Non Thermal Plasmas – New frontiers in Control of Biofilm Infections.

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Abstract: Biofilms, surface adhered microbial communities embedded in a self produced matrix of extracellular polymers, represents the predominant mode of growth of microorganisms in almost every niche, including chronic infections. The biofilm provides a privileged environment for the survival of pathogens during persistent and chronic infections, acting as a nidus of infection. Considerable variability in tolerance to plasma exposure has been described across various species of bacteria when grown as biofilms. This presentation focuses on elucidation of the mechanisms by which bacterial biofilms tolerate plasma exposure and describes recent research into understanding and overcoming phenotypic resistance for improved biofilm control.

1. Introduction

The first observations of microorganisms (‘animalicules’) by Dutch scientist Antonie van Leeuwenhoek (1632-1723), led to the foundation of the field of microbiology. The validation of the ‘germ theory of disease’ by the experimental work of Robert Koch over 200 years later and the drafting of Koch’s postulates (the first of which require the causative agent of infectious disease (the pathogen) to be isolated and grown in pure monospecies culture) [1-3], defined the trajectory of classical microbiology for the next century until the accumulation of observations by a number of microbiologists led to the recognition that microorganisms grew not, for the most part, as planktonic or free floating single-celled organisms but, predominantly, as surface-associated consortia encased within a self-produced, protective matrix of extracellular biopolymers which exhibit distinct phenotypic variation from their planktonic counterparts [4,5]. A common feature of biofilm communities is an elevated tolerance to antimicrobial challenge, rendering chronic and device associated infection recalcitrant to conventional antibiotic and biocidal treatments.

The observation that bacteria form sessile communities on submerged or partially submerged surfaces was first described in the 1930s in the work of Arthur Henrici, [6] and Claude Zobell, [7,8]. However, it was the pioneering work of Bill Costerton and colleagues that eventually led, in 1978, to the proposal of the ‘Biofilm Theory’ [9]. Biofilms have been recognized for the critical roles they perform and the problems they cause in loss of industrial productivity, human, plant and animal disease, energy production and agriculture. A common

Atmospheric pressure non-thermal plasmas have proven to be effective in the eradication of a range of pathogenic biofilms, including *Pseudomonas aeruginosa* and other members of the ‘ESKAPE’ pathogen group [1]. These observations make this a promising approach for potentially controlling *P. aeruginosa* and other pathogens associated with chronic or device associated infections in the clinical environment. However, the exact mechanisms of plasma-mediated biofilm destruction (and tolerance to plasma exposure) remain poorly elucidated. Indeed, we report here widespread variation in response to plasma exposure across a range clinical strains of *P. aeruginosa* isolated from sputum of cystic fibrosis patients. In this study we have therefore investigated the role of biofilm matrix components in mediating tolerance to plasma-mediated bactericidal activity. In this presentation, discussion of the potential mechanisms by which plasma bactericidal efficacy may be attenuated during interaction with biofilm matrix components, and potential mechanisms to improve or reinstate antimicrobial sensitivity will be discussed.

2. Experimental

The dielectric barrier discharge plasma jet used in this work (figures 1a and 1b) is composed of a capillary quartz tube with annular copper electrodes assembled around it. The powered electrode is driven with a 20 kHz pulse repetition and high voltage (6 kV) power supply. The jet was operated under atmospheric pressure using helium (99.5%) and oxygen (0.5%) gas admixture with a total flow rate of 2 slm-1.

Initially 6 clinical P. aeruginosa strains (three mucoid, three non-mucoid, CF Unit, Belfast City Hospital) were grown on as biofilms on individual pegs of the Calgary biofilm Device (CBD/MBEC plate) and exposed to plasma irradiation for defined intervals. Time-kill curves were constructed for each strain tested.

The effect of gene knockouts of specific adhesion/biofilm components (f*leQ*, *pelA* and *mucA*) was evaluated and D-values calculated for both phases of killing.

Varying concentrations of alginate (0 - 1.25 %) and plasmid DNA (0 -75 µg/ml) were added to planktonic bacteria which was subsequently exposed to the plasma afterglow discharge for exposure times up to two minutes.

3. Results

Figure 1 shows the plasma jet configuretaion used in this study:

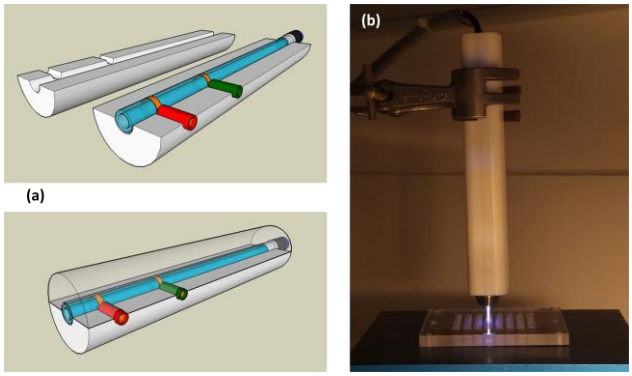

Figure (1a) *Schematic diagram of the kHz-driven plasma source used in this study and* ***(1b)*** *Photograph of hand held non-thermal plasma source used in the present study.*

As shown in Table (1) and Figure 2, mucoid strains are less susceptible to plasma inactivation in comparison with non-mucoid strain. D1 and D2 values for DC1 non-mucoid PA were 0.55 minutes and 5.15 minutes respectively whereas the D1 and D2 values for SS30-D2 mucoid PA isolates were 1.02 and 8.66 minutes respectively.

Table 1 *Decimal reduction times calculated from the biphasic Time-kill curves which constructed for each clinical strain tested*

P.aerogenosa (PA) clinical isolates	D_1-value (minute)	D_2-value (minute)
DC1 Non-mucoid PA	0.55	5.22
CW1 Non- mucoid PA	0.61	6.34
BU10 Non- mucoid PA	0.63	5.32
ES05C-F1 mucoid PA	0.90	8.50
SS30-D2 mucoid PA	1.02	8.66
W002 mucoid PA	0.75	8.34

The mucoid phenotype is characterised by production of an extensive extracellular matrix, or slime layer. The observation that mucoidy is correlated to biofilm tolerance to plasma exposure indicates that the biofilm matrix is directly influencing plasma derived activity-responsible species in vitro, attenuating the bactericidal activity of APNTP. Therefore, to examine this phenomenon further we employed a range of P. aeruginosa strains where key biofilm components were either knocked-out or overexpressed. The data from these studies are shown in Table 2.

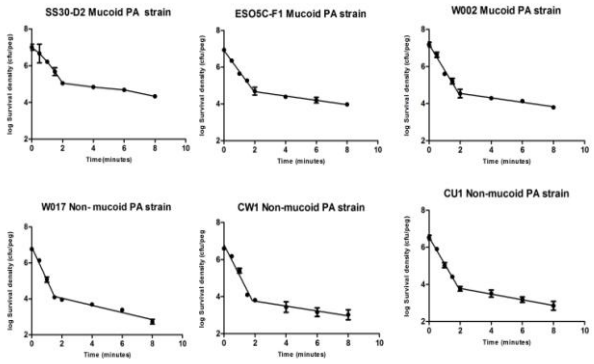

Figure 2 *Representative plasma-mediated time-kill graphs for mucoid and non-mucoid P. aeruginosa biofilms (clinical strains). (Each point represents the mean of 3 values ± SE). Time (min) versus log survival density (cfu/peg)*

Table 2 *Decimal reduction times calculated from the biphasic kill curves*

	fleQ (-)	*pelA*(-)	*mucA* (+)
D_1 – Value (minutes)	0.63	0.67	0.81
D_2 – Value (minutes)	5.59	6.68	10.53

The role of extracellular matrix in protection of Pseudomonas aeruginosa biofilm was confirmed by studying the effect of APNTP on mutant strains as shown in Table 2. D1 value for FleQ mutant strain which is mucoid deficient was 0.63 minutes while it increased to 0.81 minutes for mucA mutant which is alginate overproducer.

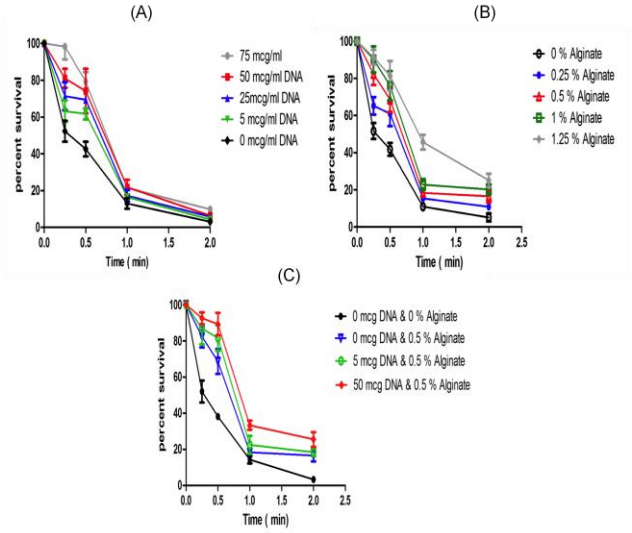

Figure 2: (a) Effect of Alginate (b) Effect of DNA (c) Synergistic effect of DNA and Alginate on Percent survival of PA01 planktonic cell after exposure to APNTP.

Figure (3a) shows that addition of alginate to planktonic PA01 resulted in the decrease in PA01 inactivation by APNTP. At 15 seconds exposures to APNTP, the percent survival increased from 52 % in absence of alginate to 91 % with addition of 1.25 % Alginate.

Presence of eDNA in planktonic PA01 reduced the inactivation efficiency of APNTP as shown in figure (3b) as PA01 percent survived increased 52 % to 98 % with addition of 75mcg/ml DNA after 15 seconds of exposure to APNTP . Figure (3c) shows the additive effect of DNA and alginate on percent survival of PA01 after exposure to APNTP. After 15 seconds treatment with APNTP , the percent survival was increased from 51 % to 82 % with addition 0.5 % alginate then to 92 % with addition mixture of 0.5% alginate and 50 mcg eDNA/ml.

Conclusions

All biofilm cultures exhibited increased tolerance to plasma exposure when compared to their planktonic counterparts, with mucoid strains exhibiting increased phenotypic resistance than non-mucoid strains, with a number of biofilm components conferring phenotypic resistance/elevated tolerance to the biofilm population as evidenced by gene knockout/overexpression studies (Table 1).

Mucoid phenotypes exhibit increased tolerance to plasma exposure, confirmed in both clinical and gene knockout/over-expression studies. The lack of elevated phenotypic resistance in persister cells (data not shown) indicates a physical destruction mechanism in atmospheric pressure, non-thermal plasma treatment.

This is the first study to determine the effect of specific biofilm components of non-thermal plasma exposure. This study will facilitate the optimisation of this technology towards clinical biofilm eradication of this important pathogen.

4. References

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