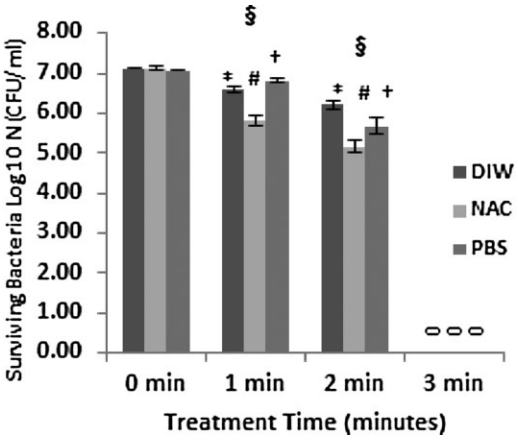
Nonequilibrium Plasma-Activated Antimicrobial Solutions are Broad-Spectrum and Retain their Efficacies for Extended Period of Time†

Utku K. Ercan, Hong Wang, Haifeng Ji, Gregory Fridman, Ari D. Brooks, Suresh G. Joshi*

By applying dielectric-barrier discharge nonthermal plasma technique, we have treated fluids and generated antimicrobial solutions, tested for properties such as changes in pH, temperature, delay time, holding time, fluid-aging, and detection and comparison of acid and hydrogen peroxide. All plasma-treated solutions showed strong biocidal activity, and among them, NAC solution was the most powerful, inactivated biofilms of tested microorganisms in 15 min of holding time. During accelerated aging experiments, plasma-treated NAC solution exhibited the equivalent of two years of shelf. These results indicate that it retained its antimicrobial properties for an extended period against a wide range of multidrug-resistant pathogens, making it an excellent candidate for further testing in vivo.



1. Introduction

Hospital-acquired infection is a major challenge for clinicians and other health care providers and has assumed increasing importance for overall public health. It is one of the most common causes of health care complications and death in the United States. According to the Center for Disease Control and Prevention, about 1.7 million infections occur annually, of which about 100 000 deaths are associated with hospital-acquired infection, which increases rates of mortality and morbidity and medical costs.[1] They estimated about $28–$336 billion in excess annual health care costs.[2] Because of their high prevalence in hospitals, *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *S. epidermidis* have become important members of the group of agents leading to nosocomial infections, partly because they are able to form biofilms on animate and inanimate surfaces in hospitals. The pathogen biofilms often act as a reservoir of contamination and infection and are likely disseminated by health care providers. Soft tissue infections, catheter-related bacteremia, urinary tract infections, hospital-

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acquired pneumonias, and open wound infections are often the result of transmission of such nosocomial pathogens.[3–5]

Although many medically approved, commercially available surface disinfectants and hand washes are used routinely, many of them are not sufficient to disinfect surfaces, and they fail to inactivate a substantial percent of the pathogens found in biofilms.[3,4,6] Also contamination of surfaces with multidrug-resistant pathogens is one of the major risk factors in hospitals, and an effective disinfection agent is required to prevent the transmission[6–8] of these pathogens. It has been reported that recent biocides containing benzalkonium chloride, chlorhexidine gluconate, and triclosan used commercially in medicine, and the powerful agents such as Acticide Bac-50 (Thor Group Ltd, Canterbury, Kent, UK) and MediHex-4 (Medichem International, Sevenoaks, Kent, UK), are not sufficient to completely decontaminate sources and reservoirs of nosocomial infections, including sessile biofilm with embedded pathogens.[7,8] Therefore, a new compound or formulation is needed to prevent hospital-acquired infection that are more effective, rapid, less deleterious to surfaces being treated, and able to prevent transmission.[6–11]

Thermal plasmas are in use in the medical field for cauterization. Recently, direct or indirect application of nonthermal, dielectric-barrier discharge (DBD) plasma (also known as cold plasma) is under investigation for its ability to disinfect and sterilize biomaterial or the surfaces likely to be damaged by thermal plasma.[12] The plasmas are mediated by an electric field; electrons receive energy faster than heavier ions and receive thousands of degrees before surrounding ions heat up. Consequently, ionized gas remains at a lower temperature (room temperature) instead of receiving energy from excited ions, and the heated ions can be cooled down efficiently.[13] It is also reported that plasma creates charged and uncharged species, which in part are responsible for the disinfection and sterilization effects. The most notable species created by treatment with plasma that play a major role in the inactivation of bacteria are superoxide, hydrogen peroxide, singlet oxygen, OH-radicals, ozone, nitric oxide, ultraviolet light, and electrons.[13,14] There are no universally accepted terminologies related to the plasma application techniques. Here, the investigators have used the terms ''direct application of plasma'' to denote the fact that plasma comes in contact with the material of interest and ''indirect application of plasma'' to denote the fact that plasma is applied by any appropriate vehicle material (such as gas blowing, treated liquids), thus conveying the effect of the plasma to the material (to be sterilized) held away from the plasma source.[15–18] Although, the physical and chemical compositions are different, and the exact differences are not yet known, both these techniques are being investigated in infection control and surface sterilization.

We demonstrated previously the unique technique of applying plasma to biological material or to the surface to be sterilized, known as the dielectric-barrier discharge (FE-DBD) plasma technique, wherein the temperature at the site of the plasma application is maintained close to room temperature and thus does not harm heat-sensitive or heat-labile surfaces, yet completely inactivates bacterial pathogens in both planktonic and embedded biofilm forms.[6,14] We also demonstrated that the FE-DBD technique generates high amounts of reactive oxygen species (ROS), which rapidly inactivate pathogens in planktonic (in $<$120 s) and embedded biofilm (in $<$180 s) forms, requiring 15.6 and 23.4 $J \cdot cm^{-2}$, respectively.[6] With the direct application of the FE-DBD plasma technique, the plasma effect is composed of physical and chemical species created at normal atmospheric air (no other gas or air under pressure) between the primary electrode and the surface contaminated with the biological material (secondary electrode).

We briefly introduced the application of indirect plasma mediated through plasma-treated fluid [phosphate-buffered saline (PBS)].[6] In the present work, we demonstrate how effectively the FE-DBD plasma-treated fluid(s) carries antimicrobial properties that retains for extended period of time, and inactivates bacterial pathogens distantly (from the creating device). We call it the fluid-mediated plasma application, wherein the fluid-mediated plasma treatment is a direct treatment of liquid but an indirect treatment of the bacteria (distantly located from plasma generating device). We evaluated the ability of the fluid-mediated, nonthermal plasma treatment to inactivate the most common nosocomial pathogens in their planktonic and biofilm (sessile) forms and its efficacy in terms of holding times, delay times, fluid aging, temperature changes, and pH changes.

2. Experimental Section

2. 1. Plasma Treatment of Liquids

To generate nonthermal plasma DBD plasma, we used an experimental setup similar to that reported previously.[6] To scale up the process, we made two changes. First, we used a bigger copper electrode (38 mm $\times$ 64 mm) covered with a 1-mm glass slide (Fischer Scientific, Inc., Pittsburgh, PA). The discharge gap was fixed at 2 mm. Second, we customized a liquid container to maintain a liquid column of 1 mm. Deionized water (MP Biomedicals, Inc., Solon, OH), PBS, or N-acetyl-cysteine (NAC; Sigma Chemical Co., St. Louis, MO; 1 ml) was treated separately at different time points. A stock solution of NAC (100 mM) was prepared in PBS and sterilized through a filter; aliquots were stored at $-20\ ^{\circ}C$ until used. A freshly prepared working solution of 5 mM NAC in PBS was used for subsequent experiments. All liquids were treated for 0, 1, 2, and

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3 min, using a custom-made glass container (referred to here as a liquid holder), as described previously.[6]

2.2. Culture and Isolates of Bacterial Pathogens

E. coli (ATCC25922), *S. aureus* (ATCC25923), *A. baumannii* (ATCC19606), and *S. epidermidis* (ATCC12228) strains were purchased from American Type Culture Collection (ATCC, Manassas, VA). All strains were maintained and used as overnight cultures in trypticase soy broth (TSB) for primary inoculations according to the supplier's guidelines. Reference strains of *Candida albicans* and *C. glabrata* (obtained from Dr. Thomas Edlind, Drexel University College of Medicine) were grown in yeast extract-peptone-dextrose medium. Hydrogen peroxide (Sigma) or 70% ethyl alcohol was used as the known biocide agent and either TSB alone or PBS alone was used as the negative control, as appropriate.

2.3. Plasma Fluid-Mediated Bactericidal and Fungicidal Activity

A given pathogen was cultured overnight, inoculated (10 μl) into TSB medium (10 ml), and incubated at 37 °C for 4 h on an orbital shaker incubator; the optical density at 600 nm (OD_{600}) was adjusted to 0.2 (to have uniform number cells every time) before use. The culture dilution thus prepared (1:100; that corresponded to $\sim 1 \times 10^7$ CFU · ml^{-1} during colony count assay) was mixed with plasma-treated liquids (50 μl:50 μl) and held together at room temperature for 0- to 15-min intervals. After holding (holding time), the culture was diluted appropriately (1:10 000) with sterile PBS and spread on trypticase soy agar plates to incubate at 37 °C for 24 h. After the culture was incubated, the colony forming units (CFU) were counted to quantify surviving pathogen cells. Some of the experiments were carried out using 10^6–10^9 CFU · ml^{-1} (as initial cell numbers) to determine cell density-dependent rates of inactivation. Plates that did not show any growth were incubated further up to 72 h and observed every 24 h for possible growth. Similarly, the cultures of 0.2 OD_{600} were diluted (1:100). The relative CFU · ml^{-1} were calculated and exposed to plasma-treated fluid (50 μl:50 μl), mixed, and held for variable times. The sample was centrifuged at 8 000 rpm for 10 min; the supernatant was removed to collect the cell pellet. The pellet was resuspended in XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma–Aldrich, St. Louis, MO) reagent to carry out the XTT assay to demonstrate cell viability, as described previously.[6,7,19] In some of the experiments, plasma-treated liquid was used to treat pathogen biofilms as described previously.[6] The biofilms were established on catheter slices and 24-well polystyrene plates. In brief, the overnight cultures were diluted to 1:100 using 1% glucose containing TSB; 500 μl was seeded in each well; and plates were incubated at 37 °C in a stationary incubator for 24 h. The biofilms were gently washed thrice with PBS and exposed to XTT reagent. The untreated or treated biofilms were processed using the XTT assay to determine whether the bacteria embedded in the biofilms had been inactivated. Ethanol (70%) was used as a positive control for the biofilm experiments.

2.4. Temperature and pH Detection of Treated Fluid

To determine whether the temperature of the fluid during plasma treatment was maintained at normal room temperature, we measured the temperature of all three fluids before, during, and after plasma treatments. A special ultrasensitive K-type thermocoupler with LCD/digital display (ThermoWorks, Lindon, UT) was used for this purpose. Similarly, a pH ultrasensitive probe attached to a Thermo Orion Research Digital pH meter (Thermo Fisher Scientific, Waltham, MA) was used to detect plasma treatment-associated pH changes in fluid over time and to find out how much of a drop in pH can occur.

2.5. Delay Time, Holding Time, and Fluid-Aging Experiments

Holding time (alternatively known as contact time elsewhere) was defined as the time that plasma-treated liquid came in contact with the bacterial suspension. To evaluate the effect of holding time, plasma-treated fluid was exposed to bacteria for variable periods of time. For 0 min holding time, plasma-treated fluid was exposed to bacteria and then immediately mixed thoroughly by micropipetting; a standard colony-counting assay was performed. For longer holding times, the plasma-treated fluid and bacteria were mixed and held together for the desired time in the same tube at room temperature; after the desired time, the standard colony-counting assay was performed.

We defined delay time as the time that starts immediately after treatment with plasma until the exposure of the plasma-treated liquid to bacteria. To evaluate the effect of delay time, different time points were selected (0 min–3 months). For 0 min delay time, plasma-treated liquid was exposed to bacteria immediately after plasma treatment, and the standard colony-counting assay was performed. For prolonged delay time points, plasma-treated liquid was stored either at +4 °C (in the refrigerator) or at room temperature in microtubes sealed with Parafilm. Once the tube containing the sample was opened, to avoid contamination of the fluid, the sample was used for the experiments at hand and never reused. All reactions after holding times, were stopped by diluting with PBS (min 1:100 onwards; depends upon the initial cell density).

For aging experiments, the plasma-treated NAC solution was kept in a thermostatically controlled incubator set to an elevated temperature (55 °C) and incubated over time. We used the protocol of the U.S. Food and Drug Administration for aging pharmaceutical compounds.[20] In brief, 1 ml of plasma-treated NAC solution was immediately transferred into 3-ml glass vials; the screw caps were replaced; the vials were sealed with Parafilm and put upright in racks kept in the incubator. At the indicated time point, one vial was removed, and we tested for the antimicrobial property of the liquid using the colony count assay described above.

2.6. Effect of Acid and H_2O_2 in Comparison to Plasma-Treated Liquid

Because we noticed a reduction in pH from neutral to acidic, we wanted to determine whether acid production was responsible for

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the strong antimicrobial effect. In a separate set of experiments, we exposed bacterial suspensions of known cell densities to sulfuric acid, hydrochloric acid, or nitric acid for exposure times equivalent to those of the plasma-treated liquid; we performed the colony assay to quantify the amount of bacterial inactivation. Similarly, various concentrations of H_2O_2 were mixed with the bacterial suspensions; after a holding time, the colony assay was performed to quantify the pathogen. In separate sets, we mixed known concentrations of acid and hydrogen peroxide and then exposed the mixture to bacterial suspensions. After a holding time, colony assays were carried out to evaluate inactivation of the pathogens. In a separate experiment, a hydrogen peroxide assay kit (Cat. # CL204, National Diagnostics, Atlanta, GA) was used according to the manufacturer's instructions to determine the amount of H_2O_2 generated in the plasma-treated NAC solution.

2.7. Data Analysis

All experiments had built-in negative and positive controls as stated. The initial concentrations (1×10^7 CFU · ml^{-1}) of bacteria (untreated samples or 0 time treatment samples) were taken as 100% surviving cells to calculate relative percent inactivation (unless specifically stated). All of the experiments were carried out thrice in triplicate. Wherever needed, Prism software v4.03 for Windows (Graphpad, San Diego, CA) was used for analysis. A *p*-value was derived using pair comparisons between two bacterial groups with the Student *t*-test and one-way analysis of variance for multiple comparisons. A *p*-value of <0.05 was considered statistically significant.

3. Results

To determine whether various plasma-treated liquids carry antimicrobial properties and inactivate surface-contaminating and biofilm-forming nosocomial pathogens, we performed a series of experiments. We analyzed the profiles of temperature, pH, holding time, and delay time of the plasma-treated liquid and found the relative antimicrobial efficacies of three different liquid systems. Figure 1A depicts the schematic setup used for this piece of research, including a plasma-generating probe and fluid holder.

3.1. Plasma-Treated Liquids Inactivate Bacteria in a Concentration-Dependent Manner

To confirm whether the antimicrobial efficacy of plasma is a function of the amount of plasma energy deposited into the liquids, we performed additional experiments. Figure 1B shows that all three plasma-treated liquids (NAC solution,

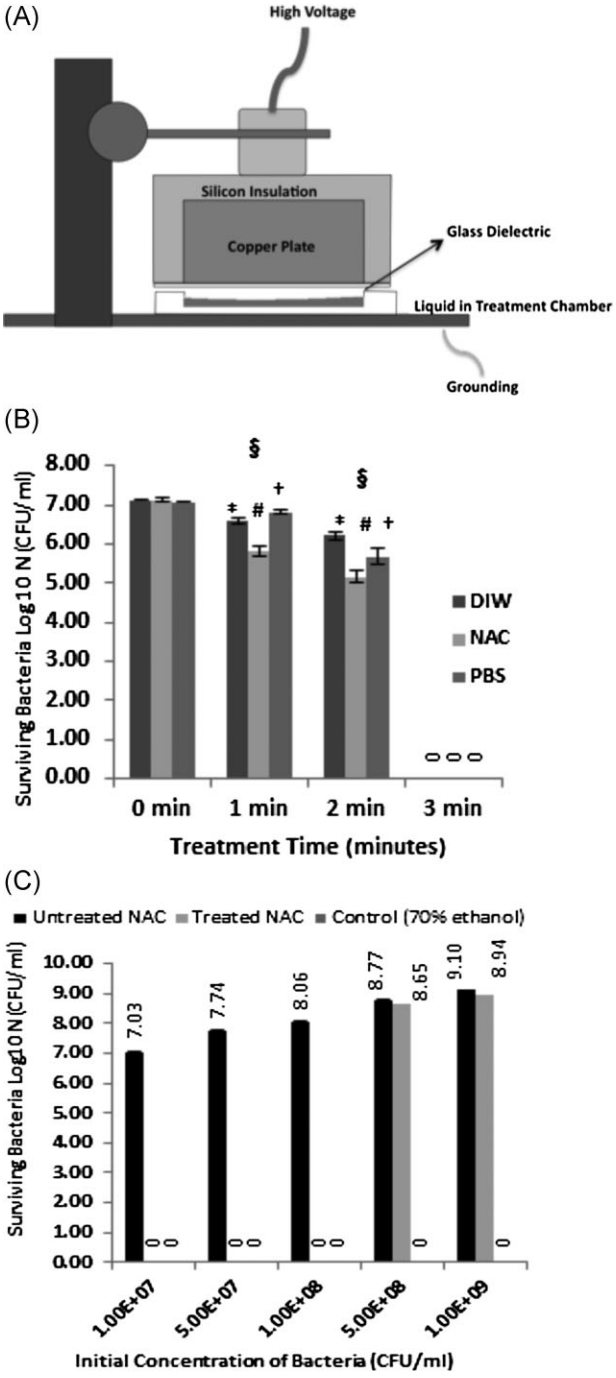


Figure 1. Application of fluid-mediated plasma. (A) A schematics of plasma-generating probe and fluid-treatment. A fluid holder is placed on a mechanical stage underneath the DBD probe. (B) Fluid-mediated plasma inactivates *E. coli* (1×10^7 CFU · ml^{-1}) in a time-dependent manner. Plasma-treated NAC (5 mM) solution has stronger antimicrobial properties and a significantly greater biocidal effect than treated deionized water (DIW) or phosphate-buffered saline (PBS) [all *p* values were <0.05 derived against respective untreated control (0 min) (*, #, †, and §, against corresponding DIW or PBS conditions); $n = 3$]. (C) Treated NAC solution causes cell density-dependent inactivation of *E. coli*. Within 3 min treatment time, it inactivated up to 1×10^8 CFU · ml^{-1}. 70% ethanol was included as positive control of inactivation during colony count assays ($n = 3$). Holding times in (B) and (C) are 15 min.

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PBS, and deionized water) carry strong antimicrobial properties and that about 3 min of treatment with plasma generated a sufficient amount of energy transferred into the liquids to effect complete inactivation of planktonic *E. coli*. Even by the end of 2 min, there was significant inactivation by the NAC solution ($p < 0.05$), PBS ($p < 0.05$), or deionized water ($p < 0.05$) compared with the respective controls (0 min or no treatment). At 3 min of plasma treatment, fluids caused complete inactivation, and therefore bars are not visible. The plasma-treated NAC solution had the most powerful antimicrobial effect. With treatment for 1 min ($14.5\,J \cdot cm^{-2}$), it inactivated a significant amount of *E. coli* ($p < 0.05$) compared to deionized water or PBS alone for comparable times. We found that free-floating planktonic bacteria (10^7 CFU $\cdot$ ml^{-1}) were inactivated during exposure to plasma-treated liquid. Therefore, we exposed cell suspensions of various CFUs of *E. coli* to plasma-treated liquids. Figure 1C shows colony assays that indicate rapid inactivation of bacterial cells. The treated liquid showed complete inactivation when $\sim 1 \times 10^8$ CFU $\cdot$ ml^{-1} exposed, and exhibited strong antimicrobial effects ($p < 0.05$) compared with the respective untreated samples. Non-significant inactivation was observed when higher cell densities (5×10^8 to 1×10^9 CFU $\cdot$ ml^{-1}) were exposed.

3.2. Plasma-Treated NAC Solution is a Broad-Spectrum Antimicrobial Agent

To determine whether NAC solution treated with plasma inactivates common pathogens such as *S. aureus*, *S. epidermidis*, *A. baumannii*, *C. albicans*, and *C. glabrata*, in addition to *E. coli*, we tested a range of concentrations of NAC (1–20 mM) with or without plasma treatment. We found that 5 mM was sufficient for bacterial inactivation studies during colony assays. Higher concentrations of NAC did not show significantly different efficacy (optimization data not shown). Studies in planktonic forms showed that the plasma-treated NAC solution completely inactivated all of the pathogens tested (Figure 2) by 3 min of treatment with plasma. Only *C. glabrata* required 3.2 min (195 s) of treatment with the liquid to achieve 100% inactivation of this fungal pathogen (data not shown). Most of the pathogens in their biofilm form were equally sensitive to the biocidal effect of the treated NAC solution. As there is a complete inactivation by 70% ethanol (positive control), the bars are not visible.

3.3. Determination of Holding Time, Delay Time, and Accelerated Aging of Solution

The holding time of the antimicrobial agent with the pathogen is critical. Often, the biocidal effect is proportional to the initial contact time. Figure 3A shows that, on

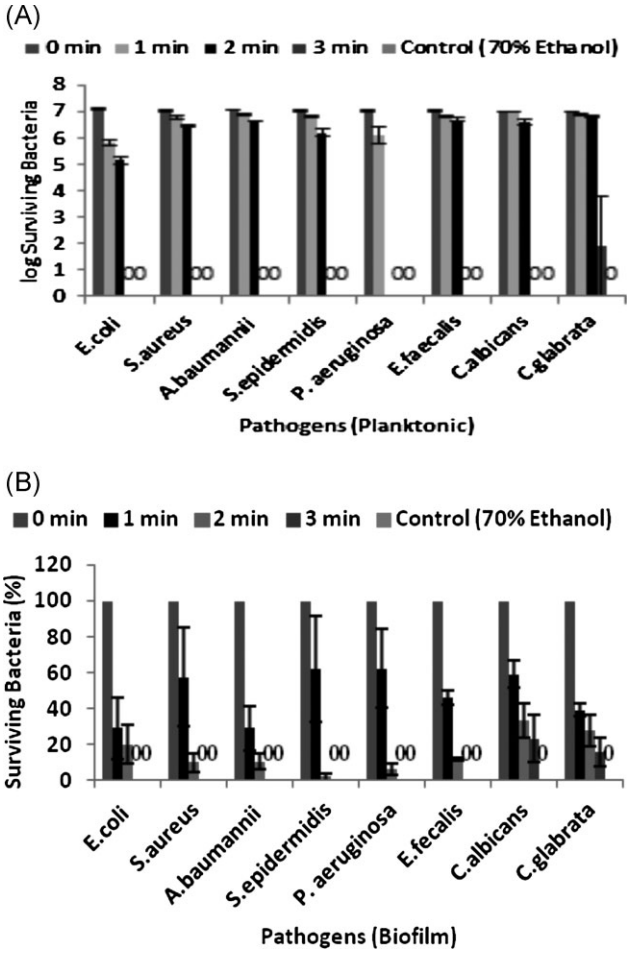


Figure 2. Fluid-mediated inactivation. Plasma-treated NAC (5 mM) solution inactivates a wide array of pathogens both in planktonic form (1×10^7 CFU $\cdot$ ml^{-1}, initial cell count at 0 min; A; colony assay) and biofilm-embedded form [B; XTT (viability) assay] with 15 min holding time. Three minutes of plasma treatment produced sufficient antimicrobial properties in solution, inactivating almost all pathogens in their planktonic and biofilm forms (*C. albicans* and *C. glabrata* biofilms required 3.2 min; data not shown). For biofilms, a relative percent was derived compared to the no plasma treatment (0 min) condition ($n = 3$).

exposure to the plasma-treated NAC solution, significant inactivation of *E. coli* occurred from 2 min of holding time onward (p values for 2, 3, and 5 min against 0 min < 0.05); in <15 min, the bacteria were completely inactivated. Similarly, to determine how long the plasma-treated fluids retained the antimicrobial effect at room temperature, we performed delay time experiments. We delayed mixing the post-treatment fluid (NAC solution) with the pathogen to determine whether the fluid lost its antimicrobial properties. Table 1 shows the antimicrobial efficacies of treated liquid delayed over time, post-treatment. It is apparent that treated fluid retained significant antibacterial effect ($p < 0.05$; against untreated

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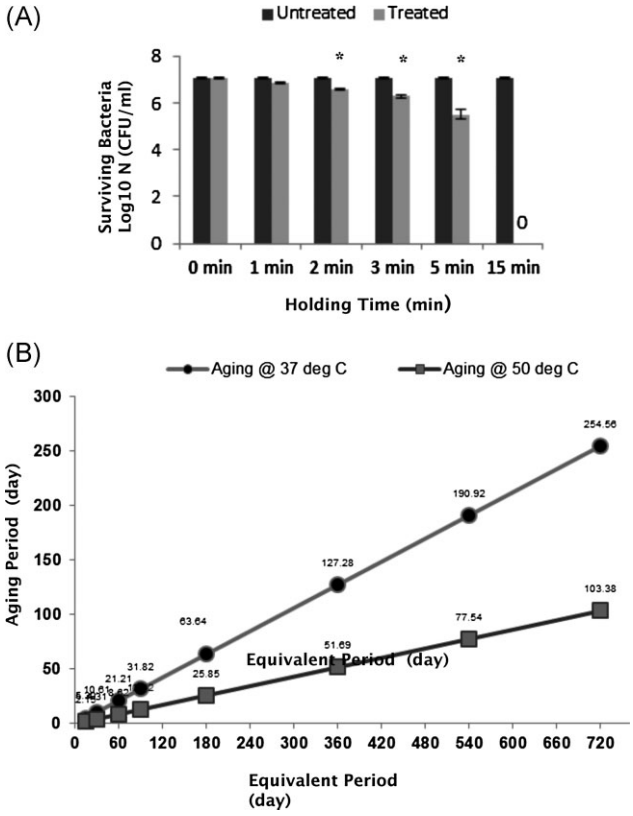


Figure 3. Inactivation of *E. coli*. (A) Holding time required by plasma-treated NAC (5 mM) solution to complete inactivation of bacteria. The graph of shows a significant, rapid decline in surviving *E. coli* as holding time increases (*, $p < 0.05$; against 0 min). By the end of 15 min holding there is a complete inactivation. (B) A graphical presentation showing the schema of an accelerated aging (maturation) experiment of treated NAC solution at elevated temperature of 50 °C which demonstrates that, for approximately two years of equivalent time to room temperature, the treated solution retains strong biocidal activity, a quality required by an ideal antimicrobial solution ($n = 3$; Please correlate to Table 1).

controls) for up to 720 d (two years) and showed complete inactivation of *E. coli*.

To assess the shelf life of the plasma-generated antimicrobial properties of the NAC solution, we performed experiments of accelerated aging for medically important solutions and devices at 37 and 50 °C over time, equivalent to two years of delay time at room temperature. Figure 3B illustrates days of accelerated aging time at the respective temperatures and equivalent delay time at room temperature. We observed complete inactivation when *E. coli* at 10^7 CFU · ml^{-1} were exposed (for 15 min holding) to the solution (Table 1). This result indicates that the plasma-treated NAC solution has the potential to remain active for extended period of time, a quality required by antimicrobial solution for local application.

3.4. Plasma-Dependent Temperature and pH Changes

The technique of nonthermal plasma is generated by micropulse in our system and is not continuous plasma. Furthermore, we apply it indirectly by fluid mediation; therefore, the fluid is not heated nor is the local temperature increased at the point of application. To demonstrate that the plasma treatment does not cause a heating effect and is cold plasma, we applied a digital thermocoupler for actual temperature measurements during generation. Figure 4A shows the temperatures of all three liquids exposed to plasma. The range of temperatures is close to room temperature (~23–26 °C), even when the plasma treatment lasts 3 min. No linear regression is seen between temperature and different treatment times.

We noted that the pH of the liquid decreased with plasma treatment time, moving toward acidity, when tested with pH strips. Therefore, we measured the pH using an ultrasensitive pH meter with a digital display system. Figure 4B shows the pH ranges of all three liquids (deionized water, PBS, and the NAC solution). We confirmed that the pH drops as the treatment time advances, up to 3 min (thereafter no change up to 5 min; may be saturation point). At 3 min of exposure to plasma, deionized water, PBS, and NAC solution showed, respectively, a pH of 2.00, 2.35, and 2.58. Thus the pH of deionized water was slightly lower than that of PBS and the NAC solution.

3.5. Comparison with the Effect of Nitric Acid and Hydrogen Peroxide

We also detected H_2O_2 as one of the ROS during direct treatment of bacterial cells with plasma. To determine whether the decrease in pH or H_2O_2 or a mixture of these components is responsible for biocidal activity, we tested the antimicrobial effects of nitric acid, H_2O_2, and a mixture of the two, having adjusted the pH to 2.5, in part to try to justify and correlate the antimicrobial property with the species or products generated. Figure 5A shows the standard curve of H_2O_2 detection, a linear relationship with the amount of H_2O_2 and absorbance at 240 nm, which is used to calculate the relative amount of H_2O_2 from the plasma-treated NAC solution. Figure 5B demonstrates that plasma treatments for 1, 2, and 3 min generated, respectively, 0.42, 1.66, and 0.91 mM of H_2O_2. Thus, we showed that saturation occurs at 2 min treatment time. Figure 5C indicates that, when *E. coli* (1×10^7 CFU · ml^{-1}) are exposed to an equivalent amount (Figure 5B) of H_2O_2, <0.2 log reduction of *E. coli* occurs. Furthermore, the bacterial inactivation was non-significant and undifferentiable at concentrations of H_2O_2 equivalent to those generated by 1, 2, and 3 min of plasma treatments. During preliminary in vitro experiments, we found that one possible major cause could be generation of a nitric acid-like product in the

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Table 1. The findings of colony count assays (CFU · ml^{-1}) show that the treated fluid NAC solution retains antimicrobial effects equivalent to 720 d at room temperature (holding time in all *E. coli* experiments, 15 min).

Equivalent days at room temperature	Incubation temperature							
	37 °C				50 °C			
	log 10 N surviving bacteria				log 10 N surviving bacteria			
	Days incubated	Control (70% ethanol)	Untreated NAC	Treated NAC	Days incubated	Control (70% ethanol)	Untreated NAC	Treated NAC
15	5	0	7.05	0	2	0	7.03	0
30	11	0	7.03	0	4	0	7.09	0
60	21	0	7.03	0	9	0	7.07	0
90	32	0	7.05	0	13	0	7.05	0
180	64	0	7.03	0	26	0	7.03	0
360	127	0	7.02	0	52	0	7.06	0
540	191	0	7.05	0	78	0	7.02	0
720	255	0	7.04	**0**	103	0	7.03	**0**

Bold values show the 0 colony numbers after two years of aging.

treated liquid, which is partly responsible for reduction in pH. Treatment of the liquid with plasma for 1, 2, and 3 min generated, respectively, 1.77, 2.89, and 3.86 mM of a nitric acid-like product. Therefore, in a separate experiment, we exposed *E. coli* (1×10^7 CFU · ml^{-1}) to an equimolar concentration of nitric acid alone. The colony assay demonstrated a non-significant reduction in CFUs (<0.05 log) as compared to untreated *E. coli* cells when tested against given concentrations of HNO_3 (Figure 5D). Because both HNO_3 and H_2O_2 molecules are generated in plasma-treated liquid, we mixed the molecules in concentrations equivalent to those generated by treatment with plasma and performed colony assays to demonstrate whether they synergize each other in *E. coli* inactivation. Figure 5E demonstrates the findings from this experiment. A <0.2 log reduction is observed in various concentrations as compared to untreated *E. coli* cells, and the findings are non-significant. This indicates that additional species or products generated from the plasma treatment play an important role in the antimicrobial properties of treated liquid. In Figure 5 on *x*-axis "C" denotes positive control condition (70% ethanol treatment) for comparison, and that have zero colonies and therefore bars not visible. The holding time was 15 min for *E. coli* experiments shown in Figure 5.

4. Discussion

It has recently been shown that atmospheric nonthermal plasmas mediated by argon gas or by a gliding arc decontaminate bacteria.[21–23] The plasma-generated reactive species are largely determined by the gas or air and the type of application technique used; thus, the antimicrobial efficacy is also likely to differ.[23–26] Treatment of water with the gliding arc-type plasma and its bactericidal effect have been reported.[23,25] Direct current (DC) electrical discharges in atmospheric air has also been tried by exposing either static or flowing water to bio-decontaminate bacteria in water solution, wherein three different types of discharges were tested viz. streamer corona, transient spark, and glow discharge.[27] Our design of plasma device and approach is differently then previously reported corona, spark, or glow plasma discharge. We use alternate current (AC) dielectric barrier discharge (micro-pulsed plasma), and we treat water first, and then expose bacterial pathogens to treated water (indirect plasma application). Thus, chemical composition of the plasma is different, and depends upon the type of device. We reported this technique on earlier occasions.[6,14] Here, our goal is to understand the uniquely different FE-DBD plasma technique and evaluate the antimicrobial efficacies of the three fluids treated by this technique (against a free living planktonic, and biofilm embedded pathogens), the concept of which we introduced earlier,[6,14] and the retention of biocidal activity over time.

We reported previously how direct plasma treatments inactivate contaminating pathogens[6] when the FE-DBD (nonthermal) plasma technique is used. We also demonstrated that the FE-DBD plasma technique generates ROS inside the bacterial cells on direct treatment with plasma.[14] Through the present studies we report that

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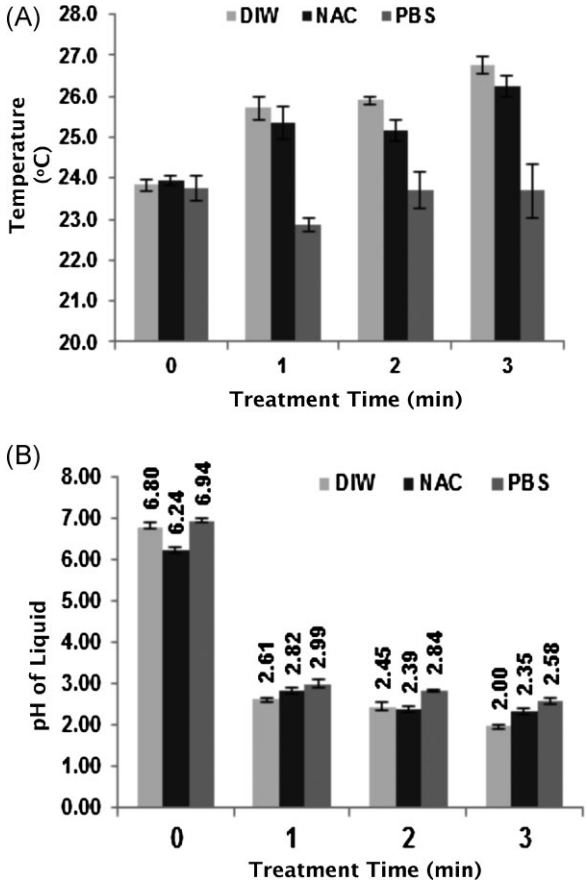


Figure 4. Effects on treated liquids. (A) No drastic increase in the temperature of treated liquids was noted, even with 3 min of plasma treatment. The fluids remained cool, close to room temperature. (B) On treatment with plasma, the pH of all three fluids decreases rapidly from 1 min through 3 min. After 3 min of plasma treatment, the pH of the liquids was 2–3. The pH drop is largely proportional to the amount of plasma energy received by the fluid, a distance between the plasma generating probe and the material being treated, and the immediate atmosphere in vicinity of discharged plasma. Even the buffer has a threshold to resist ($n = 3$).

the technique can also deliver an antimicrobial effect (indirectly) through plasma-treated liquids or solutions and that these liquids hold their antimicrobial properties for extended periods of time. The report also suggests that the liquids that turn into powerful antimicrobial solutions are highly bacteriocidal against a range of pathogens, including pathogenic fungi, and are as effective as the direct plasma treatment we reported previously.[6] This also means, we do not say in terms of chemical composition but the antimicrobial properties.

In these experiments, the plasma-generating probe was larger than reported earlier, to cover a larger treatment area. The fluid holder was also large enough to hold 1 ml of liquid (compared with 100 μl used in earlier publications), but the gap between the probe and the surface of the fluid remained the same (~2 mm). A typical schematic device set is shown in Figure 1A. All of the components were calibrated in our collaborating laboratory at the A.J. Drexel Plasma Institute of Drexel University. There are two reasons to adopt this fluid-mediated plasma application. Briefly, it is accepted that, like thermal plasma, nonthermal (cold) plasma also generates an electric field, a magnetic field, and certain physical species, such as charged particles, ultraviolet rays, and electrons in addition to chemical species such as ROS or reactive nitrogen species (RNS). Physical species are more detrimental and damaging to surfaces,[28] so it is a challenge to apply them to delicate contaminated surfaces and to biological materials. The antimicrobial effect of plasma fluids that permits one to sterilize or disinfect such surfaces without damage is an advantage. These fluids can be used to improve hospital hygiene, to treat bacterial skin diseases, and cosmetically, to control acne-causing organisms. The skin is not needed as a plasma electrode, which significantly simplifies their use in patients. Furthermore, the risks of electric current, thermal damage of tissue, and UV irradiation can be avoided by using fluid-mediated plasma with the patients or health care providers. Also, the purpose of investigations on plasma-activated liquids is to search for a better option as an antimicrobial agent over existing chemical preparations (such as chlorhexidine and silver containing compounds) which have toxicity. Protocols for investigating the toxic effects of fluid-mediated plasma on pig skin are in preparation.

In preliminary experiments, we tested a range of concentrations of aqueous solutions of NAC and found that a treated NAC solution of 5 mM holds antimicrobial properties sufficient to inactivate *E. coli* faster than other liquids, e.g., water alone (deionized water) or PBS (Figure 1B), and faster than reported previously for other plasma techniques.[23,26] The treated NAC solution could inactivate significantly more *E. coli* by the end of 1 and 2 min ($p < 0.05$) than treated deionized water or PBS, and thus is a better candidate for an antimicrobial solution. Therefore, we performed a cell density-dependent experiment with treated NAC (Figure 1C). A large quantity of CFUs (1×10^8) was completely inactivated by the treated NAC solution, again indicating its strong antimicrobial effect. The concentration of NAC (5 mM or 0.081 g%) selected for this study is considerably lower than the clinically permitted amount of NAC for therapeutic purposes. Also the bacterial load (10^7 CFU · ml^{-1} and more) used for inactivation experiments is high and therefore covers the clinically significant number of bacterial cells. Most other studies have used <6 log, which is clinically less relevant to represent severe sepsis. The exact chemical changes that occur in NAC solution needs to be studied in detail. The present work is a proof of concept.

The type and dose of pathogen are important in deciding the outcome of the processes of disinfection,

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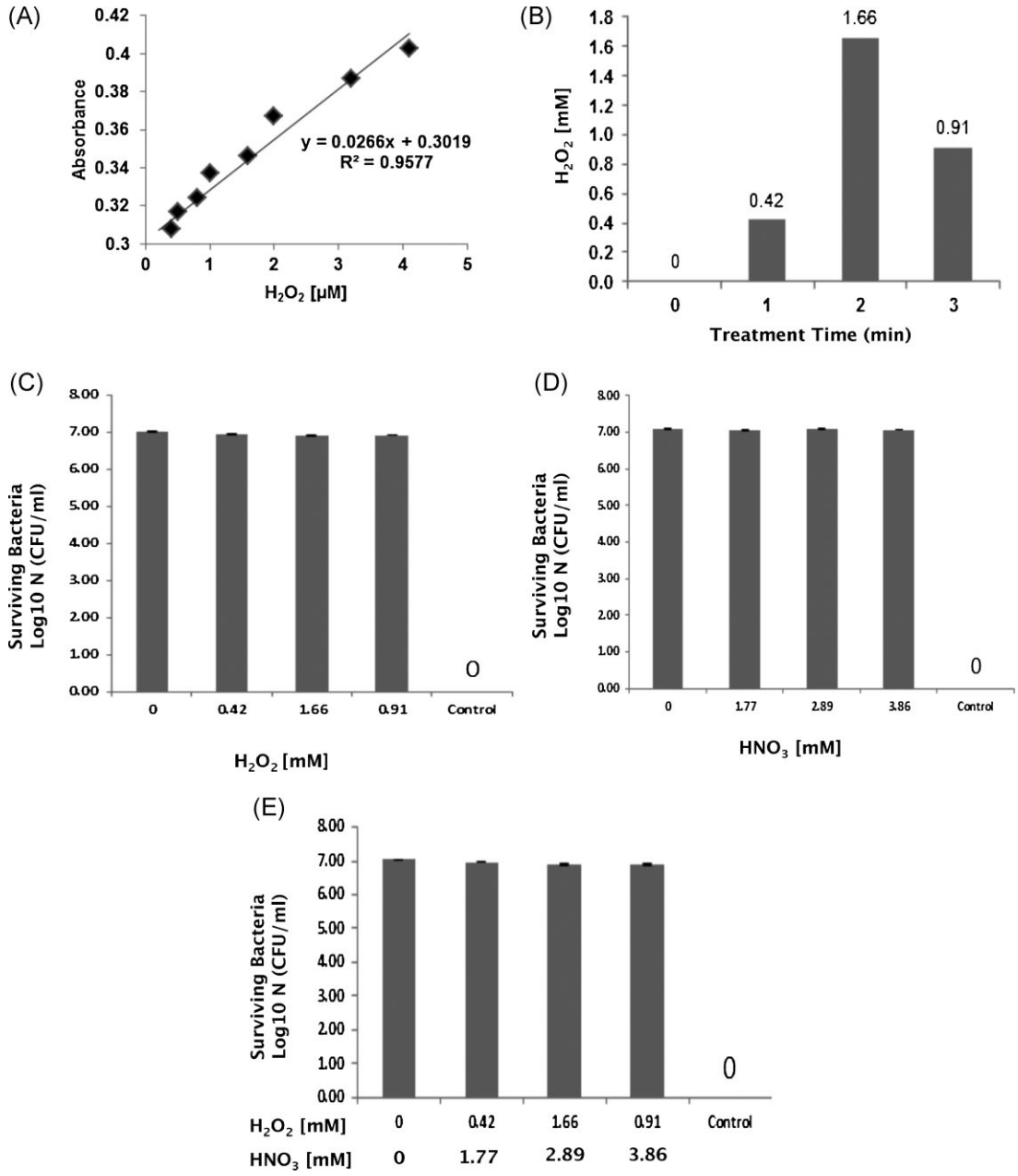


Figure 5. Is drop in pH or earlier reported, generation of H_2O_2 responsible for bacterial inactivation? (A) Standard H_2O_2, supplied in a kit, was used to compare and calculate unknown H_2O_2 generated in the NAC solution. A graph shows that kit components, standard H_2O_2, and the technique is working well, and can be comfortably used for such studies. (B) Amount of hydrogen peroxide was estimated during plasma treatment. Graph shows an increase in H_2O_2 at 2 min (saturates), but drops slightly at 3 min of treatment with the NAC solution (no further increase in H_2O_2 seen beyond 3 min). (C) Non-significant relation between inactivation of *E. coli* and challenged equimolar H_2O_2 in vitro. The findings demonstrate that plasma-generated equimolar H_2O_2 is not sufficient to inactivate *E. coli*. (D) *E. coli* response to equimolar nitric acid (HNO_3), which is equivalent to detected amounts during plasma treatment of NAC solution. The data shows that nitric acid also is not effective for inactivation of *E. coli*. (E) A mixture of H_2O_2 (C) and HNO_3 (D) with pH adjusted to 2.5, used to challenge *E. coli* with a similar holding time, did not reduce CFU by more than 0.1 log [$n = 3$; holding time of 15 min in all experiments; control (70% ethanol) is also shown, denoting 0 (zero) CFU]. The findings indicate that additional products of stabilized species might be involved in complete inactivation of bacteria during fluid-mediated plasma treatments.

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decontamination, or sterilization. Multidrug resistance is an additional hurdle in these processes and helps disseminate hospital-acquired infections, either by direct transmission or through biofilm-loaded surfaces.[11,29] Therefore, to investigate the antimicrobial efficacy of plasma-treated fluids, we chose multidrug-resistant nosocomial pathogens (which are common in hospitals) in moderately high doses (7 log CFU) known to form biofilms.[6,7] Because these pathogens also contaminate the surfaces of stethoscopes, catheters, and the hands of health care providers,[7] they are ideal candidates with which to test the decontamination regime. Figure 2 clearly demonstrates the strong antimicrobial effect of the plasma-treated fluid. The effect was comparable to that of a fresh solution of 70% ethanol, and bleach (3.8% sodium hypochlorite) solution (positive control; as zero colonies, hence not shown).

The holding time of these fluids with given bacteria and the delay time (analogous to residual effect) of the antimicrobial agent are also crucial for decontamination of surfaces. Different biocidal solutions have variable holding times and delay times.[30] Our plasma-treated solutions were as effective as the existing commercial disinfectants and required only 5–15 min of holding time to completely inactivate all of the pathogens (*A. baumannii*, *C. albicans*, *C. glabrata*, *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. epidermidis*, and *S. aureus*) (Figure 2). Holding time-dependent inactivation of pathogens can be seen in Figure 3A. These findings are comparable to bacterial responses found in earlier reports, wherein fungus was not inactivated even after 30 min of exposure.[23,31] In the present studies, both species of fungus (*C. albicans* and *C. glabrata*) were inactivated in ~15 min of exposure to (3 min) plasma-treated fluid, when present in their planktonic form, and in biofilm forms (3.2 min plasma-treated fluid; not shown; Figure 2), suggesting that the plasma-treated NAC solution is a more potent biocidal agent. Furthermore, the strains of *A. baumannii*, *S. epidermidis*, and *E. faecalis* used in this study were multidrug resistant and strong producers of biofilms. Therefore, their rapid inactivation by the plasma-treated NAC solution supports its powerful biocidal effect. It would be interesting to study the mechanisms of interactions of these antimicrobial solutions with pathogen or the biofilm matrix and embedded pathogens. We did not test yeast and therefore could not make any comparisons with results in the existing literature. In terms of antimicrobial properties, the treated solution was stable for three months of storage, both at cold (8 °C) or room (22–25 °C) temperature. Accelerated aging experiments confirmed that the antimicrobial property of this solution was retained at elevated temperatures such as 50 °C and that its shelf life was a minimum of two years (Table 1). These findings suggest that (i) both the short-lived reactive species and the long-lived species or products generated in fluid by plasma treatment are equally important and probably participate in biocidal activity and (ii) the species or the product(s) generated in fluid are stable for an extended period of time, and are responsible in part for the long-lasting antimicrobial properties of the plasma. These are novel and highly encouraging findings observed for the first time. Earlier, the treated water thought to be having effective antimicrobial effects only for few days to few weeks, and with the slightly less antimicrobial efficacies.[32] Although there could be many reasons for antimicrobial efficacy differences, the most noticeable reasons that can be correlated are the amount of fluid being treated, and the gap between primary electrode and the surface of the fluid. This gap was 42 mm versus 2 mm in the present studies.

During plasma treatments of liquid, the pH decreased from neutral to acidic (Figure 4B) and remained relatively stable during storage. The fact that the pH of deionized water, NAC, and even buffer solutions such as PBS drops to 2–2.6 suggests that a change in pH is not merely a phenomenon of the type of solute but of the oxidation properties of the plasma and of the creation of newer chemical species or products thereof. Several authors have reported such reduction in pH, and the acidification of solutions, but exact mechanism is still not fully understood.[26,32,33] It is anticipated that a drop in pH could be due in part to generation of nitric acid via interaction of RNS, ROS, and hydrolysis of water that gives rise to proton and hydroxyl radical. There are several other possibilities, and a detail chemical analysis is required to be studied. The extent of acidification of solution being treated by plasma depends, upon the amount of plasma energy ($J \cdot cm^{-2}$) being deposited, duration of the treatment, amount of liquid being treated, height (column) of liquid, immediate environment around the probe-generating plasma, and the gap between the primary electrode and the surface of the fluid. A relatively smaller amount of solution if treated with plasma for a relatively longer duration of time, with a narrow gap for plasma discharge, can make solution highly acidic, and saturated with acidifying species. We observed a significant drop in pH of PBS (inorganic buffer) too. During optimization studies we observed that unlike inorganic buffer such as PBS, organic buffers (e.g., HEPES buffer) resisted a drop in pH for relatively longer time. The previous literatures which have reported a moderate reduction in pH of PBS or water solution always had a plasma discharge gap of more than 2 mm through 45 mm, and the amount of liquid more than 1.5 ml through 10 ml.[26,32,33] This drastically changes the chemical properties and pH equation of these solutions.

We did not find any significant temperature changes in these treated fluids; in fact, the temperature was close to room temperature even after 3 min of treatment ($p > 0.05$)

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and was not related to the change in pH. Acidification of treated fluid could be the result of factors other than H_2O_2, nitric acid and nitrous acid, such as hydroperoxyl (HO_2) and peroxynitrous acid (HOONO), or a combination of all of these and other unknown species. Similar changes are reported in the gliding arc-mediated plasma technique,[30] which is different than the technique we used. The exact species and products generated in these fluids (deionized water, PBS, NAC) have not yet fully known, and detailed chemical characterization that needs authentication by several techniques is under way. Based on our previous studies, hydrogen peroxide is one of the major species generated.[14] In preliminary findings, we detected nitric acid as one of the possible products in treated deionized water. Therefore, we performed an experiment with nitric acid-mediated inactivation of *E. coli* wherein we tried to mimic equivalent concentrations of nitric acid, and the pH. However, the findings suggested that nitric acid inactivated about 10% of the total cell population (Figure 5D). The largest amount of H_2O_2 generated by plasma treatment was 1.66 mM, and we saw $<$20% of *E. coli* inactivation at this concentration with an adjusted pH of 2.5. The amount of H_2O_2 required for complete (100%) inactivation was 124 mM (0.38%); this solution had corresponded to a pH of 6.9. Even the mixture of H_2O_2 and nitric acid at low pH could not inactivate all *E. coli* cells (Figure 5E). Our findings of slightly more amounts of nitric acid or H_2O_2 were mainly due to the smaller amount of treatment fluid and prolonged plasma treatment of 3 min, with just a plasma discharge gap of 1.5–2 mm. Neither the acidity, nor nitric acid and H_2O_2 were able to inactivate pathogens significantly. Taken together, these findings suggest that the treated fluid may carries other trace long-lived species or products that are stabilized, which maintain their antimicrobial properties for extended period of time.

In brief, FE-DBD nonthermal plasma-treated fluids retain their antimicrobial effects for longer periods (three months by delay time and two years by solution aging) than earlier thought, using the present technique. This fluid-mediated plasma is faster than reported previously ($<$15 min holding time), and inactivates a wide range of multidrug-resistant bacteria and fungal pathogens, in both their planktonic and biofilm forms. The antimicrobial effect is due not only to a change in pH and in the H_2O_2 or nitric acid but also to the likely generation of additional species or products that are responsible for the powerful biocidal effect. Studies on detail chemical analysis are required to confirm these species or their products.

5. Authors' Contributions

U.E. carried out the antimicrobial assays and designed the upgraded versions of the plasma probe and fluid holders, antimicrobial solution maturation assays, and temperature/pH experiments. H.W. participated in in vitro chemical analysis assays. H.J. analyzed the chemical data and supervised chemical analysis. G.F. participated in the design of plasma probes and the related accessories of the nonthermal plasma system. A.B. participated in data analysis and corrected the manuscript. S.G. designed the overall study, supervised biological assay experiments, wrote the manuscript, and coordinated the project. All authors read and approved the final draft of the manuscript.

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