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Delivery and Quantification of Hydrogen Peroxide Generated via Cold Atmospheric Pressure Plasma through Biological Material

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Abstract

The ability of plasma-generated hydrogen peroxide (H_2O_2) to traverse bacterial biofilms and the subsequent fate of the generated H_2O_2 has been investigated. An *in vitro* model, comprising a nanoporous membrane impregnated with artificial wound fluid and biofilms of varying maturity was treated with a helium-driven, cold atmospheric pressure plasma jet. The concentration of H_2O_2 generated below the biofilms was quantified. The results showed that the plasma-generated H_2O_2 interacted significantly with the biofilm, thus exhibiting a reduction in concentration across the underlying nanoporous membrane. Biofilm maturity exhibited a significant effect on the penetration depth of H_2O_2, suggesting that well established, multilayer biofilms are likely to offer a shielding effect with respect to cells located in the lower layers of the biofilm, thus rendering them less susceptible to plasma disinfection. This may prove clinically significant in the plasma treatment of chronic, deep tissue infections such as diabetic and venous leg ulcers. Our results are discussed in the context of plasma-biofilm interactions, with respect to the fate of the longer lived reactive species generated by cold atmospheric pressure plasma, such as H_2O_2.

Keywords: cold atmospheric pressure plasma, hydrogen peroxide, bacterial biofilm

1. Introduction

The ability to treat antibiotic resistant microorganisms has become paramount in the fight against bacterial infection owing to their ever-increasing prevalence. The apparent stagnation in antimicrobial drug development has fuelled urgent initiatives from a number of prominent healthcare organisations tailored towards treating and ultimately preventing such infections [1-3]. The extent of the current crisis is exemplified according to a predicted annual death toll of 10 million by 2050 arising solely from drug resistant infections (surpassing that of cancer) [4]. In addition to antimicrobial resistance (both *de novo* and pre-existing), the ability of bacteria to form biofilms, (dense, multilayer communities of bacterial cells protected by an extracellular polymeric substance (EPS)), further encumbers effective disinfection owing to changes in the physiological state of the cells contained within. Notoriously difficult to treat, it is estimated that biofilms are associated with 65% of all bacterial infections, rising to 85% when considering chronic infections [5]. Biofilms are able to form on a multitude of surfaces; both biotic and abiotic, thus providing a significant reservoir of

pathogenic microorganisms responsible for numerous human infections. Biofilms are reported to be involved in half of all hospital-acquired infections, which the World Health Organisation estimates to affect 4.5 million people per annum in Europe, highlighting the significant challenge currently facing the global healthcare community [6, 7].

Wound infections, specifically chronic wound infections have a reported prevalence of 6% in the UK and account for at least 5.5% of the total NHS expenditure [8]. In the USA such non-healing wounds carry an annual cost of $25 billion and it has been estimated that 1-2% of the population in developed countries will develop a chronic wound in their lifetime [9]. Wounds (venous, pressure, arterial and diabetic) are the leading cause of amputation, the latter being responsible for 70% of all lower limb amputations which occur globally every 30 seconds as a direct result of non-healing diabetic ulcers [10, 11]. The protective nature of the EPS along with the induction of various quorum sensing pathways and the subsequent alteration in gene expression, has rendered biofilms largely recalcitrant to common antimicrobial agents. Hence, the need for effective treatment strategies has become imperative in reducing both the social and economic burden of wound infection [12].

Cold atmospheric pressure plasma (CAP) has been proven to deliver a potent antimicrobial cocktail of reactive species able to successfully decontaminate numerous clinically relevant single- and mixed-species biofilms, including those associated with significant drug resistance; termed the 'ESKAPE' pathogens [13-15]. The composition and delivery of reactive species generated during CAP therapy, such as reactive oxygen and nitrogen species (RONS) may be controlled according to the plasma parameters employed, providing an attractive therapeutic treatment option currently undergoing significant investigation within the scientific community [16, 17]. Extensive discussion can be found in the literature surrounding the effects of the plasma source, the variation in operating conditions and the current progress and challenges facing CAP therapy in the control of microbial biofilms and wound infection [18-22]. The interaction of non-thermal plasma with biological material has been well documented in terms of bactericidal effects, phenotypic and genetic consequences (in both eukaryotic and prokaryotic cells) and with respect to plasma-activated liquids [23-25]. Studies have been conducted using tissue surrogates such as gelatin and agarose, alongside cellular mimics such as phospholipid vesicles to track plasma delivery of RONS [26-30]. Furthermore, the biofilm penetration depth of CAP-generated reactive species has been reported as a function of cell death and via computational modelling [31-33]. However, the direct quantification of longer-lived species, such as hydrogen peroxide (H_2O_2) has yet to be conducted through living biofilms. This study reports the effects of biofilm composition on the delivery of plasma-generated H_2O_2 across the cellular interface, thus confirming the ability of biologically active plasma-generated products to traverse bacterial biofilms according to biofilm density/maturity. The presence of H_2O_2, or lack thereof, may provide insight into the fate of the longer-lived species generated by CAP and the subsequent implications this may have for the safe and effective decontamination of chronic wounds.

2. Materials and Methods

2.1 Materials

Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), Luria-Bertani (LB) broth, phosphate buffered saline (PBS) tablets (pH 7.4), Brain Heart Infusion (BHI) agar, foetal calf serum (FCS) (HyClone), sodium chloride (NaCl), potassium iodide (KI), H_2O_2, peptone, poly (vinyl alcohol) (PVA) (M_W 14600–18600 g mol^{-1}), carboxymethyl cellulose (CMC) and agarose were all purchased from Sigma-Aldrich, UK. Biofilms were formed on sterile, nanoporous polycarbonate filter membranes (Whatman), 19 mm in diameter with an average pore size of 200 nm.

2.*2 CAP jet*

The plasma jet used consists of a single electrode configuration with a 150 mm glass capillary tube and a 15 mm external ring copper electrode operating at a distance of 40 mm from the end of the tube [30]. Helium was used as the carrier gas at a fixed flow rate of 0.6 standard litres per minute (SLPM). The jet was operated at an applied voltage of 10 kV_{p-p} and a frequency of 25 kHz. The distance from the end of the capillary tube to the surface of the substrate was 5 mm. The jet used predominantly produces H_2O_2 under the described operation parameters [28]. There is evidence that the initial interaction of the plasma with ambient air in the gas phase is the primary production site for H_2O_2 [34].

2.3 Quantification of H_2O_2

Potassium iodide (KI) was used to quantify H_2O_2 concentration in solution via the generation of a standard curve. 1M KI in deionised (DI) water was added to varying concentrations of H_2O_2 at a ratio of 1:1 and incubated at 25 °C for 30 minutes. Absorbance measurements were taken at 410 nm using a microplate reader (Spectrostar Omega, BMG Labtech) and used to produce a calibration curve (Supplementary Information Figure 1) [35]. For quantification of plasma-generated H_2O_2 in PBS, 350 μL of PBS was added to each well in a 96 well microtiter plate and treated for 0.5 - 5 minutes. 100 μL of the treated solution was then removed, added to 1M KI (1:1 v/v) and incubated at 25 °C for 30 minutes. Absorbance measurements were taken as before and the concentration calculated using the calibration curve.

2.4 Bacterial strains and growth conditions

Methicillin-resistant *Staphylococcus aureus* (MRSA) 252 and *Pseudomonas aeruginosa* (*P. aeruginosa*) PA01 were sourced from a bacterial strain collection belonging to the Biophysical Research Group housed at the University of Bath, UK. Single colonies were cultured from freezer stocks (stored at -80 °C) on TSA or LB agar for MRSA 252 and PA01, respectively. Overnight cultures were grown from single colonies in TSB or LB liquid media for MRSA 252 and PA01, respectively. Cultures were incubated at 37°C with agitation (200 rpm) for 18 hours to achieve a cell density of 10^9 colony forming units (CFU) / ml.

2.5 Bacterial biofilm formation

Overnight cultures of MRSA 252 and PA01 were centrifuged at 10,000 x g for 10 minutes, washed and resuspended in fresh PBS to an optical density of ~ 0.2 (~ 10^6 CFU/ml). White sterile polycarbonate filter membranes positioned on BHI agar were inoculated with 20 μL artificial wound fluid (FCS mixed in equal volume with 0.85% NaCl and 0.1% peptone) in order to better model the wound environment and components supporting bacterial growth and adhesion. 30 μL of bacterial subculture was added to the conditioned membranes. Membranes were UV sterilised for 10 minutes prior to bacterial inoculation. Plates were incubated at 32°C for 8, 12 and 24 hours [36].

2.6 Quantification of viable bacterial cells

Following incubation, biofilms were removed from the plates and transferred into 5 mL of PBS. The solutions were vortexed at 3000 rpm for 1 minute and sonicated for 15 minutes. This process was repeated twice to ensure complete detachment of the bacterial cells from the membrane. Serial dilutions were carried out in PBS and plated on TSA or LB agar with subsequent incubation at 37°C for 24 hours to enumerate the number of viable bacteria.

2.7 CAP treatment of bacterial biofilms

Bacterial biofilms grown for 8, 12 and 24 hours were placed atop 350 μL of PBS in a 96 well microtiter plate (full experimental set-up is shown in Figure 2 Supplementary Information). Biofilms were plasma treated for 5 minutes, with manual movement of the jet around the circumference of the well similarly as *in vivo* wound treatments. Following plasma treatment, 100 μL of PBS was removed from each well and the concentration of H_2O_2 determined as before via correlation to the standard curve. In order to assess any time-dependent generation of H_2O_2 as a result of plasma treatment, additional 8 hour biofilms were subject to plasma treatment followed by a further 4 hour incubation *in situ* at 32 °C prior to H_2O_2 quantification. Separate experiments were undertaken to quantify the number of viable bacterial cells (post plasma treatment) as previously described. All experiments were conducted in triplicate for each bacterial species and each time point.

2.8 Temperature measurement of plasma jet

The temperature of the CAP jet and biofilm onto which the jet was directed was measured using a Xenics® GOBI-640-GigE thermal imaging camera. Plasma temperature was assessed when treating a PA01 biofilm grown for 24 hours and placed atop 350 μL PBS in 96 well microtiter plate.

2.9 Topical application of H_2O_2

The effect of H_2O_2 on bacterial survival was investigated via topical application of H_2O_2 to 8, 12 and 24 hour biofilms of both MRSA 252 and PA01. 100 μL of 550 μM H_2O_2 (the same concentration of H_2O_2 produced in solution by the plasma jet in 5 minutes) was applied to the surface of the biofilms. The concentration of H_2O_2 below the biofilms was quantified after 5 minutes, and after 4 hours incubation at 32 °C as previously described.

2.10 Hydrogel formulation

PVA/ CMC hydrogels were prepared by dissolving PVA (5% w/v) in DI water and heating to 97 °C with constant stirring to facilitate dissolution. The solution was supplemented with 0.5% w/v CMC, cast to a thickness of 1 mm and stored for 18 hours at -20 °C to promote cryogenic gelation. Discs of gel were cut to a diameter of ~1 cm before being placed atop 350 μL PBS in a 96 well plate and subject to CAP treatment.

Agarose hydrogels were prepared by dissolving agarose (1.5% w/v) in DI water and heating to 90 °C. The solution was cooled, supplemented with 0.5 M KI and cast to a thickness of 1 cm. Biofilms were placed atop discs of gel (~17 mm in diameter) before treatment with the CAP jet.

3. Results

3.1 CAP generation of H_2O_2 in PBS

As previously reported, an increase in CAP exposure time results in a greater production of H_2O_2 [28]. The CAP jet used and the time dependent generation of H_2O_2 in PBS is shown in Figure 1. The KI calibration curve used to calculate concentration is shown in Figure 1 Supplementary Information.

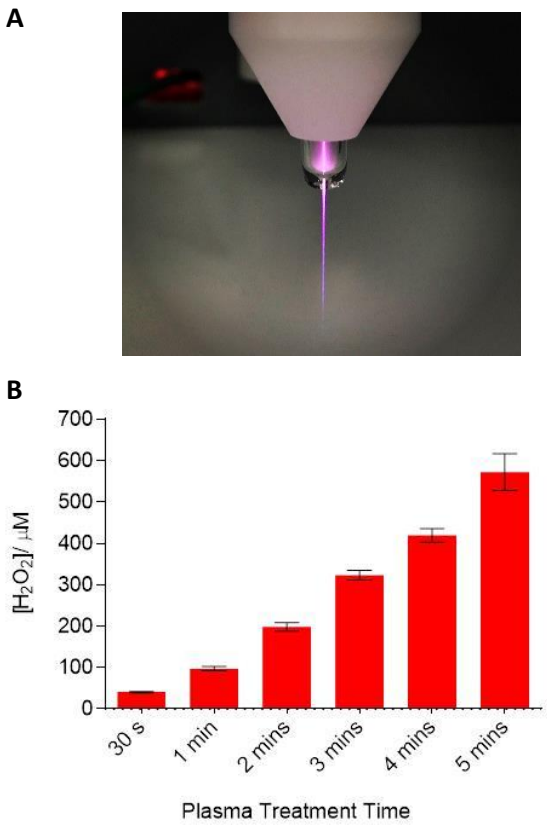

Figure 1. (A) CAP jet used in this study. (B) Generation of H_2O_2 in PBS as a function of plasma treatment time. Means and standard deviations from three independent replicates are presented.

3.2 Temperature measurement of plasma jet

Measurement of the temperature of the plasma jet in contact with a *P. aeruginosa* biofilm reached a maximum temperature of 34 ˚C (Figure 2).

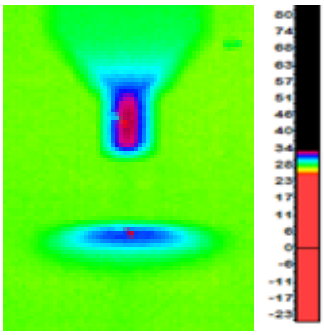

Figure 2. Temperature of plasma jet (top colour spot) showing a temperature gradient within the capillary tube and biofilm temperature at point of plasma contact (5 mm gap distance, image captured after 5 minutes, scale bar corresponds to temperature in ˚C).

3.3 CAP treatment of bacterial biofilms

The effect of biofilm maturity on the transmission of plasma-generated H_2O_2 was first evaluated by calculating bacterial cell density of untreated biofilms at various time points, corresponding to biofilm incubation time. For comparative purposes, biofilms of Gram-positive MRSA 252 and Gram-negative *P. aeruginosa* PA01 were chosen as representative isolates commonly associated with chronic wound infection [37]. Further experiments were conducted evaluating the effect of CAP treatment on cell density as a function of biofilm maturity as shown in Figure 3. A scanning electron micrograph of a 24 hour biofilm is shown in the Supplementary Information (Figure 3) in order to highlight the density of the bacterial matrix.

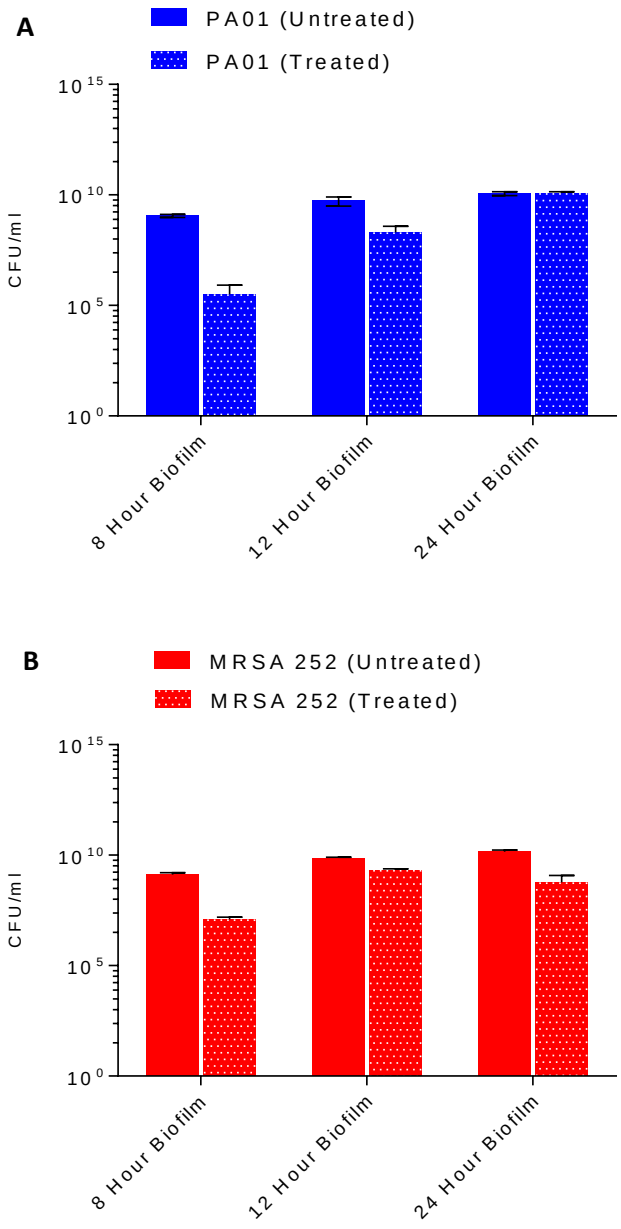

Figure 3. Effect of 5 minutes CAP treatment on cell density (CFU/ ml) of *P. aeruginosa* PA01 (**A**) and MRSA 252 (**B**) biofilms at varying maturities relative to untreated control biofilms. Means and standard deviations from three biological replicates are presented.

Owing to the high bacterial cell densities in the biofilms used in this study, it is not unexpected that the application of CAP fails to reduce the density of the mature biofilms. Plasma treatment of 8 hour biofilms reduces the cell density but does not necessarily provide a clinically significant reduction in biomass with viable cell count remaining arguably high post treatment. Previous studies have focused on the recovery of biofilms post CAP treatement as a function of biofilm maturity [38]. Additional studies have been reported in which CAP therapy is able to significantly reduce or even eliminate bacterial biofilms (albeit often when treating biofilms with a lower bioburden), however this study concerns the fate of CAP-generated RONS as opposed to their biological effect [39-40]. Therefore high density biofilms indicative of chronic infection were chosen as the cellular interface over which to monitor H_2O_2 formation.

Prior to establishing the concentration of H_2O_2 able to traverse the cellular interface, it was necessary to evaluate transmission across the polycarbonate membrane on which the biofilms were grown. Plasma treatment through the nanoporous membrane for 5 minutes resulted in a 33% reduction in H_2O_2 concentration in the PBS below the membrane (relative to direct treatment of PBS), as shown in Figure 4. This was taken into account in subsequent experiments by the introduction of a transmission factor (TF) of 0.67 to account for the interference of the membrane.

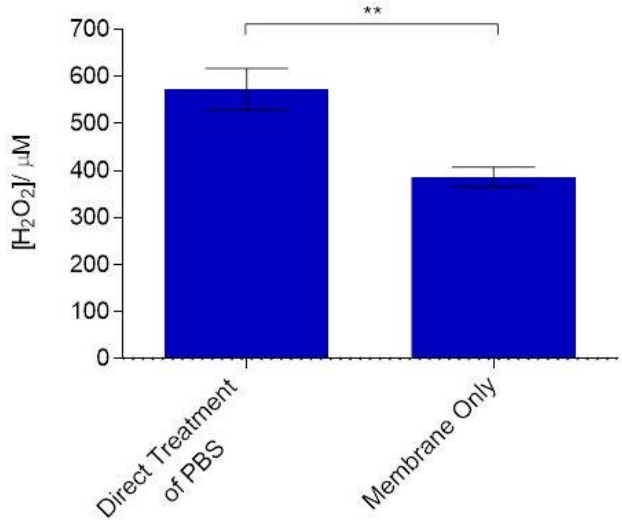

Figure 4. Effect of the polycarbonate membrane (a function of the experimental protocol) on the generation of H_2O_2 in PBS after 5 minutes, showing a significant reduction in H_2O_2 concentration. Means and standard deviations from three independent replicates are presented and have been analysed using an unpaired t test: ** $p = 0.0029$.

Considering the TF, the presence of AWF on the membranes (included as a pre-treatment prior to bacterial inoculation) was also investigated. Membranes were pre-treated with a conditioning layer of AWF and incubated at 37 °C for 8, 12 or 24 hours (in-keeping with subsequent biofilm growth conditions) to establish any interference from the conditioning layer over time. The concentration of H_2O_2 generated in the PBS below the membrane impregnated with AWF was comparable to the membrane only (data shown in Figure 4), regardless of incubation time as summarised in Table 1.

Results illustrate no statistical significance with respect to H_2O_2 concentration as a result of the inclusion of AWF or the incubation period. This suggests little measurable interference of the AWF on the transmission of H_2O_2 across the interface.

Table 1. Concentration of H_2O_2 generated across polycarbonate membranes inoculated with AWF following incubation for various time periods (taking into account the TF). Means and standard deviations from three biological replicates are presented and have been analysed using a one-way ANOVA with multiple comparisons.

Incubation Time/ hours	$[H_2O_2]$/ TF / µM
8	374.9 ± 40.4
12	520.5 ± 13.1
24	400.2 ± 96.8

However, Figure 5 shows the presence of bacterial biofilms (of all maturities) to have a significant effect on the transmission of H_2O_2 relative to the membrane + AWF only. Biofilms grown for 8 hours, corresponding to approximately 10^9 CFU/ml, reduced the concentration of H_2O_2 by approximately half, whereas biofilms approaching 10^{10} CFU/ml almost entirely prevented the detection of H_2O_2. The results show a significant difference in H_2O_2 concentration when comparing 8 hour biofilms of both MRSA and *P. aeruginosa* to the corresponding 12 and 24 hour biofilms, which may be as a direct result of the observed difference in bacterial cell death (seen in Figure 3) when comparing 8 hour biofilms to more mature biofilms. There is no significant difference observed between the 12 and 24 hour biofilms of either MRSA or *P. aeruginosa*; likewise, there is no difference in H_2O_2 concentration between the bacterial species at each time point.

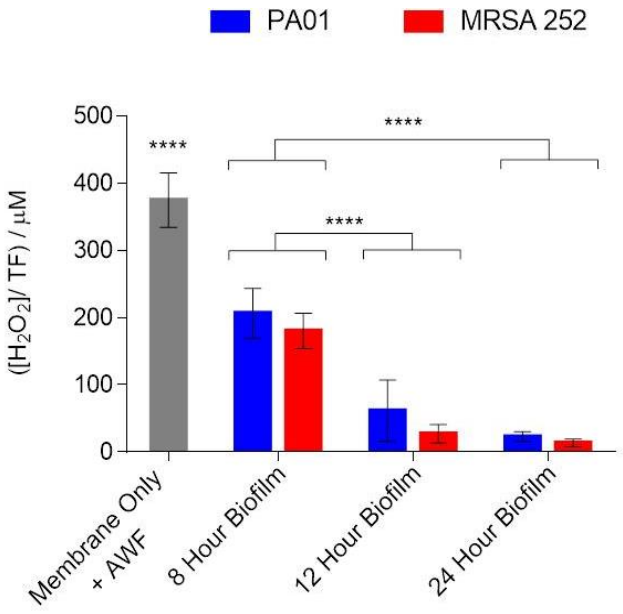

Figure 5. Effect of biofilm composition on the generation of H_2O_2 in PBS below the interface as a function of biofilm maturity. Means and standard deviations from three biological replicates are presented and have been analysed using a two-way ANOVA with multiple comparisons: **** p <0.0001.

In order to investigate time-dependent formation and/or diffusion of plasma-generated H_2O_2, PVA/ CMC hydrogels were used as a model system prior to commencing biofilm analysis. The gels were placed atop wells containing PBS and treated with the CAP jet for 5 minutes. The concentration of H_2O_2 in the PBS was then determined immediately (0 hours incubation) and following incubation at 32 °C (skin temperature) for 1 - 8 hours (Figure 5) [41]. In this case the incubation period facilitated the formation of H_2O_2 in a time dependent manner over the course of 8 hours, reaching a maximum concentration within ~ 4 hours.

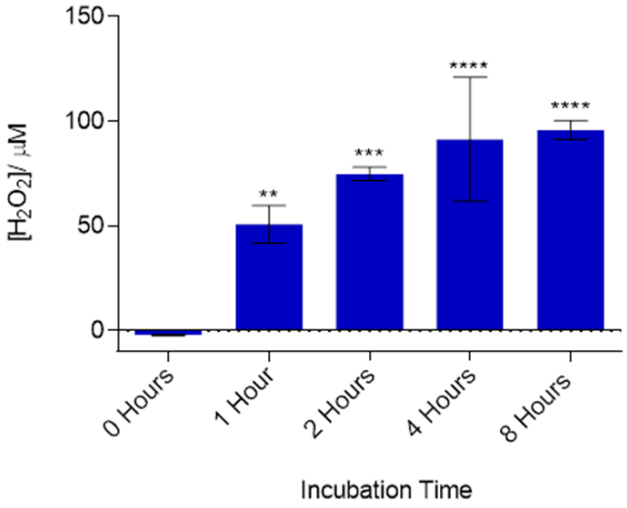

Figure 6. Effect of incubation time on H_2O_2 concentration in PBS post CAP treatment of PVA/CMC hydrogels. Means and standard deviations from three independent replicates are presented and have been analysed using a one-way ANOVA with multiple comparisons: **** p < 0.0001, *** p < 0.001, ** p < 0.0001. Statistical significance is reported with respect to the concentration of H_2O_2 detected immediately post exposure (0 hours).

However, this trend was not seen when subjecting 8 hour biofilms to the same incubation conditions. Conversely, the concentration of H_2O_2 was lower for both MRSA and *P. aeruginosa* post incubation (Figure 7). Previous studies have indicated the role of catalase in protecting biofilms from the effects of hydrogen peroxide by catalysing its decomposition into oxygen and water [42-43]. Both *S. aureus* and *P.*

aeruginosa are catalase-positive, which could explain the results seen in Figure 7 using biofilms that had been incubated for 4 hours post CAP treatment. Further experimental studies will aim to address this point in the future.

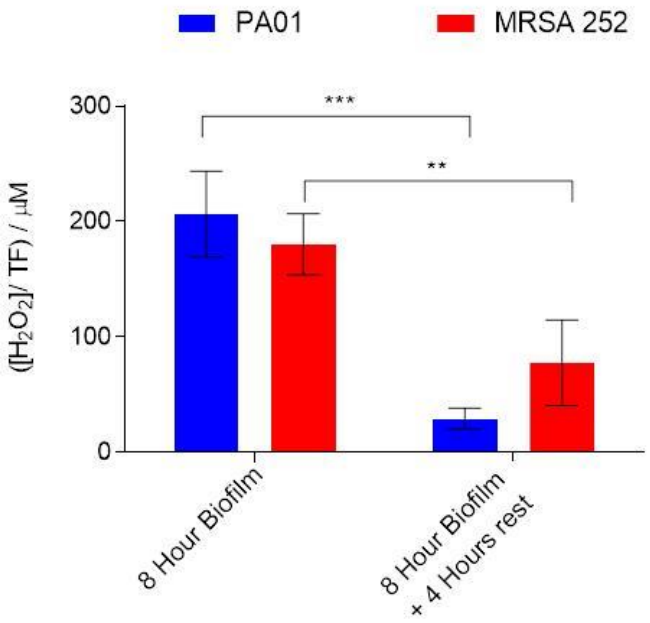

Figure 7. Effect of incubation time on H_2O_2 concentration in PBS post CAP treatment of 8 hour biofilms. Means and standard deviations from three biological replicates are presented and have been analysed using a two-way ANOVA with multiple comparisons: *** $p < 0.001$, ** $p < 0.0001$.

3.4 Visualisation of H_2O_2 formation

Qualitative assessment of H_2O_2 formation (both immediately and over time) was undertaken using agarose gels containing KI. The hydrogel reservoir was able to indicate the formation and diffusion of CAP-generated H_2O_2 through bacterial biofilms via a distinctive colour change upon the liberation of iodine. Biofilms were placed on the surface of the gels and subjected to CAP treatment. The gels were then imaged immediately following exposure, before being incubated for 4 hours at 32 °C, after which the biofilms were removed and the gels imaged once more. In agreement with previous quantitative data no colour change was observed within the gels during CAP treatment of 24 hour biofilms, whereas an immediate colour change was observed with 8 hour biofilms of both bacterial species (Supplementary Information Figures 5 and 6). However, when treating biofilms grown for 12 hours, MRSA biofilms facilitated a greater degree of iodine release within the agarose gels relative to *P. aeruginosa* biofilms grown for the same length of time. Figure 8 shows the colour change within the gels immediately post exposure (A and B) and after 4 hours incubation (C and D).

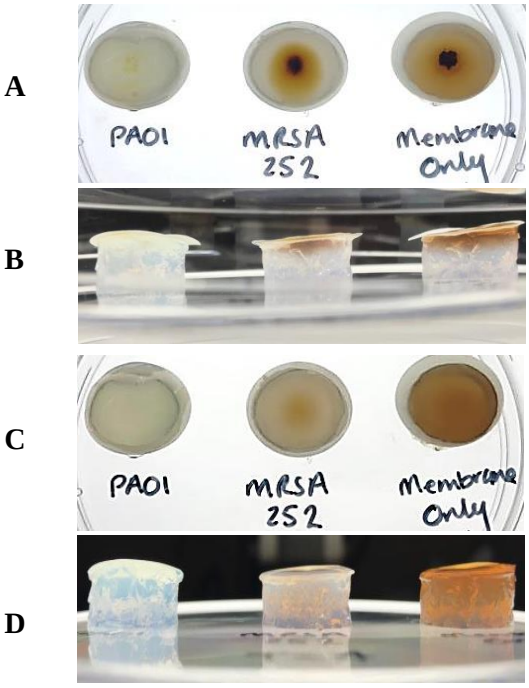

Figure 8. 12 hour biofilms of MRSA 252 and *P. aeruginosa* PA01 placed atop agarose gels containing KI and subject to CAP treatment imaged immediately post treatment (**A, B**) and after 4 hours incubation at 32°C (**C, D**), compared to the uninoculated nanoporous membrane pre-treated with AWF.

This result contrasts with the quantitative data, which indicates very little H_2O_2 generated across the 12 hour biofilms of both MRSA and *P. aeruginosa* into PBS (Figure 5). Indeed, within the range of the experimental error the concentration of H_2O_2 below the biofilms is effectively equal for both species. However, in the case of biofilms placed atop a hydrogel surface there is a negligible colour change in the gel supporting the *P. aeruginosa* biofilms, whereas there is a clear colour change for the MRSA biofilms (albeit this change is small when compared to the 'membrane only' control). This discrepancy may be due to a number of factors; the delivery reservoir (PBS vs a gel matrix) is likely to affect the diffusion of reactive species and the uniformity of the biofilms may vary (they are matured by time as opposed to thickness and therefore there will undoubtedly be some variation). The behaviour of the biofilms when placed atop a liquid or solid interface may change and/or there may be a change in the H_2O_2 catalysis under these conditions. Such considerations when designing experimental protocols for the analysis of CAP therapy should be carefully rationalised and investigated with respect to the intended application in order to more accurately predict and measure the possible outcomes.

3.5 Topical application of H_2O_2

Owing to the multitude of different processes occurring during CAP treatment which go beyond the direct operating parameters (to include gas mixing with atmospheric gases and mixing at the solution interface) it is challenging to provide a representative control for the analysis of plasma-generated species across biological interfaces [44]. Consequently, we have focused solely on H_2O_2 transmission (as this is the major long-lived species generated by the jet described). H_2O_2 at the same concentration as generated by the CAP jet in 5 minutes was topically applied to *P. aeruginosa* and MRSA biofilms determine equivalency between plasma-assisted penetration and topical therapy. Whilst it is difficult to compare the results directly, topically applied H_2O_2 was unable to traverse biofilms of all maturities indicated by the absence of any quantifiable concentration of H_2O_2 in the PBS reservoir below. This was confirmed immediately after the 5 minute exposure time and following a subsequent 4 hours incubation at 32 °C. This result suggests that plasma-generated H_2O_2 (and possibly other CAP-generated RONS), are better able to penetrate bacterial biofilms than topical application of the same chemical species. Again, this is likely a result of the additional processes occurring, including continuous gas flow as a driving force into the biofilms, localised heating and/or synergistic effects from other plasma-generated RONS.

4. Discussion and Conclusions

The bioburden of the mature biofilms used in this study is arguably much higher than seen in some other studies. However, healthy skin is reportedly permanently colonised with 10^3 – 10^4 microorganisms per cm^2 skin (rising to 10^6 in moist areas), as part of a healthy skin microflora [45]. Therefore, dense biofilms with high microbial cell counts were used to represent the exponential proliferation of opportunistic pathogens associated with untreated chronic infection. In this study the use of CAP is intended as a therapeutic treatment as opposed to a preventative measure (whereby treatment may commence at lower cell densities than those often considered indicative of progressive infection).

In contrast to other studies, this work describes the effect of bacterial biofilms on the fate of plasma-generated products rather than any direct cellular effects of CAP therapy on the biofilms - an important consideration in the future clinical application of cold plasma. This investigation highlights the ability of plasma-generated reactive species to traverse live biofilms, remaining active despite interaction with the bacterial species present. This is observed up to cell densities of 10^9 CFU/ml, above which the concentration of longer lived species is significantly reduced when evaluated across the interface of the biofilm. Determining the exact mechanism of cellular interaction with plasma-generated H_2O_2 is ongoing. Owing to the complexity in determining the exact pathway of plasma-generated H_2O_2 with respect to both the gas phase and the liquid interface, there are a number of considerations to take into account such as direct interaction of biofilm components (including catalase) with plasma-generated H_2O_2, alongside the transport and any subsequent reactions with additional plasma-generated RONS [46-47]. Nonetheless, the ability of high density bacterial biofilms to influence the transmission of such species may have implications for the application of CAP therapy in the treatment of chronic infection, whereby high bacterial loads may be readily encountered. In the context of deep-seated infections, the ability to deliver RONS directly into the infected tissues may vary with different device designs or may in fact be a ubiquitous challenge associated with CAP therapy. This should be established with the intention of improving RONS delivery and penetration in order to successfully apply this technology to highly contaminated wounds**.** Alongside ensuring the efficacy of CAP therapy via adequate penetration and delivery of plasma-generated reactive species, the effects of such species on other cellular entities cannot be underestimated. Ultimately, for the succesful therapeutic treatement of infection, a balance must be established between efficacious elimination of pathogenic organisms and implementaion of adequate controls for the protection of the surrounding host tissue.

Further research will focus on establishing the effect of catalase on the lifetime and bioavaliability of plasma-generated H_2O_2, the effect of CAP-induced bacterial cell death on the transmission of H_2O_2 and any protective effects offered by bacterial biofilms with respect to surrounding host tissues. Additional research will also aim to investigate the formation and transmission of other reactive species such as O_2·-, ·OH, ·HO_2, to determine additional transportation mechanisms of plasma-generated RONS with respect to the material interface (biotic or abiotic).

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