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Mass spectrometric study on inactivation mechanism of spore-forming bacteria by low-pressure surface-wave excited oxygen plasma

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In this letter, the etching phenomena of the spore-forming bacteria by oxygen plasma were investigated by using quadrupole mass spectrometry. The etching by-products of H₂O and CO₂ were obviously detected during the oxygen plasma irradiation by the multiple ion detection measurement. Inactivation of roughly 10⁶ spores population was achieved under almost the same reduced spore shapes for three different incident microwave powers. It is considered from the present results that the oxygen radical etching could cause damage to the germinant receptors located in the inner membrane inevitable for germination of spores, without any damage of the DNA in the cores.

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Inactivation mechanisms of spore-forming bacteria have been extensively studied to establish the plasma technique as a more effective sterilization tool. As for the low-pressure plasma sterilization, Boucher, *et al.*¹ suggested the UV photons in plasma played an essential role on sterilization, and Bol'shakov, *et al.*² reported that high densities of excited oxygen radicals induced chemical degradation of the biological materials followed by volatilization of the decomposition products. Moisan *et al.*³ insisted that chemical reactions, UV-induced damage or photodesorption and ion sputtering act synergistically, the inactivation by these processes were significantly faster than that by either process alone.

For the atmospheric pressure plasma sterilization, Park *et al.*⁴ concluded that the strong etching process observed is actually responsible for sterilization. Perni *et al.*⁵ claimed that oxygen atoms are identified as a major contributor in plasma inactivation with minor contributions from UV photons, OH radicals, singlet oxygen metastables, and nitric oxide. Kong *et al.*⁶ reviewed the recent experimental results on sterilization or biological effects of atmospheric plasmas and they also stated that the plasma-generated UV radiation and reactive species including free radicals and some ground state molecules such as peroxides and ozone play the important role on the inactivation of viruses and bacteria. At the present time, although there have been a lot of investigation on sterilization mechanisms of low temperature plasma, there are no fully understandable explanations as to the respective contributions of the various agents emanating from plasma to the bacterial killing process and this method is still under investigation.

In our previous works, we reported that a six-log reduction in spore population could be achieved only several minutes' irradiation with low-pressure oxygen surface-wave plasma.⁷ It was clearly seen from the scanning electron microscope (SEM) analysis that the sizes of spores were significantly reduced with increasing plasma irradiation time due to etching effect.⁸ But until now, the measurements were not sufficient to confirm that the irreversible damage of samples was resulted from the erosion because the observa-

tion of the samples was performed by using the SEM after plasma treatment.

In this work, therefore, we carried out the mass spectrometric studies using quadrupole mass spectrometry (QMS) (Ref. 9) to confirm the etching event directly by measuring the etching by-products of micro-organisms during oxygen plasma irradiation and discussed the relation between the spore etching and sterilization characteristics, focusing upon the etching effect by oxygen radicals on the inactivation of microorganisms.

The experimental setup consists of a stainless steel cylindrical vacuum chamber of 300 mm in diameter and 300 mm in height with a microwave launcher and 2.45 GHz microwave generator, as shown in Fig. 1. The output power of the microwave oscillator was varied from 0 to 1.5 kW. The plasma discharges sustained in O₂ at a gas flow rate of 100 SCCM (SCCM denotes cubic centimeter per minute at STP) and a pressure of 13.3 Pa. The sterilization experiments were performed using nonpathogenic spore-forming bacteria, *Geobacillus stearothermophilus* (ATCC #12980, Raven Biological Laboratories), as the biological indicator (B.I.). The population of spores pasted on an oblong polished stainless steel disk was 1.7 × 10⁶. These samples were placed in a glass Petri dish on the stage about 10 cm below the quartz window. The top of the glass Petri dish was covered with a stainless steel shutter having 100 mm in diameter and 5 mm in thickness to block the plasma particles and plasma optical

FIG. 1. (Color online) Schematic drawing of experimental setup.

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FIG. 2. (Color online) Time evolution of H_2O and CO_2 components during *Geobacillus stearothermophilus* samples were irradiated by O_2 plasma.

emission. The shutter was controlled with closing/opening time as 30 s/60 s and the shutter was closed at the beginning of experiment. The volatile compounds of microorganisms during the plasma exposure were measured by QMS (Pfeiffer Vacuum, Prisma™ QMS 200). In order to investigate the etching efficiency, we varied the net microwave power in 400 W, 600 W, and 800 W, respectively. In each condition, some of the B.I. samples were treated under a LiF filter (having the optical transmission at $\lambda > 120$ nm) to block the plasma particles and some B.I. samples were directly exposed to the plasma without the LiF filter. During plasma discharges, the stage temperature was monitored with a thermo label attached to the rear surface of the glass Petri dish. After the plasma treatment, the samples were washed with 1.5 ml of Brain-Heart Infusion solution in the test tube for colony count measurement. Morphological changes in spores by plasma treatment were observed by SEM (Hitachi, S-3000N). For the optical emission spectroscopy (OES) measurement, the vacuum ultraviolet (VUV) to IR monochromator systems (Acton Research Corporation, VM-502) were used.

Because the etching by-product was small amounts, we used the multiple ion detection operation mode of QMS, where the temporal changes in the specific mass components were simultaneously monitored. In order to obtain enough signal intensity, twenty B.I. samples were used each time. The evolutions of the appropriate mass numbers of by-products expected from the components of bacteria were measured during plasma treatment. Figure 2 shows the time evolution of the H_2O ($m=18$) and CO_2 ($m=44$) components, during the period B.I. samples were irradiated by O_2 plasma at a microwave power of 600 W. As opening the shutter first after getting stable oxygen plasma, it is clearly seen that the ion currents of the H_2O and CO_2 significantly increased from original values to each maximum. Through several rounds with shutter closing/opening times of 30 s/60 s, the changes in H_2O and CO_2 were gradually reduced, and the signals were almost back to the original levels at last. These by-products were apparently originated from the components of spore coats via the chemical etching process by oxygen radicals. In addition to the mass components of $m=18$ and 44,

FIG. 3. (Color online) Intensities of O atoms at 130.5 and 777.2 nm observed by OES as functions of net input power.

we also observed a component of $m=28$, which is plausibly considered as CO.

In order to investigate the effect of reactive species on the spore inactivation, the power dependence of oxygen emission lines were studied by the OES. The two main lines of atomic oxygen which correspond to the transition $3s^3S \rightarrow 2p^4P$ at 130.5 nm (Ref. 10) and the transition $3s^5S \rightarrow 3p^5P$ at 777.2 nm were used for monitoring the excited oxygen atoms. The net microwave power controlled in operational ranges of 300–900 W raised the intensity of O at 130.5 and 777.2 nm, as shown in Fig. 3. With an increase in input microwave power, optical emission intensities were roughly linearly increased. Thereby, it is expected that the production of the reactive oxygen species promotes the process of chemical etching with an increase in net microwave power.¹¹

Figure 4 shows the results of colony forming units (CFUs) of the *Geobacillus stearothermophilus* spores treated by oxygen plasma without and with the LiF filter at 400 W, 600 W, and 800 W, respectively. The treatment temperature in all the cases was maintained below 65 °C by on/off plasma operation, where plasma on- and off-time were 1 min/2 min, respectively. It is noted here that the survival curves show a multislope, the first segment is related to the

FIG. 4. (Color online) Survival curves of *Geobacillus stearothermophilus* treated with and without a LiF filter by O_2 plasma at different net input powers of 400 W, 600 W, and 800 W, respectively.

FIG. 5. (Color online) SEM and QMS analyses showing (a) length and (b) width of spore versus O_2 plasma treatment time at different net input powers.

inactivation of isolated spores, while the second one may have to do with clamped spores.^{12,13} When the LiF filter was set above the B.I. samples, the CFUs obviously decreased with treatment time. About 90% spores were killed by only VUV/UV radiation at each six-log reduction time for direct plasma exposure, as shown by arrows in Fig. 4. It means that the VUV emission of atomic oxygen at 130.5 nm could contribute to the spore mortality, it might be because the VUV/UV high energy photons have enough energies to break C–C (~3.4 eV) or C–H (~4.1 eV) bonds of DNA strands.^{14,15} Because the typical depth of photochemical reaction is restricted to thin layer, the deactivations by VUV/UV photons are rather restricted in the case of the clamped spores. The oxygen radicals interact with the outermost layer of the spore and the topmost material of spore to reduce any shadowing of the incident UV light.

In order to study the etching effect of the spores subjected to the inactivation process, SEM and QMS analyses were performed. The SEM statistical analysis of the spore length and width was made for 50 spores by measuring the size of spores with micrographs. The results were plotted in Figs. 5(a) and 5(b). It is clearly seen that the length and width of the spores were monotonically decreasing with the treatment times for different microwave powers of 400, 600,

and 800 W. The shapes of the initial ellipsoidal structure shrink to slender shapes like jelly beans, and only small fragments left after longer time treatment. Here, it is interesting to note that the six-log reduction of surviving spores was achieved at each plasma treatment time (plotted by dashed lines), when the spore etching was not completed, but still on the way. From Figs. 5(a) and 5(b), it is seen that the shapes of etched spores at the six-reduction times were almost the same for different powers, indicated by arrows, that is, length was 1.35–1.40 μm and width was 0.65–0.70 μm , respectively. After these erosion stages, all the spores did not indicate any germination processes even under the nutrient incubation condition. These results are very important to understand the inactivation mechanism of spore-forming bacteria by the oxygen plasma. It is well known in the biochemical field that the germination of spores occurs when the germinant receptors located in the inner membrane of the spores were triggered by the specific nutrient germinants, such as L-Alanine or a mixture of D-glucose, fructose, L-Asparagine, and KCl.¹⁶ The inner membrane inside the spore plays an important role to convert from the dormant spores into metabolically active vegetative cells. In other words, the germination of spores could occur no longer, once the inner membranes were damaged by some reason, even though the DNAs inside the core were not damaged by UV emission or radicals directly. The present results suggest the possibility that the inner membranes were directly damaged by the oxygen erosion process.

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- ¹R. M. Boucher, U.S. Patent No. 4,207,286 (10 June 1980).
- ²A. A. Bol'shakov, B. A. Cruden, R. Mogul, M. V. V. S. Rao, A. P. Sharma, B. N. Khare, and M. Meyyappan, *AIAA J.* **42**, 823 (2004).
- ³M. Moisan, J. Barbeau, S. Moreau, J. Pelletier, M. Tabrizian, and L'H. Yahia, *Int. J. Pharm.* **226**, 1 (2001).
- ⁴B. J. Park, D. H. Lee, J. C. Park, I. S. Lee, K. Y. Lee, S. O. Hyun, M. S. Chun, and K. H. Chung, *Phys. Plasmas* **10**, 4539 (2003).
- ⁵S. Perni, G. Shama, J. L. Hobman, P. A. Lund, C. J. Kershaw, G. A. Hidalgo-Arroyo, C. W. Penn, X. Deng, J. L. Walsh, and M. G. Kong, *Appl. Phys. Lett.* **90**, 073902 (2007).
- ⁶M. G. Kong, G. Kroesen, G. Morfill, T. Nosenko, T. Shimizu, J. van Dijk, and J. L. Zimmermann, *New J. Phys.* **11**, 115012 (2009).
- ⁷M. Nagatsu, F. Terashita, H. Nonaka, L. Xu, T. Nagata, and Y. Koide, *Appl. Phys. Lett.* **86**, 211502 (2005).
- ⁸Y. Zhao, M. K. Singh, A. Ogino, and M. Nagatsu, *Thin Solid Films* **518**, 3590 (2010).
- ⁹H. Y. Zhou, J. Watanabe, M. Miyake, A. Ogino, M. Nagatsu, and R. J. Zhan, *Diamond Relat. Mater.* **16**, 675 (2007).
- ¹⁰L. G. Piper, M. A. A. Clyne, and P. B. Monkhouse, *J. Chem. Soc., Faraday Trans.* **78**, 1373 (1982).
- ¹¹J. R. Chen, *Low Temperature Plasma Chemistry and its Application* (Science, Beijing, 2001).
- ¹²X. T. Deng, J. J. Shi, G. Shama, and M. G. Kong, *Appl. Phys. Lett.* **87**, 153901 (2005).
- ¹³M. K. Singh, A. Ogino, and M. Nagatsu, *New J. Phys.* **11**, 115027 (2009).
- ¹⁴N. Munakata, M. Sato, and K. Hieda, *Photochem. Photobiol.* **54**, 761 (1991).
- ¹⁵C. Lindberg and G. Horneck, *J. Photochem. Photobiol.*, **B** **11**, 69 (1991).
- ¹⁶S. J. Foster and K. Johnstone, in *Regulation of Prokaryotic Development*, edited by I. Smith, R. A. Slepecky, and P. Setlow (American Society for Microbiology, Washington, 1989), pp. 89–108.