

## Inactivation of Bacterial Spore, Endotoxin, Lipid A, Normal Prion and Abnormal Prion by Nitrogen Gas Plasma Exposure

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Review Article

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### Abstract

This review discusses the application of non-equilibrium several sorts of gases plasma discharges for the sterilization and disinfection towards spores or bioburden on/in the healthcare products or biological indicator. The basic properties of electrical discharges are briefly reviewed and thereafter it discusses the interactions of gas plasma with several sorts of biological systems such as bacteria, bacterial spores, endotoxins, lipid A and normal and abnormal prion proteins.

**Keywords:** Sterilization, Non-Equilibrium Gas Plasma, Spores, Endotoxins, Prion Proteins.

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**Received:** March 07, 2015

**Accepted:** May 29, 2015

**Published:** June 09, 2015

**Citation:** Hideharu Shintani (2015) Inactivation of Bacterial Spore, Endotoxin, Lipid A, Normal Prion and Abnormal Prion by Nitrogen Gas Plasma Exposure. *Int J Bioanal Methods Bioequival Stud*, 2(1), 23-33.

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### Introduction

The infections can be attributed to various microorganisms such as *Staphylococcus aureus*, *E. coli*, *Bacillus cereus* and so on which can be present on the surface of healthcare products as bioburden and can come into contact with human tissues during operation. Several studies have demonstrated that some of these microorganisms are resistant to antibiotics such as MRSA and VRE and so on ([http://www.betterhealth.vic.gov.au/bhcv2/bhcarticles.nsf/pages/Antibiotic\\_resistant\\_bacteria](http://www.betterhealth.vic.gov.au/bhcv2/bhcarticles.nsf/pages/Antibiotic_resistant_bacteria)) and have an impact on the patients [7] this indicates the need for improved sterilization procedures free from bioburden, airborne falling microorganisms or adhesive microorganisms as far as possible [27, 31, 32] However, some studies [18, 19] have shown that significant quantities of residues composed of salts, proteins and organic matter are left on operational instruments even after complete cleaning and sterilization in sterile service departments (SSDs). These observations raise serious health concerns because residue pathogens can be transmitted to pathogen free patients as iatrogenic diseases. Most serious pathogens included are the abnormal prion

proteins, the principal factors for the transmission of Creutzfeldt Jakob Disease (CJD); this could potentially be transmitted with contaminated instruments during brain operation.

Another concern is related to pyrogens (fever-inducing substances) deriving from gram-negative bacteria (lipopolysaccharides (LPS)), endotoxin or Lipid A (Figures 1, 6-8). These LPS, bacterial endotoxins or lipid A, can cause fever when in contact with the blood stream and provoke fever shock, which is a major cause of death among, for example, dialysis patients. Endotoxins are extremely resistant to temperature and are not removed by conventional sterilization procedures such as autoclave sterilization or  $\gamma$ -ray or e-beam sterilization. A few papers were published on the effect of endotoxin by gas plasma exposure at more than 5 log reduction during 30 min exposure by nitrogen gas plasma [28-31] (Figure 2). In general 3log reduction were required by authorities and GMP (good manufacturing practice) and according the published paper by Shintani et al in 2007 and Shintani 2012, during 10 min at 28-45°C operation, 3log reduction were completed in Figure 2, which is significant achievement.

Sterilization procedures must follow official rules such as ISO 14937 and 14161 and so on. This kind of subjects will discuss in the coming book [34]. The normal procedure followed in SSDs consists of treatments in a mechanical washing/disinfection medical devices: they are, first, pre-washed to prevent blood coagulation and adhesion of protein. The instruments are then cleaned and sonicated with an alkaline enzymatic detergent and, finally, washed at high temperature and rinsed. They are then visually inspected after drying and packaged for sterilization, which in most cases can be carried out by autoclave sterilization, which is inferior procedure of inactivation of endotoxin and abnormal prion proteins. Because these operations are not efficient in completely removing organic residues, and other sterilization methods are also unable to destroy endotoxin and abnormal prions completely. However, as mentioned later, Shintani et al succeeded in inactivation of endotoxin and prions by nitrogen gas plasma exposure [28, 30]. Moreover, the usual sterilization techniques have several drawbacks including the potential degradation of heat-sensitive

healthcare products (e.g., autoclave sterilization at 121.11°C for 20 min in normal); relatively long operation time due to toxic gas degassing (ethylene oxide gas or hydrogen peroxide gas sterilization). The autoclave sterilization is required 121.11°C for 20 min,  $\gamma$ -ray sterilization of 25 kGy is 2.5h (10 kGy/h in normal) and electron-beam sterilization is a few s, therefore sterilization procedures are not always required too long process times in every cases. It depends on sterilization procedures selected.

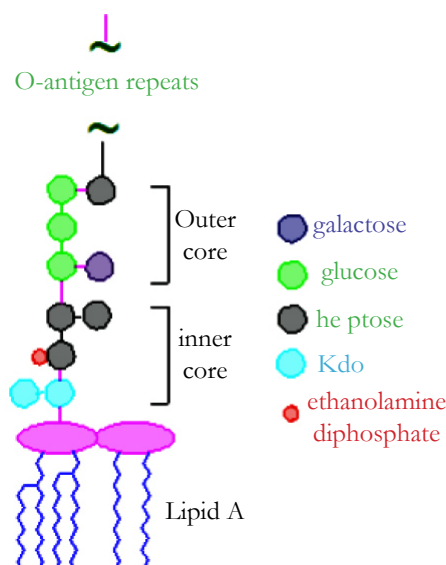
Alternative sterilization procedures are under investigation including the use of non-equilibrium gas plasma discharges: we will see that plasma processes are able to sterilize bacterial spore with sufficient degree of more than 6log reduction without residue of toxic gases [34] and conducted at relatively low temperature at 25-60°C for endotoxin inactivation for more than 5log reduction [28] (Figure 2). Non-equilibrium gas plasma sterilization was carried out to show that many pathogens can be inactivated [28-30] and mechanism and inactivation factors of gas plasma sterilization against microorganisms or biomolecules has been extensively studied. According to our recent studies, the major factors causing sterilization by nitrogen gas plasma exposure was not well clarified and defined. This will be described later in this article [34] (Figure 3). In this review we present the basic principles of atmospheric and low pressure gas plasma sterilization and the active species produced and discuss their interactions with organic materials, pathogens (endotoxins, abnormal prion proteins), and bacterial spores [28, 30] showing how these factors could be used for sterilization and inactivation.

## Brief Description of Plasma Generation

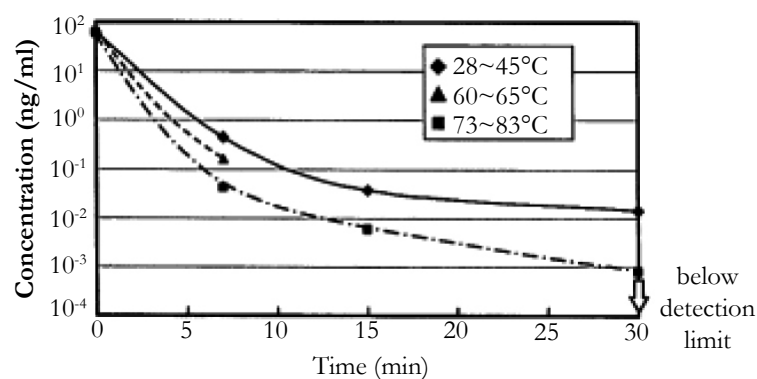
Plasma denotes in physics quasi-neutral ionized gas- that is, a gas in which a certain fraction of particles is charged (Figures 3-5). The presence of charged species turns a plasma into a highly conductive gas that responds readily to electromagnetic fields. As a consequence of this, plasma presents unique properties as compared with solids, liquids or gases and is therefore often referred to as the fourth state of matter. The fourth state of matter exists 99% in the world without recognition.

Plasma is normally generated by supplying sufficient energy to a neutral gas and induces the formation of charged species, electrons and ions (Figures 3-5). This process proceeds by means of collisions between energetic species with neutral atoms or molecules or perhaps in collisions with walls surrounding the gas. There are various potential methods of providing a gas with the necessary energy to ionize it. One possible way is based on thermal heating: the energy is produced by exothermic chemical reactions in the molecules. In this case, all the ions, electrons and neutral species constituting the plasma are in a thermodynamic equilibrium and plasmas created in this way are called thermal plasmas at high temperature such as 3000°C. The temperatures needed to create thermal plasmas are extremely high (e.g., the energy needed to ionize argon is 15.8 eV, which is equivalent to a temperature of approximately 180,000 K), which limits their practical use and of

**Figure 1. Endotoxin and lipid A of gram negative bacteria.**

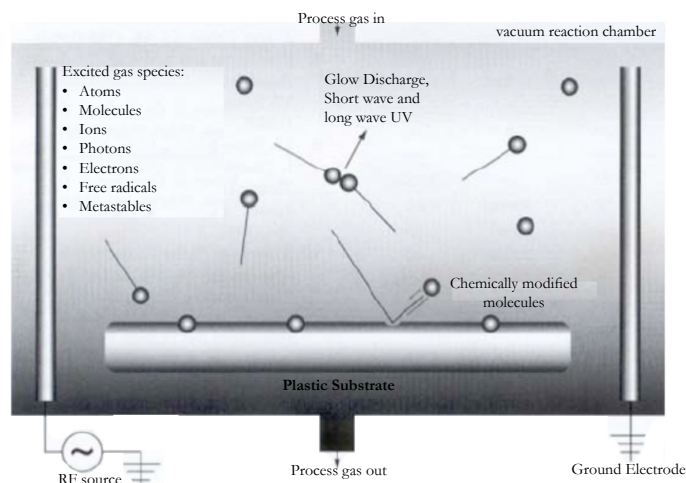


**Figure 2. Endotoxin inactivation by nitrogen gas plasma exposure at higher temperature (73-83°C), more than 5 log reduction can be observed. Three log reduction required by the authority can be attained 10 min at 28-45°C.**



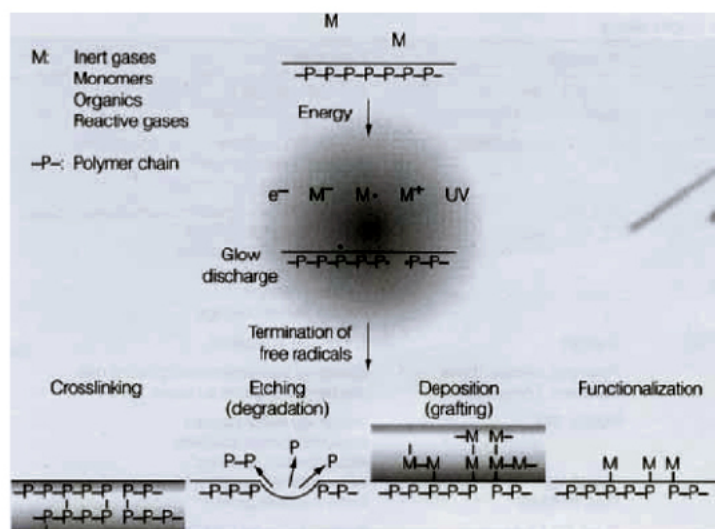
**Figure 3. Model case of gas plasma exposure.**

Cited from <http://www.astp.com/plasma-equipment/applications>

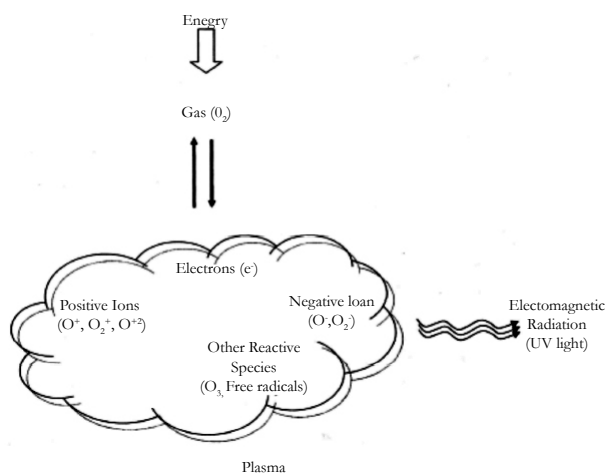


**Figure 4. Surface modification by gas plasma.**

Cited from <http://www.astp.com/plasma-equipment/applications>



**Figure 5. Example of oxygen gas plasma production.**



course it cannot apply for gas plasma sterilization.

Another way to produce plasma for technological use is based on the application of an external electric field. The basic properties of such plasmas, which are denoted as electrical discharge, will be described briefly.

### Properties of electrical discharges

Any neutral gas contains a certain amount of charged carriers created, for instance, by the interactions of cosmic rays with the gas. These charged particles are accelerated in an external electric field by the Lorentz force (<http://web.mit.edu/sahughes/www/8.022/lec10.pdf>) up to kinetic energies sufficient for ionization of atoms or molecules in the gas volume, which happens principally through electron-impact ionization.

It can occur through the emission of new charged particles from electrodes induced by the impact of energetic species. Continuous production of new charged carriers can balance their recombination losses and a steady state can be reached. However, due to the significant differences in masses of electrons and atomic or molecular ions, the electrons reach considerably higher kinetic energy compared with heavier atomic or molecular ions, whose kinetic energy remains relatively low and close to the temperature of neutral species. Since the temperatures of electrons and other neutral species (atoms, molecules, radicals, Figures 3-5) are different, such plasmas are called non-equilibrium plasmas or non-thermal plasmas.

Moreover, collisions between particles present in plasma do not lead solely to their ionization. In fact, a significant portion of energy supplied to the plasma is used for excitation of atoms and molecules to produce metastables or radicals (Figure 3). The presence of excited species has two important consequences. First, excited species, and particularly long-living metastables or radicals, can act as energy carriers: their internal energy can be released when they impact on the surfaces of objects placed into the plasma, which can lead to physical sputtering of their surface, or can contribute to volume ionization by a process known as Penning ionization (<http://www.jstor.org/discover/10.2307/3573984?sid=21106032981603&uid=4&uid=3738328&uid=2&uid=70&uid=2129>). Second, excited species can be the source of intense light emission, which is connected with their radioactive transition to lower energetic levels. Depending on the difference between energy levels of a particular atom or molecule, the radiation can be emitted in the visible (e.g., in the case of nitrogen molecules), UV (e.g., bands of NO radicals) or even VUV (e.g., spectral lines of argon) spectral range. Nitric oxide (NO) radicals in nitrogen gas plasma sterilization were detected using chemical indicator specific to NO radicals and published in Japan Patent 5465749 [32]. The life period of NO radicals was 3-6 s, so as radicals life period was long enough because for example the life period of OH radical was a few  $\mu$ s.

Furthermore, collisions between electrons and molecules can also cause their dissociation ionization. This triggers subsequent chemical reactions leading to the presence of species initially not present in a gas. Typical examples are the production of atomic oxygen in plasma sustained in O<sub>2</sub> gas, or OH radicals in discharges containing water vapor (Figures 3 and 5). Species created in this way can chemically react with the surfaces of objects placed into the plasma, which can cause modifications of their physical or

chemical properties of their surfaces (Figure 4).

Although the processes described above by no means provide a complete description of plasma, they illustrate the key features of plasma discharges that make them highly interesting for a wide range of applications and for the sterilization of bioburden existing on/in the healthcare products. They can be summarized as follows:

1. A significant fraction of species in plasma discharges is charged. Such charged species can be further accelerated by an additional electric field to reach energies sufficient for physical sputtering of the treated objects (Figures 3 and 5).
2. Plasma discharges can be operated at moderate temperatures (and in some cases even at room temperature to 80°C, Figure 2) allowing the treatment of heat-sensitive materials.
3. Plasma is a potent source of radiation, comprising germicidal UV (UV254 nm) and VUV photons.
4. In plasma discharges, species not present in the initial working gas can be created (Figures 3 and 5). Such atoms, molecules, radicals, ions or metastables can interact with treated objects, resulting in modification of their properties.
5. The interactions between an immersed object and the plasma surrounding it are limited to a thin surface layer of the treated object (around 10-20 nm level, [28, 30]) and thus do not induce significant modifications of its bulk properties.

## Interaction of Plasma with Bacteria and Bacterial Spores

### Sterilization with atmospheric-pressure plasma discharges

The use of atmospheric plasma for sterilization of different types of spores has been the major subject of researches. Different types of discharge were used, and the results were summarized. [21, 14, 15, 9, 22, 29].

However, the conditions and geometry vary to a large extent, which makes the comparison of the results difficult. Moreover, determining the main processes leading to the sterilization of bacterial spores is more difficult than with low-pressure discharges, due to the presence of additional obstacles. First, the plasma diagnostic of discharges generated at atmospheric pressure is rather complex and thus only a limited number of studies. Second, contamination by air backflow in the discharge occurs in the majority of cases unless particular precautions are clearly taken. In other words, the working gas mixture is not well defined, since it generally contains a non-negligible fraction of impurities, for instance, water vapor or air, which may markedly alter the interactions between plasma and biological pathogens.

Nevertheless, the role of UV [35, 24, 10, 17] against spores was shown to be similar to what was observed for low-pressure plasmas. In addition, further two mechanisms that were not observed with low-pressure plasma discharges are active in this case. However, in general UV and VUV contributions to sterilization of bacterial spores are considered not to be significant among plasma researchers. One example is reported by Deng et al in 2006.

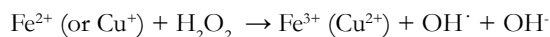
**Electrostatic disruption:** This effect is attributed to the accumulation of surface charge on spore membranes, which results in a build-up of electrostatic forces. Such forces could exceed the total tensile force on the membrane and cause it to be disrupted



[16, 37]. This mechanism has been observed with a resistive barrier discharge (RBD) on yeast [37], on gram-negative bacteria [16] and at lower extent also on gram-positive bacteria [3].

**Interaction with reactive oxygen and nitrogen species:** Reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydroxy radical (OH $\cdot$ ), hydrogen peroxide (H $_2$ O $_2$ ), the superoxide anion (O $_2^{\cdot-}$ ), nitric oxide (NO) or peroxyxynitrite anion (OONO $^-$ ) can interact with various classes of biomolecules presented in cells and interior of bacteria, leading to a complex chain of events that can cause cell death. Surface sterilization or modification (Figure 4) does not result in bacterial death. The molecular targets for ROS are DNA, RNA, lipids and proteins existing in the interior of the bacteria (Farr and Kogoma, 1991). In the case of lipids, ROS attack unsaturated fatty acids in the cell membrane, thus initiating lipid peroxidation. As a consequence, the structural integrity of the membrane is compromised and osmotic imbalance occurs, which speculates lead to cell death. The outer layer of gram-negative and gram-positive bacteria can be shown in Figure 6. Lipopolysaccharide exists only in the gram negative bacteria, not in gram positive bacteria. Teichoic acid containing in gram-positive bacterial cell wall was not lipopolysaccharide ([http://www.nature.com/nsmb/journal/v17/n5/fig\\_tab/nsmb.1819\\_F1.html](http://www.nature.com/nsmb/journal/v17/n5/fig_tab/nsmb.1819_F1.html), Figures 6-7).

The reaction of ROS with proteins has also serious implications for the function of cells, since the accumulation of injured proteins can significantly disrupt the cell metabolism. Finally, ROS and in particular, OH radical can cause a DNA strand to break. Nevertheless, OH radical cannot diffuse freely through the cell to reach DNA due to their short period of life at a few  $\mu$ s. Therefore it is assumed that OH radicals are created in the vicinity of DNA from hydroxide peroxide by the Fenton reaction indicated below. However, hydrogen peroxide is from where? Gas plasma sterilization does not produce hydrogen peroxide (Figures 3-5). This explanation is impractical to consider when seriously discussed.



## Interaction of Plasma with Endotoxins and Prion Proteins

Unlike bacterial spores, endotoxins and prion proteins are not living organisms. They are mostly consisting of lipopolysaccharide or proteins. No DNA or RNA was involved [28, 30]. As a consequence lack of targeted DNA or RNA, different strategies for their inactivation have to be followed. The main results related to the application of plasma for treatment of endotoxins and prion proteins are summarized in two sections emphasizing the effect of reactive species.

### Effect on endotoxin

Endotoxins consisting of lipopolysaccharide as shown in Figures 1 and 8 are another common surface contaminant that is not addressed in the sterilization processes at health care facilities. The outer coating of gram-negative bacteria contain endotoxins and lipid A consisting of lipopolysaccharide (Figure 6). Their presence in the blood stream leads to physiological events such as fever and so on and at higher doses to patient's death, especially at dialysis patients [2]. Endotoxins are extremely resistant to temperature and difficult to remove by conventional methods; their inactivation by low-pressure nitrogen gas plasma has been studied by [28-30] (Figure 2). Nitrogen gas plasma was the most efficient and oxygen gas plasma destroyed the outer layer of endotoxins by etching but cannot inactivate. The mechanisms of inactivation of endotoxin was the cleavage of lipid A at ester bonding or acid amide bonding due to the lower bonding energy (Figures 10 and 11, Table 1). The chemical structures of endotoxins and Lipid A are presented in Figures 1, 8 and Figure 9, respectively. Oxygen gas plasma causes etching phenomenon of the targeted material including bacterial spores, which is inferior to the real application

Figure 6. Outer layer of gram-negative and gram-positive bacteria.

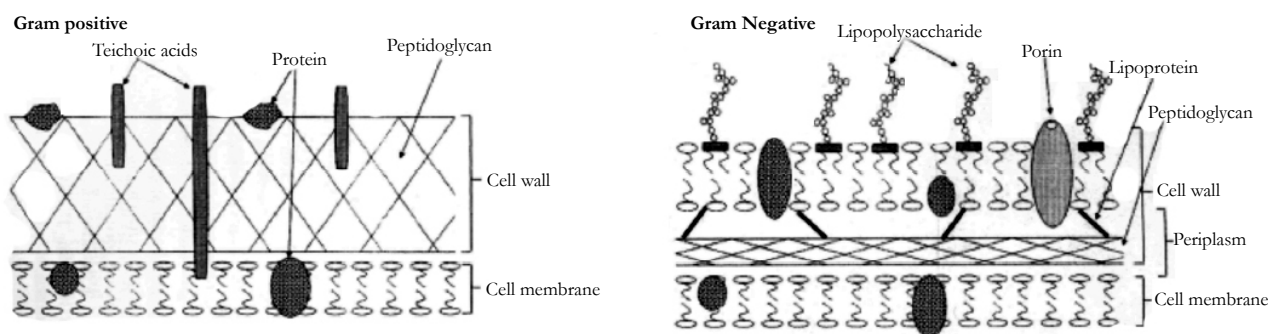


Figure 7. Chemical structure of teichoic acid containing in gram positive cell wall.

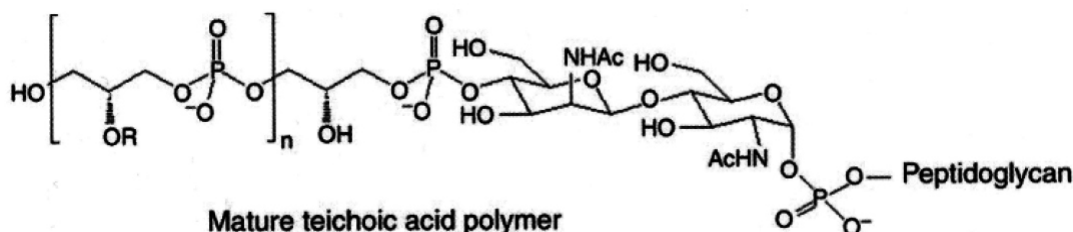


Figure 8. Chemical structure of lipopolysaccharide.

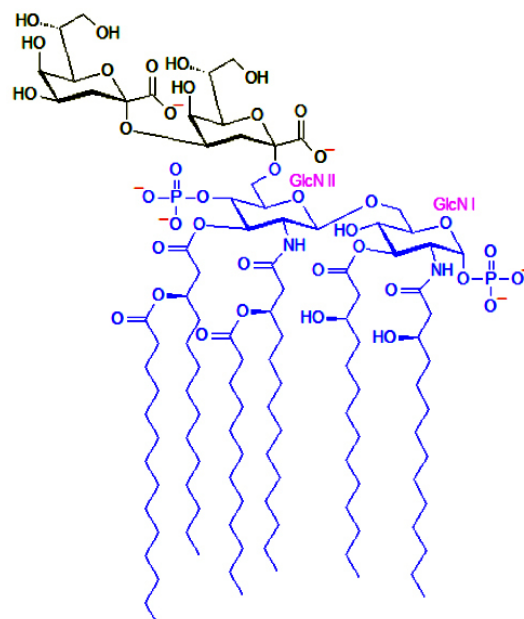


Figure 9. Chemical structure of Lipid A.

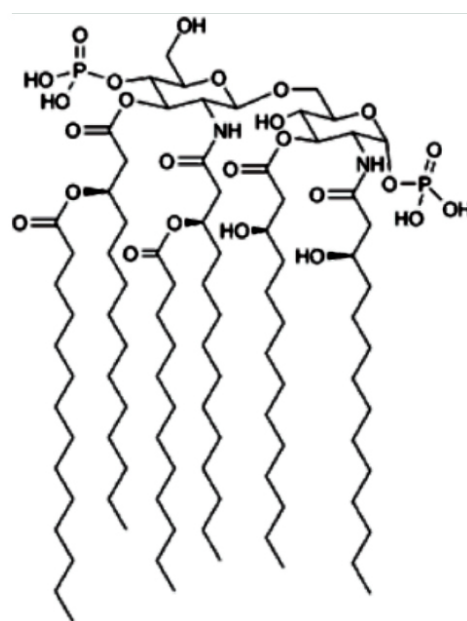


Table 1. Bonding cleavage energy (kJ/mol at 25°C)

Cited from <http://mh.rgr.jp/memo/mq0110.htm>

C-C 結合	D KJ/mol	C-H 結合	D k J/mol	-H 結合	D k J/mol
CH <sub>3</sub> -CH <sub>3</sub>	368	H-CH <sub>3</sub>	434	H-OH	499
CH <sub>3</sub> -C <sub>2</sub> H <sub>5</sub>	357	H-CH <sub>2</sub>	461	H-OH	427
CH <sub>3</sub> -C(CH <sub>3</sub> ) <sub>3</sub>	344	H-CH	427	H-O <sub>2</sub> H	376
CH <sub>3</sub> -C <sub>6</sub> H <sub>5</sub>	417	H-C	339	CH <sub>3</sub> O-H	436
CH <sub>3</sub> -CHCH <sub>2</sub>	466	H-C <sub>2</sub> H <sub>5</sub>	412	CH <sub>3</sub> COO-H	442
CH <sub>3</sub> -CCH	465	H-C(CH <sub>3</sub> ) <sub>3</sub>	387	(N-H)	
CH <sub>3</sub> -CH <sub>2</sub> OH	350	H-C <sub>6</sub> H <sub>5</sub>	460	H-NH <sub>2</sub>	432
CH <sub>3</sub> -COOH	403	H-CHCH <sub>2</sub>	455	H-NH	388
CH <sub>3</sub> -COCH <sub>3</sub>	355	H-CCH	500	H-N	352
CH <sub>3</sub> -CN	513	H-CHO	360	(S-H)	
C <sub>2</sub> H <sub>5</sub> -C <sub>2</sub> H <sub>5</sub>	346	H-CH <sub>2</sub> OH	393	H-SH	383
C <sub>6</sub> H <sub>5</sub> -C <sub>6</sub> H <sub>5</sub>	468	H-COOH	374	H-S	351

Figure 10. MS spectrum before nitrogen gas plasma exposure (a), after nitrogen gas plasma exposure, (b) and solvent alone injection, (c) From the MS fragmentation, it can be seen that ester or acid amide bonds are cleaved by nitrogen gas plasma exposure.

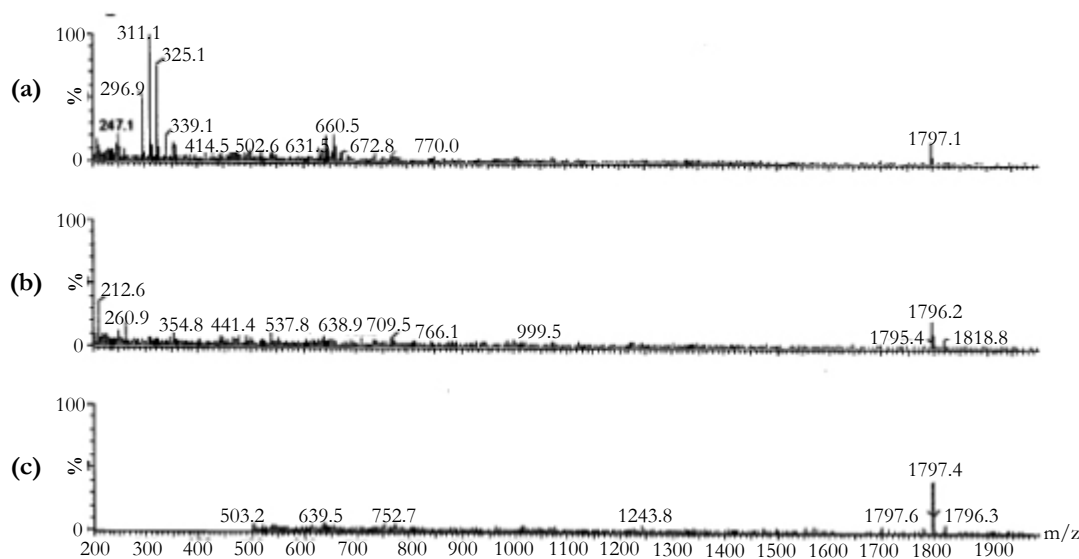


Figure 11. Major fragmentation of lipid A cleaved at ester linkage and acid amide bondage.

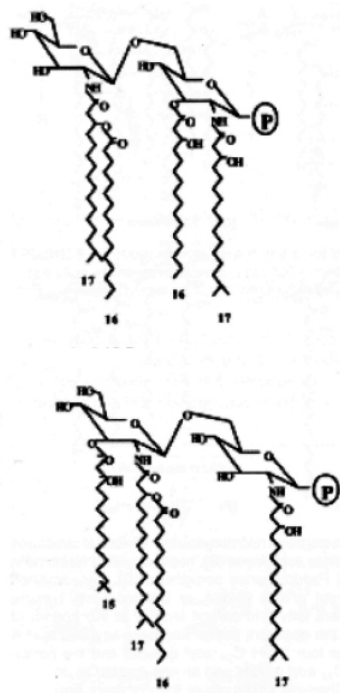
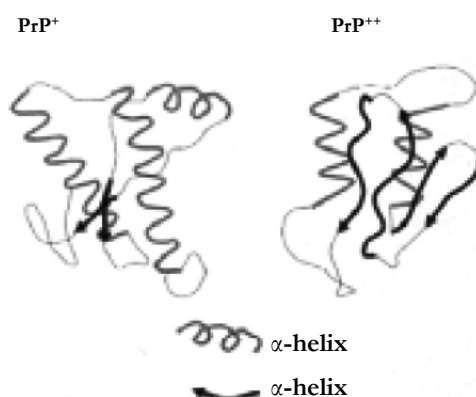


Figure 12. Chemical structure of normal and abnormal prions The left is normal prion and the right is abnormal prion. The difference of both is that normal prion has more  $\alpha$ -helix and less  $\beta$ -sheet.



to attain material/functional compatibility. The phenomenon that sterilization was finished but material is useless must be avoided, therefore oxygen gas plasma is not recommended in research as well as practical use. GMP (good manufacturing practice) required attaining both SAL (sterility assurance level) of  $10^{-6}$  and material/functional compatibility and oxygen gas plasma sterilization is not suited for following GMP regulations due to causing a significant etching and shrinkage which means material degradation [34].

### Effect on normal prions and abnormal prions

Protein residues remaining on the surface of instruments constitute another group of possible contaminants. These residues might contain pathogens, and in particular abnormal prions, since abnormal prion has been found mostly in the brain of CJD patients. Abnormal prion can be so often described as scrapie, but scrapie is the abnormal prion of CJD patients, indicating scrapie and abnormal prion does not coincide. The scrapie is one of abnormal prions. CJD could possibly be transmitted by contact with contaminated instruments with abnormal prions. Unlike bacterial spores, prions do not contain genetic material such as DNA or RNA. The chemical structure of normal and abnormal prions is presented in Figure 12 [30]. Prions have been found to be extremely resistant to conventional sterilization procedures because of their unique and stable secondary and tertiary structure that cannot be easily altered (Figure 12). As explained in Figure 12 captions, normal prions have more  $\alpha$ -helix and less  $\beta$ -sheet compared with abnormal prions.

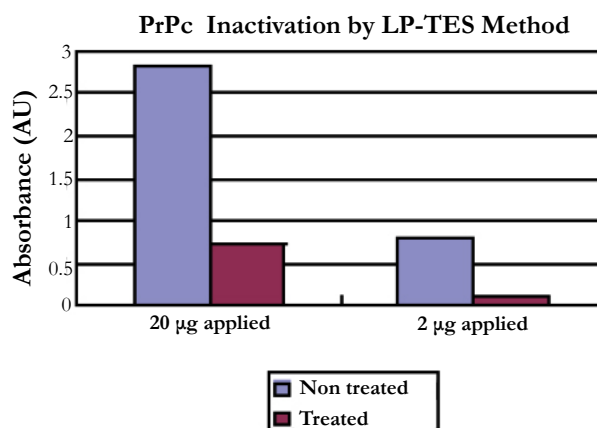
Although the possibility of removing prions by means of non-equilibrium plasma discharges has already been demonstrated [30] (Figure 13). In Figure 14, the test result of BSA (bovine serum

albumin) of electrophoresis before and after nitrogen gas plasma exposure [30]. BSA is the most popular protein used for biological study. We also tested myoglobin, which contains abundant  $\beta$ -sheet and it resembles to abnormal prion. The test result was completely destroyed by electrophoresis resemble to BSA presented in Figure 14 (data not shown as being resembled to Figure 14).

The mechanism of deterioration of abnormal prion is not completely clarified. But according to the Sakudo's paper in 2013, the higher order structural change may occur. The effects of non-equilibrium plasma discharges have been studied on non-pathogenic models of proteins in order to identify general mechanisms useful for prion elimination. The results of these studies reveal the possibility of removing proteins by nitrogen gas plasma [30], which may induce their deformation as speculation but not confirmed and fragments cannot identify, so fragmentation may not be observed, but deformation may be correct [25].

The protein removal follows a two-phase kinetics, composed of an initial fast followed by a second slow phase. The first phase was attributed to the fast deformation of the proteins, while the second phase was related to the enrichment of the surface in non-volatile elements (based, for instance, on Na, Ca, F). It was also found that the same mechanism of chemical sputtering observed for bacterial treatment is also operative for proteins, in close correlation with the measured rates between post and direct discharges [12]. Here again, synergy was observed between ion bombardment and radicals such as atomic H or O, as well as  $O_2$  or  $N_2$ . Moreover, it was also found that the initial rate did not depend on the primary structure of the polypeptides contained in the proteins leading to the speculation that most proteins could be deformed with their higher order structure of proteins [25]. The

**Figure 13. Inactivation of abnormal prion by nitrogen gas plasma exposure** 20  $\mu$ g and 2  $\mu$ g of abnormal prions were tested. Left is 20  $\mu$ g and Right is 2  $\mu$ g. In each tested, left bar is before exposure of nitrogen gas plasma and right bar is after exposure of nitrogen gas plasma. The comparison indicates that nitrogen gas plasma inactivate abnormal prion in success.



**Figure 14. Before and after of nitrogen gas plasma exposure to BSA and analyzed by electrophoresis** The left is before exposure and the right is after exposure to BSA. It can be clarified BSA was completely degraded.





most important parameters found were the radical content of the discharge and the plasma density both of which are responsible for the synergy observed during chemical sputtering.

The treatment of protein by atmospheric plasma discharge has also been the subject of researchers [4, 5, 1, 30]. By using an atmospheric pressure glow discharge with pure nitrogen gas, the authors showed that proteins could be destroyed at low temperatures, the speculated but not confirmed main agents were oxygen metastable and excited nitric oxide (NO), with a possible synergistic effect between the two species speculated. Electrophoresis of the protein films before and after plasma treatment was used to show that the proteins were completely degraded and fragmented by the treatment as Shintani presented in Figure 14, and that the plasma action could be speculated as protein degradation by sputtering effect on proteins [30] (Figure 14).

It was reported that spore inactivation kinetics was also secondary to thirdly kinetics, but it was the first order kinetics when clump-free biological indicator was used, therefore the above speculation must be further clarified.

## The Use of Plasma for Sterilization Purposes

Although there was progress in the field of plasma-based sterilization, there are still some questions. The aim of this part is to mention briefly some of un-resolved questions as well as to draw further perspectives of sterilization technique.

### Optimization of the plasma sterilization process

The results from the literature clearly show that atmospheric and low-pressure plasma can be used to sterilize bioburden in the surfaces. Different mechanisms have been clearly identified, namely reaction with reactive species as shown in Figures 3 to 5 were mostly not the major contributors excepting metastables. The speculated major contributor was peroxyxynitrite anion. Nitric monoxide radical detected by us [33] combines with superoxide anion and produce peroxyxynitrite anion on the biological indicator spores or bioburden to sterilize them. Life period of the peroxyxynitrite anion, nitric monoxide radical and superoxide anion was a few s, 3-6 s and 5 s, respectively, which are relatively long period compared with most of radicals with  $\mu$ s life period such as OH radical. As the outer layers of gram-negative and gram-positive bacteria have been charged, therefore charged contributors cannot penetrate into the interior of the bacteria to attack interior DNA or RNA. In that sense, nitric monoxide radical can be understood due to neutral, but superoxide anion has a problem to understand due to minus charge. As a whole speculation of peroxyxynitrite anion was problematic to define and required to consider another reasonable factor to consider without problems. As being indicated charged factors can be neglected as contributors. This indicates that metastables of N or O may be the major contributors due to a few s of life period, neutral and abundant energy produced from the excited state to the ground state emitted energy to destroy bacteria. Radicals may be candidate as they are not charged, but their life period is too short such as a few  $\mu$ s, so radicals with long life period may be the candidates. In that means NO radical may also be candidate, but unfortunately NO radical has no sterilization or disinfection function.

UV leads to very effective sterilization when spores are not clumped on the materials. However, UV or VUV alone is not

efficient for inactivating bacterial spores, endotoxin or prion proteins, indicating gas plasma produce UV or VUV (Figures 3 and 5), but they are not completely major contributors and even not minor contributors.

### Limitations of plasma processes

Apart from the problems related to the matrix effect, another practical difficulty in the use of plasma discharges is related to their directionality and their limited penetration at 10-20 nm in high aspect ratio holes and trenches (low-pressure plasma discharges [28, 30]. The former requires special handling of the equipment during treatment, which complicates the operation. The latter requires the procedure to be carried out at intermediate pressures: this limits the efficiency of the chemical sputtering effect, which decreases the sterilization effects.

Finally, plasma treatments are not very compatible with pre-packaged instruments, as the packaging may block the reactive species created by the plasma discharge. This is particularly crucial for atmospheric plasma treatment, since at low pressure, the plasma discharge could in principle be created inside the package, which means gas plasma sterilization is not carried out like  $\gamma$ -ray or e-beam irradiation sterilization. This is seriously obstacle to apply the gas plasma sterilization for the practical use.

### Treatment of biological pathogens in an aqueous environment

Another important aspect is the possibility of treating biological pathogens in an aqueous environment by means of atmospheric pressure plasma discharges. Bacteria can be effectively sterilized not only in a moist environment that is, when a minute amount of non-liquid water is present [6], but also when completely suspended in liquids [20, 23]. This effect was attributed to a gradual acidification of the water caused by the reactions of NO<sub>x</sub> produced in plasma with water and the subsequent oxidation of the bacterial fatty acids by per hydroxy radicals at low pH produced by nitrous acid or nitric acid formed [20]. This is not the plasma effect, but acid effect. Another explanation assumes that there is synergy between reactive oxygen and nitrogen species, particularly OH radical, NO radical and OONO<sup>-</sup> (peroxyxynitrite anion). In this reaction scheme, NO releases iron ions from intracellular metalloproteins; the Fe<sup>2+</sup> content then catalysis the Fenton reaction to produce toxic OH radicals if hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) happens to be present together with Fe<sup>2+</sup>. In real status it is quite rare to present hydrogen peroxide together with Fe<sup>2+</sup>, thus the above explanation is the quite rare story.

### Treatment of biological items

The possibility of using atmospheric pressure plasma discharges for the treatment of living tissue is currently the subject of research. It has been demonstrated that different plasma sources are capable of effectively killing bacteria, but are non-destructive to human tissues, which may be the shallow penetration depth of gas plasma at 10-20 nm level [28, 30]. Bioburden can be destroyed by gas plasma, but contributors of gas plasma sterilization may not penetrate to attack the interior DNA or RNA in humans. Another possible explanations have been proposed as below:

1. Mammalian cells have a defense mechanism against oxidative stress. For instance, the presence of NO radical induc-

es cellular synthesis of antioxidative enzymes in cells. This mechanism, which is absent or considerably lower in bacteria, then counterbalances the increased production of  $\text{Fe}^{2+}$  or  $\text{Cu}^+$  ions responsible for the formation of toxic OH radicals through Fenton reaction if hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) happens to be present together with  $\text{Fe}^{2+}$  in this case.

2. There is a size effect. The bacteria are much smaller than mammalian cells and thus the dose of toxic compound needed for their inactivation is lower [6]. Moreover, electrostatic forces that can eventually lead to the rupture of membranes are considerably lower in the case of cells as compared with bacteria since the charging is in the first approximation inversely dependent on the diameter of the treated object.
3. There is a complexity factor. The mammalian cells in tissue communicate with each other, which may lead to a lower toxic effect than that observed on single cell bacteria [6].

Moreover it has been found that in addition to its ability to inactivate bacteria, plasma treatment of living tissues also has therapeutic effects in some cases. It can be used for wound healing, tissue regeneration, blood coagulation or even for killing of cancer cells [36]. This information, if reproducible which is important, is the use of plasma discharges not only as a sterilization procedures, but also as an important tools in medical applications.

## Conclusions

This review article summarized the work carried out to date on low-pressure and atmospheric-pressure sterilization. The main mechanisms of plasma action on various biological pathogens have not well been identified and it has partly been shown that plasma treatment is a process for sterilization at low temperatures [28, 30]. However, several unclear points still need to be clarified before plasma technologies can be applied in practical use. For example, the sterilization mechanism is not completely clarified and the contribution factors of sterilization are not still identified. In this status, application to the real status is too early. For medical application, it is required official approval beforehand, however in this status, approval may be quite difficult to obtain.

Apart from general process problems, the first issue is linked to the variability in efficiency of the sterilization mechanisms, which leads to difficulties in treating large loads with different shapes and sizes homogeneously. The second issue is linked to the matrix effect, which underlines the need to integrate plasma technology in a complete washing/cleaning process. The economics of the whole process including plasma discharge will need to define, otherwise practical application of gas plasma cannot be utilized in the public in future.

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