Pharmaceutical Regulatory Affairs: Open Access

ISSN: 2167-7689

Shintani, Pharmaceut Reg Affairs 2015, 4:1
DOI: 10.4172/2167-7689.1000129

Review Articles Open Access

Inactivation of Endotoxin and Lipid A by Nitrogen Gas Plasma Exposure Running title: Inactivation Endotoxin and lipid A

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Abstract

The gases plasma from several different gases has shown a sporicidal activity. From these gases, nitrogen gas was most difficult to produce atomic nitrogen radicals. However, these radicals have a high energy, indicating that nitrogen gas plasma could be used to sterilize microorganisms and inactivate endotoxins and lipid A. The sterilization mechanism of nitrogen gas plasma may be the synergistic effect of free radicals such as (OH radical and NO radical) and metastables (N_2 or O_2 metastable). Thus, the target microorganisms such as bioburden were damaged by degradation, which resulted in death. The biological indicator BI used in this study was *Geobacillus stearothermophilus* ATCC 7953 at an initial population of 106 CFU/carrier. Sterility assurance level (SAL) of 10^{-6} was confirmed by using the BI. In addition endotoxins were successfully inactivated by nitrogen gas plasma expossure. More than 5 log reduction of endotoxins could be attained within 30 minutes of nitrogen gas plasma exposure. Lipid A degradation by nitrogen gas plasma exposure was confirmed by LC-MS-MS. Material/functional compatibility was also maintained by nitrogen gas plasma exposure due to shallower penetration depth of 10-20 nm. No deterioration of polymers could be observed by nitrogen gas plasma exposure. Residual gases, if any, are non-toxic. From the above description, it can be said that nitrogen gas plasma sterilization is an alternative future sterilization method.

Keywords: Plasma; Endotoxins; Peptide; Sterilization; Validation

Introduction

It was confirmed that the degree of endotoxin inactivation was more than 5 log reduction in 30 minutes by nitrogen gas plasma (Figure 1) and that by Sterad[R] was around l log reduction [1-3], indicating under experimental conditions, performance characteristics of nitrogen gas plasma was superior to those of Sterad[R] using hydrogen peroxide gas plasma. Sterad[R] is not true gas plasma sterilization [3,4]. Our method of endotoxin inactivation was conducted using nitrogen gas plasma exposure. Concerning Pub Med research or Google research with key words of endotoxin, gas plasma, inactivation or depyrogenation, it is only a few articles to be matched, respectively, indicating gas plasma study of endotoxin inactivation/depyrogenation was very limited [3,5-9].

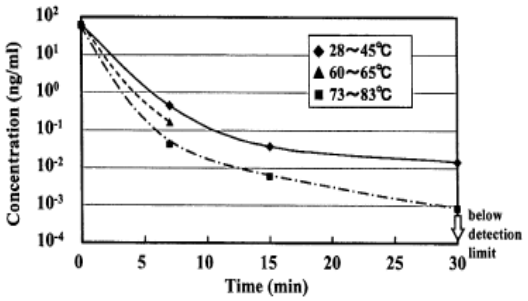
Inactivation of endotoxin

As shown in Figure 1, the higher the temperature, the greater the degree of endotoxin inactivation was obtained. The degree of inactivation was dependent on temperature, which indicates that the inactivation follows the Arrenius equation. Inactivation of endotoxins was confirmed from the results of Limulus ES-II test Wako and Toxinometer ET-2000/J System, Wako. Endotoxins were from LPS (lipopolysaccaride) of *E. coli* 0111 (Figure 2) and LPS was inoculated onto modified PS (polystylene) evenly at the nm level and thereafter exposed to nitrogen gas plasma. After inactivation of endotoxins by nitrogen gas plasma, no deformation or deterioration of PS of the carrier material was observed, which indicated PS successfully maintained its material functionality (Tables 2 and 3) [3,10-12]. The convenient method of endotoxin inactivation is by dry heat treatment at 250°C for more than a half hour i.e. 45 min or gamma-ray irradiation at 100 kGy after l N NaClO immersion [2]. By these procedures material/functional compatibility may fail to be maintained. However by the treatment with nitrogen gas plasma exposure, endotoxins at more than 5 log reduction can be attained in around 20-30 minutes while simultaneously maintaining material/functional compatibility. In that sense, we could find that nitrogen gas plasma was superior to the conventional procedures of endotoxin inactivation [13].

Mechanism of endotoxin inactivation using 1ipid A

The mechanism of endotoxin inactivation was studied by using lipid A and HPLC-MS-MS (High performance liquid chromatography-Mass spectrometry-Mass spectrometry). Synthesized lipid A from Peptide Institute, Inc. (Osaka, Japan) was used for confirmation of the endotoxin inactivation mechanism. This is because lipid A is an active site of endotoxins [14-21]. Chemical structure of endotoxin and lipid A from *E. coli* is presented in Figures 2 and 3, respectively.

In Figure 4, M-l peak (negative peak) was observed as 1797 m/e as the mother peak of lipid A. In addition, peaks at 209, 247, 297, 311, 325, 339, 415, 429, 502, 603, 632, 645, 660, 673 and 770 m/e were observed in the non-treated sample which dissolved in DMSO (Figure 4 upper).



LP conditions Input power, 84 W; frequency, 2.5kHz; temperature, 28-73 oC, Vacuum 45kPa; Gap 40 mm; N2 flow rate, 15L/min; Voltage 19 kV.

Figure 1: Temperature dependence of endotoxin inactivation by low pressure (LP)

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Received December 22, 2014; **Accepted** January 07, 2015; **Published** January 12, 2015

Citation: Shintani H (2015) Inactivation of Endotoxin and Lipid A by Nitrogen Gas Plasma ExposureRunning title: Inactivation Endotoxin and lipid A. Pharmaceut Reg Affairs 4: 129. doi:10.4172/2167-7689.1000129

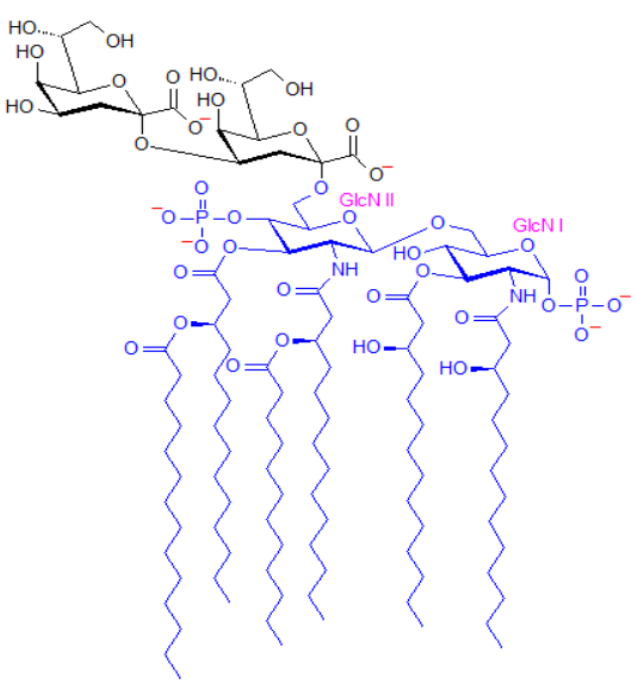

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Figure 2: The basic lipopolysaccharide of *E. coli*, incorporating lipid A (lower portion of the structure)

Chemical bonding	Bonding energy (kL/mol)
H-H	436
C-C	344
C=C	615
C≡C	812
O-O	143
C-O	350
C=O	725
C-H	415
N-H	391
O-H	463

Table 1: Averager bonding energy (kJ/mol)

Sample	C(%)	O(%)	N(%)	Si(%)
PS (before treatment)	98.7	1.3		
PS (nitrogen gas plasma treatment)	82.9	14.2	2.2	0.7

Condition of nitrogen gas plasma treatment:1/2 atmospheric pressure, 60°C, 6 min

Table 2: XPS result for PS (polystylene)

However, in the sample treated by the nitrogen gas plasma (Figure 4 middle), these peaks were totally diminished and moreover lower molecular weight peaks significantly decreased. The tiny height of MS peaks remained in the MS spectrum of the plasma-treated sample. This indicates that in the treated sample, any cleavage at several portions in lipid A such as acid amide bondage or ester bondage can be speculated to occur due to the nitrogen gas plasma exposure, As shown in (Figure 4(c)), the mother peak can be observed even in the DMSO solvent alone injection, which means the mother peak may remain in the injection port and/or LC column as an artifact. Thus, due to the appearance of the artifact we unfortunately could not discuss the comparison of the peak height of the mother peak before and after nitrogen gas plasma exposure. However it is clarified that the peaks of around 200 and 700

Disposition	Before of After Treatment to PS	CO[1] (v/v, ppm)	NOx[2] (v/v, ppm)	HCN[3] (v/v, ppm)	O_3[4] (v/v, ppm)	N_2O[5] (v/v, ppm)
Low Pressure	Beforer	<2	<0.5	N.D.	N.D.	N.D.
Low Pressure	After	3.9	1.1	<0.1	<0.05	2.6

1. UV-absorbance spectroscopy
2. Chemical luminescence method
3. Piasoron light absorption method
4. Ozone detector
5. GC-MS

N.D. not detected

Table 3: Analysis of the exhaust gas from PS (polystylene) treated with nitrogen gas plasma

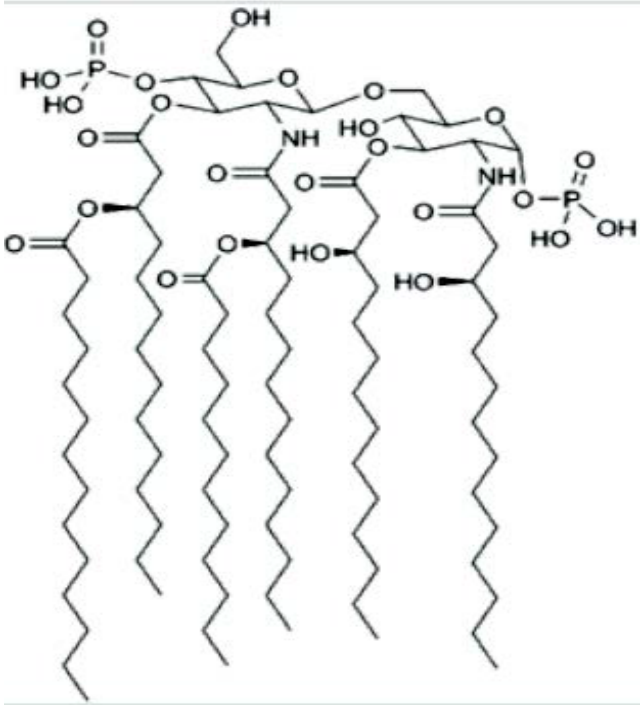

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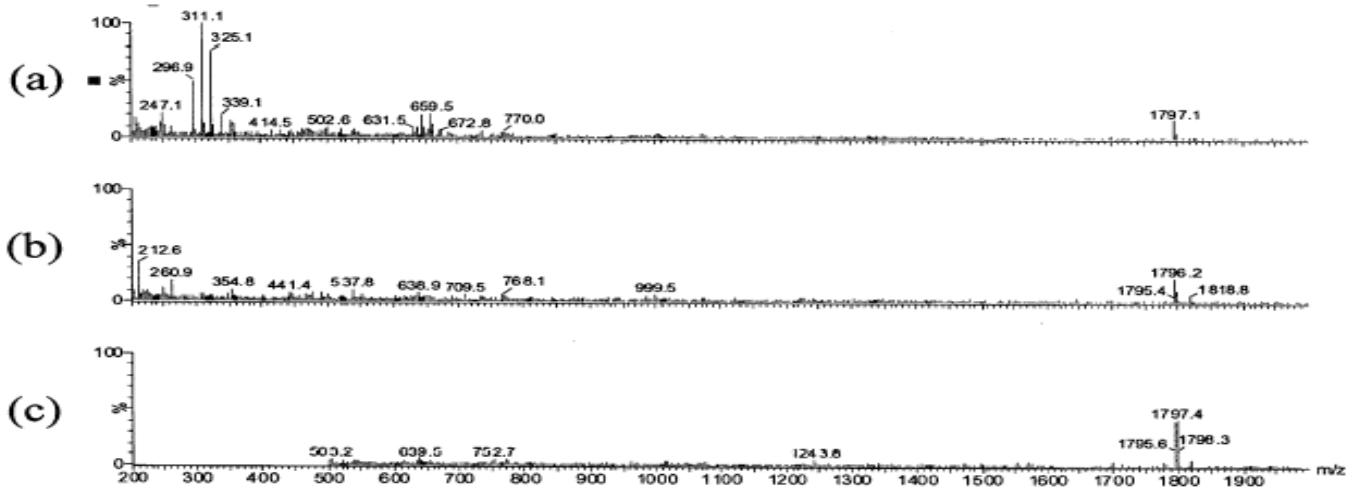
Figure 3: Chemical structure of lipid A

m/e in lipid A decreased by degradation with nitrogen gas plasma treatment (Figure 4 (a) and (b)).

The mass of peaks at around 200-300 m/e may be single or double chains of fatty acids of C 14 linkage with ester bonding and peaks at around 600,700 m/e may be multiple chains of fatty acids of C14 linkage with ester bonding. The definite portion cleaved in lipid A by nitrogen gas plasma exposure was not clearly clarified [21-23]. It can be speculated that the bonding portion with lower bonding energy such as ester bond or acid amide may be cleaved (C-O, N-H) (Table 1).

Degradation of lipid A by nitrogen gas plasma exposure was observed by atomic force microscopy (AFM) (Figure 5) and X-ray photoelectron spectroscopy (XPS) (Figure 6). By AFM only a trace of lipid A inoculated onto glass remained after nitrogen gas plasma exposure. Moreover, XPS analysis presents an identical result in (Table 2) and an increase in organic nitriding due to increased binding energy at organic nitrogen peaks of around 400 to 404 eV after treatment was observed. Anyhow lipid A at the nm level was almost completely



(a) is before exposure (b) is after exposure and (c) is DMSO injection.

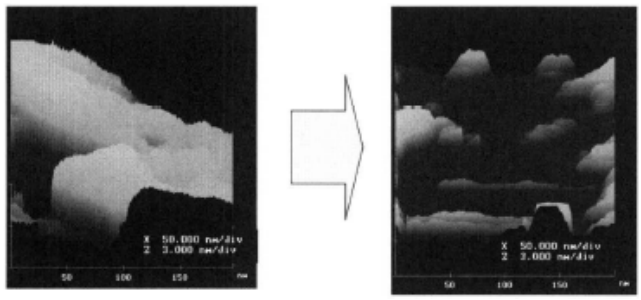
LC-MS from Waters was used. LC was Waters 2695 and MS was Waters Quattro PremierR XE.LC column was Golf PackR.

HR 3.5μm 2.1×150mm (polymer base C18 column) Mobile phase was acetonitrile, Flow rate was 0.2 mL/min, Injection volume was 5μL.

MS mode

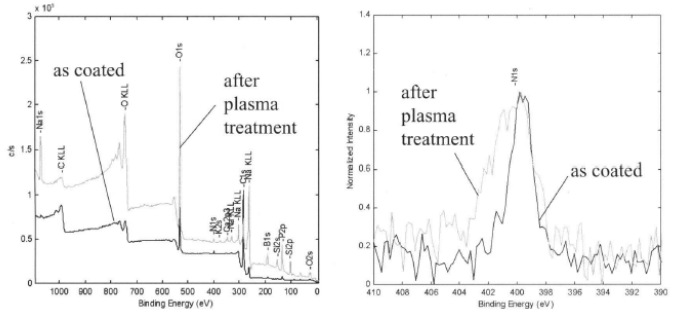
ESI negative mode was used for ionization. Capillary voltage was 4.0kv, Desolvent gassing rate was 900L/hr, Cone gas rate was 50 L/hr and Ion source heater was 120 oC. MS data scanning conditions were as follow, MS scanning range was 200-2000 Da, scan time was 0,5 seconds and cone voltage was 150 V.

Figure 4: MS spectrum of lipid A before and after nitrogen gas plasma exposure.



LP stands lower pressure.

Figure 5: MAFM analysis of LP plasma treated Lipid A (1,25 μg), which was inoculated onto giass.



LP stands lower pressure.

Figure 6: XPS analysis of LP plasma treated lipid A (1.25μg), which was inoculated onto glass.

degraded by nitrogen gas plasma exposure (Figure 5).

Endotoxin inactivation is essential in implanted medical devices to avoid pyrogen shock in patients which may cause even death in the worst case. In that respect the attainment of endotoxin inactivation at a reduction of more than 5 log by nitrogen gas plasma exposure in 30 minutes has an important meaning for applications to medical devices prior to implantation into the human body. It has significant benefit to patients requiring medical devices implantation by ensuring endotoxin-free devices to prevent disease conditions associated with contaminated endotoxins such as pyrogen shock. The authority requires less than 20 EU (endotoxin unit) reduction of endotoxin together with maintaining material/functional compatibility, so according to our method authority's requirement can be achieved in success without any difficulty.

As we have confirmed the destruction of spore-type microorganisms and endotoxin inactivation by nitrogen gas plasma exposure, we will conduct experiments on prion inactivation by nitrogen gas plasma exposure without carrier material deterioration [3,4]. According to our preliminary experiment normal prions were totally destroyed as had been the case with spores and endotoxins. From this we speculate abnormal prions causing Creutzfeldt-Jakob disease to humans would also be destroyed without deteriorating support material using nitrogen gas plasma exposure, which would bring significant benefits to our life. We speculate abnormal prions can destroy in success maintaining material functionality because abnormal and normal prions differs only the higher dimension structure and primary amino acids sequence is identical. Abnormal prion has more beta sheet structure than normal prion. We already confirmed myoglobin degradation which enriches beta sheet in that third structure by the nitrogen gas plasma exposure maintaining material/functional compatibility. Additionally, we will conduct experiment on the validation of the sterilization of the interior of the endoscope, expensive artificial heart valves, expensive surgery devices used for brain or nerves and so on by nitrogen gas plasma to attain benefits for patients using these devices in the coming study to avoid iatrogenic diseases caused at health care facilities.

Conclusion

Endotoxin inactivation can be successfully conducted after nitrogen gas plasma exposure for 30 minutes. After 30 minutes exposure, more

than 5 log reduction of endotoxin can be confirmed. The authority requires less than 20 EU (endotoxin unit) reduction of endotoxin together with material/functional compatibility, so according to our method authority's requirement can be achieved in success without any difficulties.

Lipid A is an active site of endotoxin. Lipid A degradation after nitrogen gas plasma exposure was observed using high performance liquid chromatography-mass spectrometry. From the HPLC-MS-MS data, fragmentation and dergradation of lipid A by nitrogen gas plasma was confirmed. Normal prion and myoglobin degradation was also confirmed.

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