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Antimicrobial Efficacy of PBS Pretreated with Plasma Using N_2 and Air as Gas Source

Taejung Kim,[a] Hyemi Seo,[b] Hanjun Bae,[a] Sangsik Yang,[a,*] & Eunpyo Moon[b]

[a]Department of Electrical and Computer Engineering, Ajou University, Suwon, 443-749, Republic of Korea; [b]Department of Biological Science, Ajou University, Suwon, 443-749, Republic of Korea

*Address all correspondence to: Sangsik Yang, Department of Electrical and Computer Engineering, Ajou University, Suwon, 443-749, Republic of Korea; Tel.: +32-31-219-2481; Fax: +32-31-212-9531, E-mail: ssyang@ajou.ac.kr

ABSTRACT: Nonthermal atmospheric pressure plasma produced by a micromachining technique is shown to have sterilizing effects caused by the created active species. We modified the plasma system reported previously by our group, in which the components and antimicrobial activity of the plasma were confirmed for biomedical applications using a nonthermal atmospheric plasma system. Phosphate-buffered saline (PBS) solution was pretreated with the modified plasma system using N_2 and air as the gas source. We investigated components of the active species, amount of dissolved ozone produced according to the gas, and residual amount with respect to time. In the case of dissolved ozone, which is the most important component of antimicrobial activity, N_2 was confirmed to perform much better than air in both the residual and produced amount. The PBS solution pretreated with plasma, that is, PBS/N_2 plasma and PBS/air plasma, were shown to be effective at inhibiting pathogenic bacteria such as *Staphylococcus* and *Pseudomonas*. In particular, PBS/N_2 plasma showed a strong antibacterial effect and the duration of the effect was considerably long, > 24 h, implying that it is a promising gas source for practical use in plasma medicine.

KEY WORDS: nonthermal atmospheric pressure plasma jet, dissolved ozone, PBS solution pretreated with plasma, pathogenic bacteria, plasma medicine

I. INTRODUCTION

Plasma can be classified into thermal and nonthermal. Nonthermal plasma can be produced in lower pressures with lower energies, in comparison to thermal plasma. Nonthermal plasma is used in illuminants, displays, underwater discharge, and thin-film deposition.[1] Furthermore, the temperature of the reaction gas is similar to the atmosphere, which facilitates biological applications. Studies concerning the latter have been conducted by other groups. However, most of the studies reported unstable plasma discharge or small amounts of active species, indicating insufficient effects.[2] But Y. Lee et al. created nonthermal plasma equipment using micromachining processes, analyzed creation ingredients of the active species, and applied them to destroy cancer cells and verified the effects.[3]

The suppression of pathogenic bacteria growth is a vital task, in which molecular, biological, and biochemical knowledge of microbial control in science, medicine, and agriculture have been applied. To date, efforts have been made to control bacteria, and a variety of applications have been offered in various fields. Molecular and cellular effects were examined using the two pathogenic bacteria *Staphylococcus* and *Pseudomo-*

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nas, respectively. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are opportunistic pathogens, causing a variety of infections in hosts. They survive in a wide variety of environments and cause disease in humans, plants, and animals. *S. aureus* is one of the most common pathogenic bacteria that causes hospital-acquired bacterial infections and a range of illnesses, from minor skin infections to life-threatening diseases such as pneumonia, meningitis, and bacteremia.[4] In particular, *P. aeruginosa* is an opportunistic human pathogen that causes infections in burn wounds and the lungs of patients suffering from a genetic disease called cystic fibrosis.[5] Traditional antibacterial methods are based on heat treatment, chemical treatments with exposure to chlorine, ultraviolet (UV) radiation, or any combination of these.[6] However, these currently used conventional antibacterial methods are unsuitable for heat- and drug-resistant bacteria and can induce emission of toxic residual gases or radiation.[7] Thus, innovative methods must be developed to provide alternative strategies for human well-being. Recently, the use of plasma operating at atmospheric pressure in a variety of gas sources has gained prominence for medical and biological applications. Nonthermal plasma was generated using a number of gas sources, such as N_2 and air, either individually or in combination.[8] These atmospheric-pressure plasmas have been reported to efficiently deactivate microorganisms including pathogenic bacteria.[9] The advantages of nonthermal plasma include the stability of the low-temperature effect, nontoxicity, and a broad spectrum of deactivation efficiency in pathogenic bacteria.

In this article, we use creation ingredients of postactive species that change diameter, number, and arrangement of electrode holes in nonthermal plasma equipment, as reported by Lee et al.[3] Further, phosphate-buffered saline (PBS) solution was processed to confirm the created and remaining amounts of dissolved ozone, which is effective for sterilization. Additionally, PBS/N_2 plasma and PBS/air plasma, where PBS solution was pretreated with N_2 or air plasma for 5 min, were effective for pathogenic bacteria inhibition. Nonthermal plasma using this new mechanism is needed for the development of next-generation microbial control, where pathogen-controlled possibilities are ample.

II. EXPERIMENTAL SETUP

A. Structure of the Plasma-Jet Electrode

Figure 1 shows the structure of a nonthermal atmospheric plasma-jet electrode. The anode substrate is a 400-μm-thick glass wafer of 10-mm diameter with a 100-μm-thick nickel colayer. The cathode is a cylindrical metal gas inlet. The structure of the plasma jet is similar to the previous model in Lee et al.,[3] except that the electrode has 37 holes with a diameter of 400 μm arranged in a honeycomb shape.

B. Plasma Treatment and Measurement

Figure 2(a) shows the nonthermal atmospheric plasma system consisting of a plasma nozzle, power supply generating an alternating current voltage of 15 kV at 15 kHz, and

FIG. 1: Cross-sectional schematic view of the plasma jet system and image of the plasma jet electrode

gas supply with a gas flow meter. N_2 gas and air are supplied to the device at a flow rate of 8 L/min. Figure 2(b) shows the electrode and light intensity profile during N_2 and air plasma discharge. We treated 1 mL of PBS solution with plasma jet for 5 min, with the nozzle 1 cm away from the liquid surface. Active species were dissolved in the PBS during plasma discharge, including dissolved ozone after a lapse of time following plasma treatment, which was measured with a dissolved ozone detector (OZ-20; DAKK-TOA, Japan).

III. RESULTS

A. Dissolved Ozone

Figure 3 shows the amount of dissolved ozone generated after plasma treatment in PBS solution as well as the residual amount according to time. Results are based on three experimental runs, to confirm reproducibility. When N_2 gas was used, dis-

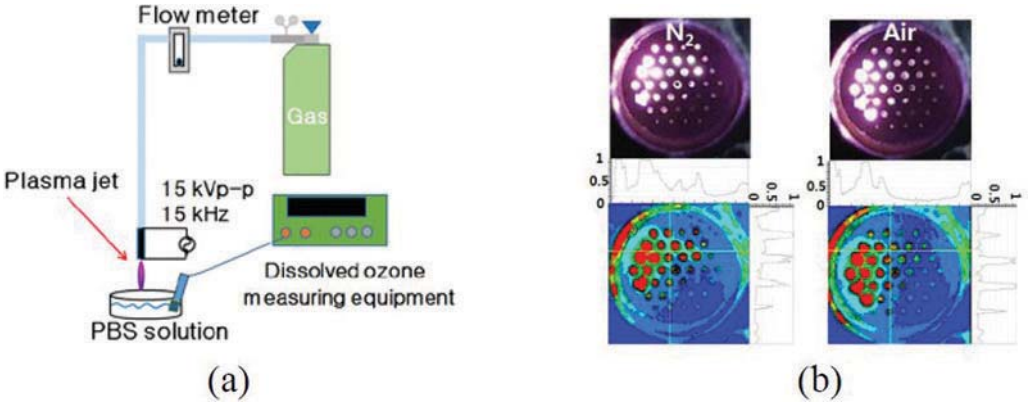

FIG. 2: (a) Schematic view of the experimental setup; (b) images of N2 and air during discharge

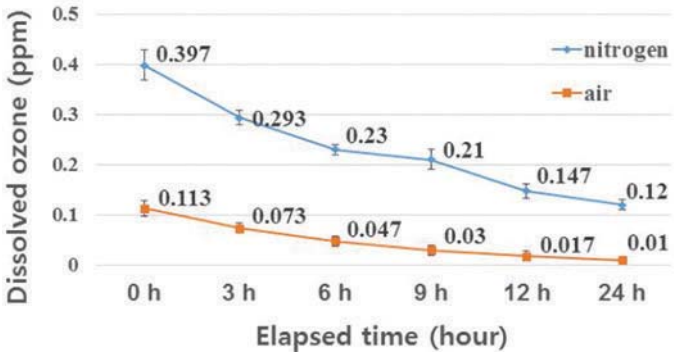

FIG. 3: Amount of dissolved ozone in PBS solution treated with plasma; ppm, parts per million

solved ozone of about 0.4 parts per million (ppm) was generated immediately after the plasma treatment. As time passed, the amount of dissolved ozone decreased, but ~ 0.12 ppm of dissolved ozone remained after 24 h. When air was used, the amount of dissolved ozone was insufficient. Dissolved ozone of ~ 0.11 ppm was generated immediately after the plasma treatment, but the residual amount of dissolved ozone decreased with time, and it was confirmed that dissolved ozone of about 0.01 ppm did not remain after 24 h.

More ozone occurred in nitrogen gas compared to air because the nitrogen molecule from the nitrogen gas has higher energy and collides with the oxygen molecule to separate the molecule into an oxygen atom.[10] The oxygen atom recombines with the atomic molecule or atom to create ozone, with outstanding antibacterial effects.[11] Based on this experiment, antimicrobial activity was confirmed from the bacterium treatment experiment.

B. Bacterial Viability Assay

For Fig. 4(a) and (b), the control is untreated, but plasma using N_2 or air was treated. We also carried out a cell viability assay using a LIVE/DEAD staining kit to visualize the antibacterial activity of the PBS/N_2 plasma and PBS/air plasma. Propidium iodide, a nucleic acid probe that is red fluorescent in color, is impermeable to the cell membrane and thus only stains dead bacteria with damaged cell membranes.[12] When used alone, the SYTO 9 labels bacteria with both intact and damaged membranes. However, propidium iodide penetrates only bacteria with damaged membranes, competing with SYTO 9 for nucleic acid binding sites when both dyes are present.[13] When mixed in recommended proportions, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. We used a LIVE/DEAD Bacterial Viability assay to show inactivation of pathogenic bacteria using nonthermal PBS/N_2 and PBS/air plasma, as shown in Fig. 4. Here, we see images of *P. aeruginosa* PA01 [Fig. 4(a)] and *S. aureus* [Fig. 4(b)] in merged images (top

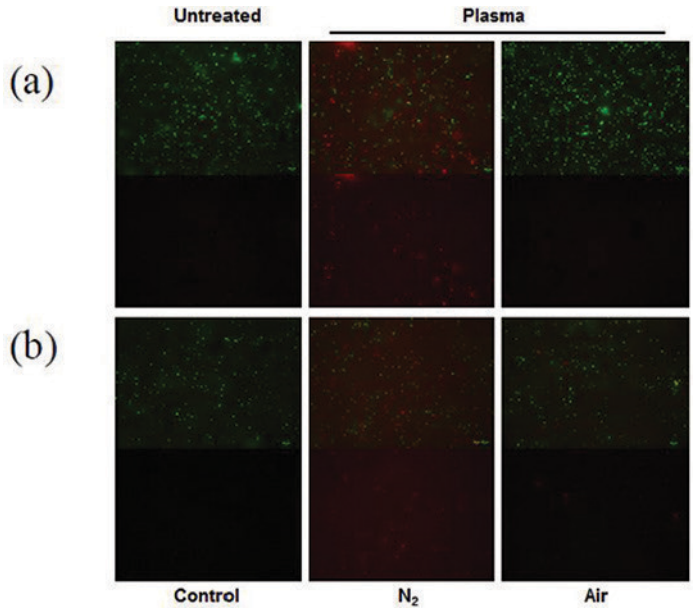

FIG. 4: Live/dead bacterial viability assay of (a) *P. aeruginosa* PA01 and (b) *S. aureus*

panel) and red images of dead cells (lower panel). The number of dead cells increased by more than 50% after 5 min of PBS/N_2 plasma and almost exhibited resistance after 5 min of PBS/air plasma treatment.

C. Duration Test

The duration of antimicrobial activity of PBS/N_2 and PBS/air plasma was examined for up to 24 h (Fig. 5). Under the PBS/air plasma condition, *P. aeruginosa* PA01 underwent a weak antibacterial effect for up 3 h, which was similar to the untreated control after 6 h [Fig. 5 (a)]. *S. aureus* has a weak antibacterial effect for up to 6 h [Fig. 5 (b)]. In the PBS/N_2 plasma condition, both pathogenic bacteria showed strong antibacterial effects that lasted up to 24 h. In other words, we confirmed that the antibacterial effect of PBS/N_2 plasma lasted for 24 h.

IV. CONCLUSION

This research confirms the antibacterial effects of nonthermal atmospheric pressure plasma with respect to time. For the dissolved ozone, we confirmed that it has a persistency of > 24 h, and its antibacterial effects also show persistency. In addition to the amount of dissolved ozone, the remaining amount with respect to time was larger when we used nitrogen gas instead of air.

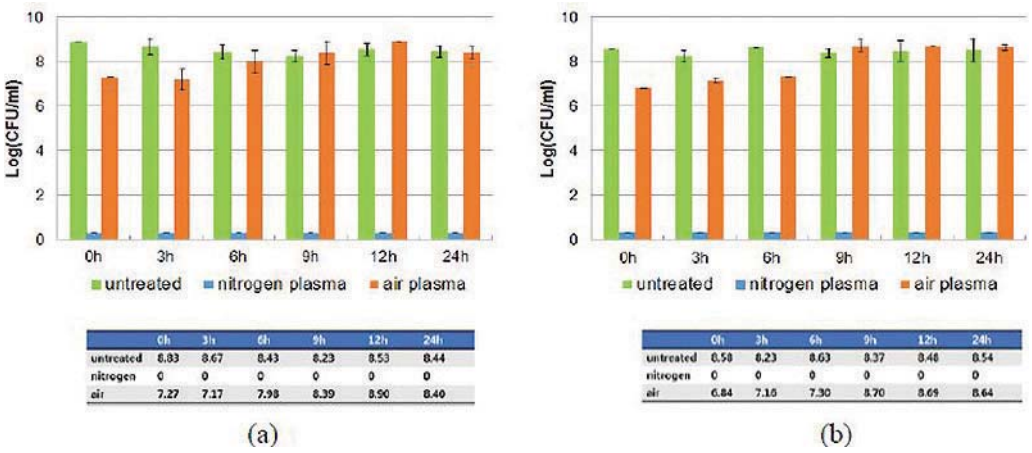

FIG. 5: Duration test of (a) *P. aeruginosa* PA01 and (b) *S. aureus*

It identified PBS solution exposed to N_2 or air plasma antibacterial activity and cellular characteristics through various gas sources conditions. The LIVE/DEAD bacterial viability assay and statistical analysis results suggest that this nonthermal atmospheric pressure plasma can be a potential candidate for future biological control. PBS/N_2 plasma facilitates more effective control of pathogens such as *S. aureus* and *P. aeruginosa* PA01, both of which affect infections.

Our method will have an important role in the next-generation of microbial control because it is highly effective compared to traditional bacteria control methods, such as UV and antibiotics. Moreover, this study identified that PBS solution exposed to N_2 or air plasma is needed for the development of next-generation microbial control, and that pathogen-controlled possibilities are ample and we plan to perform follow-up studies on improving the plasma treatment of solutions.

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