Plasma Medicine, 2(4): 237–247 (2012)

Bacterial Inactivation in Liquids Using Multi-Gas Plasmas

Toshihiro Takamatsu,[1,*] Akitsugu Kawate,[1] Kodai Uehara,[1] Takaya Oshita,[1] Hidekazu Miyahara,[1] Danil Dobrynin,[2] Gregory Fridman,[3] Alexander Fridman,[4] & Akitoshi Okino[1]

[1]Department of Energy Sciences, Tokyo Institute of Technology, Yokohama, Japan; [2]Department Electrical and Computer Engineering, College of Engineering, Drexel University, Philadelphia, Pennsylvania; [3]School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, Pennsylvania; [4]Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, Pennsylvania

*Address all correspondence to: Toshihiro Takamatsu, Department of Energy Sciences, Tokyo Institute of Technology, J2-32, 4259 Nagatsuta, Midori-ku, Yokohama, Japan, 226-8502; toshihiro@plasma.es.titech.ac.jp

ABSTRACT: Using a multi-gas plasma jet, we generated plasmas of various gas species such as argon, oxygen, nitrogen, carbon dioxide, and air. Photometric measurements of color-forming reactions were used to identify singlet oxygen, OH radicals, hydrogen peroxide, NO radicals, nitrite, and nitrate, which are important sterilization agents that are generated in the liquid phase. Oxygen plasma generated the largest amount of singlet oxygen, OH radicals, and hydrogen peroxide. Air plasma generated NO radicals, nitrite, and nitrate. The pH of air plasma–treated water for 120 s dropped below 3.0. The air plasma sterilized *Escherichia coli* in distilled water after 120 s of treatment. In addition, when the initial pH was fixed below 3.6, *E. coli* was more effectively sterilized by oxygen plasma. Furthermore, dimethylsulfoxide, which is an OH radical scavenger, suppressed the sterilization effect of oxygen plasma.

KEY WORDS: multi-gas plasma, nonthermal plasma, sterilization, pH measurement, reactive oxygen and nitrogen species, plasma medicine

I. INTRODUCTION

In recent years, atmospheric nonthermal plasma has attracted attention in the medical field because of its use in effective and fast sterilization,[1] blood coagulation,[2] and wound treatment.[3] It is thought that the active species, which are generated by the plasma,[4] significantly contribute to these end results.[5] However, the generated active species depend on the plasma gas species, with conventional plasma sources placing limits on the generation of active gas species. In addition, the effect of gas composition on the plasma is not well studied. Our group developed a multi-gas plasma jet source in 2010. The developed plasma source can generate a stable atmospheric plasma jet using different gases such as helium, argon, oxygen, nitrogen, carbon dioxide, air, and their mixtures without damaging the target materials either thermally or by electric discharge. In surface treatments using the plasma jet, we observed hydrophilization due to the plasma gas species.[6] We anticipate that the sterilization effect will

1947-5764/12/$35.00

depend on the plasma gas species. Thus, in this study, we used the multi-gas plasma jet to investigate generated active species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), the pH of plasma-treated liquids, and the inactivation of bacteria.

II. MATERIALS AND METHODS

A. Atmospheric Multi-Gas Plasma Jet

The experiments were conducted using the multi-gas plasma jet source.[6] The body of the device (83 mm in length) is grounded and the interior high-voltage electrode is connected to an AC power supply (Plasma Concept Tokyo Inc.) of 16 kHz and 9 kV. The generated plasma flows through a 1-mm hole with a flow rate of 1 L/min. The plasma jet source can generate stable atmospheric plasma of various gas species such as argon, oxygen, nitrogen, carbon dioxide, air, and their mixtures at low gas temperature (<57 °C) and around 10 W.

B. Characterization of the Various Plasmas

The active species can be identified using photometric measurements of color-forming reactions.[7] A fluorescence spectrophotometer (LS-55; Perkin Elmer) and an ultraviolet/visible (UV-VIS) spectrometer (U-2910; Hitachi High-Technologies Co.) were used to estimate the amount of the generated ROS (singlet oxygen, OH radicals, and H_2O_2) and RNS (NO radicals, nitrite, and nitrate).

Solutions of Amplex UltraRed Reagent, fluorescent probe Singlet Oxygen Sensor Green (Molecular Probes Inc.), and terephthalic acid (Sigma-Aldrich) were used to investigate the ROS. These solutions react with specific ROS and produce fluorescence at a particular wavelength. The Amplex UltraRed Reagent can detect H_2O_2 at excitation and emission settings of 530 nm and 590 nm, respectively.[8] The reagent was adjusted to 100 μM in phosphate-buffered saline (PBS) that contained 0.1 U/ml of horseradish peroxidase, and the intensity of fluorescence was calibrated with 3% hydrogen peroxide solution (Merck Millipore). Singlet Oxygen Sensor Green can detect singlet oxygen at 504 nm of fluorescence produced by 525 nm of excitation and it was adjusted to 10 μM in PBS.[9] Terephthalic acid converts to 2-hydroxyterephthalic acid by reacting with OH radicals[10,11] at emission and excitation settings of 425 nm and 310 nm, respectively. Terephthalic acid was prepared by dissolving the acid in distilled water containing NaOH (Sigma-Aldrich). The concentrations of terephthalic acid and NaOH were 0.2 mM and 1.4 mM, respectively.

A solution of DAF-2 (Cayman Chemical), which was adjusted to 1 μM in PBS, was used to identify the RNS. The reagent can detect nitric oxide (NO) radicals at emission and excitation settings of 538 nm and 485 nm, respectively. The nitrite and nitrate concentrations in liquid were measured using nitrite and nitrate test kits (TNTplus 840 and TNTplus 835; Hach Company). The nitrite and nitrate in the solution react with these

reagents and the respective absorbances are 515 nm and 330 nm. For plasma treatment, these reagents were treated with nitrogen, oxygen, carbon dioxide, argon, and 20% oxygen in nitrogen (dry-air gas) plasmas. The solutions were added to 96-well culture dishes diluted to 100 µl and directly treated as shown in Fig. 1. The multi-gas plasma jet outlet was fixed at a distance of 9 mm from the liquid surface.

The pH is also an important factor in sterilization.[12] Distilled water was treated from 0 to 120 s using the different gas plasmas, and the pH was measured using a pH meter (Orion DUAL STAR meter; Thermo Scientific).

C. Inactivation of Bacterial Pathogens by the Various Plasmas

The inactivation of *Escherichia coli* (MG1655) was investigated using the multi-gas plasma jet. Incubated *E. coli* cells in Luria-Bertani medium were added to the 96-well culture dishes and diluted about 100 times with distilled water from the initial population of 8 digits. The population was finally fixed at 3.0×10^6 in 100 µl. The bacteria in the liquid were treated with argon, oxygen, nitrogen, carbon dioxide, and 20% oxygen in nitrogen (dry-air conditions) plasma.

To investigate the dependency of bacterial inactivation on pH, *E. coli* in 100 µl of citrate buffer fixed at various pH levels from 3.2 to 4.8 was irradiated with the different gas plasmas for 60 s. In addition, to void the inactivation effect owing to ROS, 10 mM of dimethylsulfoxide (DMSO), which is an OH radical scavenger, was added and ex-

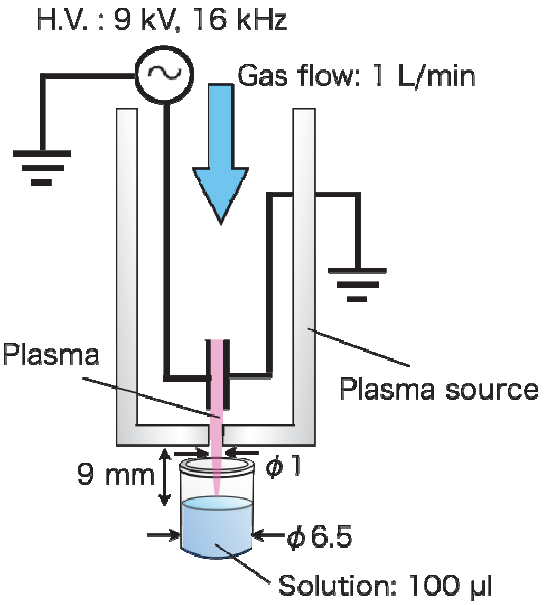

FIG. 1: Plasma irradiation of liquid. The liquid volume is 100 µl and the distance from the liquid surface is 9 mm.

posed to oxygen plasma for 60 s at pH 3.2. Colony counting was performed to quantify the bacterial inactivation. After the plasma treatment, *E. coli* in the culture dish were distributed to agar medium and incubated for 18 h at 37°C.

III. RESULTS AND DISCUSSION

A. Active Species Generation by Various Gas Plasmas

There are many active species in plasmas such as (1) to (4) in Table 1.[4] The ROS are generated by plasmas such as (5) to (9) in Table 1. Figures 2–4 show the fluorescence intensity of the reagent after reacting with singlet oxygen and OH radicals and the amount of hydrogen peroxide after each gas plasma treatment. The production of active species depends on the gas species. The oxygen plasma generated the largest amount of singlet oxygen, OH radicals, and hydrogen peroxide. The amount of hydrogen peroxide after 60-s oxygen plasma treatment was 53 mg/L. The carbon dioxide, argon, and nitrogen plasmas also generated reactive species. However, the generation of OH radicals and hydrogen peroxide after the air plasma treatment were the lowest.

Figures 5–7 show the fluorescence intensity of the reagents after reacting with the NO radical and the generated amounts of nitrate and nitrite for each gas plasma. Air plasma generated NO radicals, nitrite, and nitrate. The amounts of nitrite and nitrate after 60 s air plasma treatment were 82 mg/L and 160 mg/L, respectively. However, the generated amounts by nitrogen gas plasma treatment were lower than the detection limit, indicating that air plasma can generate RNS such as NO radicals and NO_2 in gas phases such as (10) in Table 1. Moreover, the NO radical reacts with DAF-2 and the RNS change to nitrite and nitrate by reacting with water as in (11) to (13) in Table 1.

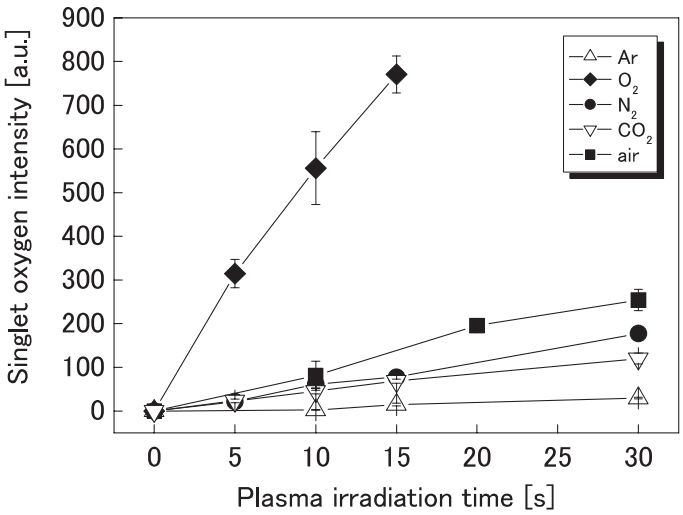

FIG. 2: Singlet oxygen generation per plasma (excitation/emission of 504/525 nm).

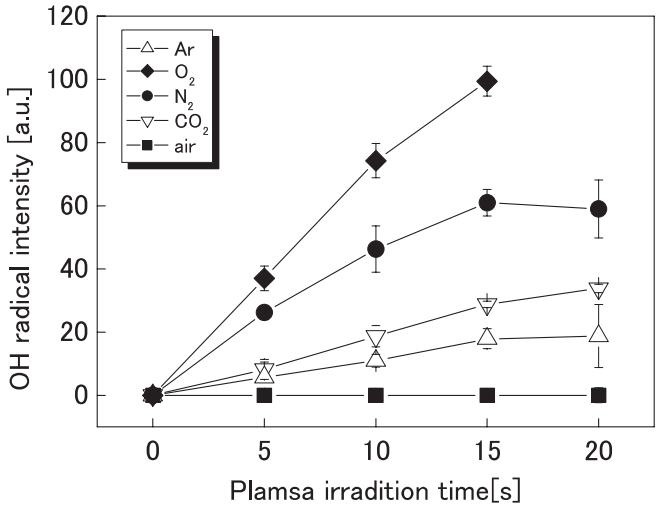

FIG. 3: OH radical generation per plasma (excitation/emission of 310/425 nm).

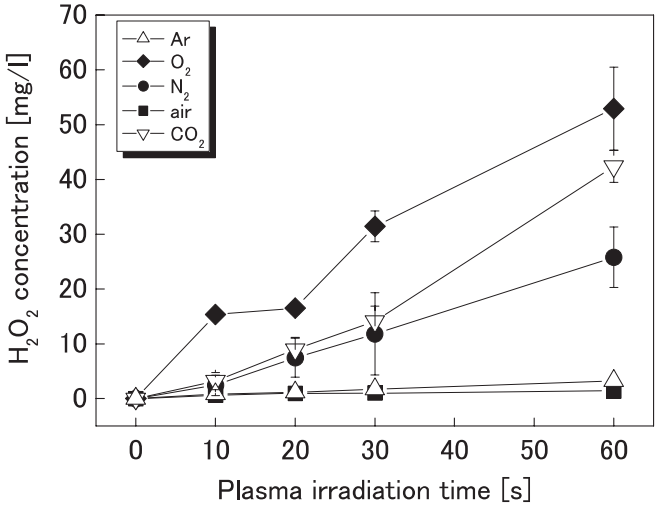

FIG. 4: Hydrogen peroxide generation per plasma treatment (excitation/emission of 530/590 nm).

B. pH Change by Irradiation with Different Plasmas

Figure 8 shows the pH change in distilled water after each gas plasma treatment. The initial pH of the distilled water was approximately 5.3. In the case of argon and nitrogen plasma treatment, pH increased to around 7.0 and 6.0 after 120 s, respectively. It is thought that dissolved hydrogen carbonate ions were removed by the flowing gas. By contrast, oxygen, carbon dioxide, and air plasma decreased pH to approximately 4.7,

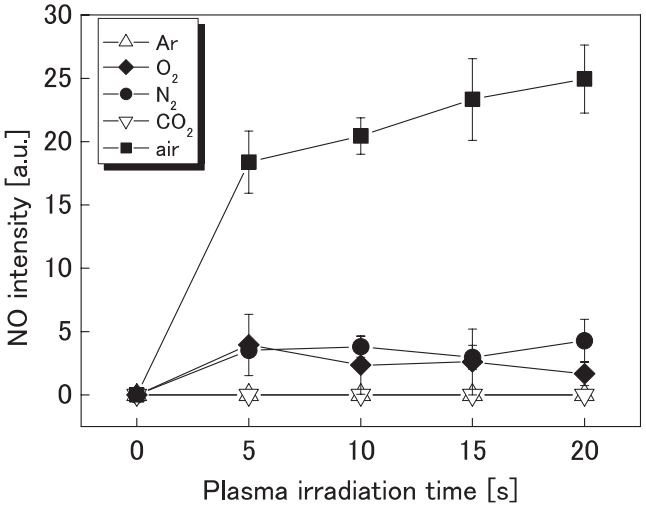

FIG. 5: NO radical generation per plasma treatment (excitation/emission of 485/538 nm).

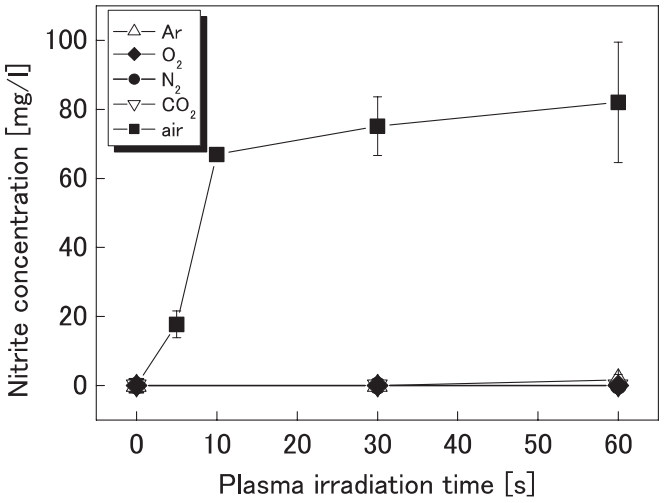

FIG. 6: Nitrite generation per plasma treatment (absorbance of 515 nm).

3.5, and 2.6 after 120 s, respectively. In the case of oxygen plasma treatment, the data suggest that H_2O changes to H_3O^+ and the OH radical reacts with the oxygen plasma to produce H_2O_2 via two OH radicals reactions such as (9), (14), and (15) in Table 1. The concentration of the hydrogen carbonate ion increased after the carbon dioxide plasma treatment. Conversely, the generated nitrite and nitrate in water decreased the pH after the air plasma treatment.

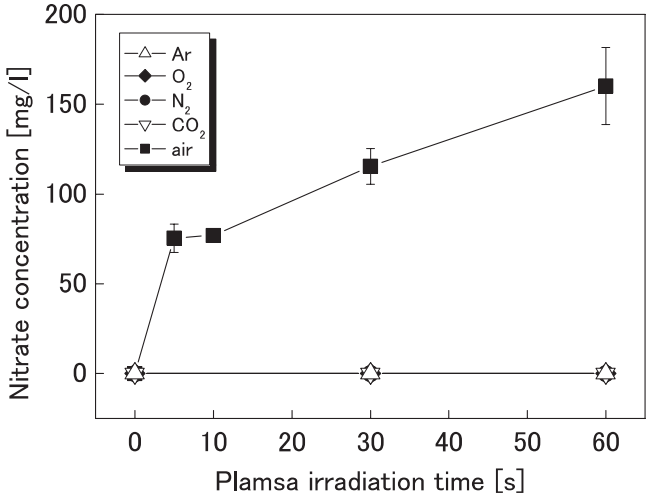

FIG. 7: Nitrate generation per plasma treatment (absorbance of 330 nm).

TABLE 1. Key reactions leading to the formation and destruction of active species in the plasma jet

Reaction	Number
Electron impact reactions	
$Ar + e \rightarrow Ar^* + e$	(1)
$O_2 + e \rightarrow 2O + e$	(2)
$N_2 + e \rightarrow 2N + e$	(3)
$CO_2 + e \rightarrow CO + O + e$	(4)
Radical involved reactions	
$2O \rightarrow {}^1O_2$	(5)
$Ar^* + H_2O \rightarrow Ar + HO\bullet + H\bullet$	(6)
$O + H_2O \rightarrow 2HO$	(7)
$2N + 2H_2O \rightarrow N_2 + 2HO\bullet + 2H$	(8)
$HO\bullet + HO\bullet \rightarrow H_2O_2$	(9)
$O + N \rightarrow NO$	(10)
Ion reactions	
$4NO + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^-$	(11)
$2NO_2 + H_2O \rightarrow 2H^+ + NO_2^- + NO_3$	(12)
$NO_2^- + HO\bullet \rightarrow H^+ + NO_3$	(13)
$O_2^+ + H_2O \rightarrow H_2O^+ + O_2$	(14)
$H_2O^+ + H_2O \rightarrow H_3O^+ + HO\bullet$	(15)

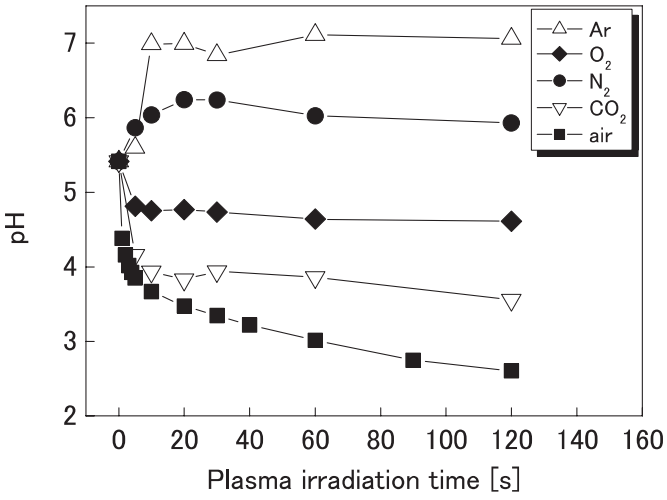

FIG. 8: pH change per plasma treatment.

C. Inactivation of *E. coli* by Various Plasmas

The bacterial inactivation effect was investigated by plasma treatment of distilled water containing 3×10^6 of *E. coli*. Figure 9 shows the inactivation effect of the various plasmas on *E. coli* in distilled water. Air and carbon dioxide plasmas resulted in the inactivation of *E. coli*. By contrast, gas species such as argon, oxygen, and nitrogen had a bacterial inactivation effect of less than an order of magnitude on *E. coli*. The generation of ROS such as OH radicals and hydrogen peroxide by air plasma were low. On the other hand, the plasma can generate RNS such as NO radicals, nitrite, and nitrate. Therefore, our results suggest that a RNS such as $ONOO^-$ was generated and has a bacterial inactivation effect[7] under low pH.

To investigate the pH dependence of the bacteria inactivation, *E. coli* in 100 µl of citrate buffer fixed at different pH levels was treated with various gas plasmas for 60 s. The results are shown in Fig. 10. When the pH is 3.2, *E. coli* is inactivated by all gas plasmas used. Specifically, oxygen plasma more effectively sterilizes *E. coli* when pH is <3.6. For oxygen plasma, we investigated the bacterial inactivation effect of the ROS for a bacterial suspension of 3.2 pH that contained 10 mM DMSO, which is an OH radical scavenger. We found that the bacterial inactivation effect was suppressed by DMSO as shown in Fig. 11. This suggests that low pH enhances the bacterial inactivation effect of the ROS and that ROS are more effective than the RNS.

IV. CONCLUSION

We investigated the characteristics and bacterial inactivation capacity of multi-gas plasma. We used a fluorescence spectrometer and UV-VIS spectrometer to identify the reactive species and quantify the amounts of singlet oxygen, OH radicals, hydrogen

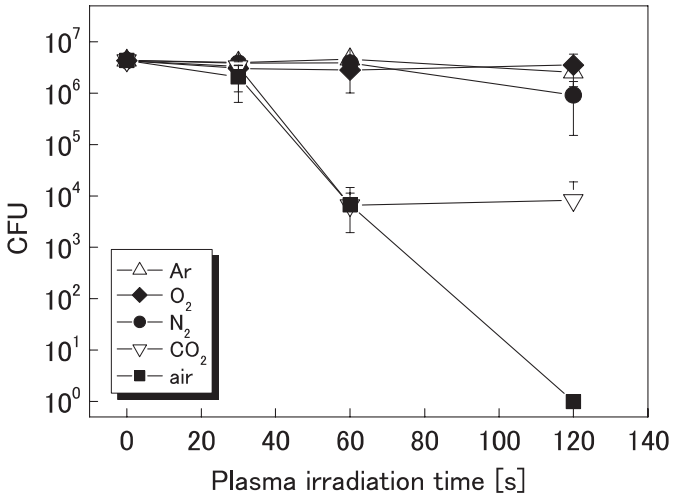

FIG. 9: Sterilization effect on *E. coli* in distilled water per plasma treatment.

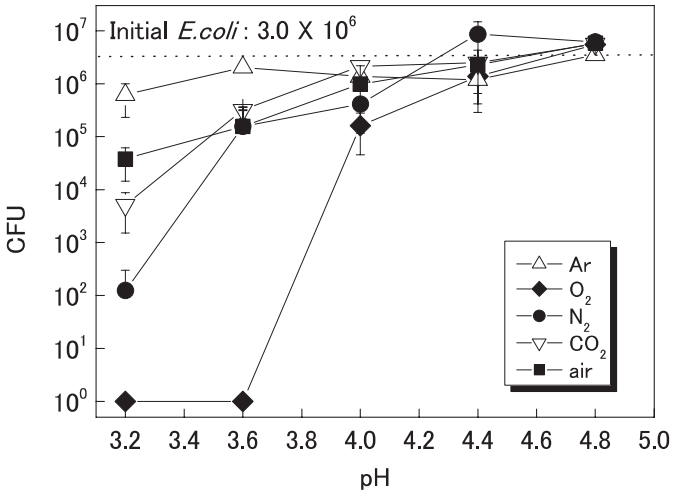

FIG. 10: Dependence of the bacterial inactivation on the initial pH.

peroxide, NO radicals, nitrite, and nitrate. We found that the amount of reactive species depended on the plasma gas species. The oxygen plasma generated the largest amount of singlet oxygen, OH radicals, and hydrogen peroxide. However, RNS such as NO radicals, nitrite, and nitrate, which are generated by the oxygen plasma, were below the detection limit. The generated OH radicals and hydrogen peroxide by air plasma were also below the detection limit; however, the NO radicals, nitrite, and nitrate were not below the detection limit.

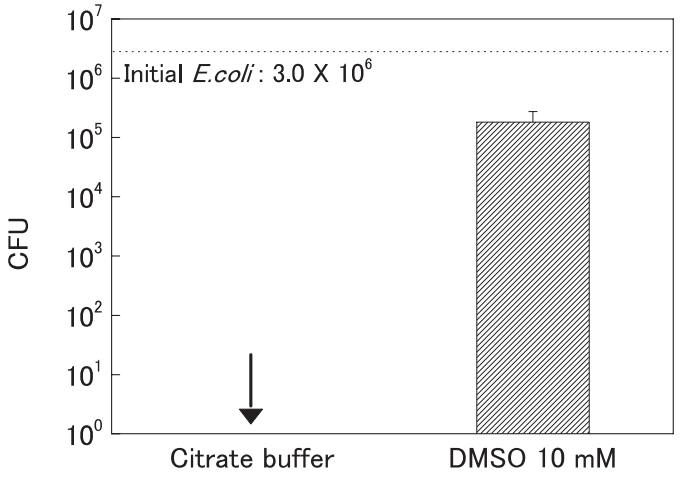

FIG. 11: Dependence of bacteria inactivation on the OH radical scavenger. The oxygen plasma exposure time is 60 s and the pH is 3.2.

The pH value of plasma-treated water also depended on the gas species. The pH of air plasma-treated water decreased to 2.6 after 120 s. In addition, air plasma sterilized *E. coli* in distilled water. When pH was 3.2, *E. coli* was inactivated by all of the gas plasmas after 60-s treatment. *E. coli* was especially more effectively sterilized by oxygen plasma when pH was <3.6. In addition, the sterilization effect was suppressed by 10 mM DMSO, which is an OH radical scavenger. Therefore, we suggest that low pH and ROS is the effective combination for sterilization.

ACKNOWLEDGMENTS

This work was partially supported by Plasma Concept Tokyo Inc. The authors thank the company for support and collaboration.

REFERENCES

1. Dobrynin D, Fridman G, Mukhin YV, Wynosky-Dolfi MA, Rieger J, Rest RF, Gutsol AF, Fridman A. Cold plasma inactivation of bacillus cereus and bacillus anthracis (anthrax) spores. IEEE Trans Plasma Sci. 2010;38:1878–84.
2. Kalghatgi SU, Fridman G, Cooper M, Nagaraj G, Peddinghaus M, Balasubramanian M, Vasilets VN, Gutsol A F, Fridman A, Friedman G. Mechanism of blood coagulation by nonthermal atmospheric pressure dielectric barrier discharge plasma. IEEE Trans Plasma Sci. 2007;35:1559–66.
3. Isbary G Morfill G, Schmidt HU, Georgi M, Ramrath K, Heinlin J, Karrer S, Landthaler M, Shimizu T, Steffes B, Bunk W, Monetti R, Zimmermann JL, Pompl R, Stolz W. A first prospective randomized controlled trial to decrease bacterial load using cold atmospheric argon plasma on chronic wounds in patients. Br J Dermatol. 2010;163:78–82.

4. Wang C and Srivastava N. OH number densities and plasma jet behavior in atmospheric microwave plasma jets operating with different plasma gases (Ar, Ar/N2, and Ar/O2). Eur Phys J D. 2010;60:465–77.
5. Perni S, Shama G, Hobman JL, Lund PA, Kershaw CJ, Hidalgo-Arroyo GA, Penn CW, Deng XT, Walsh JL, Kong MG. Probing bactericidal mechanisms induced by cold atmospheric plasmas with Escherichia coli mutants. Appl Phys Lett. 2007;90:073902.
6. Takamatsu T, Hirai H, Sasaki R, Miyahara H, Okino A. Surface hydrophilization of polyimide films using atmospheric damage-free multigas plasma jet source. IEEE Trans Plasma Sci. 2013;41:119–25.
7. Oehmigen K, Winter J, Hahnel M, Wilke C, Brandenburg R, Weltmann KD, Woedtke T. Estimation of possible mechanisms of Escherichia coli inactivation by plasma treated sodium chloride solution. Plasma Processes Polym. 2011;8:904–13.
8. Zhu A, Romero R, Petty HR. Amplex UltraRed enhances the sensitivity of fluorimetric pyruvate detection. Anal Biochem. 2010;403:123–5.
9. Ragàs X, Jiménez-Banzo A, Sánchez-García D, Batllori X, Nonell S. Singlet oxygen photosensitisation by the fluorescent probe Singlet Oxygen Sensor Green. Chem Commun. 2009;20:2920–2.
10. Ishibashi K, Fujishima A, Watanabe T, Hashimoto K. Quantum yields of active oxidative species formed on TiO_2 photocatalyst. J Photochem Photobiol A. 2000;134:139–42.
11. Kanazawa S, Kawano H, Watanabe S, Furuki T, Akamine S, Ichiki R, Ohkubo T, Kocik M, Mizeraczyk J. Plasma Sources Sci Technol. 2011;20:034010.
12. Ikawa S, Kitano K, Hamaguchi S. Effects of pH on bacterial inactivation in aqueous solutions due to low-temperature atmospheric pressure plasma application. Plasma Processes Polym. 2010;7:33–42.