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A COLD ATMOSPHERIC PRESSURE PLASMA DISCHARGE DEVICE EXERTS ANTIMICROBIAL EFFECTS

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Abstract- A cold atmospheric pressure plasma device was developed using two parallel plates of Low Temperature Co-fired Ceramic with embedded electrodes. The 2.4 cm wide by 1 mm deep plasma discharge operates at 20 kHz with a 2-5 kV AC drive signal across a 0.25 mm gap. Mixed Argon/oxygen plasmas were directed between the plates to flow toward a bacterial biofilm sample for treatment. Results showed that at 4-5 kV the plasma etched away a bacterial biofilm on glass in 10 minutes. In addition, we showed that short plasma treatments rapidly killed biofilm resident bacteria with ED90 values of <15s.
Keywords – Cold atmospheric pressure plasma, biofilms, antimicrobial, wound debridement, food safety

1. INTRODUCTION

Microbial biofilms are difficult to remove from wounds and represent a major impediment to healing through continued stimulation of host inflammatory responses that lead to a chronic wound state [1,2]. While the exact cost of chronic wounds is unknown, analysis of Public Health Service and Medicare data suggest that the prevalence rate is 1-2% in the general population, with annual treatment costs that exceed $20 billion in the U.S., alone [3,4]. Effective wound debridement enhances healing by reducing the resident bioburden that sponsors the non-resolving inflammation at the site [1,2,5]. Biofilms are removed from chronic wounds by surgical "sharp" debridement, hydro-surgery, sonication, and/or extensive topical and systemic treatment with antiseptics and antibiotics in the wound care clinic [5]. However, sharp debridement and sonication can lead to incomplete biofilm removal or may disperse the associated microbes to other parts of the wound leading to continued contamination.

Outside the healthcare industry, microbial biofilms are also a serious concern. For instance, in the food processing industry, biofilms that grow on industrial surfaces that contact food can serve as reservoirs for food-borne illness caused by organisms such as Salmonella enterica and Listeria monocytogenes, or contaminate foodstuffs with organisms that cause spoilage [6]. Subsequent human illness, product recalls, and loss of shelf-life are detractors from the food processing industry since they reduce profitability and consumer confidence in specific products. In recent years, cold atmospheric-pressure plasma (CAP) has been proposed as a method to treat chronic wound biofilms and sanitize/sterilize surfaces in food processing [7,8]. Plasma delivered reactive species have been shown to kill a variety of bacteria and inactivate biofilms [7-15]. However, further development of these CAP sources is needed to improve their effectiveness in wound debridement and surface sterilization, and to better understand biofilm removal mechanisms. More work is also required to produce viable devices with increased plasma coverage area that can deployed in clinical or industrial settings. Here, we report the initial development of a linear format CAP device capable of a low frequency (20 kHz) 2-5 kV capacitive discharge using embedded electrodes in a parallel plate configuration. The design and fabrication of the CAP discharge device, and the experimental setup to demonstrate its utility in removing medically/industrially relevant biofilms are also described. The design is scalable, so it is amenable to the creation of linear arrays and other configurations that may be useful in future commercial devices.

2. EXPERIMENTAL PROCEDURES

2.1 Plasma Device

A schematic of the CAP device configuration is shown in Fig. 1 with an accompanying photograph of the experimental system. The system (Fig. 1 [top]) consists of the parallel plate discharge structure, two mass flow controllers (MFCs), high voltage alternating current (HV AC) power supply, adjustable sample stage, oscilloscope, current transformer, and a HV probe. The flow rate of Argon (5 lpm) and Oxygen (0.5 lpm) gases were controlled with two MFCs (Kelly Pneumatics, Costa Mesa, CA). A PVM/DDR AC power supply (Information Unlimited, Amherst, NH) operating at 0-10 kV and 20-50 kHz was used in our studies. A high voltage probe (B&K Precision, Yorba Linda, CA) was used to measure AC voltage from 0-10 kV and 0-50 MHz, and model US3F-1 current transformer (Ravi Electricals, Maharashtra, India) placed on the ground side of the discharge to measure the discharge current. All experiments fixed the operational frequency at 20 kHz, used a computer

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digital-to-analog output to control MFC settings, and an oscilloscope to display the HV AC probe and current transformer signals. The CAP source was held ≈1 mm above the biofilm substrate placed on a rotating platform to distribute the plasma exposure over the entire sample.

CAP sources were constructed as diagrammed in Fig. 1 [middle left]. Briefly, parallel plates fabricated from multiple layers of 250 µm thick (before firing), DuPont 951 Low Temperature Co-fired Ceramic (LTCC) [16] were printed with silver paste traces. Laser machined holes in each layer are filled with silver paste to create vertical conductive connections between the traces on the individual LTCC layers which form the parallel plates. These traces are connected to two discharge electrodes positioned on each parallel plate, which are covered with a 50 µm thick layer of LTCC (35 µm after firing). The LTCC covering avoids direct exposure of the silver paste traces to the plasma and prevents the appearance of silver particles in the plasma discharge. Each LTCC parallel plate is then pressed at 70°C for 20 min and fired in a furnace using a standard heating profile with a peak at 850°C for 10 min. After firing, the resulting rigid ceramic structure contains embedded metal electrodes (24 mm x 1 mm) with traces that extend outside the discharge zone. To create the central gas flow channel and define the discharge gap, LTCC spacers (250 µm thick) are placed between the two opposing plates (Fig. 1 [middle left]). Traces from the opposing plates are attached to wires (red and black in Fig. 1 [middle right]) connected to the high voltage power supply, and a clear plastic gas delivery line is attached to an inlet on the front of the device.

The final structure consists of two dielectrically covered electrodes separated by a discharge gap. The gas flows through a hole in the front plate and then is directed downward between the two plates to the discharge zone and outward to the sample. The discharge gap and the width of the discharge structure can be varied. For these experiments the device discharge width was set at 24 mm, and the discharge gap was 250 µm. Plasma discharge occurred at voltage settings between 2.5 – 5.0 kV when the frequency was held at 20 kHz. Gas flow was 5 lpm Ar and 0.5 lpm O2 with an inlet pressure of ~0.1 psig. The resulting device is a "line discharge" as opposed to the more common "pencil discharge" used in many CAP systems. The engineering benefit of the line discharge structure is that arrays of these devices can be "stacked" so that electrodes are on both sides of a substrate resulting in closely packed arrays of linear discharges spaced closely together (< 2 mm). These stacked arrays are then able to cover an area of biofilm without the need to move or dither the source or substrate.

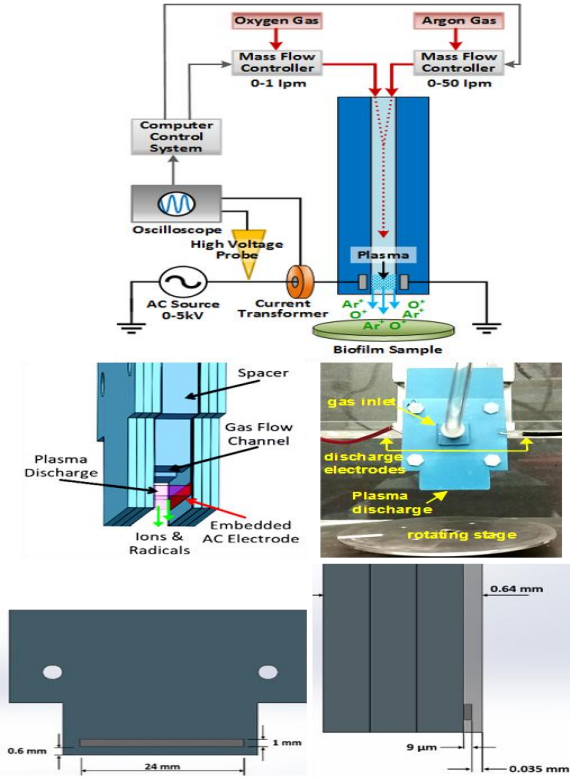

Figure 1. Schematics and photograph of the CAP system. The schematic [top] shows a parallel plate source fed by 2 mass flow controllers for Ar and O2 gas feeds. An AC high voltage source drives the plasma, and a current transformer and HV probe measure the discharge current and voltage, which are displayed on an oscilloscope. Closeup schematic [middle left] of the plasma source. Silver AC electrodes are embedded below a 35 µm layer of LTCC. Spacers create a 250 µm gap between the plates and frame the sides of the gas flow channel. The plasma discharge is generated near the bottom edge. The photograph [middle right] shows the CAP source (blue) fed by a clear Ar/O2 gas mix tube. The discharge is directed toward the rotating sample platform to distribute plasma exposure. Cross-sectional views of a single side of the source are shown [bottom] with the embedded electrode shown. The electrode is 9 µm thick, 24 mm wide, and 1 mm tall. One source substrate is 0.64 mm thick.

2.2. Biofilm Sample Preparation

Cultures of E. coli O157:H7 (ATCC 43894) and Staphylococcus aureus (ATCC BAA-44) were prepared by inoculating 10 mL Luria-Bertani broth (LB) with isolated bacterial colonies grown on LB agar plates. Bacterial cultures were grown aerobically overnight at 37 °C with shaking at 225 rpm. To prepare biofilms on glass substrates, autoclaved 2.5 cm x 2.5 cm borosilicate glass microscope coverslips (ThermoFisher Scientific) were aseptically inserted vertically into sterile 12-well Corning tissue culture plates [17]. Wells were partially filled with 1 mL LB broth inoculated with 10 µL overnight culture. Plates were covered and incubated for 48 hr at 37 °C with shaking at 100 rpm. Following incubation, the coverslips were rinsed briefly three times by immersion in sterile phosphate buffered saline (PBS) to remove loosely bound planktonic cells. Coverslips were then stored on filter papers moistened with PBS in sterile 47 mm petri dishes until the plasma treatment commenced.

2.3. Biofilm Etch Removal

To measure the ability of plasma treatment to etch (or sputter) a channel into biofilms, glass coverslips containing 48 hr E. coli biofilms were placed 1 mm below the fixed position plasma discharge, with the discharge oriented 90° to the edge of the biofilm. The sample was exposed to the discharge at 4.5-5 kV for up to 600 s without rotation of the stage to create an etched channel across the biofilm. Subsequently, the sample was air dried, imaged, and the depth and width of the etched channel in the biofilm measured using a Bruker Dektax XT-A stylus profilometer.

2.4. Plasma Antimicrobial Effects

The ability of plasma treatment to kill bacteria residing in biofilms was measured using samples of 48-hr E. coli and Staphylococcus aureus biofilms grown on glass coverslips (above). Briefly, the rinsed biofilm samples were placed on the rotating stage 1 mm below the discharge. A slow rotation (7 rpm) of the sample stage was set to distribute the exposure to the plasma discharge across the entire sample. Following treatment for 0-60 sec, the coverslip was transferred to a sterile 50 mL conical polypropylene tube containing 7-10 glass beads (6 mm diam) and 10 mL sterile PBS, and vortexed 3 min to disrupt the biofilm. The procedure was previously confirmed to remove >85% of the attached biofilm (data not shown) using crystal violet staining [18]. The samples containing bacterial cells from disrupted biofilms (106-107 cells/sample) were serially diluted in sterile PBS and plated onto LB agar plates to determine the cell concentration. The inoculated plates were incubated overnight at 37°C and the bacterial colonies enumerated. The total number of viable cells or "colony forming units" (CFU) per biofilm sample were calculated from the average of at least three replicate biofilms, using CFU counts from plates containing 30-300 colonies and the dilution factor of the sample.

The percent of total viable cells were determined using the equation:

% total viable cells = [Ave CFUtreated/Ave CFUuntreated] x 100

Control experiments consisted of biofilm samples treated with Ar/O2 gas flow alone without plasma discharge. All experiments were replicated 3 to 5 times and graphed as the average of the % total viable cells ± the standard error on the mean (SEM). The effective dose of plasma required to reduce the biofilm CFU by 90% (ED90) was determined from graphs constructed using GraphPad Prism 6.0 software.

3. EXPERIMENT AND RESULT

Photographic images of the plasma discharge can be seen in Fig. 2 [top]. The plasma appears as a purple glow discharge in the gap between the parallel plates. Diagnostic oscilloscope traces (Fig. 2 [bottom]) operating at 20 kHz during the plasma discharge showed an average (RMS) discharge current of 0.5-1 mA with an AC voltage of 2 kV. This discharge current is typical for the device. Plasma discharges typically started at voltage settings between 1.5 – 2.5 kV when the frequency was held at 20 kHz. The power dissipated in the discharge is ~1-2 W with a power density ~2.5-5 W/cm2. Overall, the LTCC material appears to be very robust to plasma, with no noticeable degradation after hundreds of hours of discharge operation. The temperature was measured on the surface of the dielectric covering one of the AC electrodes and on a substrate sample with continuous discharge operation at regular intervals up to 60 minutes. The temperature saturated at 28±1 °C after 15 minutes on the surface of the electrode dielectric and at 26±1 °C on the substrate.

The CAP device was capable of etching channels in E. coli biofilms that were held in a static position relative to the HV plasma discharge. A magnified photomicrograph (Fig. 3 [top]) shows the channel was etched down to the surface of the glass substrate. Stylus profilometry across the sample channel supports this observation, with the bottom of the channel appearing mostly smooth and approximately double the width (~500 µm) of the discharge gap in the device (Fig. 3 [bottom]). Control experiments were operated with gas flow only, and these experiments showed no formation of a channel or any in indication of the gas flow in the biofilm. This supports that either the device propels particles with sufficient energy to ablate or sputter the biomolecules from the glass substrate or that reactive etching removes the biofilm. Such a process may be a useful approach for biofilm removal from industrial surfaces such as in food processing.

Rotating the sample and reducing the discharge voltage to 2.5-3.5 kV caused a rapid decrease in resident viable cells in both gram-positive Staphylococcus aureus and gram-negative E. coli biofilms (Fig. 4). For both bacterial strains, a 90-99% reduction in biofilm associated viable cells occurred within 15 seconds of plasma exposure. The estimated ED90 values were

5 seconds for E. coli and 14 seconds for S. aureus biofilms. For control samples that were only treated with Ar/O2 gas mixture, even extended exposure (up to 60 s) did not produce an equivalent loss in cell viability.

The reduction in CFU in plasma treated or control samples did not appear to be the result of biofilm ablation, since crystal violet staining of treated and untreated samples did not reveal a significant difference in the amount of biofilm present in any of the samples (data not shown). The gradual loss of cell viability observed in the control non-plasma gas stream treated E. coli biofilms probably reflects both their sensitivity to drying and experiment to experiment variation in biofilm cell numbers. No loss of cell viability was seen with the control non-plasma gas stream treated S. aureus biofilms. In both treatment studies, the temperature of the substrate did not rise significantly during the 60 s time course of the experiment. No ED90 value could be determined for control treated biofilms of either species. As well, in the plasma treated samples, the ED90 values could only be estimated since shorter plasma exposure time points (< 10 s) were technically difficult to obtain, and a 10 s exposure was required to ensure that the entire sample received treatment by allowing at least one full rotation of the sample under the plasma discharge. In this latter regard, it should be noted that only the center of the biofilm received constant plasma treatment, while areas farther away from the axis of sample rotation were exposed only a portion of the treatment time. This suggests that the plasma ED90 estimates are potentially high, and even shorter exposures could cause 90% (or better) cell killing provided the entire sample could be treated simultaneously. This will be examined in future design iterations of the CAP device incorporating stacked arrays of plasma discharges that will create larger treatment areas thus allowing simultaneous exposure of the entire sample.

Work by Fricke, et al. reported the necessity for the addition of oxygen gas to the argon plasma discharge in order to etch 10-20 µm thick Candida albicans biofilms [10]. In their work, a 2-5 minute treatment with an Ar (5 lpm)/O2 (0.05 lpm) plasma discharge removed 80-95% of the established biofilm. Based on spectroscopic evidence, it was proposed that high energy reactive oxygen species were responsible for the biofilm removal. Later work by this research group supported this assertion by demonstrating that the production of redox protective phenazine increased in Pseudomonas biofilms following treatment with the same plasma device [14]. However, it should be noted that in both cases the device produced a plasma jet with a pinpoint discharge that extended 12 mm beyond the tip of the device and was in direct contact with the biofilm. This is quite different from our device configuration, which produces a line plasma that does not extend beyond the discharge gap.

In another study, Mathes et al.[12] showed that treatment of Pseudomonas and Staphylococcus biofilms with an array of air plasma discharges (rather than Ar/O2) could cause 1-7 log decreases in biofilm cell viability over the course of a 10 minute treatment. A minimum treatment of 60 sec (at 2 mm distance) was required to see similar reductions in viable cell number that are comparable to our 10-30 second exposure results. In addition, electron microscopy studies indicated that the array device did not etch the biofilm, but instead caused a dense layer of dead cell and biofilm detritus to accumulate on the surface that may prevent further plasma effects on the biofilm interior.

However, in general it is difficult to make direct comparisons of the results of our studies to those reported by others investigating the antimicrobial activities of cold atmospheric pressure plasmas [7,10,12-15] since the device operating parameters (e.g. voltage, wave frequency, discharge gas mix, etc.), characteristics (e.g. plasma jet, array, discharge configuration), experimental conditions (biofilm type, plasma distance, treatment time, etc.), and readout (CFU reduction, metabolism, microscopy, etc.) are not uniform among the research groups investigating this technology. Certainly, all of the devices developed to date appear to exert beneficial antimicrobial effects. It is likely that the plasma technology will assume a variety of device configurations and parameters that depend ultimately on the desired application.

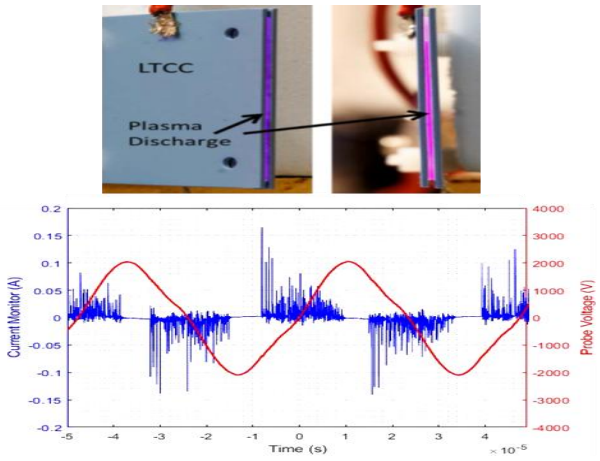

Figure 2. Plasma discharge device. [Top] Two photographic views of the CAP glow discharge in the gap between the parallel plates. [Bottom] Diagnostic oscilloscope traces at 20 kHz during the plasma discharge. Traces for the HV AC probe and current transformer are indicated in red and blue, respectively.

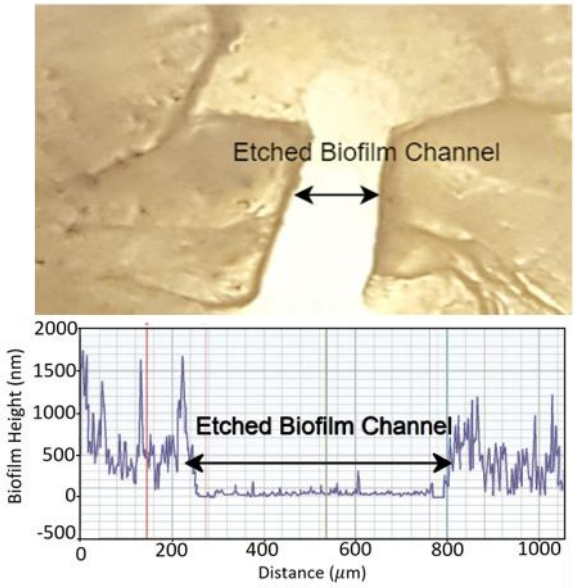

Figure 3. Biofilm etching by CAP treatment (4.5-5 kV/600 sec). [Top] Photomicrograph of etched channel in biofilm surface. [Bottom] Profilometry trace showing the biofilm was etched down to the surface of the glass substrate.

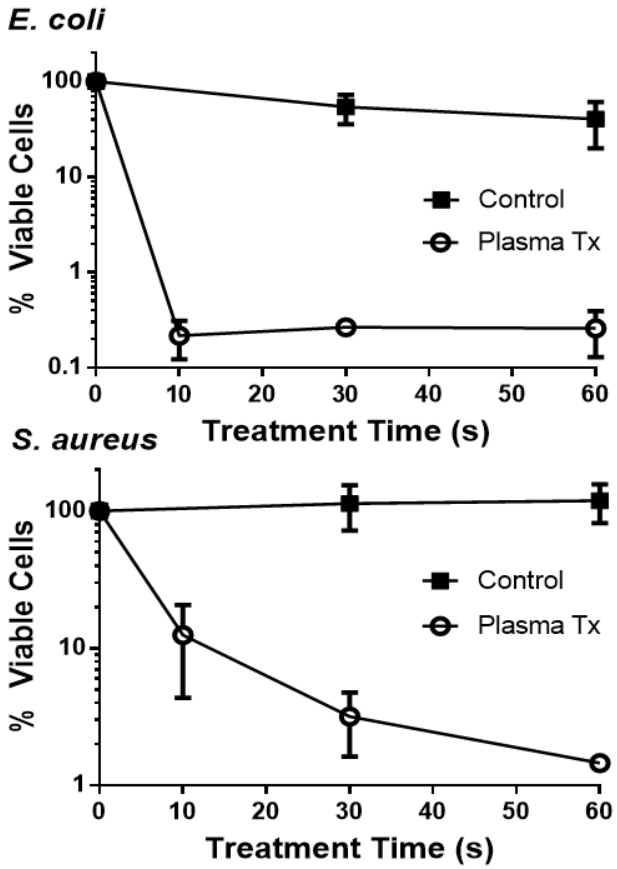

Figure 4. Plasma treatment rapidly kills bacterial cells in biofilms. E. coli and Staphylococcus aureus biofilms were treated with the CAP for up to 1 min (Plasma Tx) and the viable cells determined from CFU outgrowth on LB agar plates. Controls were exposed only to the Ar/O2 gas mixture in the absence of plasma discharge. The results shown are the average of 3-5 independent experiments (± SEM).

4. CONCLUSION

Our preliminary work has shown that our parallel plate CAP source operating at 20 kHz and 3.6 kV across a 250 µm gap can kill bacteria embedded in biofilms with ED90 values of less than 15 s. At higher voltages (4.6 kV), the CAP device can

remove the biofilm from a surface through molecular sputtering or etching. In the future, we will explore the ability of the CAP device to eliminate pathogenic microbes responsible for chronic wound biofilms and foodborne illness from a variety of substrates (e.g. steel, plastic, rubber, etc). The CAP design will also be modified to create stacked arrays of plasma discharges capable of simultaneous treatment of much larger surface areas (e.g. 9 cm2) with the goal of developing a device that could be useful in eliminating biofilms from chronic wounds or be used to treat surfaces to reduce the spread of foodborne pathogens.

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6. REFERENCES

[1] A. Omar, J.B. Wright, G. Schultz, R. Burrell, P. Nadworny, “Microbial biofilms and chronic wounds.” Microorganisms. Vol 5(1): p 9 (2017). doi: 10.3390/microorganisms5010009 PMCID: PMC5374386.

[2] S.L. Percival, S.M. McCarty, B. Lipsky, “Biofilms and wounds: an overview of the evidence.” Adv Wound Care (New Rochelle). Vol 4(7): p 373-381 (2014). doi: 10.1089/wound.2014.0557 PMCID: PMC4486148.

[3] S.R. Nussbaum, M.J. Carter, C.E. Fife, J. DeVanzo, R. Haught, M. Nusgart, D. Cartwright, “An economic evaluation of the impact, cost, and medicare policy implications of chronic nonhealing wounds.” Value Health. Vol 21(1): p 27-32 (2018). doi:10.1016/j.val.2017.07.007 PMID: 29304937.

[4] J.F. Guest, N. Ayoub, T. McIlwraith, I. Uchegbu, A. Gerrish, D. Weidlich, K. Vowden, P. Vowden, “Health economic burden that wounds impose on the National Health Service in the UK.” BMJ Open. Vol 5: p e009283 (2015). doi:10.1136/bmjopen-2015-009283 PMCID: PMC4679939.

[5] I.C. Hoppe, M.S. Granick, “Debridement of chronic wounds: a qualitative systematic review of randomized controlled trials.” Clin Plast Surg. Vol 39: p 221-228 (2012). doi:10.1016/j.cps.2012.04.001 PMID: 22732371.

[6] A. Alvarez-Ordóñez, L.M. Coughlan, R. Briandet, P.D. Cotter, “Biofilms in food processing environments: challenges and opportunities.” Ann Rev Food Sci Technol. Vol 10: p 173-195 (2019). doi:10.1146/annurev-food-032818-121805 PMID: 30653351.

[7] N. Mohd Nasir, B.K. Lee, S.S. Yap, K.L. Thong, S.L. Yap, “Cold plasma inactivation of chronic wound bacteria,” Arch Biochem Biophys. Vol. 605: p 76-85 (2016). doi:10.1016/j.abb.2016.03.033 PMID: 27046340.

[8] P. Bourke, D. Ziuzina, D. Boehm, P.J. Cullen, K. Keener, “The potential of cold plasma for safe and sustainable food production.” Trends Biotechnol. Vol 36(6): p 615-626 (2018). doi:10.1016/j.tibtech.2017.11.001 PMID: 29329724.

[9] M. Laroussi, M.G. Kong, G. Morfil, W. Stolz, “Plasma Medicine: applications of low-temperature gas plasmas in medicine and biology.” (1st Ed.) Cambridge University Press (2012).

[10] K. Fricke, I. Koban, H. Tresp, L. Jablonowski, K. Schroder, A. Kramer, K.D. Weltmann, T. von Woedtke, T. Kocher, “Atmospheric pressure plasma: a high performance tool for the efficient removal of biofilms.” PLOS ONE. Vol 7(8): p e42539 (2012). doi:10.1371/journal.pone.0042539 PMCID: PMC3412829.

[11] G. Isbary, T. Shimizu, Y.F. Li, W. Stolz, H.M. Thomas, G.E. Morfill, J.L. Zimmerman, “Cold atmospheric plasma devices for medical issues.” Expert Rev Med Devices. Vol 10(3): p 367-377 (2013). doi:10.1586/erd.13.4 PMID: 23668708.

[12] R. Matthes, C. Bender, R. Schlüter, I. Koban, R. Bussiahn, S. Reuter, J. Lademann, K.D. Weltmann, A. Kramer (2013) “Antimicrobial efficacy of two surface barrier discharges with air plasma against in vitro biofilms.” PLoS One. Vol 8(7): p e70462 (2013). doi:10.1371/journal.pone.0070462 PMCID: PMC3722131.

[13] J.-W. Lackmann, S. Schneider, E. Edengeiser, F. Jarzina, S. Brinckmann, E. Steinborn, M. Havenith, J. Benedikt, J.E. Bandow, “Photons and particles emitted from cold atmospheric-pressure plasma inactivate bacteria and biomolecules independently and synergistically,” J R Soc Interface. Vol 10(89): p 1-12 (2013). doi: 10.1098/rsif.2013.0591 PMCID: PMC3808546.

[14] A. Mai-Prochnow, M. Bradbury, K. Ostrikov, A.B. Murphy, “Pseudomonas aeruginosa biofilm response and resistance to cold atmospheric pressure plasma is linked to the redox-active molecule phenazine,” PLoS One, Vol 10(6): p e0130373 (2015). doi: 10.1037/journal.pone.0130373 PMCID: PMC4483161.

[15] D. Ziuzina, D. Boehm, S. Patil, P.J. Cullen, P. Bourke, “Cold plasma inactivation of bacterial biofilms and reduction of quorum sensing regulated virulence factors,” PLoS One, Vol 10(9): p e0138209 (2015). doi: 10.1371/journal.pone.0138209 PMCID: PMC4577073.

[16] DuPont, "Green Tape 951 Low Temperature Ceramic System," (2011). Available: https://www.etsmtl.ca/Unites-de-recherche/LTCC/Services-offerts/Dupont_951.pdf. [Accessed 2 September 2018].

[17] J.H. Merritt, D.E. Kadouri, G.A. O’Toole, “Growing and analyzing static biofilms,” Curr Protoc Microbiol, Chapter 01: Unit-1B.1 (2005). doi:10.1002/9780471729259.mc01b01s00 PMCID: PMC4568995.

[18] G.A. O’Toole, “Microtiter dish biofilm formation assay,” JoVE, 47 (2011). doi: 10.3791/2437 PMCID: PMC3182663.