Limiting *Pseudomonas aeruginosa* Biofilm Formation using Cold Atmospheric Pressure Plasma

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ABSTRACT

We investigate the ability to disrupt and limit growth biofilms of *Pseudomonas aeruginosa* using application of cold atmospheric pressure plasma (CAP) plasma. The effect of the biofilm's exposure to a helium (CAP) jet was assessed at varying time points during biofilm maturation. Results showed that the amount of time during biofilm growth that CAP pressure was applied has a crucial role on the ability of biofilms to mature and recover after CAP exposure. Intervention during the early stages of biofilm formation (0 – 8 h) results in a 4 -5 log reduction in viable bacterial cells (measured at 24h of incubation) relative to untreated biofilms. However, CAP treatment of biofilm at 12 h and above only results in a 2-log reduction in viable cells. This has potentially important implications for future clinical application of CAP to treat infected wounds.

KEY WORDS: plasma, biofilm, *Pseudomonas aeruginosa*

INTRODUCTION

Owing to the increasing disparity between the rate of antimicrobial resistance and the discovery of new antibiotics, interest has grown for the use of novel antimicrobial technologies. One such field of research surrounds the use of cold atmospheric pressure plasma (CAP), often referred to as plasma medicine. CAP therapy has proven itself a promising alternative to traditional antimicrobial therapies, demonstrating its ability to inactive a wide range of pathogens including significantly drug resistant isolates, termed the 'ESKAPE' pathogens. [1, 2] CAP therapy relies on the delivery of a range of reactive oxygen and nitrogen species (RONS), including longer lived species such as hydrogen peroxide (H_2O_2).[3-5] Already well documented are the effects of plasma-generated reactive species, including the ability to control both the composition and delivery of such species according to the plasma parameters used.[6-9] As such, the versatility of CAP therapy has facilitated its use in a wide range of applications from surface decontamination (both biotic and abiotic), equipment sterilisation, microbial and spore inactivation and cancer therapy.[10] Of particular relevance to this study is the application of CAP to wound healing. In addition to its proven antimicrobial effects, studies have shown that CAP therapy may further enhance wound healing (at appropriate doses), via stimulation of fibroblast/ keratinocyte proliferation and migration or by its pro-angiogenic effects, thus making it an attractive alternative treatment option for chronic wound infection.[8, 11, 12]

It is estimated that between 65-80% of all wound infections are biofilm associated.[13] A biofilm occurs when "free living" planktonic cells adhere to a surface to form a dense community of biologically active, surface-bound microbes. Such bacterial communities are frequently encased in a polymeric layer consisting of proteins, glycoproteins and polysaccharides collectively known as the extracellular polysaccharide (EPS) matrix.[14] In addition to the protective nature of the EPS, it also confines the cells in close proximity to one another, facilitating the activation of quorum sensing networks via the secretion of specific signalling molecules. The subsequent alteration in gene expression may control the production of extracellular virulence factors and regulate specific intracellular metabolic functions, both of which contribute to the enhanced resistance of biofilms towards many forms of antibiotics.[15, 16] Indeed, biofilm formation can increase the concentration of antimicrobial that is required by 100-1000 times relative to planktonic cells.[17]

Pseudomonas aeruginosa (P. aeruginosa) is an opportunistic, Gram-negative bacterium, responsible for 85% of all nosocomial infections. It is particularly prevalent in burns causing 57% of all infections and in cystic fibrosis patients, causing 30% mortality in ventilator-associated pneumonia.[18, 19] *P. aeruginosa* employs multiple antimicrobial resistance strategies (e.g. efflux pump mediated resistance), exhibiting the highest levels of resistance to fluoroquinolones, ranging from 20-35% and increasing each year according to epidemiological trends.[20] As a result of the increasing prevalence of biofilm-associated infection, there is a growing requirement within the scientific and medical community for the development of therapeutic treatment strategies aimed at limiting and ultimately eradicating bacterial biofilms. An important consideration in the development of such technologies surrounds the recalcitrant nature of many antimicrobials towards biofilms when compared to planktonic cells. This study reports the ability of CAP treatment to effectively reduce the formation of *P. aeruginosa* biofilms, potentially increasing susceptibility to conventional treatment strategies (such as antibiotics), which, if used in conjunction may facilitate total infection clearance.

MATERIALS AND METHODS

Materials

We obtained *P. aeruginosa* strain PA01 from a strain collection belonging to the Biophysical Chemistry Research Group at the University of Bath. Lysogeny broth (LB), LB agar, brain heart infusion (BHI) agar, fetal calf serum (HyClone) and LIVE/DEAD™ BacLight™ Bacterial Viability Kits were all purchased from ThermoFisher Scientific (Loughborough, UK). The polycarbonate membranes (19 mm diameter and 0.22 nm pore size) used to cultivate biofilms were purchased from Whatman (Kent, UK). Phosphate buffered saline (PBS), sodium chloride (NaCl) and peptone were all purchased from Sigma Aldrich (Dorset, UK).

Bacteria and Growth Conditions

P. aeruginosa PA01 was taken from freezer stocks and grown on LB agar overnight at 37 °C to obtain single colonies. Bacteria cultures were grown from single colonies at 37 °C overnight with agitation (200 rpm) in LB, resulting in 10^9 colony forming units per ml (CFU/ ml) in final culture. Bacterial aliquots were stored at -80 °C in LB supplemented with 15% (v/v) glycerol.

Bacterial Biofilm Formation

Polycarbonate membranes were positioned on BHI agar and sterilised with ultraviolet light for 10 minutes. We aliquoted 20 µl of wound fluid mimic (fetal calf serum mixed in equal volume with 0.85% NaCl (w/v) and 0.1% peptone (w/v)) onto membrane surfaces. Artificial wound fluid was added to the membranes prior to bacterial inoculation to more closely mimic the wound environment. The membranes were inoculated with 30µl of overnight bacterial culture diluted 1:1000 into fresh LB broth. Membranes were incubated statically for 24 hours at 37 °C. Following treatment and incubation the biofilms were stripped from the membranes into sterile PBS via sonication (2 x 15 minutes with 1 minute vortex prior to and in between sonication steps). The value of CFU/ ml was then determined via serial dilution into sterile PBS and plating on LB agar to colony count.[21]

Plasma Treatment

The plasma source used in this study was a helium-driven plasma jet, as previously described.[22] Gas flow was fixed at 2 standard litres per minute (SLPM) and the plasma was operated at 10 $kV_{peak\text{-}peak}$ and 25 kHz. We used a treatment distance of 5 mm between the end of the capillary tube and the surface of the bacterial biofilms ('contact mode'). The *P. aeruginosa* biofilms were all incubated for a total time of 24 hours, removed from incubation at varying time points (0, 4, 8, 12, 20 and 24 hours) and subjected to 5 minutes plasma treatment before being re-incubated for the remaining time (with the exception of the 24 hour biofilms that were assessed immediately after treatment).

Scanning Electron Microscopy

Biofilms were fixed overnight in glutaraldehyde (1.5%) and paraformaldehyde (3%) in phosphate buffer (pH 7.3). Samples were rinsed with osmium tetroxide and dehydrated in ethanol/water mixtures at increasing concentrations. Biofilms were sputter coated with gold and imaged via a scanning electron microscope (SEM) JEOL SEM6480LV (Tokyo, Japan) operated at 10 kV.

Live/ Dead Staining and Confocal Microscopy

Biofilms were washed three times in PBS in order to remove planktonic bacteria. We prepared BacLight™ stains (consisting of two nucleic acid dyes: SYTO-9 and propidium iodide) according to the manufacturer's instructions. Each biofilm was immersed in 1.5 ml of the mixture of stains and incubated for 15 minutes in the dark. After staining the biofilms were rinsed once with PBS. Biofilms were then fixed onto a microscope slide and imaged using a confocal microscope to obtain Z-stacked images of the bacterial biofilms.

RESULTS AND DISCUSSION

The effect of plasma jet treatment of *P. aeruginosa* biofilms at varying time points during biofilm maturation is shown in Figure 1. Relative to the untreated control, we found a significant reduction in the number of viable bacterial cells at each treatment intervention point, demonstrating a clear disruption in the formation of mature biofilms as a result of CAP exposure.

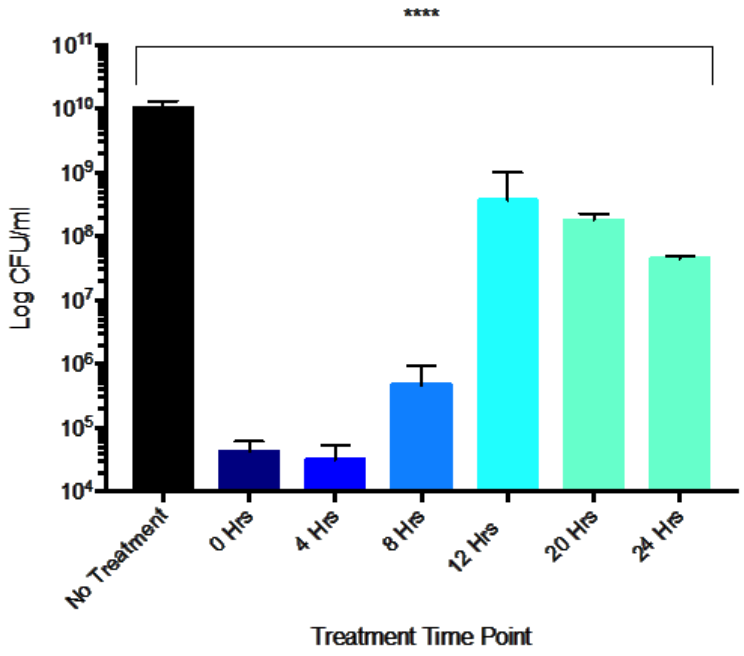

Figure 1 – Effect of treatment intervention time on bacterial viability after 24 hours incubation. CAP treatment was carried out as previously described at the time points shown. **** $p < 0.0001$ (one-way ANOVA with multiple comparisons).

CAP treatment at 0 and 4 hours produces a 5-log reduction in CFU/ ml, reducing bacterial load below the clinically relevant 10^6 CFU/ ml.[23, 24] However CAP treatment at later stages during biofilm maturation (12, 20 and 24 hours) reduces the CFU/ml by only 1-2 log units. From these data it would appear that there is a 'critical time-frame' for treatment intervention in order to limit bacterial proliferation within a biofilm. Although the exact reason for this is unclear at this point, a number of possible factors may play a part in the resistance of mature biofilms to plasma exposure, for example EPS production and/ or a change in bacterial genotype/ phenotype within the biofilms. The difference in cell counts at the varying stages of intervention may indeed play a role in the susceptibility of bacteria to plasma treatment. However, owing to the fact that each biofilm is incubated for 24 hours regardless of treatment time, the results suggest that not only does CAP treatment reduce the number of viable cells, it also prevents the recovery of biofilms into the mature state.

We carried out qualitative analysis of the biofilms before and after CAP treatment using SEM to look more closely and evaluate the effect of CAP exposure on a cellular level. Figure 2A shows an untreated *P. aeruginosa* biofilm grown for 24 hours. The bacterial cells are present in high density reflecting the high CFU/ ml calculated in the previous quantitative data (Figure 1). The presence of the EPS matrix is clearly visible, holding the cells in close proximity to one another. Figure 2B shows a *P. aeruginosa* biofilm incubated for 24 hours but treated with the CAP jet at 8 hours of growth. Relative to Figure 2A, this shows a distinct reduction in bacterial cell density alongside an accumulation of cellular debris, likely the result of bacterial cell death during CAP treatment. There is also a clear reduction in the EPS matrix. Figure 2C shows a *P. aeruginosa* biofilm grown for 24 hours treated with the CAP jet at 12 hours of growth. As expected from the quantitative data, we see a higher density of cells relative to the biofilms treated at 8 hours. However cell morphology suggests significant bacterial cell death and a clear disruption to the EPS relative to the untreated control (Figure 2A). Interestingly, despite the higher

number of viable cells when treating the biofilms at 12 hours relative to 8 hours, the ability of the former to recover to full cell density as expected in a mature biofilm is reduced, potentially reflecting the disruption to both the bacterial cells and the EPS matrix as shown in Figure 2C.

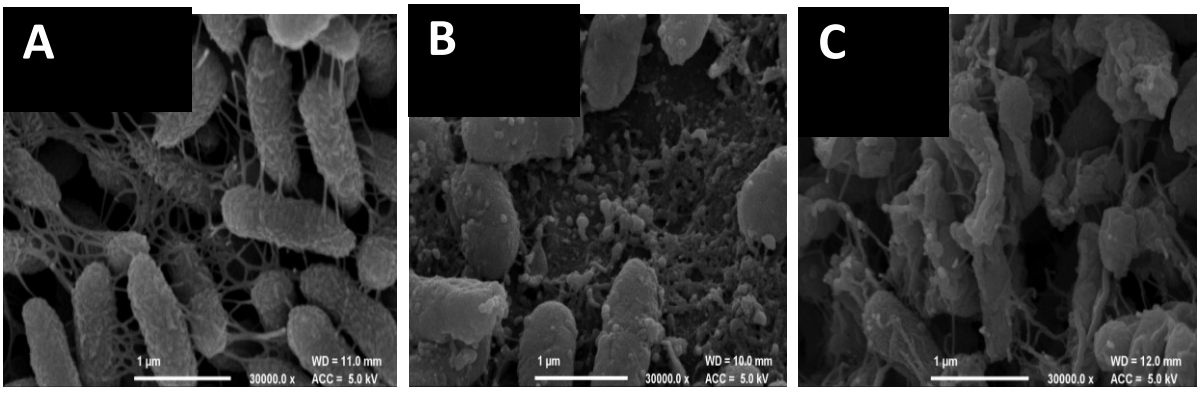

Figure 2 – SEM images of CAP treated biofilms **(A)** Untreated 24 hour *P. aeruginosa* biofilm(control) **(B)** 24 hour *P. aeruginosa* biofilm treated with the CAP jet for 5 minutes at 8 hours growth **(C)** 24 hour *P. aeruginosa* biofilm treated with the CAP jet for 5 minutes at 12 hours growth.

To further investigate the 3-log difference in the CFU/ ml between biofilms treated at 8 hours and 12 hours, we carried out live/dead staining to assess the difference in viable bacteria. Figure 3 shows the difference in cell density between biofilms treated at the two different intervention points. As expected from the previous quantitative and qualitative data, a significantly higher density of cells can be seen in the biofilms treated at 12 hours (Figure 3B).

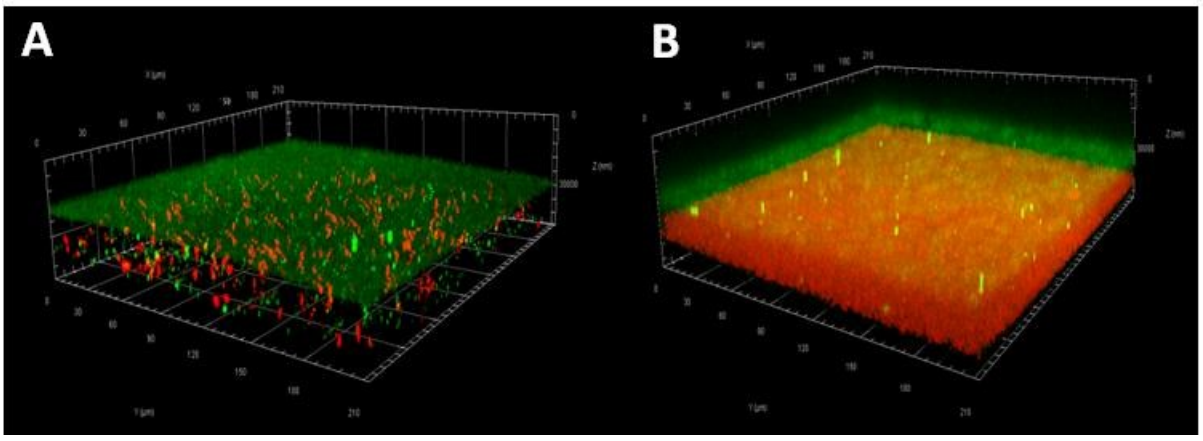

Figure 3 BacLight ™ LIVE/DEAD staining of 24 hour *P. aeruginosa* biofilms **(A)** Treated with CAP jet for 5 minutes at 8 hours growth **(B)** Treated with CAP jet for 5 minutes at 12 hours growth. Images are inverted, representing the biofilms from the base of the membrane downwards.

Figure 3A shows a thin layer of healthy viable cells, likely the result of the 16 hour post-treatment recovery period to which the biofilm was subjected, supporting the presentation of healthy cells in Figure 2B. Early treatment of the biofilms (8 hours and less) provides adequate time for the recovery of viable bacterial cells (albeit not to the full cell density seen in untreated, mature biofilms over the

same time period). However, in Figure 3B there is clearly a larger proportion of dead bacteria, suggested by SEM (Figure 2C). The density of the bacterial biofilm provides an impenetrable layer of biological material that protects the cells in the lower levels of the biofilm from the plasma jet action. Despite the ability of CAP treatment to cause significant cellular lysis, the protective nature of the more established biofilms shields the cells in the lower layers of the biofilm, thus retaining cell viability (Figure 1) despite the presence of dead cells (Figures 2 and 3).

CONCLUSION

using CAP therapy in a time-dependent manner is crucial in reducing the formation of mature *P. aeruginosa* biofilms. Whilst CAP therapy is able to cause significant bacterial cell death, the presence of both dead and living cells contained within an established biofilm offers protective effects relative to the cells in the lower layers of the biofilm, resulting in the retention of viable cells. However, treating biofilms in the early stages of development (< 12 hours) can significantly reduce bacterial loads to levels whereby traditional treatment strategies may become effective. Using CAP therapy as a tool to limit biofilm formation may prove to be clinically advantageous by increasing the potential for immune system clearance without the need for pharmaceutical intervention. Furthermore, CAP treatment could be effectively used in tandem with antibiotics by disrupting biofilm formation thus reducing the concentration of antimicrobial required. This technology therefore has the capacity to contribute to the global aim of decreased reliance on antibiotic use.

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