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Dielectric Barrier Discharge Atmospheric Cold Plasma for Inactivation of *Pseudomonas aeruginosa* Biofilms

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ABSTRACT: In recent years, atmospheric cold plasma (ACP) has been widely investigated for potential application as an alternative decontamination technology in biomedical and healthcare sectors. In this study, the antimicrobial efficacy of ACP against *Pseudomonas aeruginosa* biofilms was investigated. The 48-h biofilms were treated inside sealed polypropylene containers with a high-voltage dielectric barrier discharge (DBD) ACP (80 kV_{RMS}) and subsequently stored for 24 h at room temperature. Treatment for 60 s by either the direct or indirect mode of ACP exposure (inside or outside plasma discharge, respectively) reduced bacterial populations by an average of 5.4 log cycles from an initial 6.6 log_{10} CFU/mL. Increasing the treatment time from 60 s to 120 s and 300 s reduced biofilms to undetectable levels. According to XTT assay (a metabolic activity assay), an extended treatment time of 300 s was necessary to reduce metabolic activity of cells in biofilms by an average of 70%. Further investigation of biofilm viability by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) demonstrated that extended ACP treatment had a detrimental effect on the viability of *P. aeruginosa* through disintegration of both bacterial cells and the biofilm matrix. The results of this study demonstrate the potential of a novel, in-package, high-voltage ACP decontamination approach for the inactivation of bacterial biofilms.

KEY WORDS: Atmospheric cold plasma, *Pseudomonas aeruginosa*, biofilm, CLSM, SEM

I. INTRODUCTION

Pseudomonas aeruginosa is one of the most abundant microorganisms in the natural environment and is a common opportunistic nosocomial pathogen, which causes a wide variety of acute and persistent infections in immunocompromised patients. This microorganism has become increasingly important due to associated prevalence, pathogenicity, and increasing resistance to antimicrobial treatments, causing morbidity and mortality.[1–2] *Pseudomonas* associated health care infections include respiratory tract infections,[3–4] urinary tract infections,[5] surgical and burn wound infections[6,7] and bloodstream infections.[8,9] According to Neonatal Infection Surveillance Network, between 2005 and 2011, in the United Kingdom, *Pseudomonas* spp. were responsible for 93% of neonatal infection outbreaks and with 18% of associated deaths.[10]

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Water is the main environmental reservoir of *P. aeruginosa*, and the water distribution system is one of the possible sources of *P. aeruginosa* in healthcare settings (taps, sinks, etc.).[2,11] In addition, *P. aeruginosa* has a tendency to form biofilms. Recently, Walker et al.[12] demonstrated that, within hospital facilities in Northern Ireland, *P. aeruginosa* exists predominantly in the form of biofilm in water tap outlets, which are therefore a possible cause of infection. Biofilms are broadly described as a microbially derived sessile community with altered phenotype with respect to growth rate, in which cells are attached to a substratum or to each other and are embedded in a matrix of extracellular polymeric substances (EPSs).[13] Material secreted by bacteria EPS (i.e., polysaccharides, proteins, lipids, and nucleic acids) act as a complex defensive mechanism protecting bacteria from unfavourable environmental conditions. Due to the heterogeneous nature and multiple mechanisms of resistance, biofilms have an enormous impact on medicine.[14] It has been estimated that up to 80% of human bacterial infections are associated with biofilms.[15] Once biofilms are established, different mechanisms account for resistance to antimicrobial treatments. In addition, inappropriate use or overuse of antibiotics may also result in increased bacterial resistance.[16,17] Therefore, alternative antimicrobial measures are required as a key component of the prevention of healthcare-associated infections (HAIs) to ensure effective reductions and elimination of pathogens and their associated biofilms while simultaneously averting bacterial resistance.

Atmospheric cold plasma (ACP) technology is an innovative field that is intensively researched for a range of different medical applications. Von Woedtke et al.[18] classified plasma for medical application according to two general principles: indirect use of plasma to treat surfaces, materials, or devices for medical applications, and application of physical plasma on or within the human body. Sterilization of medical materials or devices (e.g., medical implants, catheters, or materials in blood purification systems) is the main use of indirect plasmas for medical purposes;[20] whereas physical plasmas are under intense study for applications in wound healing,[19,20] blood coagulation and skin regeneration,[21] dentistry,[22] and apoptosis of cancer cells.[23]

To date, research on ACP sterilization has demonstrated effective reduction of clinically important microorganisms in their planktonic state or in biofilms.[24,25] However, the antimicrobial effects of plasma were reduced against biofilms by comparison with their planktonic counterparts.[26–27]

The aim of this study was to evaluate the potential of novel, in-package, high-voltage air DBD ACP for elimination of *P. aeruginosa* biofilm. The effects of treatment time and mode of plasma exposure were investigated. Surviving bacterial populations in biofilm were estimated by colony count and XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) [phenyl-amino)car-bonyl]-2H-tetrazolium hydroxide) assays for determination of cell viability and metabolic activity, respectively. The ability of the contained ACP reactive species to penetrate complex biofilm structures was observed using a LIVE/DEAD bacterial viability kit followed by confocal scanning laser microscopy (CSLM). Scanning electron microscopy (SEM) was conducted to observe morphological changes of biofilms caused by ACP treatment.

II. MATERIALS AND METHODS

A. Bacterial Strains and Inocula Preparation

Pseudomonas aeruginosa ATCC 27853, a blood culture isolate, was obtained from the microbiology stock culture of the School of Food Science and Environmental Health of the Dublin Institute of Technology and was utilized in all experiments. A single isolated colony of the culture was inoculated in tryptic soy broth without glucose [TSB (-G), ScharlauChemie, Spain] and was incubated at 37°C for 18 h. The overnight culture was diluted in TSB to a density of 7.0 $\log_{10}$ colony-forming units per milliliter (CFU/mL), using the McFarland standard. This bacterial cell concentration was further used as an inoculum for biofilm formation.

B. Biofilm Formation

Bacterial biofilms were produced in either 96-well plates for the colony count and XTT assay, on glass coverslips (24 x 32 mm) for CLSM analysis, or using tissue culture-treated inserts containing polycarbonate membrane (0.45 µm, Millicell, Millipore, Ireland) for SEM analysis. Biofilms in the 96-well plates were obtained by adding 200 µl of TSB bacterial suspension (7.0 $\log_{10}$ CFU/ml) to the wells of the plates. For production of biofilms for CLSM, coverslips were positioned inside the wells of the 6-well culture plate, and 8 ml of TSB bacterial suspension was added. For production of biofilms on polycarbonate membranes, inserts were placed inside the wells of the 6-well plate, which were filled with 2 ml of TSB bacterial suspension. The plates were incubated at 37°C for 48 h. After 24 h of incubation, the supernatant from each well of the 96-well and 6-well plates containing inserts or glass coverslips was replaced with fresh TSB, with further incubation for 24 h. After incubation, the TSB containing suspended bacterial cells was removed and the wells were extensively rinsed with sterile PBS, leaving only bacterial biofilms for further investigation. Negative controls were obtained using TSB without inocula. Prior to each experiment, the biofilms were air dried for 60 min.

C. Experimental Design

The ACP system utilized a high-voltage DBD system previously described in Ziuzina et al.[28] with a maximum voltage output of 120 kV at 50 Hz. The distance between the two 15-cm diameter aluminium disk electrodes was 22 mm, which was equal to the height of the polypropylene container (310 x 230 x 22 mm) utilized as both a sample holder and as a dielectric barrier. For this study, ACP was generated in air at 80 kV_{RMS}.

The 96-well plates, glass coverslips or membrane inserts containing biofilms were placed in the center of the plastic container directly between the electrodes within the

plasma discharge for direct plasma treatment (Fig. 1A). For indirect plasma treatment, a separate container was used, and either substrate containing biofilm was placed to achieve treatment outside the plasma discharge (Fig. 1B). After sample loading, each container was sealed with a high-barrier polypropylene bag (Cryovac, B2630, USA) and placed between the aluminium electrodes of the transformer. The biofilms were treated with direct/indirect ACP for 0, 60 s, 120 s, and 300 s and were subjected to a post-treatment storage for 24 h at room temperature. Control samples were untreated. All experiments involving biofilm formation in 96-well plates were conducted using three independently grown cultures and were repeated at least twice. Experiments were repeated three times when biofilms were grown on glass coverslips and membrane inserts.

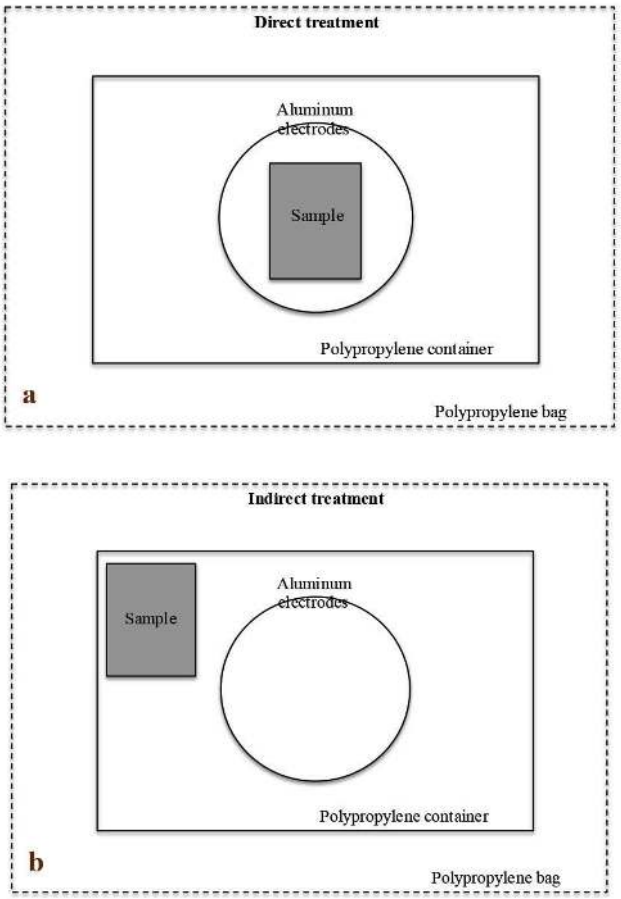

FIG. 1: Schematic diagram of samples distributed within polypropylene container with respect to the electrodes: **(a)** direct treatment and **(b)** indirect treatment

D. Biofilm Assays

1. Crystal violet assay (CV)

Crystal violet (CV) staining was used to monitor biofilm formation capacity using 24 h and 48 h incubation times. Biofilm mass was quantified as described previously.[29] The absorbance was measured at 590 nm on a microplate reader (Synergy HT, Biotek Instruments, Inc.). Each absorbance value was corrected by subtracting the means of absorbance of a blank (uninoculated) TSB. Experiments were carried out in triplicate and were repeated at least twice.

2. Colony count assay

Following ACP treatment and 24 h post treatment storage, 200 µl of sterile PBS was added into the biofilm containing wells of the 96-well plate. To disrupt biofilms, the plates were sonicated using a water table sonicator (Bransonic 5510E-MT, Branson, USA) for 5 min. Suspensions were pooled together from the wells into sterile Eppendorf tubes, serially diluted in maximum recovery diluent (MRD, ScharlauChemie, Spain), if necessary, and 0.1-mL aliquots of appropriate dilutions were surface plated on TSA and incubated at 37°C for 24 h. Results are presented as surviving bacterial population in $\log_{10}$ CFU/mL units.

3. XTT assay

Prior to each assay, fresh solution of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) [phenyl-amino)car-bonyl]-2H-tetrazolium hydroxide (XTT, 1 mg/ml, Sigma-Aldrich, Ireland) was prepared as described previously.[32] The wells containing biofilms and negative controls (TSB without inocula) were filled with sterile PBS (100 µl), then XTT-menadione (100 µl) was added to all wells. Plates were incubated for 5 h at 37°C in the dark. After incubation, the supernatant (100 µl) from each well was transferred into the wells of a new 96-well microtiter plate, and the absorbance was measured at 486 nm on a microplate reader. The percentage of the surviving bacterial population was calculated as $((A_{ACP} - A_C) / A_0) \times 100\%$, where A_{ACP}, A_C, and A_0 are the absorbance of ACP treated, the negative control, and untreated control biofilms, respectively.

4. Confocal laser scanning microscopy (CLSM)

Prior to analysis, to differentiate viable and dead bacteria, untreated controls and biofilms treated with either direct or indirect ACP for 300 s were stained using a LIVE/DEAD

bacterial viability kit containing SYTO9 and propidium iodide (PI) (Sigma-Aldrich, Ireland). Biofilms grown on glass coverslips were covered with 200 μL of SYTO9, and the PI solution was prepared according to the manufacturer's instructions and was incubated for 15 min in the dark. The samples were rinsed with sterile deionized water, placed on the microscope glass slide, and imaged using a Zeiss confocal microscope (Carl Zeiss, Model: LSM 510, Germany). The quantitative parameters to characterize biofilm thickness were obtained from CLSM stack images and the sample images were further analyzed by IMARIS image analysis software (Bitplane, Inc.). The maximum thickness of the biofilm was determined from the value of the Y-axis.

5. Scanning electron microscopy (SEM)

For SEM analysis, untreated controls and *P. aeruginosa* biofilms treated directly or indirectly with ACP for 300 s were selected and prepared as described by Gratao et al.,[30] with minor modifications. The cells were fixed in ice-cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) (SCB) for 2 h. The cells were washed with the same buffer three times and were then fixed in 1% osmium tetroxide for 2 h at 4°C. After 2 h of fixation, bacterial cells were washed with SCB followed by three washes with distilled water. The samples were treated using increasing concentrations of ethanol (30%, 50%, 70%, 80%, 95%, and 99.5%) and dehydrated as described by Srey et al.[31] using 33%, 50%, 66%, and 100% (v/v) hexamethyldisilazane (Sigma-Aldrich, Ireland). To prevent surface charging by the electron beam, the samples were sputter-coated with gold particles using Emitech K575X Sputter Coating Unit resulting in a coating of 10 nm after 30 s. The samples were examined visually using a FEI Quanta 3D FEG Dual Beam SEM (FEI Ltd, Hillsboro, USA) at 5 kV.

E. Statistical Analysis

Statistical analyses were performed using SPSS 19.0 (SPSS, Inc., Chicago, USA). The surviving populations of *P. aeruginosa* biofilms following ACP treatments were compared using analysis of variance (ANOVA). Means were compared according to the method of Fisher's least significant difference (LSD) at the 0.05 level.

III. RESULTS

A. Evaluation of Biofilm Biomass by CV Assay

According to the scheme described by Stepanovic et al.,[32] 24-h- and 48-h-old *P. aeruginosa* demonstrated strong ability to produce biofilms (Fig. 2A). Notably, after 48 h of incubation, *P. aeruginosa* biofilms were clearly visible on the surface of the wells of the

96-well microtiter plate (Fig. 2B). Based on these results, only bacterial biofilms grown for 48 h were used in further studies aimed at investigating the efficacy of ACP to inactivate bacteria in not only the realistic but also the more resistant biofilm form.

B. Effects of ACP on *P. aeruginosa* biofilms Assessed by Colony Count and XTT Assays

The influence of ACP treatment time and mode of exposure on viability of *P. aeruginosa* biofilms based on colony count assay is presented in Fig. 3A. Generally, both direct and indirect modes of plasma exposure effectively reduced bacterial populations in biofilms after short treatment times. Prior to ACP treatment, an average population of bacterial biofilm attached on the surface of the wells was 6.6 $\log_{10}$ CFU/mL. ACP treatment for 60 s reduced bacterial cells by an average of 5.4 $\log_{10}$ CFU/mL; increasing treatment time to 120 s and 300 s reduced the numbers of viable cells within the biofilms to levels below detection limits (1.0 $\log_{10}$ CFU/mL).

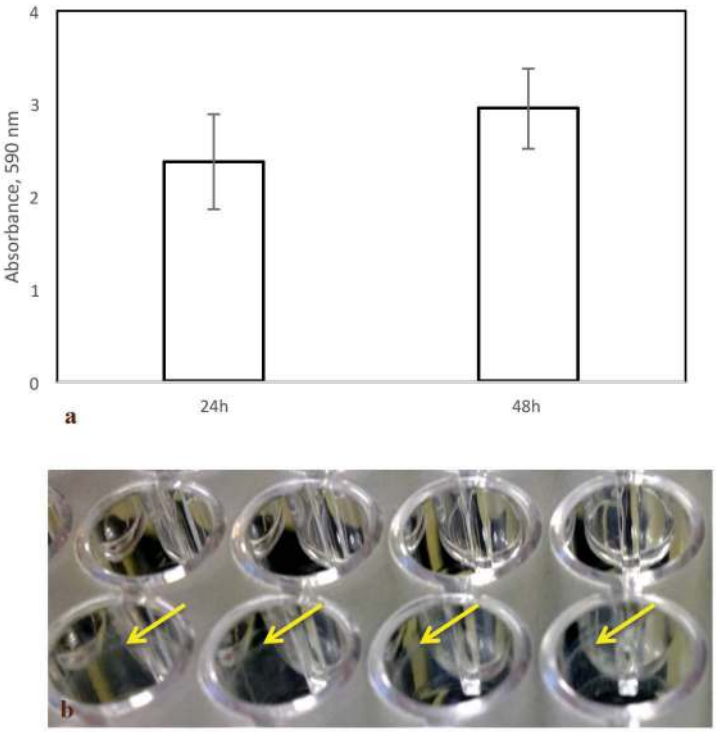

FIG. 2: (a) *P. aeruginosa* biofilm formation evaluated by CV assay after 24 h and 48 h of incubation at 37°C; **(b)** *P. aeruginosa* biofilm formed in the wells of a 96-well microtiter plate. Top row: uninoculated control wells. Bottom row: 48-h biofilm (yellow arrows)

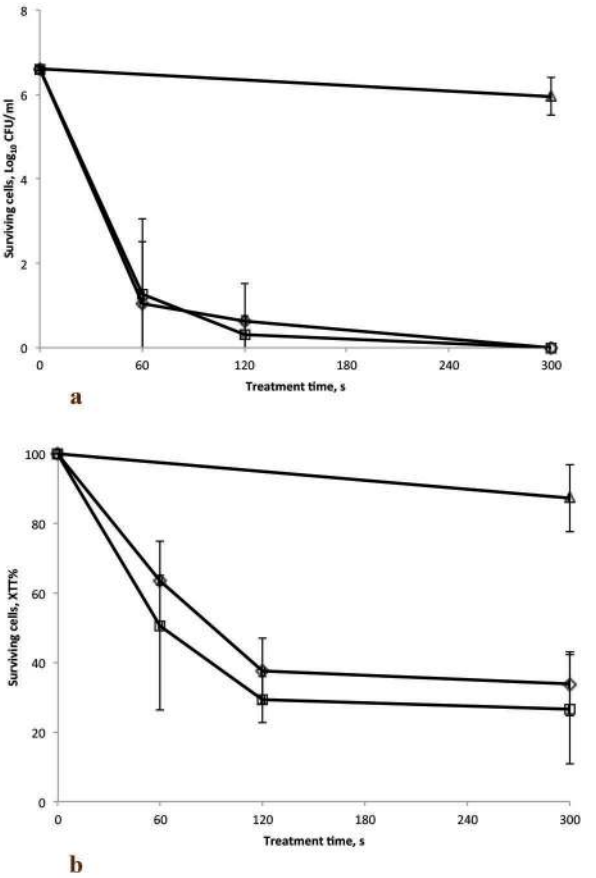

FIG. 3: (a) Surviving populations of *P. aeruginosa* 48-h biofilms assessed by colony count assay: untreated control biofilms (Δ) after direct (◊) and indirect (□) ACP treatment. Vertical bars represent standard deviation. Limit of detection, 1.0 log10 CFU/sample **(b)** Percentage surviving populations of *P. aeruginosa* 48-h biofilms assessed by XTT assay: untreated control biofilms (Δ), after direct (◊) and indirect (□) ACP treatment. Vertical bars represent standard deviation

Percentage survival of *P. aeruginosa* biofilm after ACP exposure based on XTT assay is presented in Fig. 3B. XTT absorbance values demonstrated that 60 s of direct and indirect treatment reduced metabolic activity of cells by 37% and 49%, respectively. Increasing treatment time from 60 s to 120 s further reduced cell metabolic activity by 63% and 70% when exposed to direct and indirect treatment, respectively. However, no further reductions were observed by applying extended treatment time of 300 s, with a maximum average reduction of 70% recorded. Notably, post treatment storage time of 24 h had a minor effect on biofilm activity for this microorganism based on both colony count and XTT assays.

C. Confocal Laser Scanning Microscopy (CLSM)

To evaluate the effect of ACP on viability of bacterial cells embedded in biofilm structures, CLSM analysis of untreated control and *P. aeruginosa* biofilms treated directly/indirectly for 300 s was conducted. This analysis utilizes the mixture of SYTO9 (green fluorescent) and PI (red fluorescent) nucleic acid dyes. SYTO9 labels bacterial cells with both intact and damaged membrane, whereas PI penetrates only cells with damaged membrane. Therefore, this analysis allows determination of bacterial viability and cell membrane integrity on the basis of different colors, where green and red represent live and dead cells, respectively. The results of the CLSM analysis are presented in Fig. 4. As can be seen from Fig. 4A, the untreated control sample exhibited completely green fluorescence, i.e., only live cells were present. Maximum biofilm thickness of 23 μm was recorded. Confocal images of biofilms treated with either direct or indirect ACP (Fig. 4B, 4C, respectively) displayed only red fluorescence, with no viable cells remaining after treatment. Moreover, images of ACP-treated samples also indicated a substantial reduction of biofilm thickness when compared with control biofilms. The thickness of biofilms was reduced to 8 μm and 6 μm after direct and indirect ACP treatment, respectively.

D. Scanning Electron Microscopy (SEM)

To examine the effects of ACP on *P. aeruginosa* biofilm distribution and morphology, SEM analysis of untreated control and samples treated directly or indirectly for 300 s was conducted. Figure 5A represents untreated controls, in which clustered rod-shaped bacterial cells are interconnected with each other by the complex biofilm matrix components. Significant damage of bacterial cells and EPS was observed after both direct

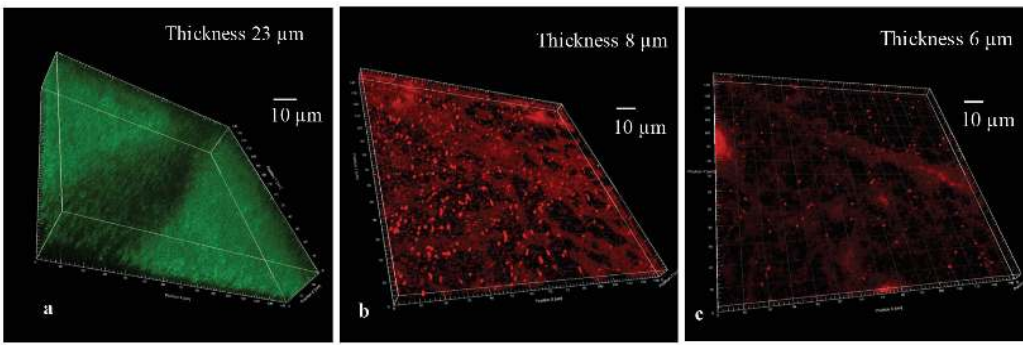

FIG. 4: CLSM images of *P. aeruginosa* 48-h biofilm stained with LIVE/DEAD bacterial viability kit: **(a)** untreated control; **(b)** 300 s of direct ACP treatment; **(c)** 300 s of indirect ACP treatment. Cells stained green are alive and cells stained red are dead

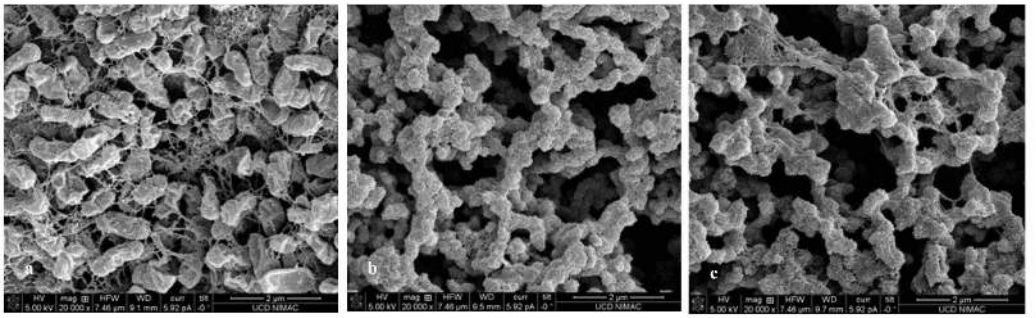

FIG. 5: SEM images of *P. aeruginosa* 48-h biofilm: **(a)** untreated control; **(b)** 300 s of direct ACP treatment; **(c)** 300 s of indirect ACP treatment

and indirect ACP treatments. Complete disintegration of cells and biofilm matrix was noted after direct ACP treatment: biofilms were converted into the 'sponge-like,' irregularly shaped debris compared with untreated controls (Fig. 5B). Although indirect ACP had similar destructive effects on biofilm structures, small fractions of EPS components were still detected after the treatment (Fig. 5C).

IV. DISCUSSION

In recent years, cold atmospheric plasma (ACP) has been widely investigated as an alternative sterilization technology for potential application in medicine. This technology has been demonstrated to have great bactericidal effects against a wide range of microorganisms with high potential to avoid any thermal damage to both living and non-living biomedical structures.[33] Our previous studies demonstrated that high-voltage DBD ACP in conjunction with treatment of contaminated objects inside sealed packages and following post treatment storage was very effective for inactivation of bacteria in their planktonic form, reducing the numbers within seconds.[31] However, bacterial biofilms, as a predominant mode of microbial growth,[34] represent major challenges in industrial and health care settings due to their increased mechanical stability and antimicrobial resistance.[35] Therefore, in the present study, the potential of ACP to inactivate bacterial pathogens was investigated against challenge *P. aeruginosa* biofilms, as one of the major causes of healthcare-associated infections.

P. aeruginosa is considered a model microorganism for biofilm research due to its excellent ability to form biofilm.[36] Because bacteria in older biofilms are more resistant to antimicrobial treatments,[37,38] only biofilms grown for 48 h were utilized to present a realistic challenge to the ACP treatment. The effect of ACP treatment on *P. aeruginosa* biofilms was investigated utilizing widely accepted plate-count and XTT assays. According to the results obtained from both assays, no significant difference between the effects of direct and indirect modes of exposure on bacterial biofilms was found. According to colony counts, rapid inactivation of cells in biofilm was achieved after

relatively short treatments (60 s), resulting in reduction of bacterial levels by 5.4 $\log_{10}$ CFU/ml. With lower inactivation rates, however, bacterial populations were below detection limits after exposure to 120 s and 300 s of treatment. Similarly, Alkawareek et al.[39] reported biphasic reductions of *P. aeruginosa* biofilms due to ACP treatment, where initial 60 s of plasma treatment resulted in rapid decline in bacterial levels, causing slower reduction between 60 s and 240 s of treatment. In general, our data suggest that 80kV air in-package DBD ACP treatment exhibits considerably high inactivation potential against the most abundant and resistant bacterial form—biofilm. Notably, based on our preliminary data, treatment for only 30 s was required to completely eliminate *P. aeruginosa* (~7.0 $\log_{10}$ CFU/ml reduction) in the planktonic form (data not shown). In general, according to previous reports, focusing on inactivation efficacy of ACP against biofilm, complete inactivation of *P. aeruginosa* biofilm could be achieved after longer treatment times, from 5 to 10 min.[27,40] Utilization of different ACP systems and different approaches to biofilm formation are probably the main reasons for variations in the published reports, which reduces the possibility of drawing adequate conclusions regarding the overall plasma treatment effectiveness against bacterial biofilms. Comprehensive system, process, and target comparative studies are still required.

To examine the effects of ACP on the metabolic state of *P. aeruginosa* biofilms, the XTT assay was utilized, which involves intracellular reduction of XTT to a water-soluble formazan, the absorbance of which is proportional to the number of metabolically active bacterial cells.[32] This method has been successfully utilized to measure the respiratory activity of bacteria after application of different sanitizers as well as ACP.[27,41] According to the results obtained from the XTT assay, even after extended treatment time of 300 s, an average of 30% of cells in biofilms were still metabolically active. This outcome was also observed by Alkawareek et al.,[42] who reported that ACP inactivated 85% of *P. aeruginosa* cells according to colony counts, while the XTT absorbance value corresponded to 36%. Borges et al.[42] observed similar bacterial response to stress, when *P. aeruginosa* biofilm was subjected to naturally derived compounds with 30% of cell metabolic activity remaining after the treatment. In general, the plate count assay accounts only for culturable bacterial cells and does not take into account cells that might still be metabolically active. Bacteria that fail to grow on the bacteriological media but are still alive are described as a viable but nonculturable (VBNC) state of bacteria. This result has been demonstrated for many microorganisms when facing environmental stress, including *P. aeruginosa*.[43,44] Bacteria in a VBNC state can regain culturability and can retain virulence, even when they are in the nonculturable state. Therefore, VBNC bacteria may contribute to further contamination.[45]

CLSM, in conjunction with nucleic acid dyes SYTO9 and PI, was used to evaluate the effects of ACP treatment on viability and cell membrane integrity. Microscopic observations demonstrated that the bacterial cell membrane was completely ruptured following treatment, which indicates the potential destruction abilities of the high-voltage system used. Although high sterilization potential is among the main characteristics and advantages of every antimicrobial technique, physical removal of bacterial biofilms is another important criteria that is gaining increased attention, specifically in plasma

biofilm research. Confocal microscopy analysis also demonstrated that ACP treatment was able not only to successfully inactivate cells through penetration inside complex bacterial aggregates but also to substantially remove the biofilm from the associated surface, which was identified by measuring the biofilm thickness.

The results of this work also point to the complex interaction between reactive species generated by air plasma and biofilms. As is commonly known, air plasma is an excellent source of electrons and positive and negative ions, free radicals, stable conversion products (e.g., ozone), excited atoms and molecules, and ultraviolet radiation photons.[46] The main mechanism of action of ACP reactive species is the diffusion of oxygen species through the bacteria cell wall, which causes local damage to the cytoplasmic membrane, protein and DNA strands, as well as physical effects by causing microbial etching and erosion.[47,48] In this study, to monitor the physical changes caused by ACP treatment, SEM analysis of biofilms was conducted. The microscopic images enabled observation of the differences between control (untreated) samples, which proved biofilm complexity and the resulting challenge linked to antimicrobial resistance, and treated samples, where cells and biofilm components were significantly damaged by the action of ACP.

Notably, the in-package, high-voltage ACP treatment used a 24-h post treatment storage time. This allowed the retention of plasma reactive species inside the pack over time, thus facilitating bactericidal action of these species on biofilm samples. This approach is more likely to show increased antimicrobial effects of plasma, the mechanism of which is mainly based on the reaction of long-lived and recombined species rather than on charged particles or short-lived species, which are more relevant to effects noted using treatment without post treatment storage. This approach could contribute to an increased potential of plasma species to penetrate complex biofilm matrices further inactivating the cells. However, there are several limitations in this work. It is known that formation of biofilms is influenced by environmental conditions and characteristics of substrates to which bacteria attach. In this study, the model system, a 96-well microtiter plate, was utilized for biofilm development, which cannot holistically describe the broad range of materials implicated in biofilm formation within clinical environments. Moreover, it is more likely that, in real clinical environments, biofilm communities may be inhabited by numerous different species and that interactions among these species could contribute to the organization of multispecies biofilms. Multispecies biofilms are usually more resistant against antimicrobial treatments than monospecies biofilms.[49] Therefore, further studies on the efficacy of ACP against multispecies biofilms developed on a wider range of relevant materials may be warranted.

IV. CONCLUSION

The results of this study clearly demonstrate that in-package, high-voltage air DBD ACP in conjunction with post treatment storage is very effective against complex bacterial biofilms. With relatively short treatment of 60 s, it was possible to achieve substantial

reductions (5.4 $\log_{10}$ CFU/ml) of 48-h-old *P. aeruginosa* biofilms. In this study, a series of methods were utilized to monitor the changes in biofilms caused by ACP treatment, namely, colony count, XTT assay, CLSM, and SEM, elucidating information regarding different parameters associated with the viability of cells in biofilms. In terms of metabolic activity, 30% of cells survived extended treatment for 300 s, whereas, according to colony count, there were no bacterial survivors. These results indicate a possible induction of VBNC state of bacteria due to the stress caused by ACP treatment. However, CLSM and SEM analyses indicate that extended treatment caused severe damage to *P. aeruginosa* cell membranes and effectively disrupted heterogeneous biofilm structures. However, better understanding of interactions between plasma ACP reactive species and biofilm components and identification of specific biofilm targets will allow further optimization of ACP treatment parameters toward more effective and less damaging sterilization required in the field of medicine.

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REFERENCES

1. Lambert ML, Suetens C, Savey A, Palomar M, Hiesmayr M, Morales I, Agodi A, Frank U, Mertens K, Schumacher M, Wolkewitz M. Clinical outcomes of health-care-associated infections and antimicrobial resistance in patients admitted to European intensive-care units: a cohort study. Lancet Infect Dis. 2011;11:30–38.
2. Sydnor ERM, Perl TM. Hospital Epidemiology and Infection Control in Acute-Care Settings. Clin Microbiol Rev. 2011;24:141-173.
3. Venier AG, Leroyer C, Slekovec C, Talon D, Bertrand X, Parer S, Alfandari S, Guerin JM, Megarbane B, Lawrence C, Clair B, Lepape A, Perraud M, Cassier, Trivier D, Boyer, Dubois V, Asselineau J, Rogues AM, Thiébaut R. Risk factors for *Pseudomonas aeruginosa* acquisition in intensive care units: a prospective multicentre study. J Hosp Infect. 2014;88:103–8.
4. Weingrow D. Update on emerging infections: news from the Centers for Disease Control and Prevention. Infectious disease/CDC update. 2012;60:374–6.
5. Döring G. Prevention of *Pseudomonas aeruginosa* infection in cystic fibrosis patients. Int J Med Microbiol. 2010;300:573–7.
6. Fujii A, Seki M, Higashiguchi M, Tachibana I, Kumanogoh A, Tomono K. Community-acquired, hospital-acquired, and healthcare-associated pneumonia caused by *Pseudomonas aeruginosa*. Respir Med Case Rep. 2014;12:30–3.
7. Mittal R, Aggarwal S, Sharma S, Chhibber S, Harjai K. Urinary tract infections caused by *Pseudomonas aeruginosa*: a minireview. J Infect Public Health. 2009;2:101–11.
8. Tredget EEa, Shankowsky HA, Rennie R, Burrell RE, Logsetty S. *Pseudomonas* infections in the thermally injured patient. Burns. 2004;30:3–26.
9. Jombo GT, Akpan S, Epoke J, Denen Akaa P, Odey F. Multidrug resistant *Pseudomonas aeruginosa* infections complicating surgical wounds and the potential challenges in managing post-operative wound

infections: University of Calabar Teaching Hospital experience. Asian Pac J Trop Med. 2010;479–82.
10. Scheetza MH, Hoffmanc M, Bolond MK, Schulerte G, Estrelladof W, Baraboutisd IG, Sriramd P, Dinhd M, Owensg LK, Hauser AR. Morbidity associated with *Pseudomonas aeruginosa* bloodstream infections. Diagn Micr Infec Dis. 2009;64:311–9.
11. Donlan R. Biofilm Elimination on intravascular catheters: important considerations for the infectious disease practitioner. Clin Infec Dis. 2011;52:1038–45.
12. Kadambari S, Botgros A, Clarke P, Vergnano S, Anthony M, Chang J, Collinson A, Embleton N, Kennea N, Settle P, Heath PT, Menson EN. Characterizing the burden of invasive Pseudomonas infection on neonatal units in the UK between 2005 and 2011. J Hosp Infect. 2014;88:109–12.
13. Loveday HP, Wilson JA, Kerr K, Pitchers R, Walker JT, Browne J. Association between healthcare water systems and Pseudomonas aeruginosa infections: a rapid systematic review. J Hosp Infect. 2014;86:7–15.
14. Walker JT, Jhutty A, Parks S, Willis C, Copley V, Turton J, Hoffman PN, Bennett AM. Investigation of healthcare-acquired infections associated with *Pseudomonas aeruginosa* biofilms in taps in neonatal units in Northern Ireland. J Hosp Infect. 2014;86:16–23.
15. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15:167–93.
16. Mah T-FC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Review. Trends Microbiol. 2001;9:34–40.
17. Römling U, Balsalobre C. Biofilm infections, their resilience to therapy and innovative treatment strategies. J Intern Med. 2012;272:541–61.
18. Mai-Prochnow A, Murphy AB, McLean KB, Kong MG, Ostrikov K. Atmospheric pressure plasmas: Infection control and bacterial responses. Int J Antimicrob Ag. 2014;43:508–17.
19. Sharma G, Rao S, Bansal A, Dang S, Gupta S, Gabrani R. Pseudomonas aeruginosa biofilm: Potential therapeutic targets. Biologicals. 2014;42:1–7.
20. Von Woedtke Th, Reuter S, Masur K, Weltmann K-D. Plasmas for medicine. Phys Rep. 2013;530:291–320.
21. Kramer A, Lademann J, Bender C, Sckell A, Hartmann B, Münch S, Hinz P, Ekkernkamp A, Matthes R, Koban I, Partecke I, Heidecke CD, Masur K, Reuter S, Weltmann KD, Koch S, Assadian O. Suitability of tissue tolerable plasmas (TTP) for the management of chronic wounds. Clin Plasma Med. 2013;1:11–18.
22. Wu AS, Kalghatgi S, Dobrynin D, Sensenig R, Cerchar E, Podolsky E, Dulaimi E, Paff M, Wasko K, Arjunan KP, Garcia K, Fridman G, Balasubramanian M, Ownbey R, Barbee KA, Fridman A, Friedman G, Joshi SG, Brooks AD. Porcine intact and wounded skin responses to atmospheric nonthermal plasma. J Surg Res. 2013;179:1–12.
23. Emmert S, Brehmer F, Hanßle H, Helmke A, Mertens N, Ahmed R, Simon D, Wandke D, Maus-Friedrichs W, Daschlein G, Schon MP, Viol W. Atmospheric pressure plasma in dermatology: Ulcus treatment and much more. Clin Plasma Med. 2013;1:24–29.
24. Pan J, Sun K, Liang Y, Sun P, Yang X, Wang J, Zhang J, Zhu W, Fang J, Becker H. Cold Plasma Therapy of a Tooth Root Canal Infected with Enterococcus faecalis Biofilms In Vitro. J Endodont. 2013;39:105–10.
25. Kim C-H, Bahn JH, Lee S-Ha, Kim G-Y, Jun S-I, Lee K, Baek SJ. Induction of cell growth arrest by atmospheric non-thermal plasma in colorectal cancer cells. J Biotechnol. 2010;150:530–8.
26. Traba C, Chen L, Liang JF. Low power gas discharge plasma mediated inactivation and removal of biofilms formed on biomaterials. Curr Appl Phys. 2013;13:S12–18.
27. Alkawareek MY, Algwari QT, Graham B, Gorman S, O'Connell D, Gilmore B. Application of atmospheric pressure nonthermal plasma for the in vitro eradication of bacterial biofilms. FEMS Immunol Med Microbiol. 2012;65:381–4.
28. Maisch T, Shimizu T, Isbary G, Heinlin J, Karrer S, Klämpfl TG, Li YF, Morfill G, Zimmermann JL. Contact-free inactivation of *Candida albicans* biofilms by cold atmospheric air plasma. Appl Environ

Microb. 2012;78:4242–7.
29. Joshi SG, Paff M, Friedman G, Fridman G, Fridman A, Brooks, AD. Control of methicillin-resistant *Staphylococcus aureus* in planktonic form and biofilms: a biocidal efficacy study of nonthermal dielectric-barrier discharge plasma. Am J Infect Control. 2010;38:293–301.
30. Ermolaeva SA, Varfolomeev AF, Chernukha MY, Yurov DS, Vasiliev MM, Kaminskaya AA, Moisenovich MM, Romanova JM, Murashev AN, Selezneva II, Shimizu T, Sysolyatina EV, Shaginyan IA, Petrov OF, Mayevsky EI, Fortov VE, Morfill GE, Naroditsky BS, Gintsburg AL. Bactericidal effects of non-thermal argon plasma *in vitro*, in biofilms and in the animal model of infected wounds. J Med Microbiol. 2011;60:75-83.
31. Ziuzina D, Patil S, Cullen, PJ, Keener KM, Bourke P. Atmospheric cold plasma inactivation of *Escherichia coli* in liquid media inside a sealed package. J Appl Microbiol. 2013;114:778–87.
32. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Meth. 2008;72:157–65.
33. Gratao PL, Monteiro CC, Rossi ML, Martinelli AP, Peresc LEP, Medici LO, Lea PJ, Azevedo RA. Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium. Environ Exp Bot. 2009;67:387–94.
34. Srey S, Park SY, Jahid IK, Ha S-D. Reduction effect of the selected chemical and physical treatments to reduce *L. monocytogenes* biofilms formed on lettuce and cabbage. Food Res Int. 2014;62:484–91.
35. Stepanovic S, Vukovic D, Dakić I, Savić B, Švabić-Vlahović M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Meth. 2000;40:175–9.
36. Cheruthazhekatt S, Cernák M, Slavícek P, Havel J. Gas plasmas and plasma modified materials in medicine. J Appl Med. 2010;8:55–66.
37. Costerton JW, Lewandowski Z. Microbial biofilms. Annu Rev Microbiol. 1995;49:711–45.
38. Gurung J, Khyriem AB, Banik A, Lyngdoh WV, Choudhury B, Bhattacharyya P. Association of biofilm production with multidrug resistance among clinical isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from intensive care unit. Indian J Crit Care Med. 2013;17:214–18.
39. Wei Q, Ma LZ. Biofilm Matrix and Its Regulation in *Pseudomonas aeruginosa.* Int J Mol Sci. 2013;14:20983–1005.
40. Traba C, Liang JF. Susceptibility of *Staphylococcus aureus* biofilms to reactive discharge gases. Biofouling. 2011;27:763–72.
41. Shen Y, Stojicic S, Haapasalo M. Antimicrobial efficacy of chlorhexidine against bacteria in biofilms at different stages of development. J Endodont. 2011;37:657–61.
42. Alkawareek MY, Algwari QT, Laverty G, Gorman SP, Graham WG, O'Connell D and Gilmore BF. Eradication of *Pseudomonas aeruginosa* biofilms by atmospheric pressure non-thermal plasma. PLoS ONE. 2012;7: e44289.
43. Zelaya AJ, Stough G, Rad N, Vandervoort, K, Brelles-Marino G. *Pseudomonas aeruginosa* biofilm inactivation: decreased cell culturability, adhesiveness to surfaces, and biofilm thickness upon high-pressure nonthermal plasma treatment. IEEE T Plasma Sci IEEE Nucl Plasma Sci Soc. 2010;38:3398–403.
44. Sun Y, Yu S, Sun P, Wu H, Zhu W, Liu W, Fang J, Li R. Inactivation of *Candida* biofilms by non-thermal plasma and its enhancement for fungistatic effect of antifungal drugs. PLoS ONE. 2012;7:e40629.
45. Borges A, Simões LC, Saavedra MJ, Simõe M. The action of selected isothiocyanates on bacterial biofilm prevention and control. Int Biodeter Biodegr. 2013;86:25–33.
46. Moritz MM, Flemming HC, Wingender J. Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials. Int J of Hyg Envir Heal. 2010;213:190–7.
47. Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev. 2010;34:415–25.
48. Oliver JD. The viable but nonculturable state in bacteria. J Microbiol. 2005;43:93–100.
49. Stoffels E, Sakiyama Y, Graves DB. Cold Atmospheric plasma: charged species and their interactions with cells and tissues. IEEE T Plasma Sci. 2008;36:1441–57.

50. Gallagher MJJr, Vaze N, Gangoli S, Vasilets VN, Gutsol AF, Milovanova TN, Anandan S, Murasko DM, Fridman AA. Rapid inactivation of airborne bacteria using atmospheric pressure dielectric barrier grating discharge. IEEE T Plasma Sci. 2007;35:1501–10.
51. Moreau M, Orange N, Feuilloley MGJ. Non-thermal plasma technologies: New tools for bio-decontamination. Biotechnol Adv. 2008;26:610–17.
52. Yang L, Liu Y, Wu H, Høiby N, Molin S, Song Z. Current understanding of multi-species biofilms. Review. Int J Oral Sci. 2011;3:74–81.