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Short Communication

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

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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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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

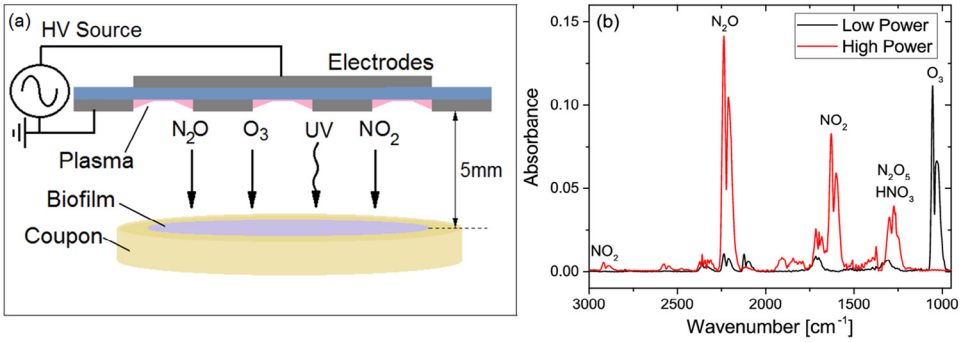

Fig. 1. (a) Schematic of plasma system and sample position. (b) Fourier transform infrared spectroscopy (FTIR) spectra obtained after 120 s of plasma generation under reactive oxygen species (ROS)-dominated (black) and reactive nitrogen species (RNS)-dominated (red) conditions.