Sterilization in liquids by air plasma under intermittent discharge

Kairi MURAMATSU*,**, Takehiko SATO*, Tomoki NAKAJIMA*, Toshikatsu NAGASAWA***, Tatsuyuki NAKATANI**** and Shigeru FUJIMURA*****

*Institute of Fluid Science, Tohoku University, 2-1-1 Katahira, Aoba, Sendai, Miyagi 980-8577, Japan
E-mail: sato@ifs.tohoku.ac.jp
**Graduate School of Engineering, Tohoku University, 6-6-01 Aoba, Aramaki, Aoba, Sendai, Miyagi 980-8579, Japan
***Hirayama Manufacturing Corporation, 2-6-5 Toyono, Kasukabe, Saitama 344-0014, Japan
****Okayama University of Science, 1-1 Ridaicho, Kita, Okayama, Okayama 700-0005, Japan
*****Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba, Sendai, Miyagi 981-8558, Japan

Abstract

In recent years, cold atmospheric plasma has received attention for medical applications like sterilization, injury treatment, and cancer cell treatment. Plasma sterilization is effective against a wide range of microorganisms. Plasma sterilization devices for contact lens have developed over the years. The purpose of this study is to clarify sterilization characteristics under high ozone, low nitric acid, and nitrous acid concentration in liquids. These concentrations are caused by a new cycle of intermittent discharges. Sterilization efficacy is evaluated by inactivating bacillus spores. Concentrations of HNO3, HNO2 and O3 in water were measured using a pack test. Dielectric surface temperature is measured using a thermocouple and thermography to study relationships between the concentration and the temperature. Sterilization of spores in water was achieved for 80 min. Increasing ozone concentration promotes the generation of hydrogen peroxide, which is a well-known factor for sterilization, and •OH, which has strong oxidizing power, however, the concentration of hydrogen peroxide is too low for sterilization, and the life time of •OH is as short as microseconds. Meanwhile, a decrease in the concentration of nitric acid and nitrous acid suppresses the formation of peroxynitrite, which has very strong oxidizing powers and a lifetime of 1 sec. These results suggest a critical role of peroxynitrite in sterilization.

Keywords : Plasma, Dielectric barrier discharge, Sterilization, Spore, Reactive species

1. Introduction

In recent years, cold atmospheric plasma has received attention for medical applications like sterilization, injury treatment, and cancer cell treatment. Plasma sterilization is effective against a wide range of microorganisms. It is reported that the shape of Escherichia coli (E. coli) shrinks and is sterilized after plasma treatment (Shimizu et al., 2012). It is also reported that the H2O2 and nitrite levels correlated with bacterial log reductions from 15 min exposures with cells in inactivation of E. coli (Traylor et al., 2011). E. coli was inactivated with a log value reduction of more than 4 by species in plasma activated water including H2O2, NO2-, NO3- and peroxynitrite which is shown to originate mainly from the reaction of H2O2 and NO2- with concentration of around 20 µM under exposure to 30 min air plasma (Zhou et al., 2018). Once inside the cell, ONOOH can damage DNA, lipids, and proteins via direct oxidation reactions or by decomposing to OH and NO2• (Huang et al., 2018). In addition, Bacillus spore, which has extreme resistance towards wet and dry heat, irradiation, UV, high pressure, and chemicals, (Setlow, 2014) is inactivated after plasma treatments with air, N2, O2 and CO2 (Hertwig et al., 2017). Furthermore, it is done that adenovirus, a non-enveloped double-stranded DNA virus, is inactivated within 240 sec of plasma treatment in the solution (Zimmermann et al., 2011). At atmospheric pressure, bacterial spore is inactivated by chemically reactive species such as free radicals, metastable atoms, and molecules, while UV photons play only a minor role or no role in the inactivation (Boudam et al., 2006). For atmospheric pressure air plasmas, oxygen and nitrogen-based reactive species such as O, •OH, O3, NO, and NO2 play the most
important role in the inactivation process (Laroussi and Leipold, 2004). *Staphylococcus aureus* (*S. aureus*) was inactivated after pH values decreased to about 4.5 and this was attributed to the high oxidizing potential of perhydroxyl radical (*HOO•*) (Liu et al., 2010). It was found that there existed a critical pH for bactericidal effects which is approximately 4.7 and that the presence of superoxide anion radicals is essential for the bacterial inactivation (Ikawa et al., 2010). The inactivation mechanism was reported that reactive oxygen species in plasma-activated water affect and damage the redox state of antioxidants first, penetrated the membrane of *S. aureus*, then damaged the cell structure, and resulted in the death of *S. aureus* (Zhang et al., 2013). Furthermore, acidification is mainly a result of the generation of nitric acid induced by reactive nitrogen species from the plasma phase, but for antimicrobial activity additional action of reactive oxygen species must be necessary (Oehmigen et al., 2010). However, it was reported that the antimicrobial effect correlates well with aqueous-phase ozone concentration, but not with pH or concentration of hydrogen peroxide, nitrite, or nitrate (Pavlovich et al., 2013).

Plasma sterilization devices for contact lens has developed over the years (Sato et al., 2018). In the development, generation of nitrogen oxides in liquids has been one of issues and these are likely to be generated if the surface temperature of the dielectric is high. It is necessary to suppress the temperature rise because the temperature rise of the electrode enhances the decomposition of ozone and increases the generation of nitrogen oxides (Kobayashi et al., 2012). Furthermore, we think that intermittent discharge can suppress the temperature rise regardless of the electrode material and it is easy to control intervals, so it has a wide range of future applications to reduce the concentration of nitrogen oxides. Hence, intermittent discharge with a cycle of a minute of discharge and a break of 2 min is adopted in the study. This discharge extends the completion time of sterilization the spore from 20 min to 58 min in water compared to continuous discharge. Furthermore, concentrations of nitric acid decreased from 270 mg/L to 110 mg/L, nitrous acid from 1.7 mg/L to 0.6 mg/L, and ozone from 5 mg/L to 2.5 mg/L in water. This result suggests that the decrease of ozone...
concentration in water extended the time taken for sterilization. It has not been clarified that the sterilization time can be shortened with high concentration levels of ozone in water, and low concentrations of nitric and nitrous acid. The change of sterilization characteristics with the change of ozone concentration in water is not clarified.

The purpose of this study is to increase the ozone concentration and suppressing the generation of nitric acid and nitrous acid in liquids while changing the intermittent discharge conditions. Furthermore, we elucidate the sterilization characteristics under that condition.

2. Experimental Method

A schematic diagram of the experimental setup is shown in Fig. 1. Plasma was generated using dielectric barrier discharge. The electrode had a circular aluminum tape bonded to both sides of the alumina plate, and the electrode was placed inside the cap. A sine wave with a frequency of 40 kHz and ± 1.6 kV was supplied to the electrode from the power source (HARCLON, HR191919). The power controller (TAKASAGO LTD., KX-S-100-L) was used to control the discharge time and supply voltage intermittently. There were three discharge intervals: 1 sec discharge with a 2 sec break, 3 sec discharge with a 6 sec break and 5 sec discharge with a 10 sec break. Plasma was generated around the lower electrode (Fig. 2). The electrode was insulated with an insulating tape except in the region of plasma generation. The vessel (MENICON) was a contact lens storage container. For the air test, there was only air in the 5 ml vessel, and for the underwater test, 2 ml of pure water was injected into the vessel.

Details of the electrode structure are shown in Fig. 2. The dielectric was a 15 mm wide square, 0.2 mm thick alumina plate as shown in (a). An aluminium electrode with a diameter of 4 mm was placed on the dielectric, and the ground side was an aluminium electrode with a diameter of 8 mm. Insulating tape was used for fixing the alumina plate and thermocouple to the cap and for insulating the linear part of aluminium electrode, as shown in (b).

Voltage and current waveform were measured using an oscilloscope (LeCroy, WaveSurfer 104MXs-B), voltage was measured using a voltage probe (LeCroy, PPE 20 kV), and current was measured using a current probe (PEARSON, CURRENT MONITOR). The power input to the plasma discharge region was measured by inserting a 470 pF capacitor on the ground electrode side and measuring the Q-V Lissajous figure. Dissolved ozone, nitrite ion, and nitrate ion concentration in water are measured using a pack test (KYORITSU CHEMICAL-CHECK Lab., WAK-O_3, WAK-NO_2, WAK-NO_3) and digital pack test (KYORITSU CHEMICAL-CHECK Lab., DPT-MT). When measuring ozone concentration in water, 30 µL of catalase (24000 U/mL) was added to pure water after the plasma discharge, erased hydrogen peroxide, following which the ozone concentration was measured. Under the same conditions, the chemical concentration was measured nine times and the average value and standard deviation were shown. The humidity value of room air was 50 - 60 % and room temperature was 20 - 25 ℃. The air was controlled by an air conditioner.

Efficacy of sterilization was evaluated using a biological indicator (3M Health Care, Attest 1291) coated with the spore, Geobacillus Steaothermophilus (ATCC 7953) as an indicator bacteria. Attest 1291 guaranteed a 5-log reduction. A sterilization judgment device (3M Health Care, 290 Auto reader) was used to judge the sterilization of the indicator bacteria. As shown in Fig. 1, in both air and water, a piece of paper coated with the spore was put into the vessel. After irradiation with plasma, the piece of paper was returned to the biological indicator, where it was determined whether sterilization was complete. This process was performed in a clean bench to maintain sterility. One piece of paper was used in each test, the test was performed 12 times under the same conditions, and if all the pieces of paper were sterilized, the sterilization was defined as complete.

Dielectric surface temperature is measured by thermocouple (ISHIKAWA TRADING, T/T-E-36-2) and thermography (NIPPON AVIONICS, S25W). As shown in Fig. 2, the thermocouple is attached to the surface of the alumina plate and covered with insulating tape. Temperature measurement with thermocouple is performed 3 times and shows the standard deviation of the average value.

3. Experimental Results and Discussion

3.1. Waveform and Power consumption

Fig. 3 shows the waveform of the applied voltage and discharge current along with the Lissajous figure. Pulsed discharge current was repeatedly generated as voltage rose and fell. Power consumption in the plasma generation region calculated from the figure was 0.26 W.
3.2. Chemical species concentration in water and pH

Fig. 4 shows the change in concentrations of dissolved ozone, nitrous acid, and nitric acid in water, along with pH vs. discharge time. After 80 min of intermittent discharge (the plasma discharge time was about 27 minutes), the ozone concentration was 3.9 mg/L, nitric acid concentration was 55.3 mg/L, and nitrous acid concentration was 0.3 mg/L, if the intermittent discharge was a cycle of 1 sec discharge with a 2 sec break. In addition, the ozone concentration was 4.5 mg/L, nitric acid concentration was 37.7 mg/L, and nitrous acid concentration was 0.2 mg/L, if the intermittent discharge was a cycle of 3 sec discharge with a 6 sec break. Furthermore, the concentration of ozone was 6.1 mg/L, nitric acid was
48.6 mg/L, and nitrous acid was 0.3 mg/L, if the intermittent discharge was a cycle of 5 sec discharge with a 10 sec break. After 80 min of intermittent discharge (the plasma discharge time was about 27 minutes), the value of pH decreased to 2.8. Compared to a cycle of a minute discharge with a 2 min break (Sato et al., 2018), the ozone concentration was higher and the nitric and nitrous acid concentrations were lower. In Fig. 4 (a), the concentration of nitric acid decreased from 110 mg/L to 55.3 mg/L, nitrous acid from 0.6 mg/L to 0.3 mg/L, and that of ozone increased from 2.5 mg/L to 3.9 mg/L in water, after 80 min of intermittent discharge. In Fig. 4 (b), the concentration of nitric acid decreased from 110 mg/L to 37.7 mg/L, nitrous acid from 0.6 mg/L to 0.2 mg/L, and that of ozone increased from 2.5 mg/L to 4.5 mg/L in water, after 80 min of intermittent discharge. In Fig. 4 (c), the concentration of nitric acid decreased from 110 mg/L to 48.6 mg/L, nitrous acid from 0.6 mg/L to 0.3 mg/L, and that of ozone increased from 2.5 mg/L to 6.1 mg/L in water, after 80 min of intermittent discharge.

### 3.3. Sterilization efficacy

Tables 1-3 show the results of the sterilization test in both air and water, with every cycle. In air, the sterilization was completed in 30 min with a cycle of 1 sec ON, 2 sec OFF, in 40 min with a cycle of 3 sec ON, 6 sec OFF and in 30 min with a cycle of 5 sec ON, 10 sec OFF. NO and NO₂ were not generated after 43 min of discharge, and the ozone concentration exceeded 1000 ppm after 12 min, with a cycle of 1 min ON, 2 min OFF (Sato et al., 2018). In the same situation, it was reported that sterilization was completed in 10 min in air. The increase in the sterilization time in this study was considered to increase the ozone concentration, because continuous discharge time was short and the discharge stopped before the ozone concentration increased sufficiently.

| Table 1 Result of sterilization test with a cycle of 1 sec ON, 2 sec OFF. |
|-------------------------|---------------------------------|
| Discharge time [min]    | Result [+: NOT sterilized, —: Sterilized] |
| In air                  |                                  |
| 10                      | + + + + + + + + + + + + + + + + — — |
| 20                      | + + + + + + + + + + + + + + + + — — |
| 30                      | + + + + + + + + + + + + + + + + — — |
| In water                |                                  |
| 40                      | + + + + + + + + + + + + + + + + — — |
| 60                      | + + + + + + + + + + + + + + + + — — |
| 80                      | — — — — — — — — — — — — — — — — — — |

| Table 2 Result of sterilization test with a cycle of 3 sec ON, 6 sec OFF. |
|-------------------------|---------------------------------|
| Discharge time [min]    | Result [+: NOT sterilized, —: Sterilized] |
| In air                  |                                  |
| 20                      | + + + + + + + + + + + + + + + + — — |
| 30                      | + + + + + + + + + + + + + + + + — — |
| 40                      | + + + + + + + + + + + + + + + + — — |
| In water                |                                  |
| 40                      | + + + + + + + + + + + + + + + + — — |
| 60                      | + + + + + + + + + + + + + + + + — — |
| 80                      | — — — — — — — — — — — — — — — — — — |

| Table 3 Result of sterilization test with a cycle of 5 sec ON, 10 sec OFF. |
|-------------------------|---------------------------------|
| Discharge time [min]    | Result [+: NOT sterilized, —: Sterilized] |
| In air                  |                                  |
| 20                      | + + + + + + + + + + + + + + + + — — |
| 30                      | + + + + + + + + + + + + + + + + — — |
| 40                      | + + + + + + + + + + + + + + + + — — |
| In water                |                                  |
| 40                      | + + + + + + + + + + + + + + + + — — |
| 60                      | + + + + + + + + + + + + + + + + — — |
| 80                      | — — — — — — — — — — — — — — — — — — |
In water, sterilization was completed in 80 min with every cycle. Sato et al. reported that sterilization was completed in 58 min with a cycle of 1 min ON, 2 min OFF. Moreover, it was reported that the ozone concentration was 4.3 mg/L at the completion of continuous discharge sterilization (after 20 min discharge), and 2.5 mg/L after 80 min discharge with the cycle of 1 min ON, 2 min OFF. This result suggested that the increase in ozone concentration did not contribute to shortening the sterilization time.

O, N, ’OH, O₂⁻, HO₂, O₃, H₂O₂, NOₓ, and ONOO⁻ were said to play an extremely important role in the sterilization of spores (Hojinik et al., 2019). However, the concentrations of H₂O₂, NO₂⁻, and O₃, which had half-lives of several tens of minutes and below several tens of mg/L, were too low to inactivate the spores in water (Hayashi et al., 2012). This report was consistent with the result that the increase in ozone concentration did not contribute to shortening of sterilization time. ’OH was reported to play an important role in sterilization in many studies, but had a very short lifetime of microseconds. O also had a lifetime of milliseconds (Hayashi et al., 2012). ’OH and H₂O₂ were produced by reaction (1), (2) (Hojinik et al., 2019), (3) and (4) (Dobrynin et al., 2014).

\[
\begin{align*}
(O_3)^{\text{-}}_{\text{liq}} + (H^+)^{\text{liq}} &\rightarrow (O_2)^{\text{liq}} + (\text{OH})^{\text{liq}} \\
(HO_3)^{\text{liq}} &\rightarrow (O_2)^{\text{liq}} + (\text{OH})^{\text{liq}} \\
(e^-)^{\text{liq}} + (O_2)^{\text{liq}} &\rightarrow (O_2)^{\text{liq}} \\
2(O_2)^{\text{liq}} + 2(H^+)^{\text{liq}} &\rightarrow (H_2O_2)^{\text{liq}} + O_2^{-}
\end{align*}
\]

Dissolved ozone produced ’OH and H₂O₂. It was assumed that ’OH and H₂O₂ were generated sufficiently because the ozone concentration was high, but specifically ’OH, with strong oxidizing powers did not contribute to shortening the sterilization time because the lifetime of ’OH is as short as microseconds.

Meanwhile, peroxynitrite was produced by the reaction of reaction (5), (6), (7), (8) (Liu Z. C. et al., 2015), (9), (10), (Liu D. X. et al., 2016), and (11) (Lukes et al., 2012). Peroxynitrite has a lifetime of 1 second (Kirsch et al., 1998); it penetrated the cell membrane, reached the cytology site, was a powerful oxidant, and was thought to contribute greatly to sterilization (Hojinik et al., 2019).

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\begin{align*}
(HNO_2)^{\text{liq}} &\leftrightarrow (NO_2)^{\text{liq}} + (H^+)^{\text{liq}} \\
(HNO_3)^{\text{liq}} &\leftrightarrow (NO_3)^{\text{liq}} + (H^+)^{\text{liq}} \\
2(NO_2)^{\text{liq}} + (H_2O)^{\text{liq}} &\rightarrow (NO_2)^{\text{liq}} + (NO_3)^{\text{liq}} + 2(H^+)^{\text{liq}} \\
(NO_2)^{\text{liq}} + (NO_2)^{\text{liq}} + (H_2O)^{\text{liq}} &\rightarrow 2(NO_2)^{\text{liq}} + 2(H^+)^{\text{liq}} \\
(O_3)^{\text{liq}} + (NO_2)^{\text{liq}} &\rightarrow (O_2)^{\text{liq}} + (NO_3)^{\text{liq}} \\
(NO_3)^{\text{liq}} + (NO_2)^{\text{liq}} &\rightarrow (NO_2)^{\text{liq}} + (NO_3)^{\text{liq}} \\
(NO_2)^{\text{liq}} + (H_2O)^{\text{liq}} &\rightarrow (ONOO^-)^{\text{liq}} + (H_2O)^{\text{liq}}
\end{align*}
\]

The formation of peroxynitrite required the presence of NOx. The results of this study indicated that the concentrations of nitric acid and nitrous acid were low, suggesting that the amount of peroxynitrite that was produced decreased. The decrease in peroxynitrite was thought to have led to an increase in sterilization time, suggesting the importance of peroxynitrite in sterilization.

Fig. 5 The change of dielectric surface temperature with a cycle of 1 sec ON, 2 sec OFF, using a thermocouple.
Fig. 6 The change of dielectric surface temperature with a cycle of 3 sec ON, 6 sec OFF, using a thermocouple.

Fig. 7 The change of dielectric surface temperature with a cycle of 5 sec ON, 10 sec OFF, using a thermocouple.

Fig. 8 The change of dielectric surface temperature measured using thermography.
3.4. Dielectric surface temperature

Figs. 5 to 7 show the temperature change of the dielectric surface using a thermocouple in each discharge cycle. The surface temperature before the start of the discharge was 26.0 °C. The surface temperature after 80 min of intermittent discharge was 35.7 °C with the cycle of 1 sec ON, 2 sec OFF (Fig. 5). Temperature change was constant. In addition, the surface temperature after 80 min of intermittent discharge was 36.6 °C with the cycle of 3 sec ON, 6 sec OFF (Fig. 6). The reason for the periodic drop in temperature by about 2 °C was assumed to be because of the timing at which the discharge stopped and temperature measurement overlapped. The surface temperature after 80 min of intermittent discharge was 32.8 °C with the cycle of 5 sec ON, 10 sec OFF (Fig. 7).

Meanwhile, in Fig. 8, an orange colour representing 39.9 °C was confirmed on the surface of the dielectric, 4.5 min after the start of the discharge. The temperature at the centre of the dielectric was low because the aluminium tape surface reflected infrared rays. The temperature difference between the thermography and thermocouple was small; thus, the thermocouple was measured accurately.

Sato et al. reported that the dielectric surface reached 47.0 °C after 1 min of continuous discharge. In this study, it was confirmed that the temperature rose to 36.6 °C even after intermittent discharge for 80 min. It was thought that the suppression of temperature rise contributed to the reduction of nitrogen oxide production, and increase of ozone production. Furthermore, as spore bacteria were sterilized at 2 atm and at a temperature higher than 120 °C, it was unlikely that the sterilization time increased owing to the temperature decrease.

4. Conclusion

In this study, a new intermittent discharge cycle was introduced to verify the effect of sterilization, to elucidate sterilization characteristics at high ozone, low nitric acid, and nitrous acid concentrations in liquids. In any intermittent discharge cycle, the nitric acid concentration decreased significantly, nitrous acid concentration decreased, and ozone concentration increased significantly, compared with the intermittent discharge cycle of 1 min ON, 2 min OFF. The dielectric surface temperature decreased by about 10 °C compared to that cycle. This contributed to the reduction of the nitrogen oxide production and the increase of ozone production.

Sterilization time was 20-40 min in air and 80 min in water, which was an increase compared to the intermittent discharge cycle of 1 min ON, 2 min OFF. From the measurement results of the chemical species in water, an increase in ozone concentration and a decrease in nitric and nitrous acid concentrations promoted •OH which had an ultra-short lifetime, and H₂O₂ and O₃ which were insufficient for sterilization at concentrations of several tens of mg/L. This also suppressed the generation of peroxynitrite, which had a lifetime of 1 sec, and had very strong oxidizing powers. This was believed to cause an increase in the sterilization time, suggesting that the peroxynitrite played a particularly important role in the sterilization of spores.

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