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Potential cellular targets and antibacterial efficacy of atmospheric pressure non-thermal plasma

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ABSTRACT

Atmospheric pressure non-thermal plasma (APNTP) has been gaining increasing interest as a new alternative antibacterial approach. Although this approach has demonstrated promising antibacterial activity, its exact mechanism of action remains unclear. Mechanistic elucidation of the antimicrobial activity will facilitate development and rational optimisation of this approach for potential medical applications. In this study, the antibacterial efficacy of an in-house-built APNTP jet was evaluated alongside an investigation of the interactions between APNTP and major cellular components in order to identify the potential cellular targets involved in plasma-mediated bacterial destruction mechanisms. The investigated plasma jet exhibited excellent, rapid antibacterial activity against a selected panel of clinically significant bacterial species including *Bacillus cereus*, meticillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* and *Pseudomonas aeruginosa*, all of which were completely inactivated within 2 min of plasma exposure. Plasma-mediated damaging effects were observed, to varying degrees, on all of the investigated cellular components including DNA, a model protein enzyme, and lipid membrane integrity and permeability. The antibacterial efficacy of APNTP appears to involve a multiple-target mechanism, which potentially reduces the likelihood of emergence of microbial resistance towards this promising antimicrobial approach. However, cellular membrane damage and resulting permeability perturbation was found to be the most likely rate-determining step in this mechanism.

1. Introduction

As part of the evolving field of plasma medicine, the use of atmospheric pressure non-thermal plasmas (APNTPs) has been emerging as a potential alternative approach for microbial inactivation. APNTP is a partially ionised gas that is created by flowing gas through a relatively high electric field. This electric field accelerates the few naturally occurring free electrons which then ionises the gas atoms and molecules creating more electrons and hence creating an 'electron avalanche', and if conditions are right, sustaining a plasma. The energetic electrons will also undergo excitation and dissociative collisions with the gas particles, with the final result being a mixture of electrons, negative and positive ions, excited gas species, free radicals and electromagnetic radiation [1,2]. Part of this mixture, especially in the presence of molecular gases such as O_2 and N_2, are reactive oxygen and nitrogen species such as ozone, superoxide, hydroxyl radicals, singlet oxygen, atomic oxygen, nitric oxide, nitrogen dioxide, nitrite and nitrates [1,3–5], which can exert a high oxidative stress and are believed to be able to interact with microbial cells, damaging them in a rapid and effective manner. As an antimicrobial approach, APNTP also offers many favourable features including simple and flexible designs, operation at or near ambient temperature and pressure, tuneable output chemistry, relatively low capital and operational cost, utilisation of virtually non-toxic gases, and absence of long-lived harmful residues.

Numerous groups have reported promising results regarding the use of APNTPs in microbial inactivation in a range of medical applications including dental care, chronic infections, wound healing and medical device sterilisation [6–10]. Nevertheless, use of different plasma systems with no universal set up and high variability in operating parameters make direct comparison difficult; differences in plasma device design, gas mixture, electrode configuration, electric frequency, power input and mode of exposure can result in significant differences in plasma properties and efficacy. Although plasma source variability extends the possibility of tailoring this approach to suit different applications both with

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inanimate objects and viable tissue, independent efficacy evaluation for each type of APNTP source is necessary. Indeed, it is important not only to evaluate the antimicrobial efficacy of different plasma systems, but also to perform comprehensive investigations of the plasma-mediated microbial destruction mechanism. Mechanistic-level investigation and valid comparisons between the different plasma systems have manifold importance, not least elucidation of APNTP-mediated destruction mechanisms in general but also in explaining the variability in results reported by different research groups using different APNTP systems. Furthermore, it facilitates guided development and optimisation of more efficient application-directed plasma devices with minimum side effects, facilitating successful translation into the clinic.

In this study, the antibacterial efficacy of an in-house-built APNTP jet was evaluated against a set of clinically significant bacterial species, including *Pseudomonas aeruginosa* and meticillin-resistant *Staphylococcus aureus* (MRSA). Survival curves were plotted for all bacterial strains using the colony count method, and viability reductions were confirmed by measuring the metabolic activity of surviving cells using the XTT assay. To investigate the plasma-mediated destruction mechanism(s), the effects of plasma exposure on critical cell structural and functional components were also explored, including: DNA damage; enzymatic activity reduction; and lipid content peroxidation and membrane permeability disruption reflected by cellular leakage of adenosine triphosphate (ATP).

2. Materials and methods

2.1. Bacterial strains and growth conditions

The microbial strains used in this study were *Bacillus cereus* NCTC 2599, MRSA NCTC 12493, *Escherichia coli* NCTC 10418 and *P. aeruginosa* PA01. All microbial strains were stored at −20 °C and were subcultured in Mueller–Hinton broth (MHB) (Oxoid Ltd., Basingstoke, UK) before testing. Mueller–Hinton agar (Oxoid Ltd.) was used as the solid growth medium where appropriate.

2.2. Plasma source

The in-house-built kHz-driven plasma source used in this study (shown in Fig. 1) has been described previously [11]. The plasma jet consists of a dielectric quartz tube with inner and outer diameters of 4 mm and 6 mm, respectively. Two copper electrodes (2 mm wide) encircle the tube with inter-electrode distance of 25 mm. For this study, the output of a high-voltage pulse source (Haiden PHK-2k; Haiden Laboratory Inc., Hyogo, Japan), operating at a repetition frequency of 20 kHz and voltage amplitude of 6 kV, was applied to the downstream electrode, which is 10 mm from the end of the plasma tube, i.e. 20 mm from the sample to be treated. 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 standard litres per min. Under these conditions, an intense core plasma was formed between the two electrodes, and a luminous plume, with a rotational gas temperature of ca. 39 °C [11], extended out of the tube end reaching the treated sample.

2.3. Bacterial exposure to plasma

Bacterial suspensions were prepared in phosphate-buffered saline (PBS) from overnight culture and each was adjusted to an optical density equivalent to 5×10^6 CFU/mL. Aliquots (20 μL) from each standardised bacterial suspension were exposed to the plasma plume for up to 120 s at a distance of 10 mm between the sample and the end of the plasma tube. After plasma exposure, each sample was transferred to 180 μL of fresh PBS and the resultant suspensions were used for the surviving cell viability determination using the colony count method and XTT viability assay (Sigma–Aldrich Company Ltd., Gillingham, Dorset UK). Volume loss caused by liquid evaporation was assessed at the longest exposure time (i.e. 120 s) and was found to be 4 ± 2 μL, however this was not considered to affect the surviving cell number as the whole sample volume, and therefore all the treated cells, was transferred to the fresh PBS that was used in viability determination. Gas-only-treated controls were performed and showed no significant reduction in bacterial viability compared with non-treated controls (data not shown).

2.4. XTT viability assay

A stock solution of the tetrazolium salt XTT (Sigma–Aldrich Company Ltd.) was prepared by reconstituting 5 mg of XTT in 5 mL of PBS. Aliquots (50 μL) of the recovered bacterial suspensions (after plasma exposure) were transferred to the wells of a 96-well microtitre plate (Nunc, Roskilde, Denmark) containing 50 μL of MHB growth medium, after which 20 μL of XTT stock solution was added to each well. After incubating the microtitre plate at 37 °C for 4–5 h in an orbital incubator (AGB Scientific Ltd., Dublin, Ireland), the absorbance at 450 nm was measured against blank controls (containing 50 μL of PBS, 50 μL of MHB and 20 μL of XTT solution) using a microplate reader (BioTek EL808; BioTek Instruments Ltd., Potton, UK) in order to quantify the XTT metabolic product, the intensity of which is proportional to the number of respiring cells. The fraction of cells killed at each time point was calculated by comparing the absorbance of the relevant sample with that of the untreated control.

2.5. Assessment of plasmid DNA damage

Plasmid DNA (pBR322; Thermo Fisher Scientific, Loughborough, UK), which was originally isolated from *E. coli* and is 4361 bp in length, was used. A working plasmid solution (20 ng/μL) was prepared in PBS and 20 μL aliquots of this working solution were exposed to the plasma jet for up to 90 s. After plasma exposure, 15 μL from each sample was mixed with 3 μL of DNA loading dye and loaded into individual lanes of an agarose gel. Then, 10 μL of DNA marker (MassRuler™ High Range DNA Ladder; Thermo Fisher Scientific) was loaded into the first lane of the gel. Gel electrophoresis using 1% agarose gel in 1× TAE (Tris–acetate–ethylene diamine tetra-acetic acid) buffer with 0.5 μg/mL ethidium bromide was run for 30 min (30 V, 30 mA, 1 W) and then for 1 h (100 V, 100 mA, 10 W). Digital images were taken of the gel after electrophoresis using a bench-top ultraviolet transilluminator (BioDoc-It® Imaging System; UVP, Cambridge, UK). Three distinct bands corresponding to supercoiled, linear and open circular plasmid conformations were observed on the gel. Their relative band intensities were measured using Image J software (National Institutes of Health, Bethesda, MD) and the data were analysed to calculate the relative abundance of each plasmid conformation at each plasma exposure time.

2.6. Inactivation of proteinase K

The effect of plasma exposure on the enzymatic activity of the serine protease, proteinase K EC 3.4.21.64 (proteinase K from *Tritirachium album*; Sigma–Aldrich Company Ltd.) was evaluated by fluorogenic assay using a fluorogenic peptidyl substrate (Suc-Ala-Ala-Ala-MCA; PeptaNova GmbH, Sandhausen, Germany). Proteinase K aliquots (20 μL) of working solution (100 μg/mL in PBS) were exposed to the plasma jet for up to 240 s. After plasma exposure, protease-mediated hydrolysis of the fluorogenic substrate was assayed in PBS buffer (pH 7.4) with a proteinase K concentration of 500 ng/mL and a substrate concentration