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Physicochemical properties of bactericidal plasma-treated water

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Abstract

Plasma-treated water (PTW), i.e. distilled water (DW) exposed to low-temperature atmospheric pressure helium plasma, exhibited strong bactericidal activity against *Escherichia coli* in suspension even within a few minutes of preparation. This effect was enhanced under acidic conditions. The bactericidal activity of PTW was attenuated according to first-order kinetics and the half-life was highly temperature dependent. The electron spin resonance (ESR) signal of an adduct of the superoxide anion radical ($O_2^{-\bullet}$) was detected in an aqueous solution using a spin-trapping reagent mixed with PTW, and adding superoxide dismutase to the PTW resulted in a loss of the bactericidal activity and weakening of the ESR adduct signal of $O_2^{-\bullet}$ in the spin-trapping. These results suggest that $O_2^{-\bullet}$ plays an important role in imparting bactericidal activity to PTW. Moreover, molecular nitrogen was required both in the ambient gas and in the DW used to prepare the PTW. We, therefore, suggest that the reactive molecule in PTW with bactericidal effects is not a free reactive oxygen species but nitrogen atom(s)-containing molecules that release $O_2^{-\bullet}$, such as peroxynitrous acid (ONOOH) or peroxynitric acid (O_2NOOH). Considering the activation energy for degradation of these species, we conclude that peroxynitric acid stored in PTW induces the bactericidal effect.

Keywords: plasma sterilization, plasma process in liquid, plasma-treated water

(Some figures may appear in colour only in the online journal)

1. Introduction

Low-temperature atmospheric pressure plasma (LTAPP) is a source of various types of chemical species with high reactivity, including free radicals [1, 2]. Many studies have investigated the potential applications of LTAPP; in particular, the field of plasma medicine has attracted intense interest [3–5] with regards to enhanced wound healing [3, 4, 6], cancer therapy [7, 8], inactivation of pathogenic amyloids [9, 10], disinfection of the human body or teeth [3, 5, 11, 12], sterilization of medical equipment [13, 14], and so on.

Disinfection is a critical issue in the treatment of infectious diseases and prevention of surgical site and secondary infections. However, it is difficult to achieve sufficient sterility owing to the undesirable side effects to healthy tissue associated with potent agents and the emergence of resistant bacteria. Plasma sterilization is a new method of disinfection that can be used as an alternative to conventional microbicides.

For medical applications of plasma disinfection, it is necessary to develop techniques to inactivate bacteria in an aqueous environment since most microorganisms grow under wet conditions. Inactivation of bacteria in liquid by direct plasma irradiation has been reported by many researchers, and extremely strong bactericidal species such as the hydroxyl radical ($OH^{\bullet}$) have been used in some cases [15–19]. Nevertheless, most of the bactericidal species supplied by plasma irradiation may also induce toxic side effects due to their high reactivity and/or stability. Therefore, to avoid side effects, anti-biological activity limited to a moderately short period and/or under

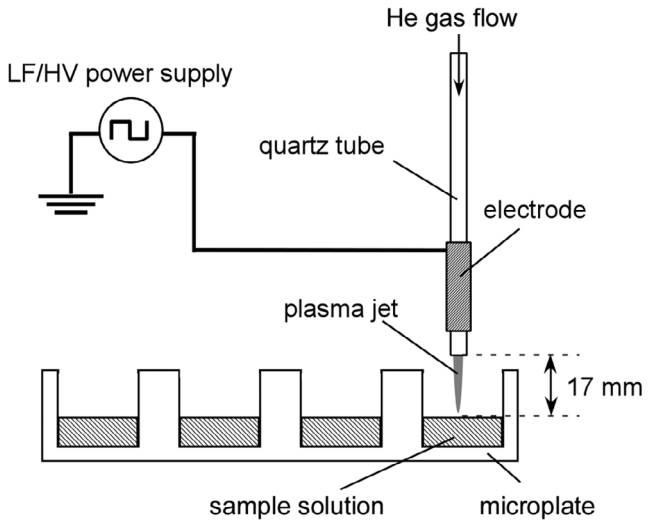

Figure 1. LF plasma jet discharge system in open air.

specific conditions is required for plasma disinfection of biological surfaces.

In a previous report, we described the reduced-pH method for enhancing the bactericidal effect induced by direct plasma exposure by acidifying the solution. This was achieved by directly exposing a bacterial suspension, for which the pH was adjusted to 4.8 or lower, to plasma. Although many theories have been proposed to explain the mechanism of bacterial inactivation by LTAPP [15–21], the superoxide anion radical ($O_2^{-\bullet}$) is thought to be the key molecule for the inactivation of bacteria in the reduced-pH method [22]. Under acidic conditions, $O_2^{-\bullet}$ is protonated and forms the hydroperoxy radical ($HOO^\bullet$) [23], which is an uncharged radical species that can easily permeate bacterial cells by traversing the hydrophobic cell membrane [23]. $HOO^\bullet$ can then denature proteins inside the cell by chemical modification, causing bacterial inactivation. It is, therefore, expected that bactericidal activity by direct plasma exposure to bacterial suspension should be active only during the exposure of bacterial suspensions to plasma, since free $HOO^\bullet$ molecule supplied by plasma discharge has a short half-life (equal to or less than a few seconds) [24].

As an alternative to direct plasma irradiation of bacterial suspensions, application of LTAPP-exposed aqueous solutions to bacterial inactivation has been studied. In those reports, the plasma-irradiated liquid was found to maintain the bactericidal activity for a long period of up to a few days. Zhang *et al* reported that reactive oxygen species (ROS), such as $OH^\bullet$, $O_2^{-\bullet}$, hydrogen peroxide (H_2O_2), and ozone, played an important role in bacterial inactivation [25]. Traylor *et al* also proposed that H_2O_2 was involved in conferring bactericidal activity to plasma-irradiated liquid [26]. On the other hand, several reports suggested that the dominant species involved was peroxynitrite ($ONOO^-$) [27–31]. Certain studies even demonstrated that the bactericidal activity of plasma-irradiated water could be reproduced by mixing H_2O_2 and nitrite (NO_2^-) under acidic conditions [30, 31]. They proposed that bacterial inactivation was achieved by $ONOO^-$ generated continuously from H_2O_2 and HNO_2, although $ONOO^-$ has an extremely short life-time under acidic conditions. However, it is unlikely that $ONOO^-$ is the main contributor to bactericidal activity under weakly acidic conditions (pH 3.5–4.5), because $ONOO^-$ is unstable and its generation from H_2O_2 and HNO_2 is very slow under these conditions.

Herein, we demonstrate that water exposed to plasma—referred to as plasma-treated water (PTW)—maintains bactericidal activity for several minutes at room temperature (RT). The bactericidal activity of PTW was enhanced under acidic conditions and was inactivated after a brief period, in contrast to well-known bactericidal compounds such as ozone, nitrous acid, or hydrogen peroxide. Although enhancement of the bactericidal activity under acidic conditions was observed in the reduced-pH method with direct plasma irradiation, it is unlikely that $O_2^{-\bullet}$ itself is directly involved in the bactericidal activity of PTW, considering the limited half-life of $O_2^{-\bullet}$ in solution. The life-time of the bactericidal activity of PTW is longer than that of $O_2^{-\bullet}$, and the bactericidal activity of PTW is more rapidly attenuated than previously reported for plasma-irradiated solutions, suggesting that the key chemical compound involved in bacterial inactivation by this PTW differs from any reported species such as ozone (O_3), H_2O_2, or ONOOH. In this paper, we report in detail the physicochemical properties of PTW and show that the bactericidal species present in this PTW are oxygen- and nitrogen-containing molecules that release $O_2^{-\bullet}$. Moreover, based on the activation energy for the degradation reaction, we propose that the active species in PTW is peroxynitric acid.

2. Materials and methods

2.1. Preparation of PTW using a low-frequency (LF) plasma jet

A low-frequency (LF) plasma jet was used as previously described [22]. Helium gas flowing at 2.0 l min^{-1} through a quartz tube was ionized by application of a high voltage (ranging from −3.5 to +5.0 kV at a frequency of 13.9 kHz) to a single-sided electrode (figure 1). The waveform of voltage applied to the electrode was shown in the previous report [22]. The plasma jet was extended from the quartz tube to make contact with the liquid surface. The discharge power of the plasma is 3 W. For experiments in which the ambient atmosphere was controlled, the LF plasma jet was used in conjunction with an airtight chamber system (figure 2). Ambient gas was supplied from the side port of the chamber. Distilled water (DW) with dissolved gas, controlled by 15 min gas-bubbling replacement, was prepared and supplied using a syringe transfer system so as to prevent contact of the water with air.

PTW was prepared in an open air system by exposing 1 ml DW in a 24-well microplate to the tip end of an LF plasma jet glass tube placed 17 mm above the water surface for 2 min at RT (figure 1). A plasma exposure system (figure 2) was used to prepare PTW under airtight conditions; 1.0 ml DW with dissolved gas was exposed to the LF plasma jet for 5 min under a controlled atmosphere. Helium, nitrogen, oxygen, and synthetic air—consisting of 80% nitrogen and 20% oxygen—were used as dissolved and atmospheric gases. PTW was collected in polypropylene microtubes and stored on ice until

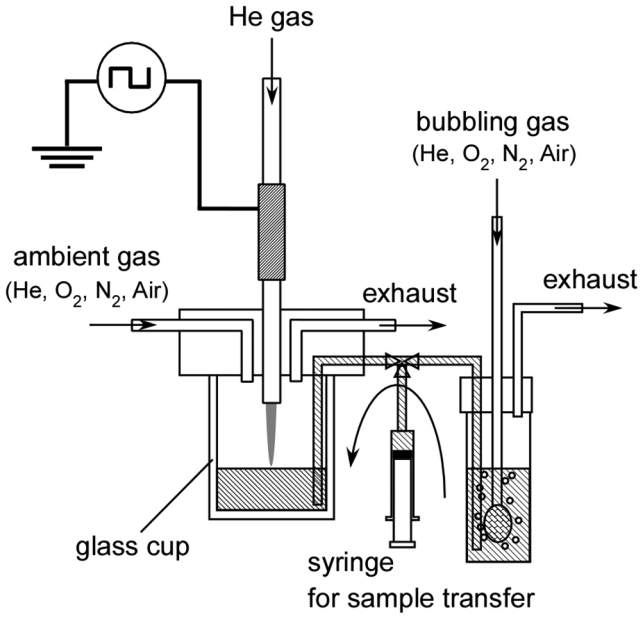

Figure 2. LF plasma discharge system in a chamber. Helium, oxygen, nitrogen and synthesized air (80% nitrogen + 20% oxygen) were used as ambient and bubbling gases.

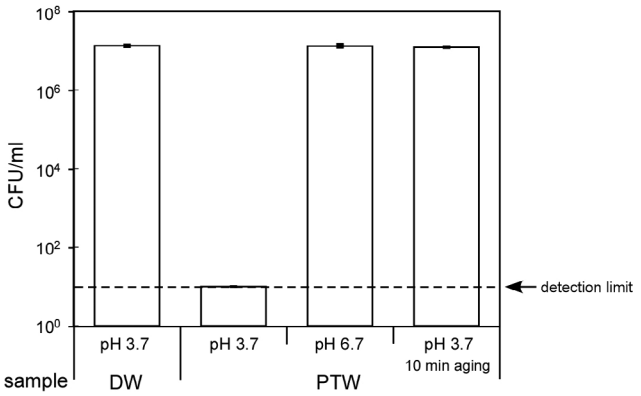

Figure 3. Bactericidal effects of PTW. PTW was prepared by exposing DW to plasma and was mixed with *E. coli* suspension (pH 3.7 or 6.7). The number of surviving cells decreased to below the detection limit when PTW was added to the pH 3.7 suspension, while no bactericidal effect was observed at pH 6.7. PTW aged for 10 min at RT had no bactericidal activity at pH 3.7. These experiments were performed in triplicate.

use. To examine the rate of attenuation of the PTW activity, PTW was aged at different temperatures and recovered after appropriate periods for application to bactericidal assay or electron spin resonance (ESR) analysis. The PTW samples that were frozen at −18 °C, −30 °C, and −80 °C were completely thawed at 0 °C and immediately used for bactericidal assay.

2.2. Bacterial inactivation with PTW

Escherichia coli NBRC 3301 was obtained from the NITE Biological Resource Centre (Chiba, Japan) and used for bactericidal assay as a general model bacterium. *E. coli* was cultured in Luria-Bertani (LB) broth for 18 h at 30 °C with 110 rpm rotary agitation, then harvested by centrifugation (8000 × *g* for 2 min) and re-suspended in an appropriate volume of DW. The suspension was diluted with DW to an optical density at 600 nm (OD_{600}) of 0.1. OD_{600} is often used as the concentration index of the bacterial suspension. The suspension of $OD_{600} = 0.1$ is expected to contain *E. coli* with approximately 7×10^7 cells ml^{-1}.

To assess the bactericidal activity of PTW prepared with the open air system, a colony-forming unit (CFU) assay was carried out. A 20 μl aliquot of bacterial suspension ($OD_{600} = 0.1$) was mixed with an equal volume of 200 mM sodium citrate buffer, followed by addition of 160 μl PTW. The suspension was allowed to stand at 25 °C for 10 min and then serially diluted by 10-fold with DW. Each dilution (100 μl) was spread on an LB agar plate and incubated for 24 h at 37 °C, at which point colonies were counted. The minimum detection value was 10 CFU ml^{-1}. For the airtight chamber system, a minimum inhibitory concentration (MIC) assay was carried out as follows. PTW from the airtight chamber was diluted 2-, 3-, or 4-fold with DW, and 10 μl each of bacterial suspension ($OD_{600} = 0.1$) and 200 mM sodium citrate (pH 3.5) and 80 μl diluted or undiluted PTW were mixed and allowed to stand for 10 min at 25 °C. A 20 μl aliquot of the mixture was added to 180 μl LB broth and cultured at 37 °C for 24 h. The MIC was estimated based on inhibition of visible bacterial growth, as determined by the turbidity of the medium.

The effect of superoxide dismutase (SOD from bovine erythrocytes; MP Biomedicals, Santa Ana, CA, USA) on the bactericidal activity induced by PTW was assessed as follows: 50 μl SOD solution (0.4 mg ml^{-1}) was mixed with 100 μl PTW and allowed to stand at 25 °C for 60 s. The mixture was added to 50 μl bacterial preparation ($OD_{600} = 0.04$ in 80 mM sodium citrate (pH 3.5)). After allowing the mixture to stand for 10 min at 25 °C, the number of surviving bacteria was determined by the CFU assay, as described above. Bovine serum albumin (BSA; Wako Pure Chemical Industries, Osaka, Japan) was used as a control.

2.3. ESR analysis of PTW

Radical species generated in water by atmospheric-pressure plasma are unstable and can be investigated by the spin-trapping technique, in which spin-trapping reagents react with unstable radicals (with short life-times) in liquids to form spin adducts (with longer life-times) that can be measured using ESR [32]. Previous analyses of LTAPP-induced radical species in water focused on direct and/or indirect plasma exposure of liquid with spin-trapping reagents [2, 33–37]. In these cases, we observed formation of radical species by plasma and afterglow gases; for example $OH^{\bullet}$ and $H^{\bullet}$ were formed from H_2O. In contrast, the active species remaining in PTW after plasma exposure were the target in this paper.

Following the previous study, the spin trapping reagent 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO; product No. RR-017, Radical Research, Tokyo, Japan) was used to detect short-lived radical species, because the ESR signal of the $O_2^{-\bullet}$ spin adduct

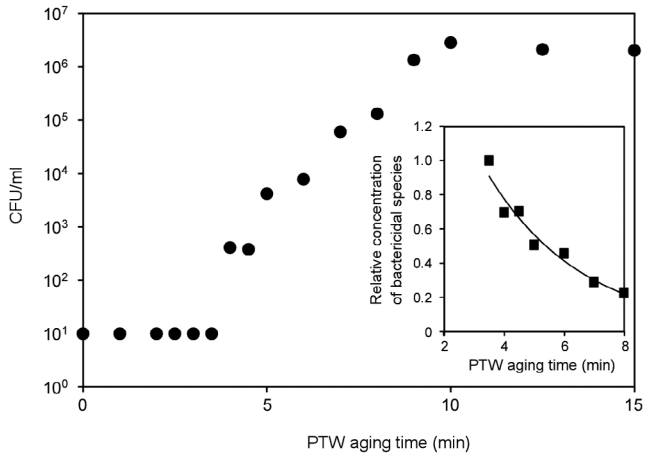

Figure 4. Attenuation time course of PTW bactericidal activity. PTW was aged at RT and mixed with *E. coli* suspensions at pH 3.7 and allowed to stand at 25 °C for 10 min. The number of surviving cells (circles) was determined by the CFU assay. Squares indicate the relative concentration of bactericidal species in PTW at each aging time; the fitted curve shows first-order decay.

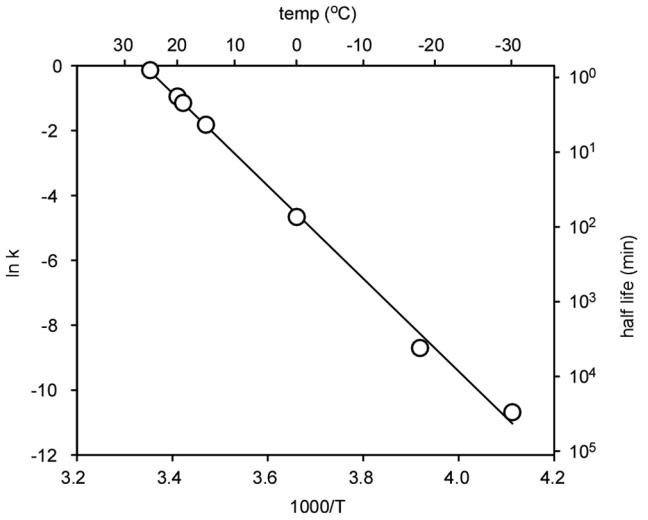

Figure 5. Arrhenius plot of the degradation of bactericidal species in PTW. Activation energy for degradation of bactericidal species was calculated as 109 kJ mol^{-1}.

is distinguishable from that of the $OH^{\bullet}$ adduct [38] and CYPMPO can detect $O_2^{-\bullet}$ in a plasma-exposed solution [39]. An aqueous solution of CYPMPO (10 mM) was prepared with DW, and 150 μl of the solution was mixed with PTW (150 μl) and analyzed at RT by X-band ESR using a flat quartz glass tube. The microwave power was 4 mW and the 100 kHz modulation field was 0.1 mT. No apparent ESR signal was detected for the blank CYPMPO solution even after gas bubbling. As in the CFU assay for bactericidal activity, the effect of SOD on the generation of an ESR signal by the active species in PTW was examined by using SOD (0.4 mg ml^{-1}) and BSA (0.4 mg ml^{-1}) as a control. PTW was first mixed with SOD or BSA solution and then mixed with CYPMPO solution. The final concentration of CYPMPO was the same in all experiments.

3. Results

3.1. Bacterial inactivation by PTW

PTW was prepared by exposing DW to plasma for 2 min in air. The PTW completely inactivated *E. coli* suspended in acidic (pH 3.7) but not in neutral (pH 6.7) buffer (figure 3). The bactericidal effect of PTW on *E. coli* in acidic buffer (pH 3.7) was eliminated when PTW was aged at RT for 10 min.

To investigate the rate of decrease of the bacteria-inactivating species in detail, PTW was aged at RT and its bactericidal activity was measured (figure 4). In general, the logarithm of the viable rate is proportional to the concentration of a bactericide, C as equation (1).

$$\ln\left(\frac{N}{N_0}\right) = -kCt, \tag{1}$$

where N is a viable bacteria concentration, N_0 is an initial bacteria concentration, k is a bactericidal reaction constant, and t is a time of bactericidal treatment. The relative concentrations of bactericidal species in PTW were estimated from bactericidal assay. The concentration of bactericidal species decreased over time, consistent with first-order reaction kinetics (figure 4); the half-life of the bactericidal species in PTW was calculated as 2.3 min at RT. The same experiments were carried out over a range of aging temperatures, including under freezing conditions. The results showed that the half-life of the bactericidal species varied significantly with temperature. At 25, 20, 19, 15, and 0 °C, the corresponding half-life was 0.8, 1.8, 2.2, 4.3 and 74 min, respectively, whereas at −18 °C and −30 °C, the corresponding half-life was 2.9 and 21 d, respectively. This temperature-dependence of the attenuation rate could be described by the Arrhenius equation, even when PTW was in the frozen state. Based on the Arrhenius plot (figure 5), the half-life of the bactericidal activity at 37 °C (human body temperature) was calculated as 7.9 s, indicating that PTW can rapidly inactivate bacteria and mitigate harmful effects to human tissue when used for disinfection of the human body.

3.2. Effect of SOD on bacterial inactivation by PTW

The bactericidal activity of PTW was dependent on the pH of the bacterial suspension. This is similar to the case of direct plasma exposure [22], suggesting that $O_2^{-\bullet}$ may also be involved in bacterial inactivation by PTW. To test this hypothesis, SOD was included in the bactericidal assay as an $O_2^{-\bullet}$ quencher. Whereas *E. coli* mixed with PTW only was completely inactivated, *E. coli* treated with PTW containing SOD remained viable (figure 6). The bactericidal effect was unaffected by the addition of BSA instead of SOD (figure 6).

3.3. Chemical properties of active species in PTW

To confirm the formation of $O_2^{-\bullet}$ in PTW, the radical-trapping compound CYPMPO was used to detect radical species that were formed. The CYPMPO solution was mixed with PTW at RT and ESR analysis was carried out. The ESR

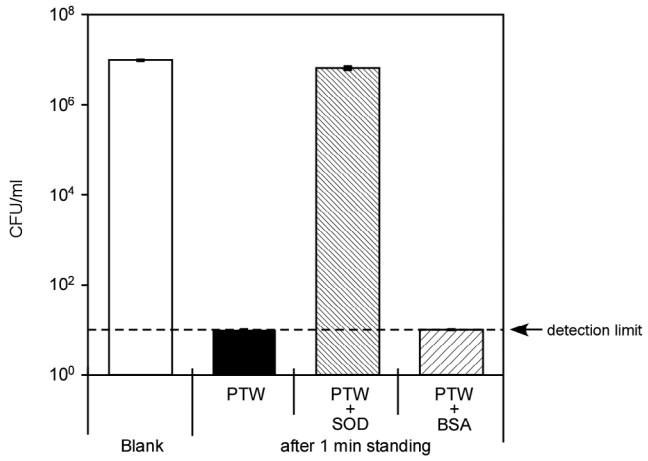

Figure 6. Inhibitory effect of SOD on the bactericidal activity of PTW. SOD solution was added to PTW and allowed to stand at 25 °C for the indicated periods. PTW was then added to the *E. coli* suspension at pH 3.7 and the mixture was allowed to stand at 25 °C; the number of surviving cells was determined by the CFU assay. BSA was used as a control protein for SOD. These experiments were performed in triplicate.

spectrum revealed the presence of the $O_2^{-\bullet}$ adduct of CYPMPO (CYPMPO-$O_2^{-\bullet}$), but the $OH^{\bullet}$ adduct was not detected (figure 7(a)). Comparison of the results obtained with SOD (figure 7(b)) and BSA (figure 7(c)) showed an obvious difference; the adduct signal of $O_2^{-\bullet}$ was very weak in PTW with SOD, whereas its intensity in PTW with BSA was almost the same as that in PTW without any additive (figure 7(a)). Although $O_2^{-\bullet}$ can be directly detected by ESR at low temperature [40, 41], no apparent ESR signal was observed at 77 K in frozen PTW. This means that $O_2^{-\bullet}$ was not stored in PTW. These results are consistent with the short lifetime of $O_2^{-\bullet}$ [24], and suggest that this radical is produced by other compounds in the PTW. In addition, the ESR signal of the adduct (figure 7(d)) was negligible in PTW stored at 30 °C for 10 min before mixing with CYPMPO solution and was not distinguishable from the initial ESR signal in CYPMPO solution. This result is concordant with the CFU assay presented in figure 3.

Given that bacterial inactivation by PTW followed first-order decay, as described by the Arrhenius equation, we investigated the attenuation rate of the activity to supply $O_2^{-\bullet}$ in PTW at various temperatures using CYPMPO (figure 8). In each experiment, PTW was maintained at 0, 10, 20, or 25 °C for up to 500 min (aging time). The intensity of the ESR signal (i.e. the amount of CYPMPO-$O_2^{-\bullet}$) was attenuated according to first-order reaction kinetics. The half-lives of the active species at 0, 10, 20, and 25 °C were 210, 29, 6.8, and 2.9 min, respectively. As observed in the bacterial inactivation assay, the temperature-dependence of the attenuation rate could be described by the Arrhenius equation.

3.4. Essential elements for the generation of bacteria-inactivating species in PTW

To evaluate the process by which active species are generated in PTW, the essential elements were identified using an airtight chamber system with dissolved gas controlled DW

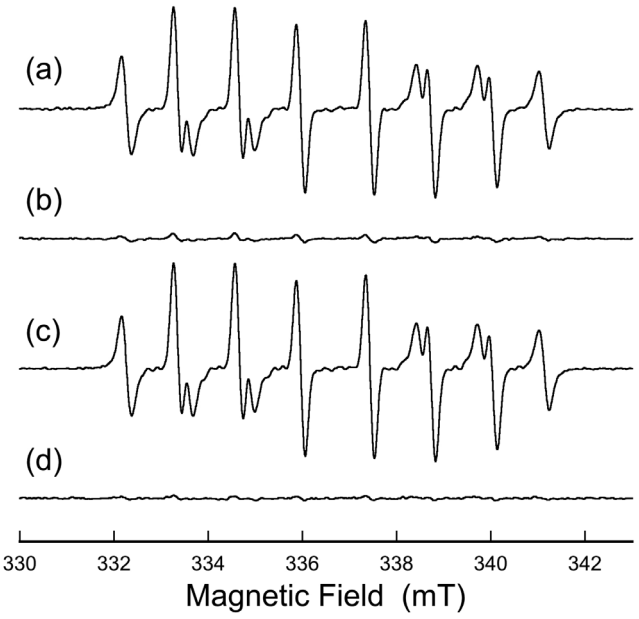

Figure 7. ESR spectra of PTW with CYPMPO. PTW was mixed with CYPMPO solution and allowed to stand at 25 °C for 5 min before ESR measurements. All main peaks are due to the $O_2^{-\bullet}$ adduct of CYPMPO. (a) PTW, (b) PTW and SOD, (c) PTW and BSA, and (d) PTW at 30 °C after 10 min.

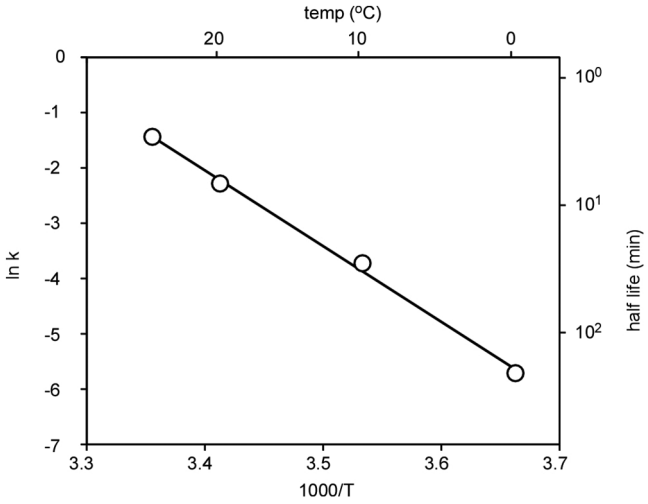

Figure 8. Arrhenius plot of the inactivation of $O_2^{-\bullet}$ adduct-formation potential in PTW. Activation energy for the attenuation was calculated as 115 kJ mol^{-1}.

(figure 2). DW was bubbled with appropriate gas for 15 min to replace the dissolved gas, and no dissolved air remained in DW after bubbling. DW in which the dissolved gas consisted of nitrogen, oxygen, helium, or air was introduced into the airtight chamber, then exposed to a helium plasma jet with controlled ambient gas. The CYPMPO-$O_2^{-\bullet}$ adduct-producing ability and bactericidal activity of the resulting PTW were measured (figure 9). Intense CYPMPO-$O_2^{-\bullet}$ ESR signals were detected when nitrogen gas or air was used as the ambient gas; the intensity of the ESR signal increased markedly for PTW samples prepared with water in which the dissolved gases contained nitrogen or air. These results indicate that nitrogen molecules in the ambient and dissolved gases are essential for

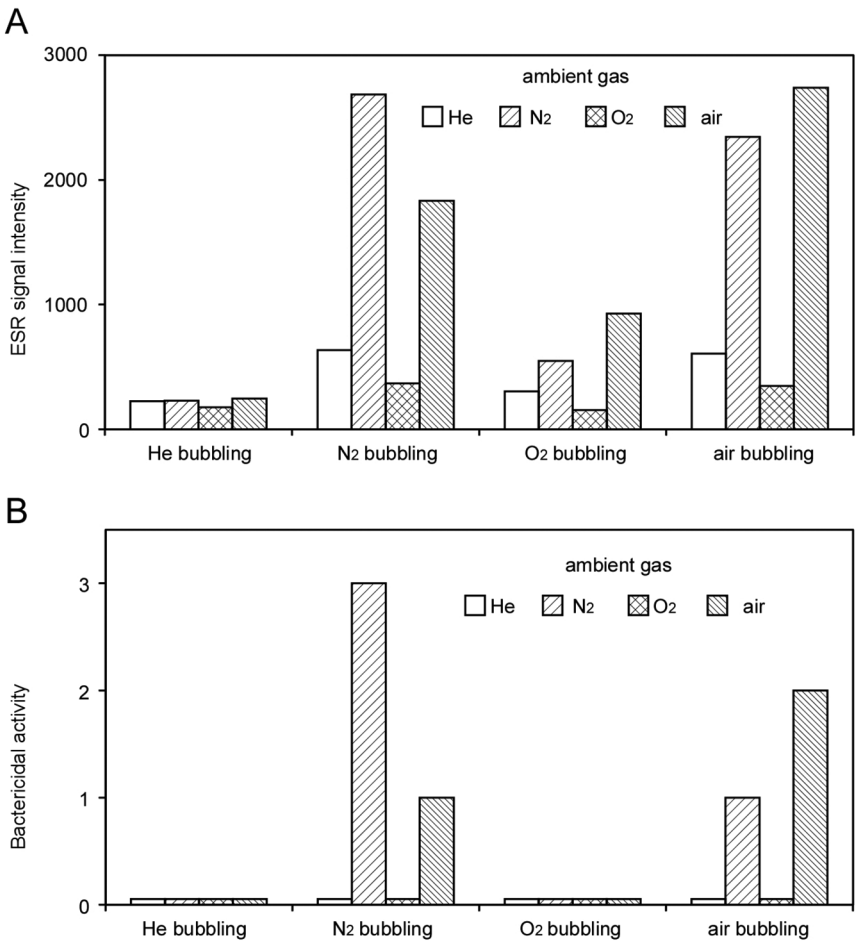

Figure 9. PTW preparations with various atmospheric and dissolved gas species in DW. PTW was prepared by discharging the LF plasma jet in the presence of controlled atmospheric and dissolved gases. $O_2^{-\bullet}$ adduct-forming potential and bactericidal activity of PTW were measured. (A) Signal intensity of the $O_2^{-\bullet}$ adduct, as determined by ESR. (B) Bactericidal activity against *E. coli*. Helium, nitrogen, oxygen, and synthetic air were used as atmospheric and dissolved gases; bactericidal activity was evaluated by MIC.

generating the key active species in PTW, and oxygen molecules are not necessary to produce the CYPMPO-$O_2^{-\bullet}$ adduct. Though O_2 gas concentration in ambient gas was clearly different between N_2 and synthesized air (0% and 20%, respectively), no significant difference was observed in the result shown in figure 9. This suggests that O_2 molecule is expected to have no or a slight effect to quench the CYPMPO-$O_2^{-\bullet}$ adduct-producing ability or bactericidal activity.

Similar results were obtained when the bactericidal activity of the same samples was assayed; that is, PTW showed strong activity only when nitrogen molecules were present in the ambient and dissolved gases.

4. Discussion

A number of studies have investigated the potential for sterilization or disinfection using atmospheric pressure plasma [3, 5, 11, 13, 19, 42–44], and reports of plasma sterilization of liquid have also increased in recent years [15–17, 20, 22, 26–28]. However, in most of these studies, the bacterial suspensions were directly exposed to plasma [15, 17, 20, 22], which is conceptually distinct from the bactericidal potential of plasma-exposed liquid described herein. Even in some studies exploring a similar concept, the bactericidal species supplied to or generated in liquids consisted of commonly used chemicals such as hydrogen peroxide, nitrous acid, and ozone [26, 27]. Nonetheless, given our observation that PTW was inactivated within a short period (i.e. several minutes) at RT, it is proposed that the bactericidal activity of PTW is not derived from these common chemicals.

Consistent with our previous report [22], the bactericidal activity of PTW was highly dependent on the pH of the solution, with high activity observed at low pH and no effect detected at neutral pH. This pH dependence is likely to be derived from $HOO^{\bullet}$ formed by protonation of $O_2^{-\bullet}$ (equation (2)), where $HOO^{\bullet}$ is an electrically neutral species that can easily penetrate the cell membrane and damage intracellular components. $O_2^{-\bullet}$ may exert bactericidal activity even when present in PTW.

$$O_2^{-\bullet} + H^+ \rightleftarrows HOO^{\bullet} \quad (2)$$

Although free $O_2^{-\bullet}$ molecules directly supplied by plasma discharge cannot be preserved in PTW for long term due to its short half-life up to few seconds, the results of the bactericidal assay with SOD presented in figure 6 support this hypothesis. When the radical-trapping reagent CYPMPO was

Table 1. Activation energy for decomposition of compound *X*, peroxynitrous acid, and peroxynitric acid.

Chemical species	Activation energy (kJ·mol^{-1})	Reference
X (bactericidal activity)	109	This study
X ($O_2^{-\bullet}$-adduct forming activity)	115	This study
Peroxynitrous acid (ONOOH)	77	[47]
Peroxynitric acid (O_2NOOH)	110	[50]

used to detect free radicals in PTW, we found that only the $O_2^{-\bullet}$ adduct was present and no other radicals such as $OH^{\bullet}$ were detected. However $O_2^{-\bullet}$ was not detected in frozen PTW by direct ESR observation at 77 K. These results mean that $O_2^{-\bullet}$ was not stored in this form but gradually released from a substance '*X*' (equation (3)).

$$X \rightleftarrows O_2^{-\bullet} + Y \tag{3}$$

This hypothetical active substance '*X*' is expected to degrade with time into inactive components '*Y*'. From the Arrhenius plots in figures 5 and 8, the activation energy for degradation of '*X*' was 109 kJ mol^{-1}, obtained based on the decrease in the concentration of bactericidal species, and 115 kJ mol^{-1} for formation of the $O_2^{-\bullet}$ adduct (table 1). These values are almost the same. We also found that both the bactericidal activity and intensity of the signal of the $O_2^{-\bullet}$ spin adduct were relatively enhanced when gases containing nitrogen molecules were used as both the plasma ambient and dissolved gases, suggesting that nitrogen molecules are necessary to generate the bactericidal species. That is, the intensity of the ESR signal of the $O_2^{-\bullet}$ adduct increased only when air or nitrogen gas was used as both the ambient and dissolved gases, and the level of bactericidal activity of PTW corresponded to the amount of $O_2^{-\bullet}$ adduct. These results suggest that the $O_2^{-\bullet}$-releasing substance *X* is a dominant species affecting the bactericidal activity of PTW, and *X* may be some type of nitrogen oxide or oxoacid.

Peroxynitrous acid (ONOOH), or the peroxynitrite ion ($ONOO^-$), is an oxoacid of nitrogen [45] and has been reported to be a major bactericidal species in plasma-exposed water [15, 27–31]. ONOOH is generated from HNO_2 and H_2O_2 (equation (4)) with a low reaction rate; ONOOH then decomposes into $OH^{\bullet}$ and $NO_2^{\bullet}$ (equation (5)) with a fast reaction rate. Thus, short-lived bactericidal ONOOH with a half-life of ~1 s under acidic conditions is continuously generated over a few days. The combination of these reactions reportedly leads to long-term bactericidal activity.

$$HNO_2 + H_2O_2 \rightarrow ONOOH + H_2O \tag{4}$$

$$ONOOH \rightleftarrows NO_2^{\bullet} + OH^{\bullet} \tag{5}$$

However, the ESR analysis of PTW presented herein showed the presence of $O_2^{-\bullet}$ and the absence of $OH^{\bullet}$ in PTW, whereas ONOOH tends to release $OH^{\bullet}$ at acidic pH rather than $O_2^{-\bullet}$ according to equation (5) [46]. The bactericidal species *X* in PTW are degraded in accordance with first-order kinetics, even under frozen conditions, suggesting that *X* is not generated continuously from two or more substances. Moreover, the activation energy for degradation of *X* was estimated as 109 kJ mol^{-1} and is clearly different from the activation energy for ONOOH decomposition (reported as 77.3 kJ mol^{-1}) [47]. These facts strongly indicate that the active species *X* in PTW is not ONOOH.

Peroxynitric acid, O_2NOOH, is another candidate for substance *X* with $O_2^{-\bullet}$ releasing ability based on equation (6) [48]. The half-life of O_2NOOH at 20 °C and pH 4.7 is estimated to be 1.6 min [49]; this is very similar to 1.8 min, which is the half-life of the bactericidal activity of PTW at the same temperature. The activation energies for degradation of O_2NOOH and substance *X* are estimated as 110 kJ mol^{-1} [50] and 109–115 kJ mol^{-1}, respectively. These values are remarkably similar. These facts suggest that the bactericidal active species *X* is O_2NOOH. Although advanced analysis, i.e. purification of substance *X* by liquid chromatography [51], is necessary to prove this hypothesis, the concentration of *X* in PTW used in this study was too low to carry out advanced analysis. It is necessary to develop another system to prepare PTW with a high concentration of substance *X*.

$$O_2NOOH \rightleftarrows HOO^{\bullet} + NO_2^{\bullet} \tag{6}$$

PTW preparation experiments with control of the ambient and dissolved gases revealed high bactericidal activity and $O_2^{-\bullet}$ adduct-forming capacity when no oxygen was present in the ambient and dissolved gases. O_2NOOH is generated by the reaction of ONOOH and H_2O_2 (equation (7)), and ONOOH is generated with H_2O_2 via equation (4), suggesting that H_2O_2 plays an important role in the formation of O_2NOOH in PTW. In fact, H_2O_2 was detected in PTW together with NO_3^- and NO_2^-. Direct plasma irradiation of DW can induce dissociation of H_2O into $H^{\bullet}$ and $OH^{\bullet}$, resulting in H_2O_2 formation, which accounts for why O_2 gas is not required to prepare PTW with bactericidal activity. Nevertheless, there are many unanswered questions regarding the involvement of dissolved nitrogen gas in the generation of bactericidal species *X*, which will be the focus of future investigations.

$$ONOOH + H_2O_2 \rightarrow O_2NOOH + H_2O \tag{7}$$

5. Conclusion

We demonstrated that PTW exerts strong bactericidal activity at low pH; the bactericidal activity was attenuated at RT, although the duration for which the bactericidal activity was maintained could be prolonged by cooling or freezing. PTW also had $O_2^{-\bullet}$ adduct forming ability, suggesting that an $O_2^{-\bullet}$ releasing substance was present in PTW. The bactericidal activity was attributed to a short-lived chemical species and was rapidly lost when SOD was added to PTW, suggesting that $O_2^{-\bullet}$ is important for this effect. These results are congruent with the hypothesis that the bactericidal species in PTW is not peroxynitrous acid, ONOOH, but peroxynitric acid, O_2NOOH. Moreover, the activation energy associated with degradation of the bactericidal activity and $O_2^{-\bullet}$ adduct formation were remarkably similar to that of peroxynitric acid, O_2NOOH. Additionally, the presence of nitrogen molecules in both the ambient and dissolved gases used to prepare PTW was essential for expression of the bactericidal activity

and O_2^- • adduct-forming capacity. Based on these findings, we propose that the bactericidal species in PTW is O_2NOOH.

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