Current Plasma Sterilization and Disinfection Studies

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Plasma sterilization methods have been studied for over 20 years in Japan and around the world, and are currently being developed for practical application. However, persisting challenges, such as the speed of the sterilization treatment, treatment of pathogenic proteins on medical equipment, and the compatibility of plasma with materials of medical equipments, currently limit the widespread application of the techniques. This paper introduces research into plasma sterilization for practical use, which is currently being carried out.

Keywords: Medical sterilizer, Active oxygen species, Plasma chemical indicator, Prion protein, Material compatibility

1. Introduction
Sterilization technology in support of modern medicine has a research and development history dating back more than 150 years. Sterilization methods using high temperature and high pressure (autoclave) and ethylene oxide gas (EOG) have some restrictions for utilization. Non-heat resistive materials cannot be applied to the autoclave, and EOG has significant toxicity for animals including human.

In recent decades, studies and developments involving the medical application of plasmas have focused on the sterilization and disinfection of medical equipment and facilities [1-12]; as well as medical treatments such as cancer therapy, treatment of cardiac disease and healing burns [13-17]. Plasma sterilization techniques, which are regarded as the first generation of medical and biotechnological applications of plasmas, have been studied in Japan and other countries. Plasma sterilization techniques are regarded as relatively new and innovative approaches in medical practice, in light of their potential to address the limitations of conventional sterilizers, such as sterilization time and treatment of infectious proteins. Early fundamental research into the plasma sterilization is now being developed for practical uses.

Since plasma can be produced at temperature lower than 60 °C, and active species in plasmas those are sterilization factors have significantly short lifetime less than ms in the low temperature circumstance, the plasma sterilization methods are promising technique for the sterilization of medical equipments, which are made of non-heat resistive materials. These techniques are now achieving the required sterilization standards, and practical application is close to becoming realized. However, there remain key areas requiring further investigation such as; the speed of the sterilization process [18-20], disinfection of abiotic materials that have pathogenicity [21-38], and understanding the compatibility of plasmas with different materials [39]. This work is necessary for the continued development of this very promising area. This report introduces current efforts to address the described challenges, which are currently being carried out in Japan. First, this summary details the low-pressure radio-frequency (RF) oxygen plasma sterilizer, which is closest to practical application. We then describe high-speed plasma sterilization...
using microwave discharge. The efficacy of plasma sterilization for the treatment of prion protein, which is abiotic but has high infectivity and persistence, and has been drawing attention as a cause of bovine spongiform encephalopathy (BSE), commonly known as mad cow disease, is then addressed. Also a process indicator, which determines an endpoint that ensures complete sterilization, is introduced. Finally, the material compatibility of oxygen plasma with non-heat resistant equipment is evaluated.

2. Sterilization of medical instruments using oxygen RF plasma

2.1. Oxygen RF plasma sterilizer

Current clinical sterilization methods use high temperature and high-pressure steam, which can cause damage to medical equipment; or chemicals such as ethylene oxide gas (EOG) that are toxic to humans and can cause environmental pollution. A plasma-based sterilization method that works at low temperature without using toxic chemical agents would therefore be a desirable alternative [1-12]. In this study, the sterilization characteristics of low-pressure RF plasma sterilization using oxygen gas are explained. Figure 1 shows the experimental setup of typical low-pressure RF capacitively coupled plasma. The plasma is generated in a vacuum chamber at a gas pressure in the order of several ten Pa and RF power in the order of several ten W, with a frequency of 13.56 MHz [1,2]. The gas for plasma production is pure oxygen. The temperature inside the vacuum chamber is kept below 60 °C to achieve low-temperature sterilization.

The active sterilization agents in the oxygen RF plasma are active oxygen species such as O(5P), O(1D) and 1Σg+, which are observed in the oxygen plasma emission spectrum of the afterglow region. Their production depends on the pressure in the chamber as shown in Fig. 2. These active species have significantly longer lifetimes under low-pressure, in the order of µs, than ions and electrons in the oxygen plasma. The chemical indicator (CI) shows that a sufficient amount of active oxygen species were obtained in the afterglow region at several tens of cm from the RF discharge electrode.

Biological indicators (BI) are used as a standard method for evaluating bactericidal efficiency. Spores of Geobacillus stearothermophilus (ATCC7953), which are heat-resistant bacterial spores, are commonly used as a test microorganism for low-temperature sterilizers. The Bi those are

<table>
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<tr>
<th>RF power (W)</th>
<th>20 min BI</th>
<th>40 min BI</th>
<th>40 min BI</th>
<th>80 min BI</th>
<th>100 min BI</th>
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<td>20</td>
<td>0/3</td>
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<td>60</td>
<td>0/3</td>
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<td>3/3</td>
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<td>80</td>
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packed into a non-woven sterilization bag located in the afterglow region of the plasma for exposure to neutral active species with longer lifetimes than charged particles. The bacterial spores are cultured after the plasma sterilization process. Sterilization is successful when there is no color change in the culture medium. Table 1 shows the sterilization results for a vial-type BI. Three BIs were used to test each set of conditions. The numerator in the table is the number of successful sterilizations. Success rate increases with RF input power and irradiation time. Sterilization of the BI completed in the shortest time (40 min) at an RF input power of 80 W, using a sterilization chamber with a capacity of 20 L. The decimal reduction values (D value) of the oxygen plasma was 21 min. The total period for the BI sterilization was approximately 90 min under these conditions, which is sufficient for practical application comparing with conventional sterilizers.

2.2. Sterilization of narrow tubes using oxygen RF plasma

Sterilization of long medical tubes with small diameters is a significant advantage of the plasma sterilization, as conventional methods using chemical agents can leave residues. Narrow tubes with length of several hundred mm and the diameter of several mm can be sterilized when placed in the afterglow region of a low-pressure oxygen RF plasma described above. A BI population of bacillus spores of $10^5$ was inserted into the tube at 100 mm intervals. The silicon rubber tube with the internal diameter of 4 mm and length of 500 mm was sterilized for 30 min with an RF power of 60 W (13.56 MHz) and oxygen gas pressure of 60 Pa.

To sterilize longer narrow tubes with length greater than 1000 mm, the glow discharge plasma can be produced inside the tube [12]. When a high voltage with a frequency of 10 kHz was applied to the discharge electrode placed on one end of a tube, an oxygen plasma was generated inside the tube, as shown in Fig. 3. The power consumption of the discharge was 15 W. The temperature inside the tube was kept below 70 °C. Complete sterilization of all BIs inside the tube was achieved by the plasma treatment for 10 min. Tube-like medical equipment can therefore be sterilized using active oxygen species generated in the equipments.

3. Sterilization using air plasma

3.1. Microwave air plasma

Inactivation of spores using low-pressure air plasmas has recently been reported as a novel sterilization method. The nature of the active species in low-pressure air plasmas depends on the plasma production method, such as CCP or ICP, and also vary depending on gas pressure and location within the plasma, discharge region or afterglow region. The species in air plasmas that are effective for sterilization have therefore not yet been identified. In this study, air plasma was generated using a torch type microwave plasma source under low-pressure conditions. The low-pressure air plasma sterilization method is expected to be safe and environmentally friendly, as no toxic chemicals are used, and only a small amount of NO$_2$ gas is released from the sterilizer. The plasma torch generates a remote plasma and performs sterilization using neutral active particles with long lifetimes. The treatment region was 20 cm away from the discharge region, and charged particles could not reach the treatment region. The temperature in the treatment region could therefore be kept below 60 °C. Sterilization using a torch type microwave plasma is therefore a promising methods for heat-sensitive medical devices.

The torch type microwave plasma source consisted of a glass tube with microwave antenna wound around the surface, and a vacuum vessel as the treatment chamber [18-20]. The frequency of the microwave source was 2.45 GHz. The glass tube comprising the plasma torch had an inner diameter of 5 mm and was set on the same axis as the vacuum chamber. The opening edge of the torch sat inside the treatment chamber. The pressure inside the glass tube and the vacuum chamber was reduced to the order of hundreds of Pa. Microwaves absorbed by the antenna produced a plasma inside the glass tube, which was blown from the opening edge. Vial-type BI was used to evaluate the sterilization efficiency, and was placed in the afterglow region within the treatment chamber.

3.2. Production of NO radical

Excited nitrogen and oxygen atoms were produced in the discharge region, and their longevity made it possible for them to pass from the torch into the treatment chamber. Yellow light emission from the plasma was observed at the opening edge of the plasma torch. Figure 4 shows the light emission spectra of the yellow plasma in the treatment chamber. The broad peak in the spectra is assigned to the NO radical, which is
produced when a metastable nitrogen atom combines with an oxygen atom. Because the intensity of this light emission was proportional to the efficacy of the sterilization, the light emission of NO was monitored to maximize NO production by changing the pressure and microwave input power. The light emission spectrum in the glass tube was the same as that of the plasma in the vacuum chamber. Therefore, NO radicals in the vacuum chamber were generated in the glass tube near the plasma production region.

To achieve low-temperature sterilization, the gas pressure and microwave power for plasma production must be controlled. As the pressure or the input power increases, the temperature of the objects being sterilized is also likely to increase. We found that a microwave power of 300 W and chamber pressure below 60 Pa kept the sterilization temperature below 60 °C. Plasmas produced under these conditions spread uniformly in the treatment chamber and allowed for the measurement of NO radicals using a strip-type chemical indicator. The CI showed that NO had higher oxidizing potential than active oxygen species, based on the speed of the CI color change being much faster than for the oxygen RF plasma. The electron density around the torch, 7 mm from the opening edge, was less than $10^5$ cm$^{-3}$, which is the detection limit of the Langmuir probe. Charged particles produced in the discharge region dissipate in the glass tube.

3.3. Sterilization efficacy of NO radical

The D value for NO radical sterilization determines the treatment period required for sterilization. A BI with Bacillus subtilis natto with the population around $10^4$ cfu/g on a glass mesh filter was placed in the diffused region below the opening edge of the torch, and exposed to the plasma for different treatment periods. The number of surviving bacillus was then counted using the colony counting method. Same sterilization tests were repeated four times to verify the reproducibility of the results. As shown in Fig. 5, the number of survived bacillus spores decreased to 10% of the initial population in 8 min, giving a D value of 8 min. However, it took 1 h for the population to decrease to 1% of the initial value. The departure from linear correlation in Fig. 5 is owing to the overlapping between spores, which makes it difficult to thoroughly permeate the BI.

4. Plasma technology for preventing iatrogenic prion disease

4.1. Prion agents

A sterility assurance level (SAL) of $10^{-6}$ is generally accepted as the definition of sterile for medical sterilization procedures [21]. This indicates that there is probably not more than one microorganism present on the sterilized item. Bacterial spores are generally used to assess the bactericidal effect of a sterilization procedure and calculate SAL, as they are thought to be resistant pathogens. However, pathogens known as prions are more resistant than bacterial spores to sterilization procedures. Therefore, even though sterilization is deemed to have been achieved with a SAL of $10^{-6}$, prions might not be inactivated. This review introduces prion agents and describes plasma technology as a potential sterilization method for prion inactivation.

Prions are pathogens that cause neurodegenerative disorders, known as prion disease or transmissible spongiform encephalopathy (TSE) [22]. Overall, sporadic
Creutzfeldt-Jakob disease (sCJD) of unknown cause accounts for 85–90% of prion disease cases. sCJD occurs at a rate of 1 case per 1 million individuals. Prion diseases can also be hereditary, such as familial CJD, fatal familial insomnia, and Gerstmann–Sträussler–Scheinker syndrome. If the prion disease is caused by infection with medical devices or materials, it is known as iatrogenic CJD. Prion disease can also occur in animals. A relevant example is bovine spongiform encephalopathy (BSE), which became well known as mad cow disease after the first report in 1987 [23]. The number of cattle with BSE peaked in 1992 and decreased thereafter [24]. In 1996, a new type of human prion disease derived from BSE, known as variant CJD (vCJD), was reported [25].

As of May 2015, 229 cases of vCJD had been recorded worldwide (including 177 in the United Kingdom) [26]. In general, individuals diagnosed with vCJD are younger than individuals with other types of CJD (age at death: vCJD 14-74 years, average 29 years; sCJD 20-95 years, average 66 years) [27].

In a recent UK study, vCJD prions were detected in the appendix at a rate of 16 cases per 32,441 individuals [28]. This suggests that there is a potential population of 30,000 individuals who are infected with vCJD and in a presymptomatic stage of the disease. It is therefore necessary to observe vCJD and BSE carefully and to take appropriate measures to prevent infection.

### Table 2. Effective treatments for prion inactivation.

<table>
<thead>
<tr>
<th>Inactivation methods</th>
<th>Representative references</th>
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<tr>
<td>Alkaline detergent (1.6%, 43°C, 15 min)</td>
<td>G. Fichert et al., Lancet 364, 521 (2004).</td>
</tr>
<tr>
<td>Hydrogen peroxide gas plasma sterilization (1.5 mg/L, 25°C, 3 h)</td>
<td>G. Fichert et al., Lancet 364, 521 (2004).</td>
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<tr>
<td>Copper (0.5 mmol/L) + hydrogen peroxide (100 mmol/L)</td>
<td>J. Tateishi et al., Microbiol. Immunol. 35, 163 (1991).</td>
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</table>

* Inactivation is insufficient by high-pressure steam sterilization (134 °C, 18 minutes) without dipping in water (if it is dried, inactivation becomes more difficult).

* Inactivation with peracetic acid (0.25%, 55 °C, 12 minutes), enzyme washing (0.8%, 43 °C, 5 minutes) plus autoclaving (121 °C, 20 minutes), enzyme detergent (0.8%, 43 °C, 5 minutes), hydrogen oxide gas (1.5 mg/L at 25 °C, 3 hours), or enzyme detergent (0.8%, 43 °C, 5 min) plus hydrogen peroxide gas (1.5 mg/L at 25 °C for 3 h) is insufficient.
infection, including neurosurgical procedures and orthopedic surgery, among others.

General sterilization procedures, such as autoclaving at 121 °C for 20 minutes, ultraviolet irradiation, γ ray irradiation, and alcohol treatment (70% ethanol treatment, etc.), are not effective for inactivation of prions [29,30]. This is because prion agents primarily comprise protein and do not contain nucleic acids. Effective prion inactivation requires treatment with a specific acid, such as formic acid, a strong base, such as NaOH, and a strong surfactant, such as sodium dodecyl sulfate (SDS), as well as autoclaving at a high temperature (134 °C or higher) (Table 2).

In the Guidelines of the Ministry of Health, Labor and Welfare of Japan, the following methods are recommended for inactivating CJD prions, based on scientific data [31]. i) Automated washer disinfection treatment with alkaline detergent (80–93 °C, 3–10 min) plus autoclaving (134 °C, 8–10 min); ii) sufficient washing with a suitable detergent plus autoclaving (134 °C, 18 min); iii) washing with an alkaline detergent (at a concentration and temperature specified in the instructions) plus vaporized hydrogen peroxide gas plasma sterilization. Many medical instruments are adversely affected by heat. In the case of instruments such as endoscopes, a combination of hydrogen peroxide and gas plasma sterilization is therefore recommended, as described in guideline (iii).

4.3. The potential of gas plasma as a method for inactivating prion agents and future perspectives

Most pathogen inactivation studies using gas plasmas focus on hydrogen peroxide gas plasma sterilization. Currently, several commercial gas plasma sterilization instruments are available. Inactivation of prions [32], human immunodeficiency virus (HIV) [33], hepatitis A virus [33], respiratory syncytial virus [33], vaccinia virus [33], herpes simplex virus [33], poliovirus [33], duck hepatitis B virus [34], and bacterial spores [35-37] have been shown using these
instruments. However, the sterilization achieved by these gas plasma instruments is primarily a result of hydrogen peroxide gas and not the gas plasma. In the case of these instruments, the gas plasma is used to decompose the hydrogen peroxide that remains after reaction. By contrast, in the case of gas plasmas derived from oxygen, nitrogen, argon, or helium, the gas itself does not have an inactivation effect on pathogens. Studies have succeeded in inactivating pathogens using gas plasmas derived from these gases possibly due to inactivation effects the gas plasma itself (Table 3). The lack of reports regarding the inactivation of prions using of oxygen, nitrogen, argon, or helium gas plasmas, means further studies using these gas plasmas would provide interesting results. In addition, it is necessary to characterize the gas plasma conditions that lead to efficient inactivation of pathogens and to clarify the mechanism by which the pathogens are inactivated. Such studies would clarify the advantages of gas plasma relative to other sterilization techniques, and would promote the practical use of gas plasma sterilization.

Recent studies have suggested that misfolded proteins responsible for neurodegenerative disorders such as Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis, frontotemporal dementia, corticobasal degeneration, and progressive supranuclear palsy may act as prions or share prion-like mechanisms and have transmissible properties [38]. This suggests that special treatments, such as the processes used to inactivate prion agents, might be required for medical devices used for patients with these neurodegenerative diseases, as well as for patients with prion diseases.

5. Material compatibilities of oxygen RF plasma

The compatibility of the plasma sterilizer with different materials is an important consideration in sterilizer development [39]. High velocity ions in the plasma can cause damage to the medical
equipment requiring sterilization. Neutral active species do not affect equipment in the same way because they are not accelerated by the electric field surrounding the discharge electrode. The compatibility of non-heat tolerant materials such as plastics and rubber with oxygen RF plasma treatment was investigated using tension tests and IR spectroscopy. Typical plastic materials, which are used for medical equipment, such as poly(methyl methacrylate) (PMMA), poly(vinyl chloride) (PVC), polyethylene terephthalate (PET), polystyrene, Teflon, silicone rubber, and natural rubber (NR) were irradiated with oxygen plasma in the sterilizer. Sample materials were formed into dumbbell-shaped specimens and enclosed in a non-woven sterilization bag. After sterilization, damage to samples caused by plasma irradiation was characterized using optical microscopy, IR spectroscopy, and tension test evaluation of mechanical strength.

With the exceptions of Teflon, NR and polystyrene (PS), all of the post-treatment plastic materials tested showed some changes in surface morphology by optical microscopy. As shown in Fig. 6(a), transparent materials such as PET and PMMA became opaque due to microscopic changes at the surface or crystallizing molecules in the bulk materials. Conversely, materials that originally appeared white, such as polystyrene and Teflon, showed no changes to their surface morphology. Figure 6(b) shows stress-strain curves obtained from the tension test for PET after 100 h of treatment. The tensile strength of PET showed no significant variation from the control samples after 100 h of sterilization. In the case of PMMA and PVC, the stress-strain curves show some differences compared with the control after 100 h of treatment. The materials tend to show softening, which could be attributed to cleavage of bonds within the structures by active oxygen species.

Figures 7(a) and (b) show the IR absorbance spectra of the surfaces of PET and polystyrene after 100 h of sterilization, respectively. The increase in CO bond and decrease in CH bond peak heights in both IR spectra indicates oxidation of the material surfaces. Because the variations in the peak heights are relatively minor in the case of PET and polystyrene, as shown in Fig. 7, any morphological changes should not greatly affect the strength of the materials. No additional peaks indicating the presence of byproducts were detected in the IR spectra. Therefore, oxygen plasma sterilization is compatible with PET and polystyrene despite slight changes to surface morphology. By comparison, when materials such as PMMA and PVC were treated with oxygen plasma for 100 h, there were noticeable differences in surface morphology.

Figure 8 shows the decomposition rates of major materials for medical equipments irradiated by oxygen plasma over varying treatment periods, which were estimated from the decrease in C-H bond peak height in the IR spectra. Significant degradations of PMMA, PVC and NR by penetration of the active oxygen species through the non-woven bag were observed after the plasma treatment for an hour. On the other hand, Teflon, polyethylene terephthalate (PET) and silicone rubber have hardly suffered modifications. The differences in the C-H bond peak height of these materials are within 10%.

Table 4 summarizes the compatibilities of the plastics used in this experiment with plasma sterilization, as evaluated by optical microscopy, tensile strength testing, and IR absorption spectra. The open circle indicates that functions as medical equipment are kept same as the control. The open triangle and cross symbols of the tension test and FTIR in the table denote differences less than 10% and more than 10% from values of untreated
materials, respectively. Also, those in the appearance indicate the small modification of the surface and generation of cracks, respectively. In the open triangle case, despite some visual differences in surface morphology due to changes in the chemical structures, the stress-strain results indicate that almost of the materials after the oxygen plasma irradiation, with the exception of PMMA, have the same strength as the untreated materials. Important materials for medical equipments such as PET, Silicon rubber and Teflon are suitable for the sterilization processes using active oxygen species giving a minor effect on plastic materials. Therefore, the oxygen plasma sterilizer has sufficient material compatibility.

6. Summary
Recent research into plasma sterilization has shown the possibility of satisfying persistent challenges. Sterilization period less than an hour, sterilization inside loing narrow tube, inactivation of prion protein, development of CI for active oxygen species, and database of material compatibility lead to full commercialization of the plasma sterilizer. It is likely that plasma sterilization methods will be put to practical use in the near future as a forerunner of plasma application in a clinical context.

Acknowledgment
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References


