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# Role of Metastable and Spore Hydration to Sterilize Spores by Nitrogen Gas Plasma Exposure and DPA Analysis by HPLC and UV

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**Research Article** 

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# Abstract

Many papers have been published so far on gas plasma sterilization. They are mostly conducted by physical researchers, so microbiology and chemical aspects are significantly insufficient. By joining the biologists and chemists, gas plasma sterilization research was outstandingly advanced. The mechanism was not clear until now and from the life time and some other reasons metastables can be the most appropriate factors for sterilization. Spore death is explained from the hydration of dipicolinic acid in the core. By attacking metastables spore surface was pin hole opened and the interior water and surrounding water penetrate into the core to hydrate DPA. DPA in the surface was collected with water and enriched with SPE (solid phase extraction) column. SPE drain was analyzed with C-18 column and eluted with an acetonitrile/water (1/4, v/v) and detected with 235 nm. Spore surface particle can be confirmed DPA. The hydration process can cause within spore, so spore figures are unchanged before and after sterilization.

**Keywords:** Nitrogen gas plasma; Radicals; Metastables; Dipicolinic acid; Hydration; Reverse phase HPLC; Ion suppression

#### Introduction

Many papers on gas plasma sterilization have ever been published so far [1-7] and most of papers and books on gas plasma sterilization were conducted by physical researchers [3-7]. Sterilization research is required a combined knowledge of chemistry, engineering and microbiology, so the past research results done by physical researchers were significantly insufficient of microbiological and chemical aspects, so it inhibits to reach the final clarification by only engineering researchers. After joining the microbiologist and chemist to gas plasma sterilization research, the content of the research is much improved and advanced [1,2]. Current understanding on mechanism of gas plasma sterilization is almost approaching to the final stage because we confirm what we do not know and what we need to clarify by joining microbiologist and chemist, so describing about what we clarified about sterilization mechanism by nitrogen gas plasma in the most recent stage.

# Experimental

# Low pressure nitrogen gas plasma apparatus

The nitrogen gas plasma sterilization chamber is what we used in citation 1. The low pressure nitrogen gas plasma apparatus can be used at around 60°C under half atmospheric pressure with 40 to 150 mm gap between the cathode and anode electrodes. The sterilization assurance level (SAL) of 10° starting from initial population of 10° CFU (Colony Forming Unit) was achieved in 9 min.

#### Sterilization process of microorganisms

Sterility assurance was confirmed by using the biological indicator (BI). As the BI *Geobacillus stearothermophilus* ATCC 7953 [1,2] was inoculated onto a surface modified SUS (stainless steel) by gas plasma and 1X 10<sup>6</sup> CFU were evenly distributed on the modified SUS surface at nm level to avoid forming clumps [2]. The BI was commercially available from Merck (Tokyo, Japan).

#### **Results and Discussion**

As shown in Figure 1, it has been reported several sorts of sterilization factors to sterilize bacterial spores and microorganisms. Among them they are atoms, molecules, minus or plus ions, photons, electrons, free radicals and metastables and UV and VUV. Among these UV and VUV contribution was denied by Kong et al. [3] (Figure 2). Free radicals, especially OH radical, are an attractive factor due to high oxidation-reduction potential (Table 1), but life period of OH radicals is too short (around a few  $\mu$ s, Table 2), therefore OH and NO radicals may contribute to sterilization as minor factors, but not as a major factors. Surely, OH radical has the greatest oxidation-reduction potential (Table 1), but the life time is too short (a few  $\mu$ s, Table 2), so OH radical was not major contributor for sterilization. Oxygen and nitrogen metastables have so and so long period (a few  $\mu$ s, Table 2), so metastable can be considered to be a most probable candidates.



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Chemical name	Chemical structure	Oxidation-reduction voltage (V)
Hydroxy radical	OH ·	2.05
Oxygen atom	0 <sub>1</sub>	1.75
Ozone	O <sub>3</sub>	1.52
Hydrogen peroxide	H <sub>2</sub> O <sub>2</sub>	1.30
Hypochlorous acid	HCIO	1.10
Oxvaen molecule	0,	0.94

Table 1: Oxidation-reduction potential (V).

Chemical name		Life time (s)
	OH radical	a few
	NO radical	a few
	Singlet molecular oxygen <sup>1</sup> O <sub>2</sub> Oxygen metastable	7
	Nitrogen metastable	2

Table 2: Life time of radicals and metastables



From exited state down to ground state, metastables emitted energy following the equation of E=h Xv=h X c/ $\lambda$ , the produced energy attack the surface layer of the spore or microorganisms and produce pin hole of the spore surface. Ions or charged ones in Figure 1 are trapped with the outer membrane of the bacterial membrane or spore outer layers (Figures 3 and 4), so charged ones are considered not to appropriate factors. In the same meaning, electrons are minus charged particle, so it will not be a major factor.

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Among metastable, we find that lifetime of singlet Oxygen is 7s and that of nitrogen metastable is 2s (Table 2). As shown in Figure 5, we can observe that the N, metastable was produced by applying pulsedamperometric discharge and we measure the lifetime of N<sub>2</sub> metastable as a few s (experimentally 2 s, Table 2 and Figure 6) [4-6]. During the excited N<sub>2</sub> down to the ground state, energy emitted and the energy is useful for sterilization. So, we can estimate N<sub>2</sub> or O<sub>2</sub> metastables may be the most favorable candidates to inactivate bacterial spores and bacterial vegetative state.

However, we have a problem. Bacterial spore death is considered by causing hydration of dipicolinic acid (DPA, Figures 3 and 8), so how can we connect N<sub>2</sub> metastable to hydration of DPA. We observed dead spores with SEM (scanning electron microscopy) carefully and found out that dead spores had several white particles on their spore surface. We consider that the white particle may be hydrated dipicolinic acid, so we carefully trapped surface white particles with water. This is because if organic solvent used in place of water, interior DPA may also recover together with surface white particle.

# HPLC analysis of DPA

Water trapped white particle was adjusted to pH 5 with acetic acid and applied to C-18 SPE (solid phase extraction column, automated SPE) and thereafter eluted with acidified acetonitrile at pH 5 with acetic acid for ion suppression of DPA. Acidified acetonitrile was evaporated and re-dissolved with mobile phase of acetonitrile/water (1/4, v/v), injected 10 µL to HPLC and detected at 235 nm. Stationary phase is C-18 (4.6 X 200 mm, Shiseido). We confirmed that only the interior (within core) DPA was successfully eluted out and detected at 235 nm.



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Figure 5: Energy of excited N<sub>2</sub> and ground N<sub>2</sub>. The upper is the excited state and the lower is the ground state.

2.0

Internuclear Distance (Å)

2.4

2.8

1.6

3.2

3.6

1.2

0.4

0.8

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Strain	Hydrophobicity (%)
Bacillus subtilis ATCC 6633	94
B. subtilis ATCC 19221	95
B. atrophaeus ATCC 9372	47
B. cereus T	95
B. coagulans ATCC 8038	49
G. stearothermophilis ATCC 9372	53
B. megaterium ATCC 12872	88
B. megaterium ATCC 33729	30
Clostridium botulinum 213B	50
C. sporogens ATCC 7955	67
C. putrefaciens ATCC 25786	78

Table 3: Hydriphobicity of several spores.



The interior DPA was hydrated with the penetrated water caused by  $N_2$  metastable and core DPA transported from the core to the surface of the spore. Water for hydration is from the interior of spore (Table 3) as well as surrounding spore.

We now speculate how the spore died with maintaining spore figure (Figure 7) [7]. The spore surface was attacked by metastables to produce pinhole of the spore surface. The water surrounding the bacterial spore and interior water of the spore (Table 3) may penetrate



into the core to hydrate DPA (Figures 3 and 8). The killing process may be caused within the spore, therefore figures of the dead spore were identical to those of the control (Figure 7) [1-3].

### Conclusion

Spore surface was produced pinhole with the energy from the  $N_2$  metastables. DPA in core was hydrated with interior water and the surrounding water of the spore. The hydrated DPA transported into the surface layer and remained as white particle. This is the mechanism of spore death by metastables and the spore figures were not changed after death because the process was caused within spore. This is the mechanism of sterilization of spore by gas plasma exposure. DPA can be analyzed by ion-suppression reversed phase C-18 combined with UV 235 nm and the mobile phase was acetonitrile-water (1/4, v/v) at pH 5 with acetic acid.

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