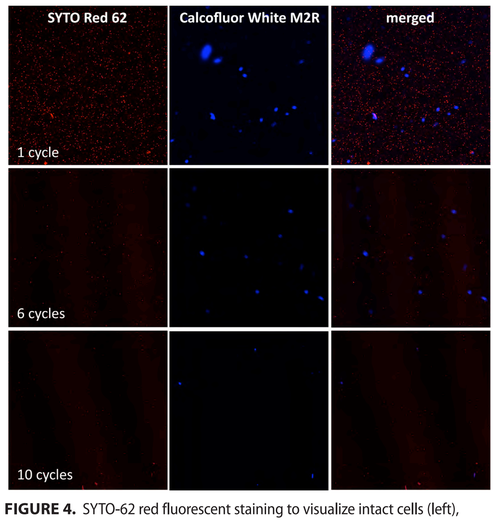

FIGURE 4. SYTO-62 red fluorescent staining to visualize intact cells (left), counterstaining of biofilms with Calcofluor White M2R (center) and merged images (right) showing the disruption of *P. aeruginosa* and their biofilms following 1, 6, or 10 cycles in the Covaris SF220 Flow System.

REFERENCES

[1] Haussler, S., Lehmann, C. Breselge, C., Rohde, M., Classen, M., Tummler, B., Vandamme, P., Steinmetz, I. (2001). Fatal outcome of lung transplantation in cystic fibrosis patients due to small-colony variants of the *Burkholderia cepacia complex. Eur. J. Clin. Microbiol. Infect. Dis.* 22, 249-253.

[2] Shih, P.C., Huang, C.T. (2002). Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance. *J. Antimicrobial Chemotherapy* 49, 309-314.

[3] Benz, I., Schmidt, M.A. (2002). Never say never again: protein glycosylation in pathogenic bacteria. *Mol. Microbiol.* 45, 267-276.

[4] Ma, B., Simala-Grant, J.L., Taylor, D.E. (2006). Fucosylation in prokaryotes and eukaryotes. *Glycobiology* 16, 158-184.

[5] Coyne, M.J., Reinap, B., Lee, M.M., Comstock, L.E. (2005). Human symbionts use a host-like pathway for surface fucosylation. *Science* 307, 1778-1781.

[6] Southey-Pillig C.J., David G. Davies, D.G., Sauer, K. (2005). Characterization of Temporal Protein Production in *Pseudomonas aeruginosa* Biofilms. *J. Bacteriol.* 187, 8114–8126.

[7] Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., Davies, D.G. (2002). *Pseudomonas aeruginosa* Displays Multiple Phenotypes during Development as a Biofilm. *J. Bacteriol.* 184, 1140–1154.

[6] Cowan, S.E., Gilbert, E., Liepmann, D., Keasling, J.D. (2000). Commensal interactions in a dual-species biofilm exposed to mixed organic compounds. *Appl. Environ. Microbiol.* 66, 4481-4485.

[7] Franklin, M.J., Nivens, D.E., Weadge, J.T., Howell, P.L. (2011). Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Frontiers Microbiology,* 2, 1-16.

AUTHOR AFFILIATIONS

[1] University of New Hampshire,
Department of Molecular, Cellular, and Biomedical Sciences, Durham, NH.

[2] University of New Hampshire,
University Instrumentation Center, Durham, NH.

[3] Covaris, Inc., Woburn, MA.