22nd International Symposium on Plasma Chemistry
July 5-10, 2015; Antwerp, Belgium

Extraction of bactericidal components in cryopreserved plasma-treated water

K. Kitano[1], S. Ikawa[2], Y. Nakashima[2] and A. Tani[3]

[1] *Center for Atomic and Molecular Technologies, Graduate School of Engineering, Osaka University, Japan*
[2] *Technology Research Institute of Osaka Prefecture, Japan*
[3] *Department of Earth and Space Science, Graduate School of Science, Osaka University, Japan*

Abstract: Plasma-treated water (PTW) prepared by the irradiation of atmospheric plasma to pure water showed strong bactericidal activity, which can be cryopreserved. Respective chemical components in PTW were isolated by ion chromatography. In addition to peaks of H_2O_2, NO_2^- and NO_3^-, a specific peak was detected and only this fraction had bactericidal activity. Based on chemical kinetics, physicochemical mechanisms in PTW are discussed.

Keywords: plasma medicine, disinfection, atmospheric pressure plasma, radicals in liquid

1. Effective plasma disinfection technique by the reduced-pH method

For disinfecting the surface of human bodies in dental [1] and surgical applications by low-temperature atmospheric-pressure plasmas (Fig. 1), the important point is the inactivation of bacteria in body fluid. The reduced pH method was developed that strong bactericidal activity by direct plasma exposure can be achieved if the solution is sufficiently acidic [2]. Drastic enhancement of bactericidal activity is obtained by controlling the pH of the solution under 4.7, and D value (decimal reduction time) became 1/100 when pH is changed from 6.5 to 3.8. D value (*Escherichia coli*) at acidic condition can be controlled to quite small (< 2 sec) under some condition. We call this technique as the reduced-pH method.

It is considered that strong bactericidal activity is brought by hydroperoxy radical (HOO•) generated from the association of hydrogen ion (H^+) and superoxide anion radical (O_2^-•). The critical pH value is associated with pKa of the dissociation equilibrium between these radicals, which is known to be approximately 4.8. This well-known chemical reaction means that O_2^-• can be changed into HOO•, which have much stronger bactericidal activity, in lower pH [3]. Here, long-lived O_2^-• has so much longer half lifetime in water than other reactive oxygen species.

Because body fluid has neutral pH buffer capacity of pH ~7.4, this reduced pH method is essential technique for plasma disinfection. Just before plasma treatment of infected area, acidic fluid should be applied to its surface.

Fig. 1. Plasma jet exhausted to a finger without burning.

2. Physicochemical mechanism of temperature dependence of Plasma-treated water (PTW) activities based on chemical kinetics

In addition to direct and remote plasma exposures to bacteria suspension, plasma-treated water (PTW) was confirmed to be effective with the reduced pH method. As shown in Fig. 2, *Bacillus subtilis* (spore) was inactivated in a rapid manner [4]. To obtain strong bactericidal activity, the reduced-pH method is also required with PTW.

Half-lives of PTW bactericidal activity were in accordance with Arrhenius equation in the liquid and the solid state (Fig. 3). From the experimental results of ESR (electron spin resonance) measurement [5] of O_2^-• with spin trapping method, half-lives of PTW were also in accordance with Arrhenius equation (Fig. 3). Both activation energies are almost equal to ~110 kJ/mol. Half-lives at deep freezer temperature (-80 degree C) and body temperature (+37 degree C) are estimated to 7 centuries and 3.9 seconds from Arrhenius equation, respectively.

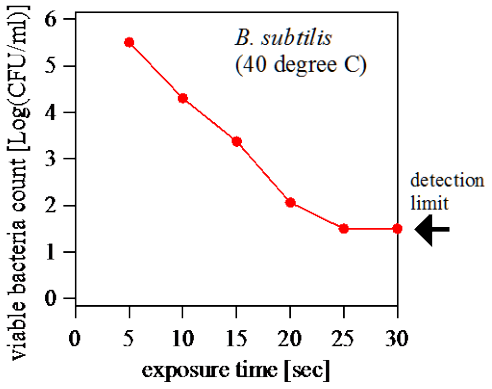

Fig. 2. Inactivation of *B. subtilis* (spore cell) with PTW.

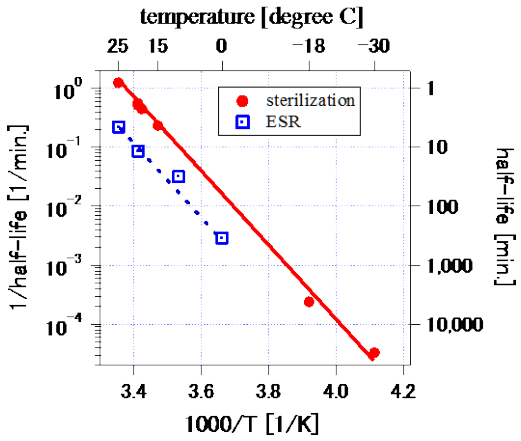

Fig. 3. Arrhenius plots for bactericidal activity and ESR measurement of PTW.

This indicates that PTW can be cryopreserved in freezer and toxicity to human body seems to be low due to fast disappearance of the bactericidal activity [6].

3. Isolation of bactericidal components in cryopreserved PTW by ion chromatograph

After high concentration PTW was prepared by 1 m long special device which can continuously irradiate atmospheric-pressure plasma to pure water flowing slowly with cooling system [7], PTW was analyzed by the ion chromatograph (IC). The analysis was carried out at low-temperature condition to maintain the bactericidal chemical species in PTW. Result of the analysis (Fig. 4) revealed that PTW contained hydrogen peroxide, nitrate and nitrite. In addition to these components, a specific peak eluted after nitrite ion was detected around at 3 min retention time. This peak was not detected in heat-treated PTW showing no bactericidal activity, suggesting that a substance contained in this peak played an important role in the bactericidal activity of PTW.

To examine the bactericidal activity of respective peaks of PTW, eluate of IC were collected by 0.5 mL and bactericidal assay (*E. coli* suspension) were performed with respective fraction. As a result, strong bactericidal activity was observed only with fractions around at a PTW-specific peak described above, and no bactericidal effects were observed in other peak fractions (Fig. 4). This result revealed that the bactericidal activity of PTW was due to single chemical substance, not a combined effect of plural components.

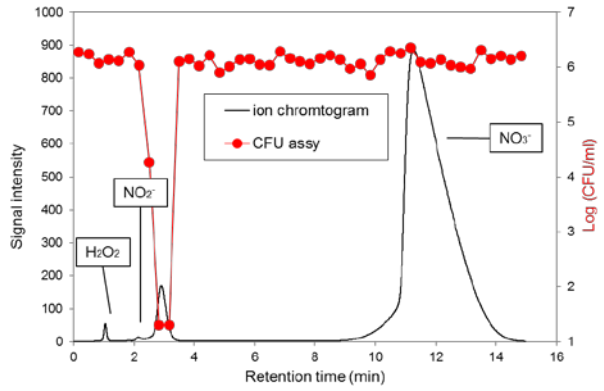

Fig. 4. Ion chromatogram of PTW and CFU assay of its fractions.

Furthermore, fractions containing bactericidal species (or its precursor) were inactivated by heating and applied to IC again. Consequently, only nitrate and nitrite were detected. This means that degradation products of the bactericidal species are nitrate and nitrite ions, strongly suggested the bactericidal species is a compound consisting of oxygen and nitrogen atoms.

We are conducting further investigations to identify this bactericidal species with absorption spectroscopy and so on. It is expected to elucidate the molecular mechanism of bacterial inactivation with PTW by additional investigations.

4. Role of nitrogen molecules in gas and liquid phases for PTW synthesis

Although PTW has such advantages for disinfection, its mechanisms are unclear at this stage. Since no O_2^-• was directly observed in PTW by ESR, another reactive species that can produce O_2^-• (hereafter called precursor X) are suspected [8]. Considering the half-lifetime of O_2^-•, specific component separated by IC seems to be precursor X. Here, we investigated the role of nitrogen molecules in preparation of bactericidal PTW for physicochemical understanding of the precursor X.

PTW was prepared by direct exposure of the LF jet to pure water in the chamber with ambient gas control, described in the reference [9]. Nitrogen, oxygen, and helium itself were used as ambient gases in the chamber because bactericidal PTW was usually prepared by plasma exposure to water in air. In addition, gas bubbling was performed before PTW preparation to control dissolved gases in water. To detect a short-lived radical

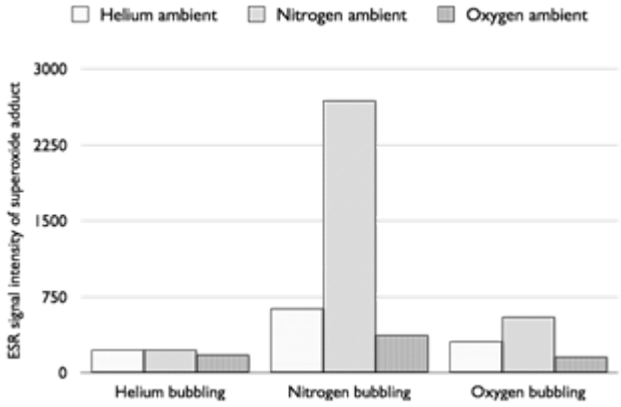

Fig. 5. ESR signal intensity of O2-• adduct in different ambient gases. The initial water samples were prepared by gas bubbling with helium (left), nitrogen (middle), and oxygen (right).

species like O_2^-•, spin trapping reagent, CYPMPO (Radical Research) was used.

ESR signal intensities of O_2^-• adduct are shown in Fig. 5. In helium bubbling water (left in Fig. 5), the signal intensities were close to noise level in all samples. In oxygen bubbling water (right in Fig. 5), O_2^-• adduct signal was not observed in oxygen ambient gas but in nitrogen ambient gas. In nitrogen bubbling water (middle in Fig. 5), the adduct signal was observed in all ambient gases. Especially, the intense adduct signal was observed in nitrogen bubbling + nitrogen ambient gas condition. Bactericidal activity of the PTW was strong in the same condition. Once LF plasma is directly exposed to CYPMPO solution in oxygen ambient, obvious O_2^-• adduct signal can be detected. However, in PTW, almost no O_2^-• adduct signal is observed in oxygen bubbling + oxygen ambient gas condition. It strongly supports that O_2^-• in PTW is not directly formed from oxygen but from another reactive species like the precursor X.

To prepare the precursor X in PTW, nitrogen is indispensable, water is possibly necessary, and oxygen is not. This means that the precursor X is a sort of reactive oxygen and nitrogen species (RONS) produced from nitrogen and water. NO and NO_2 do not match to the precursor X because of its short half-life (a few minutes) at room temperature. To identify the precursor X, further analyses are going on.

The role of nitrogen gas in preparation of bactericidal PTW was investigated for physicochemical understanding of the precursor X. Nitrogen gas is essential to prepare bactericidal PTW. The precursor X may consist of nitrogen and oxygen like RONS but not be relatively stable NOx. Further analyses should be necessary to identify the precursor X.

5. Summary

Although PTW has many chemical components, bactericidal species with the reduced pH method was isolated and purified by IC with a fraction collector. Other components in PTW showed no bactericidal effects, meaning that concentrations of these disinfectant components were low enough. The details of the observed specific peak are now under investigation. From the experiments of controlling ambient gas around helium plasma and dissolved gases in water, precursor X is one of the RONS. Observed specific peak seems to be precursor X. By cryopreservation of PTW, the degradation of precursor X would be avoided and precursor X might produce O_2^-•.

Recently, many types of PTW, containing plasma activated medium, are reported. But the controls of pH and temperature are still omitted. So many types of experimental effects reported in this paper would not be seen. We believe that isolation of key species from PTW or PAM and reactions between chemical species and biomacromolecules must be important for scientific discussions[10-12].

6. Acknowledgement

This study was supported by Grant-in-Aid for Scientific Research B (23340176) and Innovative Areas "Plasma Medical Innovation" (25108505) from Ministry of Education, Culture, Sports, Science and Technology of Japan, and the national cancer center research and development fund (23-A-15).

7. References

[1] H. Yamazaki et al., Dental Mat. Journal, 30, 384 (2011).
[2] S. Ikawa, K. Kitano, S. Hamaguchi, Plasma Process. Polym., 7, 33 (2010).
[3] E. Takai, S. Ikawa, K. Kitano, J. Kuwabara, K. Shiraki, J. Phys. D: Appl. Physics. 46, 295402 (2013).
[4] K Kitano, S. Ikawa, A. Tani, Y. Nakashima, H. Yamazaki, T. Ohshima, K. Kaneko, M. Ito, T. Kuwata, A. Yagishita, 7. 36, ISPC-21, Australia (2013)
[5] A. Tani, Y. Ono, S. Fukui, S. Ikawa, K. Kitano, Appl. Phys. Lett., 100, 254103 (2012).
[6] K. Kitano, S. Ikawa, Y. Nakashima, A. Tani, 22-AO09, 5th International Conference on Plasma Medicine (ICPM5), Nara, Japan (2014).
[7] K. Kitano, S. Ikawa, A. Tani, Y. Nakashima, T. Ohshima, 4A-PM-O3, 8th International Conference on Reactive Plasmas/31th Symposium on Plasma Processing (ICRP-8/SPP-31), Fukuoka, Japan (2014)
[8] A. Tani, S. Ikawa, Y. Nakashima, K. Kitano, 22-AO11, 5th International Conference on Plasma Medicine (ICPM5), Nara, Japan (2014).
[9] A. Tani, S. Fukui, S. Ikawa, K. Kitano, Jpn. J. Appl. Phys. 54, 01AF01 (2015).
[10] E. Takai, K. Kitano, J. Kuwabara, K. Shiraki, Plasma Processes and Polymers, 9, 77–82 (2012).
[11] E. Takai, G. Ohashi, T. Yoshida, K. M. Sorgjerd, T. Zako, M. Maeda, K. Kitano, K. Shiraki, Applied Physics Letters, in press.
[12] E. Takai, T. Kitamura, J. Kuwabara, S. Ikawa, S. Yoshizawa, K. Shiraki H. Kawasaki, R. Arakawa, K. Kitano, J. Phys. D: Appl. Physics. 47, 285403 (2014).