Hydrogen-Peroxide-Enhanced Nonthermal Plasma Effluent for Biomedical Applications

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Abstract—A novel nonthermal plasma dielectric-barrier discharge (DBD) system for decontamination, sterilization, and medical applications has been developed. The discharge is physically removed from the disinfection zone, and plasma-induced free radicals are delivered through an air stream. The physical distance between the discharge and the treatment surface can be up to 3 m, making the technology robust and flexible for applications in the medical clinic. The bactericidal properties of the free-radical effluent are enhanced by hydrogen peroxide additives. We report a 6-log reduction in Staphylococcus aureus and Pseudomonas aeruginosa bacteria strains in under 1 min of exposure in vitro and inactivation of Bacillus atrophaes spores and Escherichia coli biofilms. The concentration of hydrogen peroxide additives is seen to be a key variable in inactivation efficacy, suggesting that active species in our experiment may be different than in other DBD configurations. Precise chemical concentration measurements using direct frequency comb spectroscopy show presence of ozone (O₃), hydrogen peroxide (H₂O₂), nitrous oxide (N₂O), and nitrogen dioxide (NO₂). In vivo multiple exposures of mouse skin to the plasma effluent do not yield any adverse effects.

Index Terms—Bacteria, biomedical, decontamination, frequency comb spectroscopy, plasma medicine, reactive oxygen species (ROS).

I. INTRODUCTION

THE USE of nonthermal gas discharge plasmas in disinfection, sterilization, and various biomedical applications has received considerable attention in the last decade. Review articles written by Laroussi [1] and Moreau et al. [2] describe the major technical approaches that have been pursued to effectively inactivate a wide range of microorganisms. Specifically, there is an established distinction between so-called “direct” and “indirect” (or remote) methods of using plasma discharges in biological applications [3]. In direct methods, the target to be decontaminated or treated is physically present in the discharge region. Such direct treatment is most commonly accomplished using a dielectric-barrier discharge (DBD), where the target is a floating electrode. In indirect methods, the treatment target is physically removed from the discharge, and chemical species created in the discharge are transported to the sample by forced flow or diffusion. The advantage of direct methods is that all active species generated in a plasma discharge, including those with very short lifetimes such as free electrons, ions, hydroxyl radicals, and also UV photons, can participate in the decontamination and maximize its effectiveness. However, direct methods are inherently sensitive to the geometry of the treatment sample and, therefore, are not as robust and flexible as indirect methods in the context of applications to tissue or instrument surfaces in the medical clinic.

The need for robust and flexible engineering solutions is all the more relevant as, recently, there have been significant promising advancements in animal studies [4], [5] and also clinical studies of nonthermal plasma treatments on human patients [6], [7]. Kuo et al. [4] show evidence of air plasma treatments reducing wound bleeding, whereas Ermoloeva et al. [5] employ an argon plasma device to achieve a statistically significant increase in the rate of wound closure. Perhaps most significantly, two studies by Isbary et al. [6], [7] demonstrate that plasma treatments are effective in reducing bacterial load on chronic wounds and in treating Hailey–Hailey disease in human patients. The aforementioned clinical studies involve direct treatment hardware with capital costs in the range of tens to hundreds of thousands of dollars.

A recent comprehensive study by Joshi et al. [8] confirms previous results that hydrogen peroxide (H₂O₂) is readily generated in air plasmas and is an active species contributing to bacteria inactivation through lipid peroxidization. The bactericidal properties of H₂O₂ in plasma treatments have led to efforts to increase its concentration by direct addition. Yamamoto et al. [9] investigated injection of H₂O₂ droplets in a corona discharge and concluded that the H₂O₂ additive was one of the dominant reactive species in their sterilization experiment. It is also worth noting that sterilization technologies based on vaporized H₂O₂ have been developed by companies such as STERIS Corporation [10].

The device described herein is an indirect exposure approach with hydrogen peroxide additives that generates a plasma-induced free-radical air stream. We report sterilization results
comparable to direct methods achieved with low-cost hardware and flexibility in application. In an in vivo experiment, we demonstrate that exposure to our effluent on the time scales necessary for high-level disinfection does not create adverse effects to murine skin. We use direct frequency comb absorption spectroscopy to obtain precise real-time measurements of the concentrations of ozone (O₃), hydrogen peroxide (H₂O₂), nitrous oxide (N₂O), and nitrogen dioxide (NO₂) in our effluent.

II. DEVICE DESCRIPTION

The plasma device described herein is an upgraded version of an earlier setup described by Watts et al. [11] with the key exception that microwave discharge has been replaced with DBD, and a closed-loop flow has been implemented. Fig. 1 shows the flow diagram and main components of the system. The closed-loop flow system allows for maximum buildup of concentration of free radicals and reactive oxygen species (ROS). Two identical 100-W fans provide circulation (main fan) and exhaust (exhaust fan). The role of the exhaust fan is to keep the closed-loop system in a state of under pressure preventing any escape of free radicals or pathogens except through the exhaust exit, which contains an activated carbon ROS deactivation bed and a HEPA filter. It is, thus, possible to operate the device without the need for a chemical hood or a biosafety cabinet despite the relatively copious concentrations of ROS discussed below. Flow from the main fan is split one third to the DBD and two thirds bypassing the DBD and directly connecting to the hydrogen peroxide bubbler. The DBD component consists of two independent units, each with two concentric cylindrical electrodes. The length of the cylindrical electrodes is 15 cm. The outer diameter of the outer electrode is 2 cm with a 1-mm electrode gap. The two DBD units are in parallel in the context of the airflow. Each DBD unit is driven by a 9-kV-amplitude 24-kHz-voltage waveform. After the DBD, the flow streams connect and proceed to the hydrogen peroxide bubbler. The bubbler contains 50% H₂O₂ solution by mass. The wicking material prevents any droplets from being carried by the effluent stream into the sterilization chamber. The sterilization chamber can be up to 3 m from the bubbler exit. The flow rate through the system is 118 L/min, and the temperature is ∼25 °C. The inner diameter of the tubing connecting the main components is 2.06 cm made from Teflon and vinyl. Although operation of the device with DBD enabled but without hydrogen peroxide solution or with hydrogen peroxide solution but with DBD disabled yields a bactericidal effluent, superior bacteria inactivation rates are achieved with both components active.

Fig. 1. Block diagram of device components and effluent flow.

III. BIOLOGICAL TESTS

A. In Vitro Tests

The antimicrobial properties of the effluent were tested on Staphylococcus aureus and Pseudomonas aeruginosa bacteria strains on agar plates. The agar plates were inoculated with six distinct concentrations [10, 10², 10³, 10⁴, 10⁵, and 10⁶ colony forming units (CFUs)] of both types of bacteria, as shown in the left control panel in Fig. 2. The plates were exposed to the plasma effluent for various times. Data are presented as % bacterial cell recovery +/− standard deviation. ***(p < 0.001 compared with SA or PsA controls by two-way ANOVA of transformed data with Bonferroni’s post-test). “High H₂O₂” and “Low H₂O₂” designations refer to the heating of the hydrogen peroxide solution, as described in the text.
below. Although the results with the heated hydrogen peroxide solution are shown for completeness, and it is noteworthy that they yield higher inactivation rates than without heating, condensation of hydrogen peroxide solution was observed under this setting. Since such condensation is undesirable for most medical applications, particularly those involving live tissue, we believe that the results for the room-temperature setting ("Low H$_2$O$_2$") are more clinically relevant.

In a second more quantitative experiment, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were inoculated on plastic identification badges of the type often worn by medical personnel. We note that a similar study of disinfection of personal pagers was recently performed by Burts et al. [12]. The badges were chosen as an inoculation medium since such objects are known to be a major vector for pathogen transmission and are currently not subject to any satisfactory disinfection/sterilization procedures. Each badge was inoculated with four spots of $10^6$ CFU of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The badges were exposed for durations of 15, 30, and 60 s. Dampered cotton swabs were used to remove the bacteria and placed in a 2-mL lysogeny broth (LB) medium.

The cultures were serially diluted in phosphate-buffered saline, spotted on LB agar plates, and the viable bacteria enumerated after overnight incubation at 37 °C. Fig. 3 shows data presented as percent of bacterial cell recovery in reference to the unexposed control group. Focusing on the “Low H$_2$O$_2$” results in the left panel in Fig. 3, it is shown that a 15-s exposure results in a 2-log reduction in culturable bacteria (1% recovery), and a 60-s exposure results in a 4-log reduction (0.01% recovery) for *Staphylococcus aureus* and a 6-log reduction (0.0001% recovery) for *Pseudomonas aeruginosa*. It is useful to compare these results with those obtained by Burts et al. [12] who also used an indirect plasma exposure method to sterilize pagers but report much lower inactivation rates. Specifically, in [6, Fig. 1], it is shown that for the same initial bacterial concentrations ($10^6$ CFU), an 80%–95% survival results after a 60-s exposure with their device, and a 20%–35% survival results after a 10-min exposure. Our device is thus able to achieve inactivation rates orders of magnitude higher than other indirect devices and on par with direct methods such as those used by Fridman et al. [3]. Specifically, exposure to our effluent for times of less than a minute yields high-level disinfection as defined by the FDA to be a 6-log reduction in viable pathogens. Such a reduction is believed to eliminate enough pathogens to prevent infection [13].

In a third *in vitro* test, the plasma-induced free-radical effluent produced by our device was applied to bacteria in a spore form as pathogens in this dormant survival state are hard to eradicate. The hardness of the spores also provides an opportunity to quantitatively assess the effects of the different components of our device. Fig. 4 shows the results of *Bacillus atrophaes* spores ($10^6$ CFU) after exposure to our effluent for 5 min under five different operational modes. The control group was not exposed. For the “plasma only” mode, we operated the device without any solution in the evaporator. For the “plasma + water” mode, water was placed in the bubbler in place of the hydrogen peroxide solution. For the “H$_2$O$_2$ only” mode, hydrogen peroxide solution was placed in the evaporator, but the DBD was not activated. The “plasma + H$_2$O$_2$” mode is our normal operational mode and included both DBD and hydrogen peroxide solution as in the previously described bacteria tests. The sample size for each mode was six spore discs, and the evaporator was always at room temperature. The best inactivation rate (close to 90%) occurs for the “plasma + H$_2$O$_2$” mode, whereas the other noncontrol modes yield inactivation rates in the range of 30%–40%. The fact that the most effective combination for pathogen deactivation involves both DBD and hydrogen peroxide solution suggests that there is significant coupling between the chemical species generated in the DBD and H$_2$O$_2$.

In a final *in vitro* test, we investigated the efficacy of our device in biofilm inactivation. Biofilms are difficult to eradicate because of an extracellular matrix that protects the individual bacterial cells. Since biofilms are responsible for a significant portion of nosocomial infections in the clinical setting, there is a need for new technologies capable of effective biofilm inactivation. Biofilm samples were prepared from *Escherichia coli* in multiwell culture plates containing six wells with a volume of 16.8 mL and a surface area of 9.5 cm$^2$ per well. For these tests, we did not use the closed-loop flow of the device and set up the culture plate so that a well in the corner was directly exposed to the effluent flow. The two wells on the plate opposite from the exposed well were covered with a glass slide. The exposure time was 5 min, and the experiment was repeated three times. The geometry of the plate and exposure scheme is shown in the top panel in Fig. 5. The lower panel in Fig. 5 shows the average CFUs recovered from each well, as well as from control wells that were not exposed. The results from the directly exposed well show that 5 min of exposure leads to a 6-log reduction of culturable cells in the biofilm. The adjacent wells experience a 2–3-log reduction, whereas the covered cells are essentially not affected. The effluent produced by our device is effective against biofilms, but it is necessary that the target be fully immersed in the effluent. Dilution of the effluent as occurred in the case of wells 2–4 in Fig. 5 quickly reduces its effectiveness. These results confirm the importance of the closed-loop flow design used in the other biological tests since this ensures delivery of the effluent to all parts of the sterilization chamber and prevents its dilution.
Fig. 5. Results from the experiment with Escherichia coli biofilms prepared in multwell culture plates. The top panel shows the layout of the wells in a six-well plate. The output of the device was directed at well 1; wells 5 and 6 were covered with a glass slide. The exposure time was 5 min, and the experiment was repeated three times. The numbers in each well indicate the percentage of CFUs inactivated and the corresponding log reduction averaged over three repetitions of the experiment. The bottom panel shows a graph of average recovery of CFUs from each well. The control (well 0) was not exposed to the device effluent.

B. In Vivo Tests

Having shown that the plasma-induced effluent produced in our device is capable of achieving sterilization on time scales suitable for clinical application, the effect of the effluent on live tissue was explored in a test involving live mice. Such tests are important for all applications of plasma technology to live tissue, including wound treatments or simple alternatives to hand washing. Fig. 6 shows the special sterilization chamber that was designed allowing exposure of the body of the mice but preventing exposure of the head so that the mice would not inhale the plasma effluent. Four groups of eight CD-1 (both male and female) mice each had their backs shaved the night before the start of the experiment. Shaving allowed for maximum exposure of the skin to the effluent. The first group was a control group, which was not exposed to the effluent. The subsequent groups were exposed to the effluent for 30-s durations once per day, three times per day, and five times per day, respectively. The exposures were separated by at least 1 h, and the mice were briefly sedated with isoflurane anesthesia during the exposure. The experiment lasted for three days after which the skin of the mice was grossly examined by one of us (J. Leszczynski, University of Colorado Veterinarian). Panel (c) in Fig. 6 shows the skin of the control-group mouse, and panel (d) shows the skin of a mouse from the group exposed to five daily 30-s treatments hourly spaced for three days. From gross examination, none of the mice exposed to the plasma showed any signs of inflammation or redness as compared with the control group. The only notable damage to the skin was from small cuts from the shaving process, which were also observed on the control group. All studies were performed under a protocol approved by the University of Colorado Institutional Animal Care and Use Committee.

IV. ANALYSIS OF CHEMICAL COMPOSITION

The chemical composition of the effluent from our device was investigated using direct frequency comb absorption spectroscopy at JILA, NIST and the University of Colorado Boulder. This technique is currently at the leading edge of tools for trace gas detection requiring broadband coverage and rapid acquisition times [14], [15]. It provides high sensitivity and selectivity to molecular absorption combined with broad wavelength coverage necessary for multispecies detection [16]. The source of light in our direct frequency comb spectroscopy experimental setup is a midinfrared optical parametric oscillator (OPO) [17] pumped by a high-power femtosecond Yb:fiber laser at 1064 nm [18]. The optical spectrum of the OPO spans 150 nm around a center wavelength continuously tunable between 2.8 and 4.6 μm. The plasma effluent is flown through a multipass Herriott cell, in which the interaction length between the sample and the optical beam is 18 m. The multipass optical cell was modified so that it could be connected into the closed-loop flow of the device in place of the sterilization chamber shown in Fig. 1. The volume of the multipass cell is ∼0.3 L so that the conditions in the glass optical cell and the plastic sterilization chambers we used for the tests described above are expected to be identical. The light exiting the multipass cell is analyzed with a fast-scanning Fourier transform interferometer [19], [20].

What makes the direct frequency comb spectroscopy particularly useful for the investigation herein is the capability to simultaneously measure concentrations of multiple species to a high degree of precision with short acquisition time (less
The addition of the hydrogen peroxide solution to the DBD discharge raises the H₂O₂ concentration from below the detection limit to \(~400~\text{ppm}\). The concentration of NO₂ remains roughly constant irrespective of whether the device is operated with hydrogen peroxide. The concentrations of N₂O and O₃ are found to decrease by a factor of 2 with the addition of hydrogen peroxide. In both cases, the device requires about 10 s to reach saturation levels of the major chemical species. This quick attainment of free-radical and ROS saturation, which required considerable engineering of the fans and the evaporator to maintain a high flow rate, is likely one of the key reasons that the device is able to achieve much faster pathogen inactivation than other indirect methods.

V. DISCUSSION

Currently, the two main areas of effort in nonthermal plasma biomedical applications are understanding the exact mechanisms of pathogen inactivation and optimizing hardware solutions for specific medical applications. The primary mechanism of pathogen inactivation by nonthermal plasma-induced chemistry can be caused by multiple factors including erosion of the cell wall, interference with the cell inner membrane, or DNA damage [21], [22]. None of these mechanisms occur in isolation, and it has been shown that ROS can interact with the cell inner membrane via lipid peroxidation, which can cause the cells to leak and also create reactive byproducts that can subsequently lead to DNA damage [8]. Although polyunsaturated lipids, which are the main substrate for lipid peroxidation, are found in low levels in bacteria membranes, a growing number of experiments have reported lipid peroxidation as a mechanism of bacteria cellular damage under exposure to ROS [23]–[25]. Most relevant to the mechanisms induced by our device, a comprehensive study by Joshi et al. [8] investigated the effect of ROS produced by nonthermal plasma and found evidence that ROS scavengers such as vitamin E were able to prevent the extent of lipid peroxidation.

Although atmospheric pressure plasma discharges can produce a wide range of ROS, the DBD in our device is seen to produce mainly ozone, and comparable concentrations of H₂O₂ are achieved by direct addition through our bubbler. The reduction potential of both O₃ and H₂O₂ is sufficient for direct peroxidation (oxidative degradation) of lipids in a cell membrane, but the most oxidizing radical is known to be the hydroxyl radical (OH*) [26]. The interaction of O₃ and H₂O₂ can favorably produce hydroxyl radicals (OH*) in what is called a peroxone process [27], i.e.,

\[
\text{H}_2\text{O}_2 + 2\text{O}_3 \rightarrow 2\text{OH}^* + 3\text{O}_2.
\]

Unfortunately, the peroxone process is difficult to quantify in our device as it requires a surface or a liquid phase since O₃ and H₂O₂ are not expected to interact in the gas phase directly. In this context, we note that a subsequent measurement with the spectrometer tuned to the fundamental band of the hydroxyl radical near 2.9 \(\mu\text{m}\) did not show any OH* presence within the detection limits of the system. An experiment using direct frequency comb spectroscopy to investigate surface chemistry...
Our device’s ability to inactivate pathogens and spores on short time scales is likely due to the production of hydroxyl radicals at the biological target made possible by the presence of H$_2$O$_2$. Hydroxyl radicals produced from the interaction between ozone and H$_2$O$_2$ are a possible reason for the results in Fig. 4, where the DBD discharge and hydrogen peroxide in unison produce the most effective inactivation results. We note that ozone, by itself, cannot be the main inactivation agent since, as shown in Fig. 8, its concentration is lowered by a factor of 2 with the addition of hydrogen peroxide solution. We also note that hydroxyl radicals do not have sufficient lifetimes at atmospheric pressure to be able to reach the sterilization chamber of our device if they are directly produced in the discharge. An electron spin resonance experiment might be able to provide direct evidence of the generation of hydroxyl radicals from other ROS at the target surface upon exposure to our effluent.

Nonthermal plasmas have been seen to be slightly less effective in deactivating gram-positive bacteria than gram-negative bacteria because of a difference in the cell envelope structure [22]. The cell envelope of gram-negative microorganisms is a multilayer system composed of an inner cytoplasmic membrane made of phospholipids and proteins, a peptidoglycan layer, and an outer membrane of poly polymers such as polysaccharides. Gram-positive cells have a less complex envelope with a thicker peptidoglycan layer and the general absence of the outer membrane. As discussed above, recent works have emphasized bactericidal effects of ROS such as ozone and hydrogen peroxide to occur via disruption of envelope integrity through peroxidation of phospholipids and lipoproteins found in the inner membrane of both gram-negative and gram-positive cells. In our results, gram-negative Pseudomonas aeruginosa proved more resistant on agar plates (see Fig. 2) but less resistant on plastic badges (see Fig. 3) when compared with gram-positive Staphylococcus aureus.

We note that any interaction between the ROS produced in our device and the inner cell membrane will require passage of these species through the outer membrane and the peptidoglycan structure. It has been documented that inactivation rates of nonthermal plasma technologies depend not only on the type of bacteria but also on the type of the medium supporting the microorganisms [21]. The plastic badges represent a harsh environment with no extra nutrients in contrast to the nutrient-rich conditions on the agar plates. It is possible that Pseudomonas aeruginosa and Staphylococcus aureus differently respond to these environments in terms of their ability to directly resist ROS or perform cell repair or ROS mitigation processes. We also note that the bacteria on the agar plates were plated about an hour before exposure. Such a period of time in a nutrient-rich environment might have allowed the bacteria to direct their genome to a more biofilm type of growth, with potential differences between the two strains again playing a role. Although our device is effective against biofilms (see Fig. 5), the deactivation process requires longer time scales. The production of hydroxyl radicals OH$^*$ on the surface of either cell type by either the peroxone or Fenton processes discussed above is not able to be quantified at this time, although it is also of fundamental importance.

An important ROS species that we were not able to measure with our spectrometer is nitric oxide (NO), which can play a role in wound healing by inducing fibroblast proliferation and regulating immune deficiencies [28]. The NO absorption band at 2.8 μm overlaps with a strong absorption band of water, and another absorption band at 5 μm is outside the operating frequency range of the OPO. A new frequency comb source that will cover this spectral region is under development. In terms of chemistry dynamics, we note that the concentrations of the nitrogen oxides and other active species such as ozone are all coupled. For example, nitrous oxide (N$_2$O) gives rise to NO (nitric oxide) on reaction with oxygen atoms, and NO, in turn, reacts with ozone to produce nitrogen dioxide (NO$_2$). The
multiple and simultaneous reactions make numerical modeling of the chemical dynamics difficult, hence the need for direct measurement of simultaneous concentrations, which is a capability that the frequency comb spectroscopy uniquely provides.

The operational parameters of our device utilized in these experiments (flow rate and plasma discharge) were empirically arrived upon to produce the quickest possible pathogen inactivation rates, which we have presented above. The effects of the course variation of parameters, namely, the presence of the hydrogen peroxide solution and the activation/deactivation of the plasma discharge, are shown in Fig. 4 (spore inactivation) and in Fig. 8 (chemical concentrations). A high flow rate is key in quickly achieving the highest concentrations of ROS in the sterilization chamber and is the maximum the current hardware can supply. For a fixed geometry and flow rate, atmospheric pressure nonthermal plasmas have a small window of stable operation, therefore making significant variations of the discharge voltage unlikely to improve performance. A quantitative model of dominant chemical processes including the generation of OH* radical production will allow for future optimization of the technology.

VI. CONCLUSION

The hydrogen-peroxide-enhanced nonthermal plasma effluent produced in our device has shown for the first time that pathogen inactivation on time scales previously associated with direct plasma treatments is possible with an indirect plasma treatment. The free-radical effluent has been shown to be also effective against bacteria in the spore state and biofilms and does not produce adverse effects on mammalian tissue on time scales necessary for clinical application. The hardware for our plasma device is significantly cheaper than that of plasma treatments being used in clinical trials. The lower hardware cost and flexibility of application afforded by an indirect treatment make the technology ripe for application in the medical clinic.

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GOLKOWSKI et al.: H₂O₂-ENHANCED PLASMA EFFLUENT FOR BIOMEDICAL APPLICATIONS


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