International Journal of Antimicrobial Agents 49 (2017) 375–378

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Cold atmospheric pressure plasma elimination of clinically important single- and mixed-species biofilms

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ARTICLE INFO

Article history:
Received 31 May 2016
Accepted 25 November 2016

Keywords:
Mixed-species biofilms
Cold plasma
Decontamination
ESKAPE pathogens

ABSTRACT

Mixed-species biofilms reflect the natural environment of many pathogens in clinical settings and are highly resistant to disinfection methods. An indirect cold atmospheric-pressure air-plasma system was evaluated under two different discharge conditions for its ability to kill representative Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) pathogens. Plasma treatment of individual 24-h-old biofilms and mixed-species biofilms that contained additional species (*Enterococcus faecalis* and *Klebsiella pneumoniae*) was considered. Under plasma conditions that favoured the production of reactive nitrogen species (RNS), individual *P. aeruginosa* biofilms containing ca. 5.0×10^6 CFU were killed extremely rapidly, with no bacterial survival detected at 15 s of exposure. *Staphylococcus aureus* survived longer under these conditions, with no detectable growth after 60 s of exposure. In mixed-species biofilms, *P. aeruginosa* survived longer but all species were killed with no detectable growth at 60 s. Under plasma conditions that favoured the production of reactive oxygen species (ROS), *P. aeruginosa* showed increased survival, with the lower limit of detection reached by 120 s, and *S. aureus* was killed in a similar time frame. In the mixed-species model, bacterial kill was biphasic but all pathogens showed viable cells after 240 s of exposure, with *P. aeruginosa* showing significant survival (ca. $3.6 \pm 0.6 \times 10^6$ CFU). Overall, this study shows the potential of indirect air plasma treatment to achieve significant bacterial kill, but highlights aspects that might affect performance against key pathogens, especially in real-life settings within mixed populations.

1. Introduction

Cold atmospheric pressure plasma is an emerging technology that is currently under intense investigation for microbial decontamination applications [1]. A number of studies have highlighted the potential for plasmas to decontaminate a range of pathogens important in health care [1]. These studies have tested the efficacy of plasma against planktonic cells or single-species biofilms [2–4]. However, in the environment biofilms are likely to exist as mixed microbial communities, and studies have shown that mixed-species biofilms possess increased resistance to antimicrobial agents compared with single-species biofilms [5].

Mixed-species biofilms are important colonisers of a wide range of medical devices such as venous and urinary catheters, mechanical heart valves, prosthetic joints and endotracheal tubes [6]. Common bacterial species isolated from medical devices include those termed the 'ESKAPE' pathogens (*Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) [7]. Although by no means the only pathogens that form biofilms on medical devices, these organisms are associated with increasing levels of multidrug resistance (resistance to at least three classes of frontline antibiotics) and represent a serious public health threat [8]. A mixed-species biofilm consortium was selected for this study based on the representation of the organisms in chronic wound infections and as common colonisers on implanted medical devices [9]. As such, the outputs of the study should be informative for the use of plasma systems to generate reactive species to control these types of bacterial infection *in vivo*. Plasma systems are already used for the treatment of wound infections [10].

An advantage of plasma decontamination is that it does not rely on any one mechanism for bacterial killing; reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) photons and high electric fields are all produced simultaneously in the plasma, increasing the potential for synergistic effects. This pilot study explored how the reactive chemistry produced by an indirect cold air plasma impacts the decontamination efficacy of two clinically relevant ESKAPE bacterial species (*P. aeruginosa* and *S. aureus*) both in single- and mixed-species biofilms.

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http://dx.doi.org/10.1016/j.ijantimicag.2016.11.022
0924-8579/

2. Materials and methods

2.1. Bacterial strains and culture conditions

Four different bacterial strains were included in this study, namely *P. aeruginosa* PAO1, *K. pneumoniae* NCTC 13368, *E. faecalis* NCTC 775 and *S. aureus* ATCC 9144. Bacterial cultures were prepared in tryptic soy broth (TSB) with shaking at 37 °C or were incubated on tryptic soy agar at 37 °C.

2.2. Biofilm formation in a CDC biofilm reactor

Biofilms were generated on PVC coupons in a CDC Biofilm Reactor (BioSurface Technologies Corp., Bozeman, MT). *Pseudomonas aeruginosa* and *S. aureus* were used to form single-species biofilm models. Bacteria at a final concentration of 1.0×10^5 CFU/mL were added to 350 mL of TSB medium. Biofilms were grown for 24 h at 37 °C with stirring at 200 rpm. *Pseudomonas aeruginosa*, *K. pneumoniae*, *E. faecalis* and *S. aureus* were used in mixed-species biofilms [9].

2.3. Plasma source and sample exposure

The plasma source considered in this study is similar to that reported by Olszewski et al [11] and employs a surface barrier discharge configuration as shown in Fig. 1a. Biofilm-containing coupons were removed from the bioreactor following incubation and were rinsed twice in 25 mL of sterile phosphate-buffered saline to remove planktonic and loosely attached cells. Coupons were exposed to plasma at a distance of 5 mm between the sample and the electrode. Two plasma powers were considered: a low-power discharge ($P_{discharge}$ = 8 W), giving a ROS-dominated gas-phase chemistry; and a high-power discharge ($P_{discharge}$ = 34.5 W), giving an RNS-dominated gas-phase chemistry. Treatments were performed for 7 s up to 240 s. Coupons were treated on both sides at the same plasma conditions. Treatment at each parameter was performed in triplicate with at least three replicates per experiment.

2.4. Determination of biofilm elimination

Bacterial survival following plasma exposure was determined by serial dilution with Miles–Misra enumeration [9]. Treated PVC coupons were immediately transferred to 5 mL of TSB to quench any further inactivation and were vigorously shaken for 10 min using a vibratory shaker (VXR basic Vibrax®; IKA, Staufen, Germany) at 2000 rpm to release cells. Then, 10 µL of bacterial suspension was added to 90 µL of fresh TSB medium. This was serially diluted 10-fold to 10^{-5} in Corning® Costar® 96-well polystyrene plates (Corning Life Sciences, Tewksbury, MA). Then, 10 µL of each dilution was plated on TSA plates, with three repetitions for each dilution. Controls followed the same protocols except for exposure to plasma. Plates were incubated overnight at 37 °C. The limit of detection was ca. 5.0×10^2 bacteria.

2.5. Fourier transform infrared spectroscopy (FTIR)

FTIR was used for characterisation of reactive species in the gas phase. The plasma-generating electrodes were sealed in a box of similar volume to that used in the decontamination experiments, from where the plasma exhaust gas was drawn into a 10-cm path length gas cell and analysed with an FT/IR-4200 spectrometer (JASCO, Tokyo, Japan). A spectral resolution of 2.0 cm^{-1} was used and each absorption spectrum was acquired over 25 scans. The composition of the plasma effluent was analysed under the same conditions as those used in the biofilm deactivation experiments.

3. Results

3.1. Characterisation of gas phase

Fig. 1b highlights the FTIR absorption spectrum under low- and high-power plasma generation conditions. FTIR analysis is only capable of identifying molecules that actively absorb in the IR range; hence, the data presented in Fig. 1b should not be considered as an exhaustive characterisation of the plasma effluent. Under low-power plasma conditions, ozone was found to dominate, indicating the predominance of ROS in this regimen. Under high-power plasma conditions, the oxides of nitrogen dominated, with no measurable ozone production, indicating the predominance of RNS. The variation in gas-phase chemistry under different operating power conditions is typical for such a discharge and is attributed to elevated temperatures in the plasma, leading to thermal degradation of ozone and a thermally driven increase in nitric oxide production [11].

3.2. Effect of plasma treatment on survival of Pseudomonas aeruginosa and Staphylococcus aureus biofilms

Single-species biofilms of *P. aeruginosa* and *S. aureus* were exposed to the ROS- and RNS-dominated plasma effluent regimens over varying periods of time. The sensitivity of the two species to plasma elimination differed with respect to the respective discharge regimens and between species. Fig. 2a shows that the elimination rate of *P. aeruginosa* biofilm is strongly influenced by the dominant gas-phase chemistry. Under low-power ROS-dominated conditions, elimination of the bacteria to below the limit of detection of the

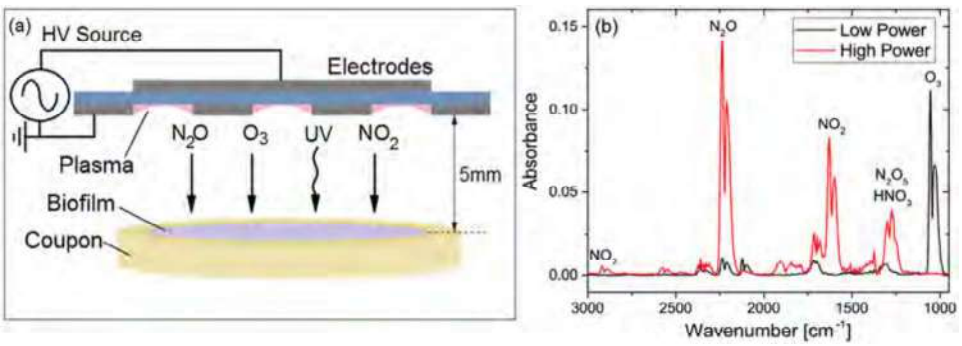

Fig. 1. (a) Schematic of plasma system and sample position. (b) Fourier transform infrared spectroscopy (FTIR) spectra obtained after 120 s of plasma generation under reactive oxygen species (ROS)-dominated (black) and reactive nitrogen species (RNS)-dominated (red) conditions.

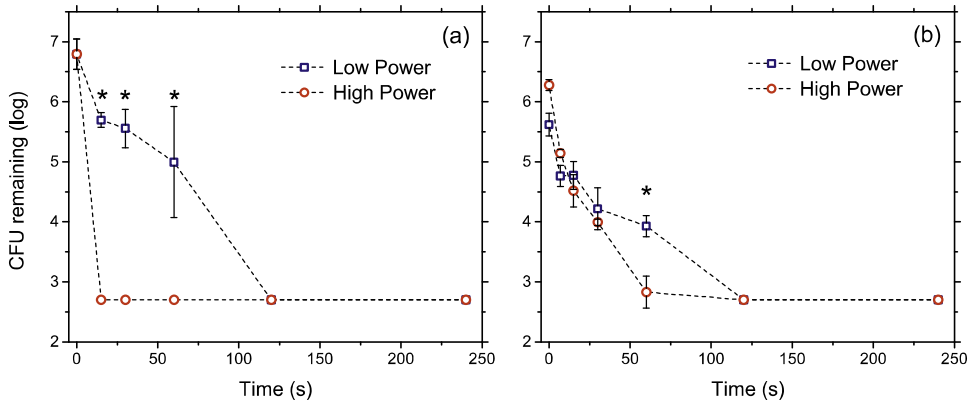

Fig. 2. Elimination of single-species biofilms under low- and high-power plasma conditions for (a) *Pseudomonas aeruginosa* and (b) *Staphylococcus aureus*. Each data point represents the mean CFU for each time point, with error bars representing the standard deviation (S.D.). The mean ± S.D. are calculated from all replicate CFU count values for each of the triplicate experiments ($n = 9$). Statistically significant differences between elimination under low- and high-power conditions were determined by one-way analysis of variance (ANOVA) using IBM SPSS Statistics v.22 (IBM Corp., Armonk, NY), with Scheffe's post-hoc test. * $P < 0.05$.

assay was achieved within 120 s. In the high-power, RNS-dominated regimen, complete elimination of *P. aeruginosa* was achieved in <15 s. Fig. 2b highlights that plasma treatment was also efficient for elimination of *S. aureus* biofilms. In contrast to *P. aeruginosa*, *S. aureus* biofilms showed little dependency on the plasma generation conditions. Thus, the rate of elimination of the bacteria was similar across the first 30 s of exposure. Thereafter, the RNS-dominated effluent was able to kill *S. aureus* to below the detection limit of the assay within 60 s, whereas this was only achieved by 120 s in the ROS-dominated case. In the comparison between the two bacteria, *P. aeruginosa* was significantly more sensitive to the RNS-dominated regimen than *S. aureus*, while the reverse was true for the ROS-dominated conditions, albeit with the two bacteria showing very different kill kinetics.

3.3. Effect of plasma treatment on survival of mixed-species biofilms

Mixed-species biofilms contained *P. aeruginosa*, *S. aureus*, *K. pneumoniae* and *E. faecalis* strains [9]. This system provides similar numbers of the four species when used at 24 h and provides a basis to compare relative elimination within a single co-culture system. The treatment conditions were the same as described for single-species biofilms. The susceptibility of *P. aeruginosa* and *S. aureus* was found to be different within the multispecies communities compared with that observed in single-species biofilms, as highlighted in Fig. 3a and b for ROS- and RNS-dominated regimens, respectively. Under ROS exposure, there was a biphasic kill of the bacteria, with only a small reduction in viable count, of the order of 1–2 logs, after 60 s of exposure. Between 60 s and 120 s, three of the species (*S. aureus*, *E. faecalis* and *K. pneumoniae*) showed a further reduction in viable count of ca. 2 log, but there were still viable bacteria after 240 s of exposure. *Pseudomonas aeruginosa* showed a much more gradual reduction in viable count and there was less than a 3 log reduction achieved in total across the 240 s of exposure. *Pseudomonas aeruginosa*, and to a lesser extent *S. aureus*, were less susceptible to kill under ROS-dominated plasma conditions in mixed culture than in single-species biofilms. Following exposure to an RNS-dominated effluent, the two Gram-negative species (*P. aeruginosa* and *K. pneumoniae*) were reduced to below the limit of detection of the assay by 30 s, and *E. faecalis* was reduced to levels only just above the limit of detection. At this time point, *S. aureus* in the mixed-species model was reduced by only 3 log.

4. Discussion

This study demonstrates that indirect air plasma exposure can achieve effective killing of a range of different clinically important pathogens even in mixed-species biofilms. As these are commonly

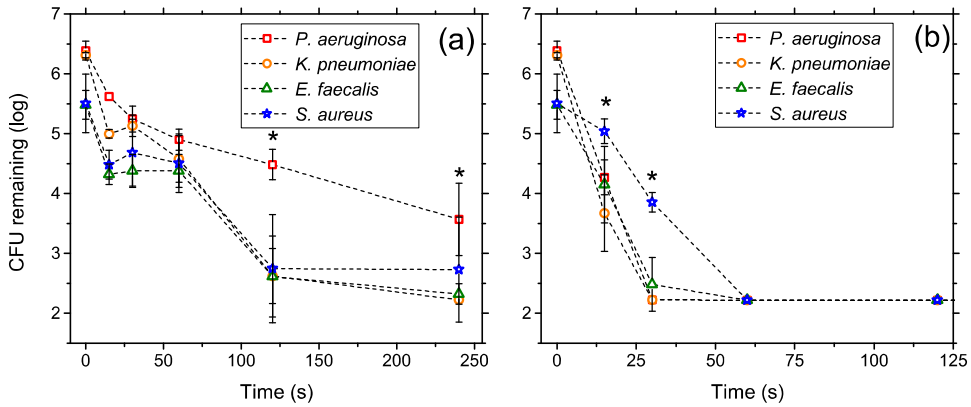

Fig. 3. Elimination of mixed-species biofilms under (a) reactive oxygen species (ROS)-dominated conditions and (b) reactive nitrogen species (RNS)-dominated conditions. Each data point represents the mean CFU for each time point, with error bars representing the standard deviation (S.D.). The mean ± S.D. are calculated from all replicate CFU count values for each of the triplicate experiments ($n = 9$). Statistically significant differences between species elimination at a given time were determined by one-way analysis of variance (ANOVA) using IBM SPSS Statistics v.22 (IBM Corp., Armonk, NY), with Scheffe's post-hoc test. * $P < 0.05$.

encountered in a variety of clinical settings and are, perhaps, the most resistant form of bacterial colonisation on surfaces, this preliminary study offers significant potential for new applications. The differential sensitivity with respect to varying gas-phase chemistries suggests that specific plasma species are important for killing specific pathogens and this may be influenced by the biofilm environment. Importantly, this information guides the potential development of plasma systems for the treatment of these complex biofilms in clinical settings, such as diabetic foot ulcers and biofilm-colonised implanted medical devices. Plasma systems that generate bactericidal reactive species of the type observed here could provide valuable alternatives to drug therapy for topical treatment of infections, which would contribute to antibiotic stewardship endeavours.

Pseudomonas aeruginosa is an archetypal biofilm-forming bacterium producing complex biofilms with significant amounts of extracellular matrix material (exopolysaccharide and DNA). Although the 24-h-old biofilms used in this study are relatively simple, it was clear that the *P. aeruginosa* biofilm was significantly more susceptible to the species released under RNS-dominated conditions than *S. aureus*. This is consistent with observations from other studies using atmospheric plasma systems (e.g. helium:oxygen plasma [4]), which showed that *P. aeruginosa* was significantly more susceptible than *S. aureus*. Under ROS-dominated conditions *P. aeruginosa* is much less susceptible, and the differences between plasma conditions in susceptibility with *S. aureus* are less distinct. There is evidence to suggest that RNS may kill *Pseudomonas* in biofilms and, at low dose, also disrupt their structure to allow improved access for other molecular species [12,13]. Similar effects generated by plasma-derived species might explain the observations in this study and why the difference is less marked under lower-power conditions, where ROS predominate, both in the gas and water phase.

The results of this study confirm that the kinetics of bacterial kill will be more complex in mixed-species biofilm systems, even in relatively immature biofilms. Under RNS-dominated conditions, in mixed-species models there is a small reduction in the susceptibility of *P. aeruginosa* but *S. aureus* is essentially unaffected. This suggests that there is free penetration of RNS into the mixed-species biofilm and that there is no significant turnover of such species by bacterial RNS defences within the time course used. This is not the case under ROS-dominated conditions, where all the species were able to survive to 240 s of exposure and there were significantly reduced levels of kill for *P. aeruginosa*. This suggests poor penetration of ROS compared with the single-species biofilms for *P. aeruginosa* and *S. aureus* and/or that catalases and superoxide dismutases expressed in the mixed-species model significantly reduce the local levels of ROS. The biphasic profile of the survival curves also suggests that there may be issues with penetration, with freely accessible cells being killed rapidly while less accessible cells are only killed by prolonged exposure, if at all. That *P. aeruginosa* is the species that benefits most from the mixed-species culture may reflect it occupying a more protected niche (e.g. closer to the solid support) within the biofilm or that its oxidative defences are activated more strongly in such biofilms than in single-species systems. The results are consistent with previous studies showing that *P. aeruginosa* was the organism most able to survive chlorhexidine exposure within the mixed-species model [9].

The study provides strong evidence that defined configurations of atmospheric pressure air plasmas can achieve rapid and highly effective bacterial killing in complex models. This brief study starts to define some of the key research questions in terms of optimising plasma discharges to generate, perhaps, more RNS species. It also identifies fundamental gaps in our understanding of the architecture of co-cultured biofilms and the cross-dependency of individual species within them.

Acknowledgements

The authors thank Dr Matthew Wand for helpful discussions on establishment of the model as well as analysis of the results.

Funding: This work was supported by Public Health England (PHE) project 109506 and the UK Engineering and Physical Sciences Research Council [grant EP/N021347/1]. MM and JLW acknowledge the support of the NATO project [SPS 984555]. The opinions expressed in the paper do not reflect those of Public Health England or the Department of Health.

Competing interests: None declared.

Ethical approval: Not required.

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