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Important Points to Attain Reproducible Sterility Assurance

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Abstract

Many papers published and conducted so far by physical researchers on sterilization using gas plasma exposure. I as microbiologist consider that they overlooked the important points to avoid clumps (overlay of microorganisms, they called clumps as stacking, which is incorrect) in biological indicator (BI) and the importance considering statistical analysis. As a characteristic, microorganisms have themselves significant variation, so number of samples utilized must be necessary to be able to conduct statistical analysis, which concept differed from concept of physical science. The aim of gas plasma sterilization is the death of bioburden and the distribution of bioburden is not seen any clumps in exact status. The penetration depth of gas plasma is around 10 nm, so if BI has clumps (the thickness of BI is around 1 μm) death of inner layer may be hard to attain or delayed, resulting in tailing phenomenon of survival curve or failure of sterility assurance. Physical researchers so often prepare BI by themselves and the prepared BI has significant so-called stacking. As microbiologist calls physical researcher's attention the importance to conduct experiment using BI free from clumps to attain correct and reproducible sterility result. Furthermore, it is necessary to attain sterility assurance together with material and functional compatibility, otherwise GMP requirement is not achieved.

Keywords: Survivor curve method; Clump; Sterility assurance; Biological indicator; Bioburden; Material compatibility; Functional compatibility; GMP

Clump formation

Many studies on gas plasma sterilization and disinfection conducted so far by physical researchers contain serious mistakes to be corrected. These points were quite important to consider about sterilization.

First, it was not seriously considered whether biological indicator (BI) have clumps or not. Clumps are the phenomenon whereby spores or microorganisms overlap (Figure 1) [1]. As can be seen in Figure 2, BI must be completely free from overlapping (clumps) to attain reproducible sterility assurance. Clumping may cause tailing in survival curves (Figure 3).

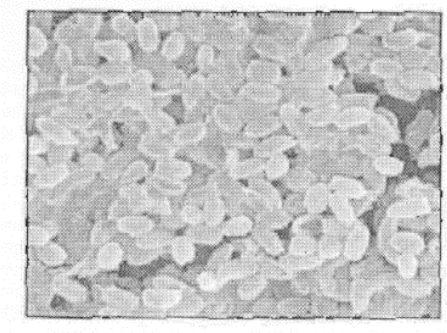

Figure 1: Clumping

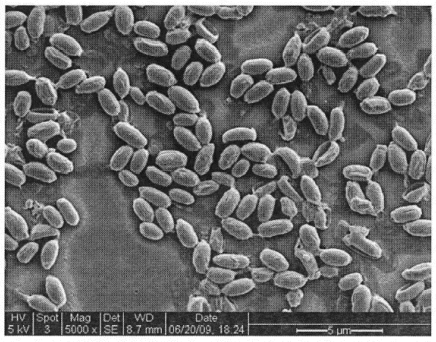

Figure 2: Mono-layer distribution of spores free from any clumps

Tailing means survival curve does not present straight line from initial population to fraction negative portion of SLR (spore log reduction) of 5 to 10^{-2} [2]. Self-prepared BIs or commercially available Bls have clumps in most cases (SEM data are skipped). BI from *Bacillus atrophaeus* ATCC 9372 was used for ethylene gas exposure sterilization and fry heating sterilization, while *Geobacillus stearothermophilus* ATCC 7953 was used for autoclaving and formaldehyde sterilization (ISO 11138-1) and gas plasma sterilization, even though gas plasma is not defined as BI officially, but it considers the most tolerance towards gas plasma exposure.

These conventional sterilization procedures have sufficient depth of penetration, therefore tailing phenomena were rarely observed even if the BIs had clumps. However, gas plasma from several sorts of gases has a quite shallow depth of penetration. The depth of penetration of gas plasma was around 10-40 nm.

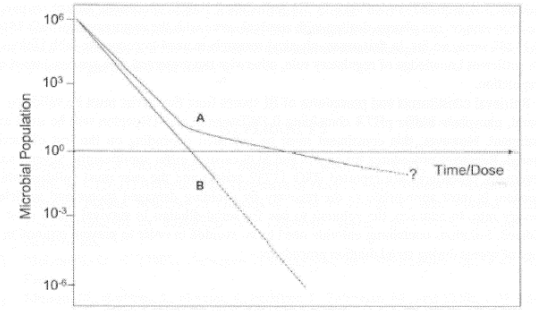

Figure 3: The survivor curve. A) The tailing phenomenon. B) Straight survival curve through the fraction negative range (SLR of 4.6 to 10-2), indicating SAL 10-6 can be achieved [2].

As one spore is around 1x3 μm (1000×3000 nm), the penetration depth by gas plasma was around one layer of spores. If multiple layers of spores on the carrier material exist as shown in Figure 1 [1], the interior layers of the clumps will be protected by the inactivated outer layer [3]. Thus, the apparent D (decimal reduction value) was, as a whole, greater than the exact D value. In addition, the tailing phenomenon in which the slope was not straight was observed (Figure 3A) [2]. If tailing phenomenon can be observed, a sterility assurance level (SAL) of 10^{-6} required for health care products can be hard to achieve. The simultaneous achievement of SAL of 10^{-6} and material and functional compatibility was also overlooked in plasma sterilization papers published so far.

Strain	% Water activity (Aw)
Bacillus subtilis ATCC 6633	94
B. subtilis ATCC 19221	95
B. atrophaeus ATCC 9372	47
B. cereus T	95
B. coagulans ATCC 8038	49
Geobacillus stearothermophilus ATCC 7953	53
B. megaterium ATCC 12872	88
B. megaterium ATCC 33729	30
Clostridium botulinum 213B	50
C. sporogens ATCC 7955	67
C. putrefaciens ATCC 25786	79

Table 1: Hydrophobicity and/or hydrophilicity of several spores

In order to avoid clumping in the BI, spores must be distributed evenly on the carrier material (Figure 2). If the hydrophobicity and hydrophilicity of spores differ (Table 1), so the characteristics of carrier materials must coincide with those of spores. Furthermore, as bioburden (bioburden means viable microorganisms on/in products) was around a few CFU (colony forming unit), so observations of clumps in bioburden could be quite rare in exact status. As an initial population of 10^6 CFU/carrier was so often required in validation study, but from the exact status the requirement of 10^6 CFU/carrier can be confirmed quite large number, so less than 10^6 CFU as an initial population was quite enough to evaluate gas plasma sterilization procedure. For another requirement of 10^6 CFU BI, an absolute bioburden method or combined BI/bioburden method can be presented in ISO 14161 and sterilization validation [4]. As BI of gas plasma sterilization, *G. stearothermophilus* ATCC 7953 must be utilized because this spore is the most tolerable to gas plasma sterilization. BI defined as the most tolerable spore towards a definite sterilization (ISO 11138-1). BI is inoculated onto a carrier material such as a polyhydroxymethylmethacrylate (PHMMA), partially modified PS (polystylene) or surface modified SUS to coincide Aw of surface and that of the spore in Table 1.

The hydrophobicity and/or hydrophilicity of PHMMA or partially modified PS or SUS are around 50/50, coinciding with hydrophobicity of *G. stearothermophilus* in Table 1.

Another way to prepare a monolayer of Bl free from clumps resembles the preparation of DNA tips. This procedure atomizes the spores onto the carrier material instead of the drop procedure, conventionally used for BI preparation. DNA tips procedure is not easily applicable to BIs used for gas plasma sterilization for both economic and technical reasons. In case of using drop procedure, the correct carrier material selection and careful dropping procedures are required to avoid clumping. DNA tipping procedure is completely free from clumps, but quite expensive and time consuming.

G. stearothermophilus ATCC 7953 does not show tailing in traditional sterilization procedures because they have no repair enzymes [5]. In the case of *Deinococcus radiodurans* (Figures 4,5), which tolerated the radiation procedure, a straight survivor curve was not attained due to the presence of repair enzymes [6]. Significant shoulder can be observed (Figure 5) [6].

Readers must keep in mind that only a limited number of bacterial spores present tailing phenomenon or shouldered survival curves [7]. As far as the author is concerned, almost all papers published so far on gas plasma sterilization presented non-straight survival curves; this is quite exceptional in the real status.

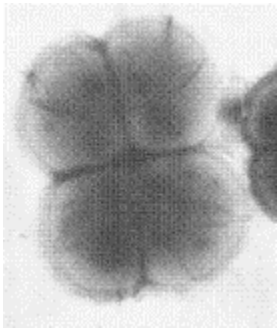

Figure 4: Micrograph of *D. radiodurans* cells in a typical tetrad formation

The reason of the non-straight survivor curve is due to clumping. You can imagine how it is too much difficult task to inoculate 10^6 CFU in the circle of 2 mm diameter without clumping. As mentioned above, most of physical researchers cannot understand the importance avoiding clumps in BIs to attain straight survivor curve when

conducting gas plasma sterilization, mostly due to quite shallow penetration depth (10 nm) of radicals and other sterilization factors from gas plasma. Up to date, no correct sterilization factors of gas plasma are defined.

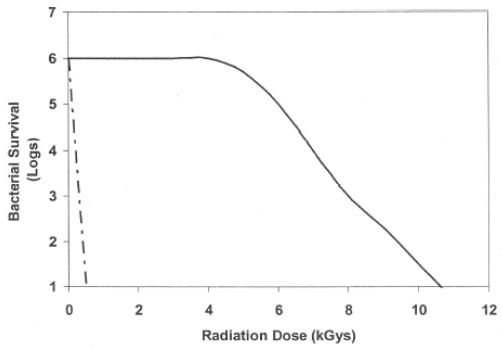

Figure 5: An example of *D. radiodurans* survival curve of radiation irradiation. The survival curve of *D. radiodurans* (solid line) is compared to that of the typical irradiation-sensitive bacterial strain (dotted line) when exposed to increasing doses of γ-irradiation

Requirement for survivor curve preparation

In addition, physical researchers must study more about statistical analysis because bacteria and microorganisms have significant variation. In the exact explanation, according to ISO 14161 and 11138 series [4,7], to prepare a survival curve, 4 BIs at an initial population and additional 4 exposes with 4 BIs at each exposure time with same interval. As a whole 4×5=20 BI sheets are required to prepare a survival curve. And the survival curve can be described with a regression line using a statistical analysis. More than 0.8 of coefficient relationship is required (ISO 11138-1), indicating no tailing approved. As far as the author is concerned, almost no papers have ever seen on gas plasma dealing with survival curve with the requirement of ISO 14161 and 11138 series so far. In that sense, physical researchers need to cooperate with biologists with sufficient knowledge of regulatory rule, otherwise the presented data are considered out of regulation.

Retrieval constituents and procedures of BI spores from the carrier must be validated. In general, phosphate buffer pH 7.4 containing 0.1% Tween 80 and 1% pepton will be used as a recovery constituent. Tween 80 is for the neutralization agent and pepoton is for enrichment for injured spore by sterilization. Depending on the degree of injury and amount of sterilizing agents, recovery constituent must also be validated depending on the sort of carrier material. Recovery procedures for papers and SUS carriers differ significantly. Several sorts of recovery procedures are reported (ISO 11737 series) and the user must validate which procedure is most appropriate to the recovery by avoiding damaged spores and sufficient recovery rate. In addition, the solution to use for seria1-dilution in survival curve must be validated. Solutions containing chloride need to be avoided in order to prevent promotion of injury of spores during serial dilution procedures.

Importance of statistical analysis

In addition, in the field of physics, variation of data may be negligible. However in the field of microorganisms, variation is significant. For example, the variation in D values was significant and often more than 10-fold. Several factors may exist to cause variation. The culture medium to prepare spores, scattering procedure of spores onto the carrier material, sorts of carrier materials, sorts of primary package, difference of lots/batch and manufacturer of spore cultivation medium (the last information must convey to the user, ISO 11138-1) [6], cultivation procedure and so on. Many factors to cause variation data must seriously be considered. In that sense, we need to evaluate data from more than 20 samples to conduct statistical analysis; otherwise we cannot obtain statistically correct data. For example in ISO 11138-1, the retrieval population from BI can be officially approved from -50 to +300% [7]. This means the sort of variation in microorganisms can be considered normal. When conducting sterilization study the readers must keep in mind that the data from microorganisms may vary significantly and need to analyze statistically, but each data is real data with only exception to be deleted statistically. This is quite different from physics data. The physical researchers may have a culture shock and the microbiologists also have a culture shock when reading the sterilization data conducted by physical researchers. It was vice versa.

Importance considering attainment of material and functional compatibility after sterilization

SAL of 10^{-6} must be attained together with material and functional compatibility. This is the requirement of sterilization validation and GPM (Good Manufacturing Practice), however no sterilization procedures was achieved this requirement in success so far. Gamma-ray and electron beam destroys material [7-15] and heating is also identical. Ethylene oxide cause alkylating phenomenon of the material.

In that sense only gas plasma sterilization can attain SAL of 10^{-6} together with functional and material compatibility because penetration depth is quite shallow, thus only bioburden on the device surface is killed and it does not deteriorate any material and functional compatibility. This is the reason gas plasma sterilization is absorbed attention and interest from biologists. We wish the future development of gas plasma sterilization.

References

1. Brandenburg R, Krohmann U, Stieber M, Weltmann KD, Woedtke TV, et al. (2007) Antimicrobial Treatment of Heat Sensitive Products by Atmospheric Pressure Plasma Sources, In Gueceri S, and Friedman A, eds, Plasma Assisted Decontamination of Biological and Chemical Agents, pp. 51-65, Springer, The Netherlands
2. McDonell GE(2007) Antisepsis, Disinfection, and Sterilization, pp.44-47, ASM Press
3. Moisan M, Barbeau J, Moreau S, Pelletier J, Tabrizian M, et al. (2001) Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. Int J Pharm 226: 1-21.
4. ISO I4161, Sterilization of health care products–Biological indicators-Guidance for the selection, use and interpretation of results.
5. Russell AD (1983) Recovery and Revival of Damaged Spores in Russell, A.D. ed, The Destruction of Bacterial Spores, pp.292-310, Academic Press, N.Y.
6. Graham GS, Boris CA (1993) Chemical and Biological Indicators, in Morrissey, R. F. and Phillips, G. B., eds, Sterilization Technology, pp. 36-70, Van Nonstrand Reinhold, N.Y.
7. Shintani H (1995) Formation and elution of toxic compounds from sterilized medical products: methylenedianiline formation in polyurethane. J Biomater Appl 10: 23-58.

8. Shintani H (1995) The relative safety of gamma-ray, autoclave, and ethylene oxide gas sterilization of thermosetting polyurethane. Biomed Instrum Technol 29: 513-519.
9. Shintani H, Nakamura A (1990) Mechanism of degradation and crosslinking of polyurethane when irradiated by gamma-rays. J Appl Polym Sci 42: 1979-1987.
10. Shintani H, Nakamura A (1989) Analysis of the carcinogen 4, 4'-methylenedianiline (MDA) in gamma-ray and autoclave-sterilized polyurethane. Fresenius' Zeitschrift für analytische Chemie 333: 637-640.
11. Shintani H, Nakamura A (1991) Formation of 4,4'-methylenedianiline in polyurethane potting materials by either gamma-ray or autoclave sterilization. J Biomed Mater Res 25: 1275-1286.
12. Shintani H (1991) Solid-phase extraction of a carcinogen, 4,4'-methylenedianiline, in serum. J Anal Toxicol 15: 198-201.
13. Shintani H, Nakamura A (1989) Analysis of a carcinogen, 4,4'-methylenedianiline, from thermosetting polyurethane during sterilization. J Anal Toxicol 13: 354-357.
14. Shintani H (2004) Study on radiation sterilization-Resistant polysulfones fabricated free from Bisphenol A. Trends Biomatr Artif Organs 18: 36-40.
15. Shintani H (2001) Determination of the endocrine disrupter bisphenol-A in the blood of uremic patients treated by dialysis. Chromatographia 53: 331-333.