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PLASMA PROCESSES AND POLYMERS

FULL PAPER

Extracellular polymeric substance-mediated tolerance of *Pseudomonas aeruginosa* biofilms to atmospheric pressure nonthermal plasma treatment

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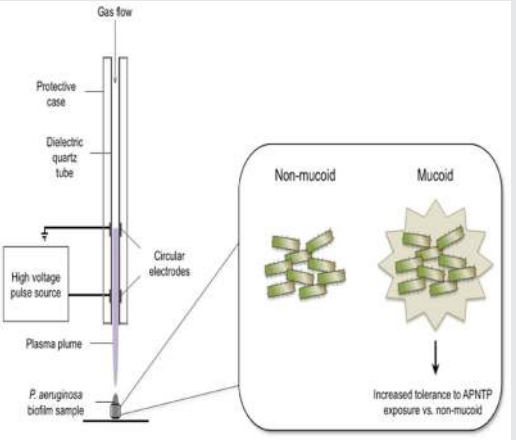
Abstract

Pseudomonas aeruginosa is an opportunistic disease-causing bacterium, with a number of strains exhibiting a mucus-forming (mucoid) phenotype during infection, producing biofilms with a surrounding matrix containing alginate. Atmospheric pressure nonthermal plasmas (APNTP) are an emerging, potential approach to control biofilms across a range of medical and industrial applications. In this study, we examine the effect of plasma treatment on *P. aeruginosa* biofilms from clinical samples of cystic fibrosis patients, exhibiting both mucoid and non-mucoid types. Biofilms of mucoid strains exhibit significantly elevated APNTP tolerance ($p < .05$). Endogenous alginate overproduction, as well as supplementation of *P. aeruginosa* cultures with exogenous alginate, results in significantly increased APNTP tolerance. Overall, this study shows how extracellular polymeric substance components mediate tolerance to APNTP, with significantly greater effects observed in mucoid strains.



KEYWORDS

alginate, biofilm, mucoid/non-mucoid, plasma, *Pseudomonas aeruginosa*

1 | INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous, Gram-negative opportunistic pathogen, which displays metabolic versatility and an ability to colonise a wide variety of environments.[1,2] The ability of *P. aeruginosa* to colonise a range of both biotic and abiotic surfaces and its widespread distribution in aquatic environments, soil and engineered water systems

make it a successful and increasingly prevalent plant, animal and human opportunistic pathogen,[1,3–5] especially in hospital-acquired infections and chronic infections of cystic fibrosis (CF) patients and patients with compromised immune function. *P. aeruginosa* exhibits resistance to a broad range of antibiotics and antimicrobial agents,[5,6] making eradication in chronic infections, including CF, difficult.[7]

Some strains of *P. aeruginosa* have the ability to exhibit a mucus-forming (mucoid) phenotype during infection, whereby the bacteria express a surrounding matrix containing alginate.[8] This facilitates the formation of protected microcolonies and eventually the formation of complex biofilms composed of alginate and other extracellular polymeric substances (EPS), including Psl and Pel, and extracellular DNA (eDNA).[9–12] Development of mucoid strains of *P. aeruginosa* in CF patients is a sign of chronic infection, which is often associated with poor prognosis.[13] Generally, biofilm formation is accompanied by a significant elevation in tolerance to antimicrobial agents and normal immune clearance, rendering conventional approaches to treatment and eradication of chronic biofilm-associated infections exceptionally challenging and problematic.[14]

Atmospheric pressure nonthermal plasmas (APNTP, also called cold plasmas) are an emerging, potential approach to control bacterial biofilms across a wide range of medical and industrial applications, which are currently the subject of intensive research in the nascent field of plasma medicine.[15] Nonthermal plasmas are capable of generating a diverse array of reactive oxygen and nitrogen species (RONS), which have a range of biological activities from stimulation of wound healing to antimicrobial activity.[15–18] Recent works in our group have demonstrated the potential of APNTP for the efficient eradication of *P. aeruginosa*[19,20] and the broader ESKAPE group, a group of pathogens with growing multidrug resistance, including *Escherichia coli* biofilms.[21,22] Furthermore, a number of studies have examined the potential cellular targets of nonthermal plasma exposure in bacteria, providing valuable information on the potential mechanisms of action[23] and tolerance to nonthermal plasma exposure, revealing a role of the redox-active molecule phenazine[24] and bacterioferritin[25] in *Pseudomonas* tolerance to nonthermal plasma. However, significant gaps exist in our current understanding of the precise mechanisms of antibacterial action of nonthermal plasma exposure and the role played by extracellular components of the bacterial biofilm in mediating tolerance to nonthermal plasma. In this study, the susceptibility of a range of clinical isolates of *P. aeruginosa*, exhibiting both mucoid and non-mucoid phenotypes, to APNTP exposure was evaluated using an in-house designed plasma jet, as previously described.[19,26] In addition, the potential role of biofilm matrix components in mediating tolerance to nonthermal plasma exposure was investigated using both *P. aeruginosa* mutant strains and evaluation of the effect of the addition of exogenous biofilm components on the biocidal efficacy of nonthermal plasma against *P. aeruginosa* biofilm was performed.

TABLE 1 Response of different *Pseudomonas aeruginosa* clinical isolates to APNTP exposure

PA isolate	D_1 value (min)	D_2 value (min)
SP1 non-mucoid PA	0.55	5.22
BC8 non-mucoid PA	0.69	6.20
JT1 non-mucoid PA	0.71	6.49
MP1 non-mucoid PA	0.61	6.34
MH10 non-mucoid PA	0.63	5.32
QB17 non-mucoid PA	0.55	5.15
BRE2 non-mucoid PA	0.72	6.80
ES05C-F1 mucoid PA	0.90	8.50
SS30-D2 mucoid PA	1.02	8.66
W002 mucoid PA	0.75	8.34

Note: D_1 and D_2 values, representing 90% reduction for the first and second phases of the kill curves, respectively, are shown for non-mucoid and mucoid *P. aeruginosa* clinical isolates after exposure to APNTP.
Abbreviations: APNTP, atmospheric pressure nonthermal plasma; PA, *Pseudomonas aeruginosa*.

2 | EXPERIMENTAL SECTION

2.1 | Bacterial strains and growth conditions

Non-mucoid and mucoid *P. aeruginosa* isolates (Table 1), cultured from the lungs of chronically infected CF patients attending the adult Cystic Fibrosis Centre at the Belfast Health and Social Care Trust, were used in the study. All isolates were cultured on Mueller–Hinton Agar (MHA) or in Mueller–Hinton Broth (MHB) at 37°C. *P. aeruginosa* PA14 and *P. aeruginosa* PAO1 were grown in MHB at 37°C. *MucA*(+) PA mutant strain[13] was grown in lysogeny broth supplemented with 75-μg/ml gentamicin. Strains used in the *mucA* mutant study, *P. aeruginosa* PA14 (wild type) and *mucA*(+) PA, were obtained from the laboratory of Dr. M. Dow, Department of Microbiology, University College Cork.

2.2 | Biofilm growth using the Calgary biofilm device (CBD)

Biofilms were prepared as described previously.[19] Briefly, *P. aeruginosa* biofilms were grown in the CBD, a 96-well plate bearing polycarbonate pegs in the lid, which

protrude into each well containing a bacterial culture. An overnight culture of *P. aeruginosa* was adjusted to a concentration of 1×10^7 CFU/ml. 150 μl of this solution was added to each well of the CBD and incubated at 37°C with rotation. After 24-hr growth, the bacterial suspension was substituted for fresh growth media. After 48-hr incubation, the lid was removed and pegs were broken off using sterile pliers. Each peg was rinsed in 200-μl phosphate-buffered saline (PBS) to remove any planktonic or loosely attached bacteria.

2.3 | Plasma source and biofilm exposure conditions

The in-house designed plasma jet configuration used in this study has been described previously,[27,28] which consisted of a dielectric quartz tube with inner and outer diameters of 4 and 6 mm, respectively. Two copper electrodes (2-mm wide) surrounded the tube, separated by a distance of 25 mm. The high-voltage pulse source (Haiden PHK-2k), operating at 20 kHz, with a voltage amplitude of 6 kV, was applied to the downstream powered electrode, 5 mm from the end of the plasma quartz tube. The upstream electrode was grounded. The plasma jet was operated with a gas mixture of 0.5% oxygen and 99.5% helium, at a total flow rate of 2 SLM (standard litres per minute) into ambient air (Figure 1). Photos of the experimental setup can be viewed in previous publications.[20,29] For treatment, either bacterial suspensions or pegs from the CBD were placed at a distance of 10 mm from the end of the plasma tube and exposed to the plasma produced for up to 8 min. At least three replicates were completed for each timepoint for each experiment, with the exception of eDNA experiments which were conducted in duplicate. Statistical analyses were performed using GraphPad Prism version 8.3.1 software.

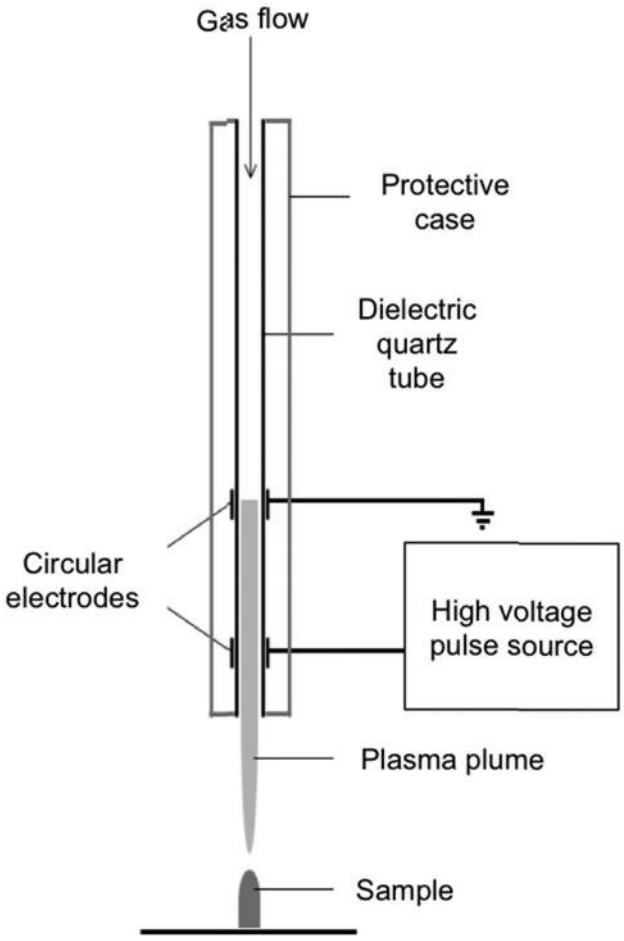


FIGURE 1 Atmospheric pressure nonthermal plasma-generating apparatus used in this study. A diagram of plasma jet configuration used for the treatment of bacterial samples, detailing the dielectric quartz tube, electrodes, high-voltage pulse source and plasma plume. Photos of the experimental setup can be viewed in previous publications[20,29]

2.4 | Biofilm recovery

Biofilm recovery was performed as described previously.[30] Briefly, after APNTP exposure, pegs were placed in 200-μl PBS and sonicated for 10 min in a dry sonicator to dislodge and resuspend the biofilms. After sonication, the pegs were discarded and the resultant bacterial suspensions were used to calculate the number of surviving bacterial cells.

2.5 | Cell viability determination

The viability of surviving cells was quantitatively determined, as described previously.[31] The recovered bacterial suspensions were 10-fold serially diluted in a 96-well microtiter plate using sterile PBS, and three aliquots (20 μl each) from each well were spotted on the surface of MHA and incubated at 37°C for 24 hr. The number of colonies from each spot was counted, and the number of surviving cells was calculated as colony-forming unit per peg (CFU/peg) for biofilm assays or CFU/ml for assays involving planktonic cultures. The decimal reduction time (D value), the time taken for one log reduction in the number of viable cells, was calculated from the resulting curves. D_1 and D_2 values were calculated, representing the biphasic nature of the kill curves produced, showing the time required for a 90% reduction in viable cells for the first linear reduction of the curve (D_1) and the second (D_2).

2.6 | Assessment of exogenous alginate and eDNA addition on tolerance to plasma exposure

Stock solutions of sodium alginate (PRONOVA™ UP LVM; NovaMatrix, Norway) and plasmid DNA, pBR322 (Fermentas), were prepared in sterile water. Varying concentrations of alginate (0–1.25%) and eDNA (0–75 μg/ml), representative of concentrations of EPS in biofilms, were added to planktonic *P. aeruginosa* PAO1 cultures, with 50 μl of these supplemented cultures exposed to APNTP for varying exposure times. The number of surviving cells was determined as described in Section 2.5.

3 | RESULTS AND DISCUSSION

3.1 | APNTP tolerance of biofilms from non-mucoid and mucoid *P. aeruginosa* clinical isolates

Ten *P. aeruginosa* clinical isolates were obtained from the Cystic Fibrosis Unit, Belfast City Hospital, including seven non-mucoid (Figure 2a) and three mucoid strains (Figure 2b). Biofilms of each of these strains were grown and exposed to APNTP for between 0 and 8 min. Plasma exposure resulted in a characteristic biphasic kill curve, allowing for the calculation of D_1 and D_2 values. These data concur with similar biphasic curves reported for *Chromobacterium violaceum*[32] and *Neisseria gonorrhoeae*.[33] The slower rate of biofilm viability observed in the second phase may result from the protection provided by the polymeric matrix surrounding cells in deep layers of the biofilm, from the shielding effect of cellular debris produced by the plasma-lysed cells at the surface of biofilms[20] or, in fact, from desiccation of the biofilm matrix upon longer exposures. D_1 values ranged from 0.55 to 0.72 min for non-mucoid strains and from 0.75 to 1.02 min for mucoid strains. D_2 values ranged from 5.15 to 6.80 min for non-mucoid strains and from 8.34 to 8.66 min for mucoid strains (Table 1). Overall, mucoid strains had a significantly higher mean D_1 value (0.89 ± 0.14 min) than non-mucoid strains (0.64 ± 0.07 min), as determined by a two-tailed *t* test ($p < .05$). Mucoid strains also had a significantly higher D_2 value (8.50 ± 0.16 min) than non-mucoid strains (5.93 ± 0.68 min), as determined by a two-tailed *t* test ($p < .05$).

The secretion of EPS by mucoid *P. aeruginosa* strains has already been associated with a protective effect against a number of common antimicrobials.[34] The data presented here suggest that EPS secretion in the biofilms of mucoid *P. aeruginosa* clinical isolates also confers tolerance to treatment by APNTP, presumably due to sequestration of RONS generated from the plasma effluent. The potential role of EPS in biofilm tolerance to nonthermal plasma is becoming increasingly recognised.[15] Recently, the protective role of the extracellular matrix in plasma tolerance has been described in biofilms of *Burkholderia cenocepacia*[27] and *Acinetobacter baumannii*,[35] where increasing biofilm biomass correlated to increased tolerance to nonthermal plasma exposure. Finally, in keeping with previous studies of biofilm susceptibility to APNTP exposure among the ESKAPE pathogens,[19–21,23,34] complete eradication was not observed even after the longest exposure time of 8 min. This indicates that a population of cells within the biofilm withstands APNTP exposure potentially through induction of dormant cells, known as persister cells, which are highly resistant to antimicrobial treatment. The induction of a viable but nonculturable state has been described previously,[21,32,36] and oxidative stress induced by APNTP exposure, leading to apparent persister cell formation in *P. aeruginosa* biofilms, has been described.[24]

3.2 | Effect of alginate overexpression on APNTP tolerance

To directly examine the effect of alginate overexpression, the plasma-exposure susceptibility of a *P. aeruginosa* mutant strain (PA *mucA*(+)) harbouring a plasmid with the *mucA* gene responsible for alginate production was compared with the reference strain, *P. aeruginosa* PA14. Planktonic cultures of both PA *mucA*(+) and *P. aeruginosa* PA14 were exposed to APNTP for between 0 and 8 min, and the remaining cell viability of each culture was determined. Log_{10} reduction in cell viability was significantly lower for PA *mucA*(+) than *P. aeruginosa* PA14 control after both 4- and 8-min APNTP exposure, as determined by two-tailed *t* tests ($p < .05$; Table 2). D_1 and D_2 values were also higher for PA *mucA*(+) (0.84 and 7.37 min, respectively), compared with *P. aeruginosa* PA14 (0.67 and 6.11 min, respectively). This indicates that the endogenous production of alginate by *P. aeruginosa* has a protective effect against the antimicrobial action of APNTP.

3.3 | Effect of alginate and eDNA addition on APNTP tolerance

Results presented in Sections 3.2 and 3.3 have highlighted the effect that endogenous production of alginate and other EPS components have on increasing tolerance of *P. aeruginosa* to treatment with APNTP. The effect of exogenous addition of alginate and eDNA on APNTP

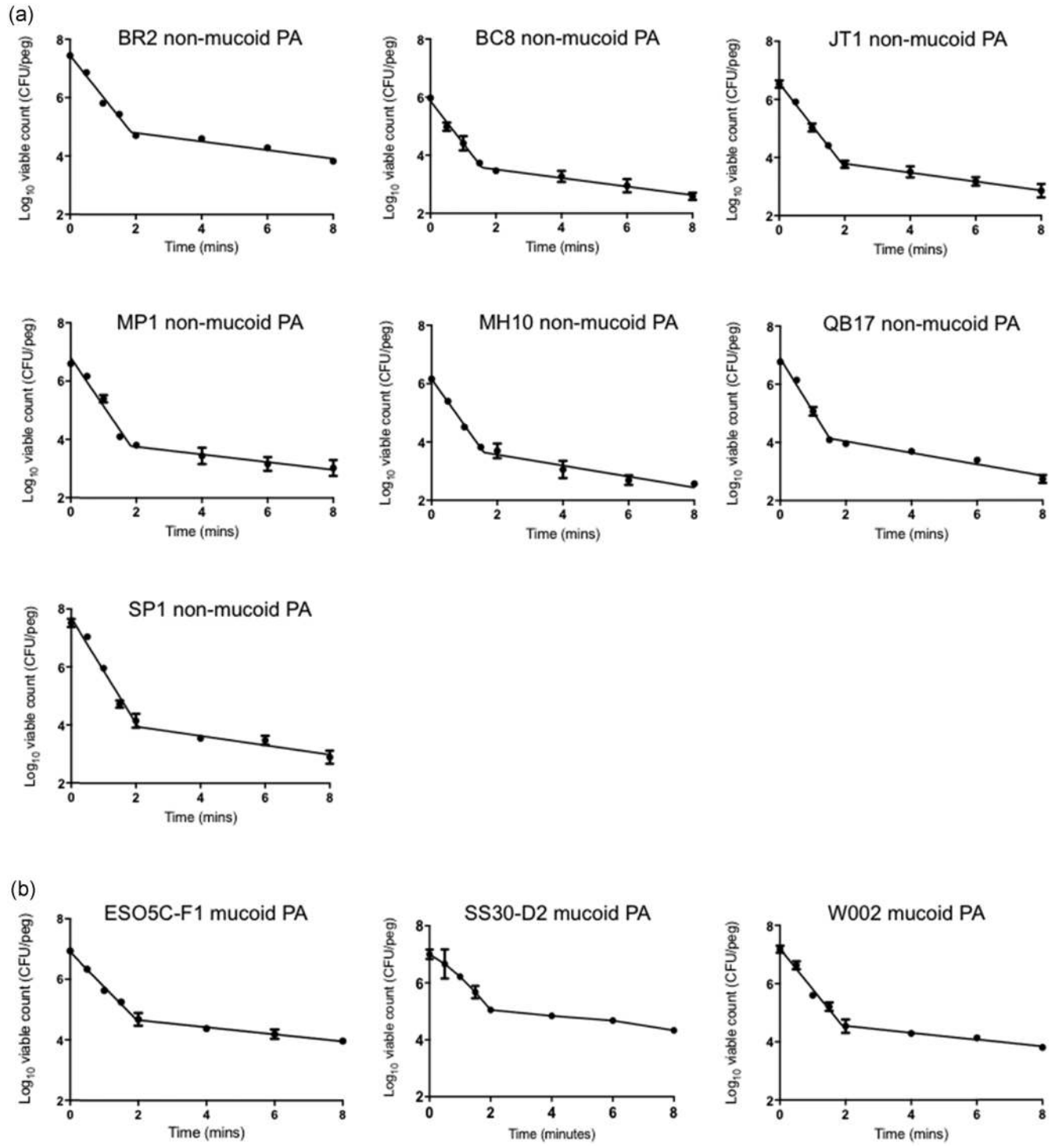


FIGURE 2 The effect of atmospheric pressure nonthermal plasma (APNTP) exposure on *Pseudomonas aeruginosa* (PA) biofilms. Log_{10} viable counts (CFU/peg) from biofilms of (a) non-mucoid and (b) mucoid *P. aeruginosa* clinical isolates after APNTP exposure

tolerance in the well-studied *P. aeruginosa* PAO1 is now considered.

Exogenous alginate was added to *P. aeruginosa* PAO1 cultures in the range of 0–1.25% (gross composition of a typical biofilm is estimated to comprise polysaccharide concentrations in the range of 1–2%),[37] with subsequent APNTP exposures between 0 and 2 min. Exogenous alginate addition conferred a protective effect on *P. aeruginosa* PAO1 against APNTP treatment, with more profound effects observed with increasing alginate concentration (Figure 3a). The biggest differences in percentage survival between the highest and lowest

TABLE 2 Response of *Pseudomonas aeruginosa* PA14 and PA *mucA*(+) mutant to APNTP exposure

APNTP exposure time (min)	Log_{10} reduction in cell viability (log_{10} CFU/ml)	
	PA14	PA *mucA*(+)
0.5	0.53 ± 0.44	0.35 ± 0.56
1.0	1.37 ± 0.44	1.28 ± 0.37
1.5	2.70 ± 0.53	1.90 ± 0.04
2.0	2.95 ± 0.15	2.31 ± 0.56
4.0	3.57 ± 0.51	2.65 ± 0.33[a]
8.0	3.94 ± 0.47	2.90 ± 0.19[a]
D_1-value	0.67	0.84
D_2-value	6.11	7.37

Note: Log_{10} reduction in colony-forming units per ml (±*SD*) along with D_1 and D_2 values for *P. aeruginosa* PA14 and PA *mucA*(+) mutant after APNTP exposure.

Abbreviations: APNTP, atmospheric pressure nonthermal plasma; CFU, colony-forming unit; PA, *Pseudomonas aeruginosa*; *SD*, standard deviation.

[a]Significant difference between *mucA*(+) strain and PA14 wild type.

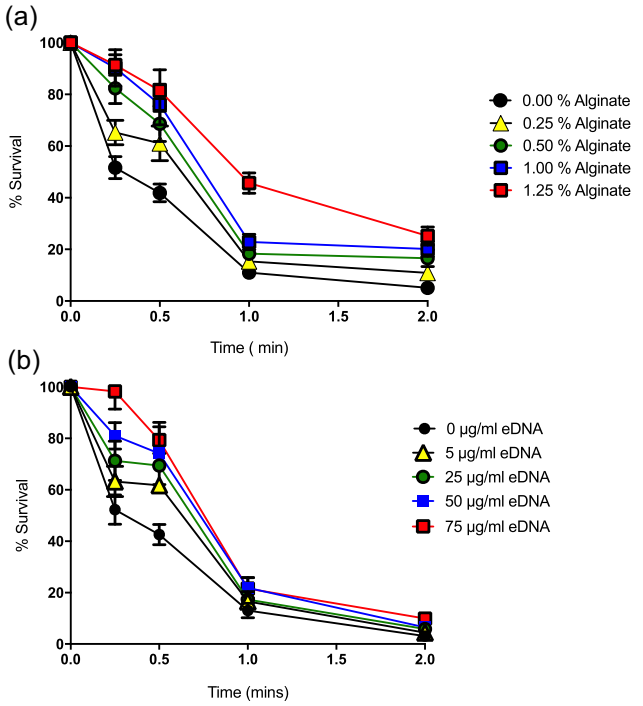


FIGURE 3 The effect of alginate and extracellular DNA (eDNA) addition on cell survival. Percentage survival of *Pseudomonas aeruginosa* PAO1 cells after addition of different concentrations of (a) alginate and (b) eDNA with subsequent atmospheric pressure nonthermal plasma exposure

concentrations of alginate were at lower APNTP exposure times. After 0.25- and 0.5-min exposure, survival rates for *P. aeruginosa* PAO1 cultures with 1.25% alginate were 91.39% and 81.46%, respectively, whereas survival rates for those with no additional alginate were only 51.63% and 41.83% at the same exposure times. After 2-min APNTP exposure, percentage survival dropped considerably across all groups; however, survival was still significantly higher for *P. aeruginosa* PAO1 cultures with the addition of 1.25% alginate as compared with no additional alginate, as determined by a two-tailed *t* test ($p < .05$). A similar protective effect was observed after the addition of eDNA to *P. aeruginosa* PAO1 cultures, and a positive correlation between eDNA concentration and percentage survival was evident (Figure 3b). As with alginate addition, the greatest differences in survival were seen at lower exposure times. Mean survival rates were 98.28% after 0.25-min APNTP exposure for *P. aeruginosa* PAO1 with 75-µg/ml eDNA, compared with just 52.27% at the same exposure time when no eDNA was added.

The effect of adding combinations of varying concentrations of alginate and eDNA to *P. aeruginosa* PAO1 cultures, followed by APNTP exposure, was also assessed (Figure 4). Supplementing *P. aeruginosa* PAO1 with alginate and eDNA together appears to have an additive protective effect as compared with either alginate or eDNA addition alone. For example, percentage survival rates after 0.25-min APNTP exposure for either 0.5% alginate or 50-µg/ml eDNA addition were 82.35% and 81.03%, respectively. However, when both 0.5% alginate and 50-µg/ml eDNA are added to *P. aeruginosa* PAO1 together, this value increased to 92.59% survival, higher than either constituent on its own. This additive effect is also evident in Table 3, with lower reductions in *P. aeruginosa* PAO1 viable counts as alginate and eDNA concentrations are increased. Alginate is an anionic polysaccharide that forms a major component of mucoid biofilms of *P. aeruginosa*,[38] which, in addition to performing important functions in cell

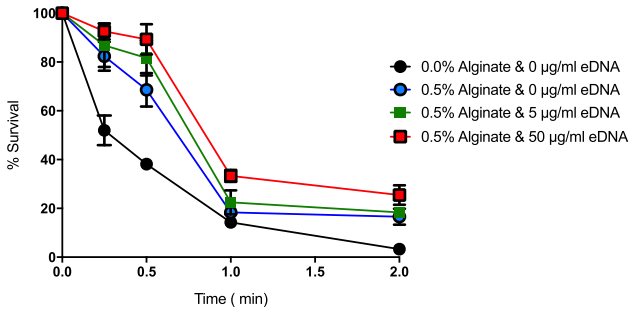


FIGURE 4 The addition of different concentrations of alginate and extracellular DNA (eDNA). The addition of different combinations of alginate and eDNA concentrations and their effect on survival of *Pseudomonas aeruginosa* PAO1 after exposure to atmospheric pressure nonthermal plasma exposure

TABLE 3 Effect of alginate and eDNA addition on tolerance to APNTP exposure

APNTP exposure time (min)	Log_{10} reduction in *P. aeruginosa* PAO1 viable counts (log_{10} CFU/ml)			
	0% Alginate and 0-μg/ml eDNA	0.5% Alginate and 0-μg/ml eDNA	0.5% Alginate and 5-μg/ml eDNA	0.5% Alginate and 50-μg/ml eDNA
0.25	0.29 ± 0.09	0.08 ± 0.06	0.07 ± 0.08	0.03 ± 0.03
0.50	0.42 ± 0.02	0.15 ± 0.08	0.09 ± 0.07	0.05 ± 0.05
1.00	0.85 ± 0.10	0.73 ± 0.70	0.67 ± 0.17	0.48 ± 0.06
2.00	1.49 ± 0.10	0.80 ± 0.17	0.74 ± 0.09	0.60 ± 0.12

Note: Log_{10} reduction in *Pseudomonas aeruginosa* PAO1 cell viability after addition of alginate and eDNA combinations and subsequent APNTP exposures.
Abbreviations: APNTP, atmospheric pressure nonthermal plasma; CFU, colony-forming unit; eDNA, extracellular DNA.

adhesion, biofilm architecture and resistance to desiccation, has the ability to scavenge reactive oxygen species (ROS),[15,39] thus limiting the exposure of cells within the biofilm to ROS. Furthermore, eDNA undergoes rapid single- and double-strand breakage events on exposure to nonthermal plasma, again primarily associated with production of ROS.[40] Taken together, the combined effect of alginate and eDNA, both in the context of *P. aeruginosa* biofilms and when added as an exogenous organic material to planktonic cultures, exerts a combined protective effect on the *P. aeruginosa* community. This happens as a result of ROS generated by the plasma interacting with the components of the biofilm matrix, thus sequestering the ROS and retarding their penetration into the biofilm. This additive protective effect, observed when alginate and eDNA are both added to *P. aeruginosa* cultures, is mirrored in *P. aeruginosa* biofilms in the clinic, where tolerance to antimicrobial therapy is increased and worse prognoses are seen for infections caused by microorganisms exhibiting a mucoid phenotype.

4 | CONCLUSION

It is well established that *P. aeruginosa* strains exhibiting a mucoid phenotype are more resistant to antibiotic therapy than non-mucoid strains. Using clinical isolates from CF patients, we have demonstrated that this is also true for treatment by APNTP, with *P. aeruginosa* biofilms of mucoid strains exhibiting significantly elevated tolerance to APNTP than their non-mucoid counterparts. Alginate, an exopolysaccharide that forms part of the biofilm matrix and is produced in large amounts by mucoid strains, appears to play a key role in mediating tolerance to antimicrobial treatment, alongside other EPS constituents such as eDNA. This was demonstrated with an endogenous overexpression of alginate by *P. aeruginosa*, as well as with supplementation of exogenous alginate. Overall, this study shows how EPS components contribute to biofilm tolerance to APNTP exposure, as they do with conventional antimicrobial chemotherapy. This also serves to highlight one mechanism by which mucoid strains of *P. aeruginosa* exhibit greater tolerance to antimicrobial treatment than those with a non-mucoid phenotype, resulting in poorer clinical outcomes.

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