

Research Article

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

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### Abstract

Many papers have been published 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 sterilization mechanism by gas plasma was not clarified until now and from the life time and some other reasons metastables or photon can be the most appropriate candidate for sterilization. Spore death is explained from the hydration of dipicolinic acid in the core. By attacking the spores with metastables or photons, it produced pin hole 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 HPLC column and eluted with an acetonitrile/water (1/4, v/v, pH5) and detected at 235nm. From the coincidence of retention time of the studied target and that of standard substance, spore surface particle can be confirmed DPA. The hydration process can cause within the spore, so spore figures are unchanged before and after sterilization.

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

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### Introduction

Many papers on gas plasma sterilization have ever been published so far<sup>[1-7]</sup> and most of papers and books on gas plasma sterilization were conducted by physical researchers<sup>[3-7]</sup>. Research in sterilization methods and mechanism of sterilization is required to integrate combined knowledge of chemical, engineering and microbiological aspects. The combined effort of these specialists in the area of gas plasma sterilization have contributed to improvement in this area of research<sup>[1-2]</sup>. The present study aims to clarify our understanding of the mechanisms of sterilization by nitrogen gas plasma.

It is said that hydration of dipicolinic acid (DPA) is the cause of spore death. We find that quite small particle on spore surface by scanning electron microscopy (SEM) and we speculate that small particle may be hydrated dipicolinic acid migrated from the core. In order to clarify this speculation and small particle is DPA or not, they are analyzed by HPLC and MS (mass spectrometry).

### Experimental

#### Low pressure nitrogen gas plasma apparatus

The nitrogen gas plasma sterilization chamber used

was similar to that described in (Figure 1 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. The sterilization assurance level (SAL) of 10<sup>0</sup>, starting from initial population of 10<sup>6</sup> CFU (Colony Forming Unit) was achieved in 9min, indicating D value (decimal reduction value, time or does to use one log reduction) is 0.75 min (ISO 11138-1).

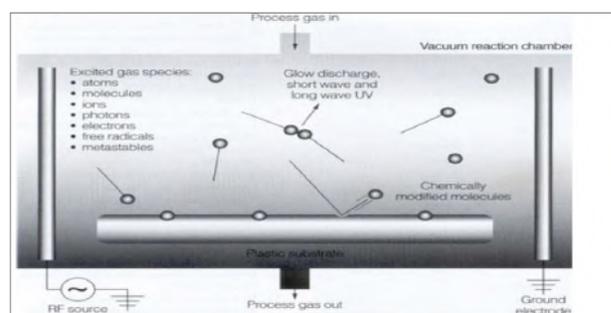


Figure 1: Several factors to connect gas plasma sterilization

#### Sterilization process of microorganisms

Sterility assurance was confirmed by using the biological indicator (BI). As the BI *Geobacillus stearothermophilus* ATCC 7953<sup>[1,2]</sup> was inoculated onto a surface modified SUS

(stainless steel) by gas plasma and  $1 \times 10^6$  CFU were evenly distributed on the modified SUS surface at nm level to avoid forming clumps<sup>[2]</sup>. Relatively clump free BI was commercially available from Merck (Tokyo, Japan).

**HPLC and automated solid phase extraction (SPE) conditions**

Sterilized spores were extracted with water. Extraction with water was used to distinguish between DPA on the surfaces and within core. As the solubility of DPA into water is only 1%, more than 100 BIs was necessary to collect sterilized spores. The collected water including sterilized spores was adjusted with acetic acid to pH5 to suppress ionization of carboxylic acid of DPA (dipicolic acid, Figure 8) and adhere to C-18 support of SPE. A pH5 adjusted water applied to C18 SPE column in order to adsorb dipicolinic acid to C18 column and eluted with acetonitrile. Acetonitrile was evaporated, condensed and re-dissolved into mobile phase of HPLC (acetonitrile/water, 1/4, v/v, pH5). The 10µL of this solution were applied into HPLC and detected with 235 nm and MS.

**Results and Discussion**

As shown in Figure 1, it has been reported several sorts of sterilization factors to sterilize bacterial spores and microorganism. Among them they are atoms, molecules, positively charged and negatively charged ions, photons, electrons, free radicals and metastables and UV and VUV. Among these UV and VUV contribution was denied by Kong et al<sup>[3]</sup> (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 µs, Table 2) and flight distance during life period of OH radical compared with N<sub>2</sub> metastable is too short (Table 3), therefore OH and NO radicals may contribute to sterilization as minor factors, but not as a major factors. Photon has also long life of 10<sup>18</sup> years, so flight distance will be infinity. Metastable and photon have an energy due to  $E=h\nu$  and long distance flight, therefore they may be major candidate of the sterilization. From excited state down to ground state, metastables and photons emitted energy as per the equation of  $E=h\nu$  (Figure 5), the produced energy attack the surface layer on the spore or microorganisms and produce pin hole on the spore surface. Ions or charged ones such as electrons in Figure 1 are trapped with the outer membrane of the bacterial or spore outer layers (Figures 3 and 4), so charged ones are considered not to major candidates.

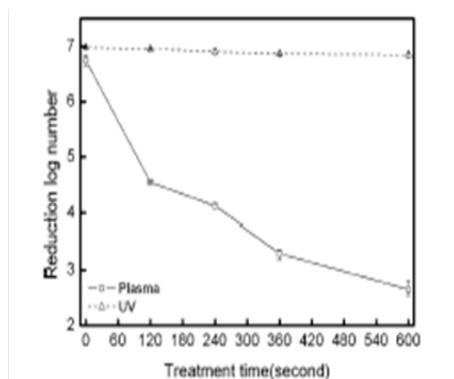
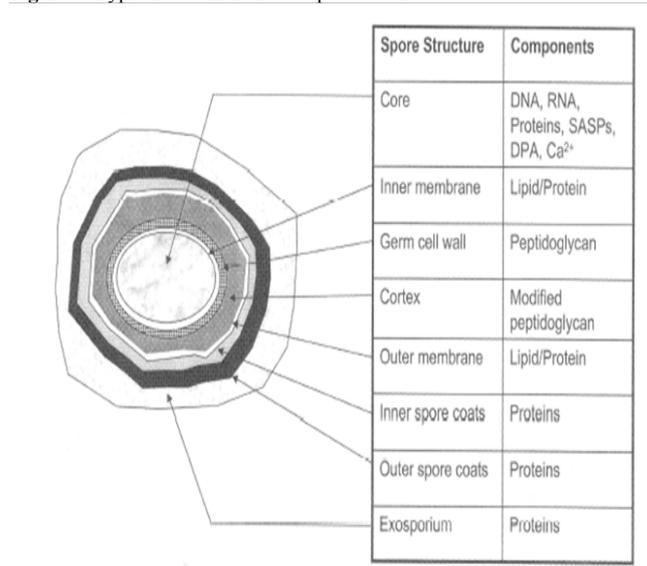


Figure 2: Effect of gas plasma and UV on BI sterilization

Figure 3: Typical bacterial-endoscope structure.



DPA is dipicolinic acid and in normal it exists as DPA=Ca (chelating).

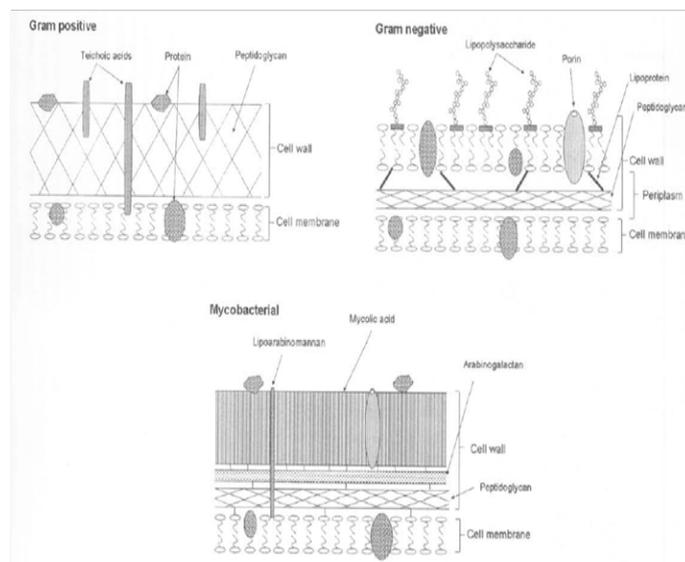


Figure 4: Typical structure of gram negative, gram positive bacteria and mycobacteria

Table 1: Oxidation-reduction potential (V)

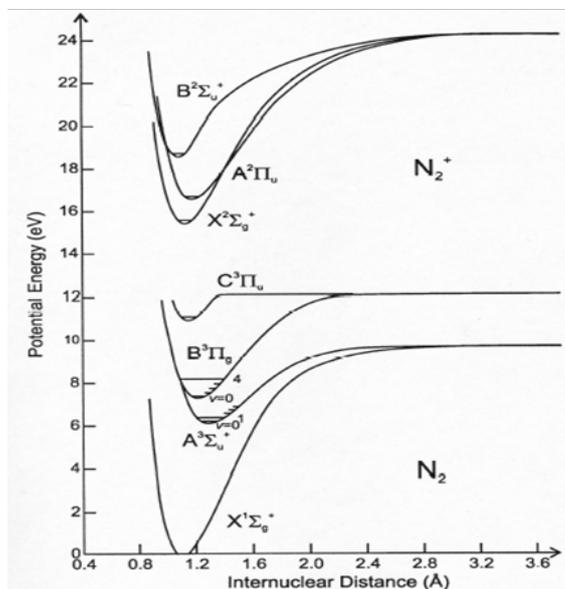
Chemical name	Chemical structure	Oxidation-reduction voltage (V)
Hydroxy radical	OH <sup>•</sup>	2.05
Oxygen atom	O <sub>1</sub>	1.75
Ozone	O <sub>3</sub>	1.52
Hydrogen peroxide	H <sub>2</sub> O <sub>2</sub>	1.30
Hypochlorous acid	HClO	1.10
Oxygen molecule	O <sub>2</sub>	0.94

Table 2: Life time of free radicals and metastables

Chemical name	Life time
OH radical	a few s
NO radical	a few s
Singlet molecular oxygen <sup>1</sup> O <sub>2</sub> Oxygen metastable	7 s
Nitrogen metastable	2 s
Photon	10 <sup>18</sup> year

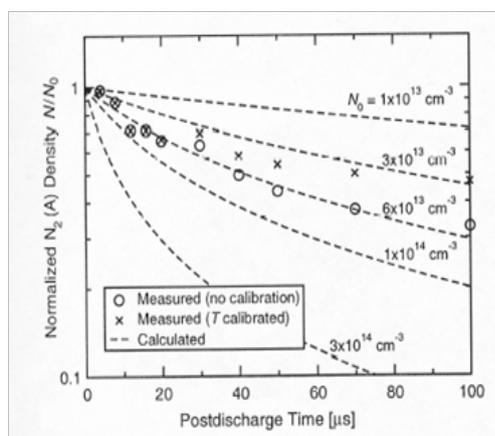
**Table 3:** Flight distance

species	Flight distance during life period
OH radical	0.003 cm/ $\mu$ s
N <sub>2</sub> metastable	144 cm/2s
Photon	infinite



**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.

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<sub>2</sub> metastable was produced by applying pulsed-amperometric discharge and we measure the lifetime of N<sub>2</sub> metastable as a few s (experimentally 2s, Table 2 and Figure 6)<sup>[4-6]</sup>. During the excited N<sub>2</sub> down to the ground state, energy emitted is useful for disruption of spores and bacterial cells. We can therefore hypothesize that N<sub>2</sub> or O<sub>2</sub> metastables and photon may be the most favorable candidates to inactivate bacterial spores and bacterial vegetative state.

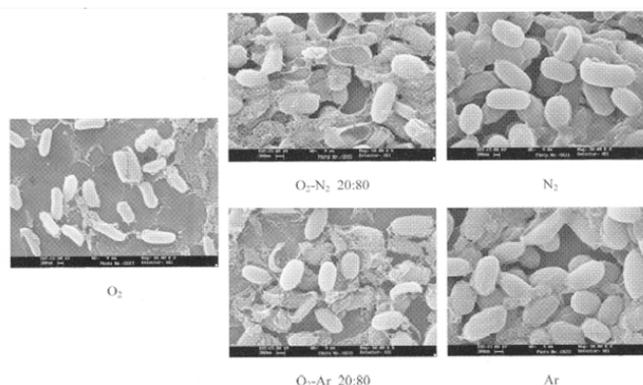
**Figure 6:** Life time of N<sub>2</sub> metastable

However, we have a problem. Bacterial spore death is considered by causing hydration of DPA (Figures 3 and 8)<sup>[8-13]</sup>, 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 par-

ticles on their spore surface. We consider that the white particle may be hydrated dipicolinic acid, so we carefully collected surface white particles with water. This is because if organic solvent is used in place of water, interior DPA may also be recovered together with surface white particle. Use of water as an extraction solvent is for the distinction of DPA in surface and in the core.

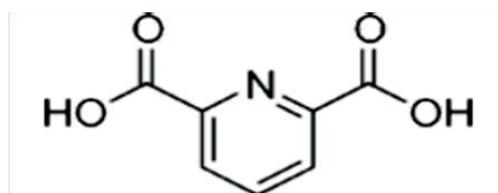
### HPLC analysis of DPA

Water trapped white particle was adjusted to pH5 with acetic acid and applied to C-18 SPE and thereafter eluted with acetonitrile. SPE was automated type SPE of Benchmate<sup>R</sup>. The acetonitrile was evaporated and re-dissolved with mobile phase of acetonitrile/water with acetic acid to pH5 (1/4, v/v, pH5). 10 $\mu$ L was injected to HPLC and detected at 235nm. Stationary phase is C-18 (4.6 X 200mm, Shiseido). We confirmed that the interior (within core) DPA was not eluted out and collected surface DPA only was extracted with water. The retention time coincided with that of standard DPA, therefore surface substance is DPA causing spore death. In addition, we confirmed both are identical from the results of MS fragmentation.



**Figure 7:** Spore structure after exposure by several gas plasmas  
 From the result, N<sub>2</sub> and Ar plasma was unchanged before and after exposure.

The core DPA was hydrated with the penetrated water caused by N<sub>2</sub> metastable and photon and core DPA transported from the core to the surface of the spore. Water for hydration is from the interior of spore (Table 4) as well as surrounding spore. We now speculate how the spore died with maintaining spore figure (Figure 7)<sup>[7]</sup>. The spore surface was attacked by metastables and photon to produce pinhole of the spore surface following the equation of  $E=h\nu$ . The water surrounding the bacterial spore and interior water of the spore (Table 4) 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 viable spore (Figure 7)<sup>[1-3]</sup>.

**Figure 8:** Chemical structure of DPA

In the core, DPA is chelated with Ca.

**Table 4:** Hydrophobicity of several spores

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

## Conclusion

Spore surface pinholes were produced by exposure to the gas plasma with the energy from the N<sub>2</sub> metastables and photon. DPA in core was hydrated with interior water and the surrounding water of the spore. The hydrated DPA migrated into the surface layer and remained as white particle. We hypothesize that this is the probable mechanism of spore death by metastables and photon. The spore structures were not changed after death because the killing process was mainly caused within core. This is the mechanism of sterilization of spores by nitrogen gas plasma exposures.

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. In addition, we confirmed both are identical from the results of MS fragmentation.

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