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Bactericidal Characteristics and Material Conformity of Atmospheric-Pressure Glow Discharge

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ABSTRACT: We studied the inactivation of microorganisms using glow discharge plasma in a mixture gas of helium and oxygen at normal atmospheric pressure. Combinations of parameters such as frequency and discharge gap are varied among a variety of solutions. We demonstrate inactivation using active species generated in the discharge space. Process compatibility was checked on the package of biological indicators, and dental impression molds in complex shapes were made of the hydrophilic polymer used in dental surgery.

KEY WORDS: atmospheric pressure glow, plasma, sterilization, *Geobacillus stearothermophilus, Bacillus atrophaeus, Escherichia coli, Salmonella enteritidis, Staphylococcus aureus, Candida albicans*

I. INTRODUCTION

Advanced schemes for the sterilization and high-grade disinfection of patient-care products has increasingly drawn attention. Increasing consideration has been required in medical institutions for nosocomial infection among patients, cross-infection among medical facilities staff, and secondary infection of immune-deficient patients. This is not only due to emerging diseases such as enterohemorrhagic *Escherichia coli* (EHECO-157), human immunodeficiency virus (HIV), severe acute respiratory syndrome virus (SARS), and Ebora hemorrhagic fever, but also to the increase in infection by normal flora in the soil and human skin, such as *Acinetobacter, Candida albicans, Staphylococcus aureus,* etc. Advanced schemes for the sterilization of medical-care materials are increasingly drawing attention.

Kogoma et al., at the University of Sophia, showed varieties of industrial applications of atmospheric-pressure glow discharge plasma (APG), such as surface cleaning and improvement of hydrophilic and hydrophobic properties.[1–3] Laroussi et al. demonstrated disinfection, i.e., complete killing of various bacteria and viruses, using atmospheric pressure discharges at frequencies below 50 kHz.[4–6] A wide frequency range is available to excite atmospheric pressure discharge, from several kHz to industrial

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frequencies of 13.56 and 27.12 MHz. At these frequencies, high-performance RF power sources are available and higher excitation is achieved compared to low-frequency discharges, but the scenario of the self-interruption effect by the dielectric barrier plate is no longer effective. In high-frequency APG plasmas, heating of neutral gas must be controlled using some artificial maneuvers. In the past, the authors have examined the approach to sterilization using RF-driven APG: the control of neutral gas temperature was achieved by pulse modulation. Some compromise was necessary with the antibacterial effects against spore-forming bacteria: *Bacillus atrophaeus, Geobacillus stearothermophilus,* and opportunistic pathogens *Salmonella enteritidis, S. aureus, C. albicans,* and *Aspergillus niger.* In this work, we aim to describe experimental development of a plasma sterilization system using an inert gas and oxygen mixture excited by RF discharge at atmospheric pressure, and to check process compatibility on the package of biological indicators and dental impression molds in complex shapes that are made of the hydrophilic polymer used in dental surgery.

II. MATERIALS AND METHODS

A. Setup of Atmospheric-Pressure Glow Discharge

Figure 1(a) shows the apparatus used in the experiment, and Fig. 1(b) shows the dielectric barrier discharge electrode. The electrode is made of an aluminum block 150 mm long and 50 mm wide. The metallic parts of the upper and lower electrodes are cooled by circulation of chilled coolant, and covered with fused quartz plates measuring 230 mm long and 115 mm wide. The volume of the discharge region is surrounded by heat-resistant glass. A working gas mixture of helium and oxygen is supplied from an array of apertures 1 mm in diameter. The flow rate of working gas is controlled with mass-flow controllers, in a range of 1 ml to 5 l/min.

The temperature of neutral gas was measured using a fluorescent-type thermometer based on the relaxation time of fluorescence coated on an optical fiber probe (FL-2000, Anritsu Measurement Co.). Emission spectroscopy is measured in the vicinity of the triplet spectrum at 777 nm, using a multichannel spectrometer (MonoSpec 18 by Thermo-Vision Colorado Inc.), installed with a Pelletier-cooled CCD camera (ST-6V, by SBIG Inc.). An optical fiber was installed facing the lateral extremity of the discharge gap.

B. Preparation of Biological Indicators

The density of microorganisms was adjusted in the following way. Selected species were incubated in soybean casein digest (SCD) fluid culture medium (Becton Dickinson Co. Ltd.). An amount of 0.01 ml of the culture medium was sampled and incubated on SCD agar culture medium. Then, a small amount of agar culture was diluted in distilled water. The final density was adjusted to approximately 10^7 CFU per ml. An amount of 0.1 ml of

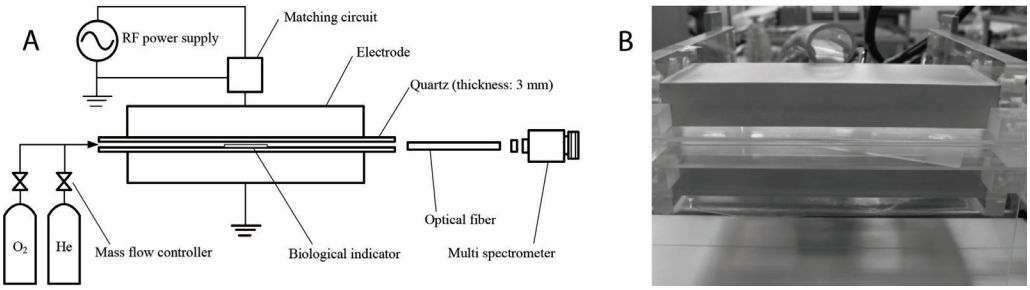

FIG. 1: Atmospheric-pressure glow plasma (APG) (a) experimental setup, and (b) discharge electrodes

the suspension was sampled and applied to a sterilized cover glass, of size 18 × 18 mm square and 150 μm in thickness. Each glass plate was dried and sealed in a sterile package, consisting of Tyvek nonwoven polyethylene fabric, 0.15 mm in thickness, and a transparent polyethylene terephthalate (PET) sheet, 0.06 mm in thickness. The selected species of microorganisms were *E. coli* ATCC8739, *S. enteritidis, S. aureus* ATCC6538, *C. albicans* ATCC10231, and spore-forming bacteria *B. atrophaeus* ATCC9372 and *G. stearothermophilus* ATCC7953.[7–11] These biological samples were supplied by Raven Biological Laboratories Co. of the United States. ATCC stands for the American Type Culture Collection, a commercially available library, and CFU stands for colony forming unit, the density of active end-spores to be detected as individual colonies after incubation. In the incubation and sterility judgments, we followed protocols for the sterility test in Ref. 12.

After the plasma treatment, the cover glass was incubated in 100 ml of SCD fluid culture medium. *G. stearothermophilus* was incubated at 55–60°C. *C. albicans* was incubated at 25°C, and other microorganisms were incubated at 30–35°C. After seven days of incubation, the result of the sterility test was judged on the basis of pH turbidity of the culture medium. The culture medium remains clear only if $6\log_{10}$-grade sterilization was performed successfully. Commercially available biological indicators of spore-forming bacteria, Attest type 1262, from 3M Co., were also used for comparative study. Each vial of Attest type 1262 containing *G. stearothermophilus* ATCC 7953, at 8.6×10^5 CFU, was carried out for 48 h in vials, and validation of sterility was judged by the change of color of the incubation medium according to a pH indicator. *G. stearothermophilus* is approved as a standard indicator for steam sterilization.

III. RESULTS

The first experiment was the antibacterial effect of APG excited with 13.56 MHz. Table 1 shows the resulting antibacterial effect obtained with a discharge gap of 10 mm be-

tween quartz plates. Neutral gas was heated to 200°C in 5 s and to 220–250°C in 30 s. An enhanced antibacterial effect was observed above 115°C.[13]

The next experiment was the antibacterial effect of APG excited with pulse-modulated RF at 27.12 MHz, with a discharge gap of 3 mm. Sterilization time strongly depended on temperature. Sterilization was achieved in shorter time in higher temperature conditions. This experiment aimed to sterilize spore-forming bacteria in the Tyvek at low temperature. Gas temperature in the discharge region was kept to no higher than 90°C.[14]

The relative population of oxygen atoms is proportional to the intensity of the spectral emission from oxygen atoms, at 777 nm. Excitation of oxygen atoms via energy transfer from meta-stable helium atoms—that is, Penning excitation—was expected. A possible hypothesis for the enhancement of the excitation process can be described as in the following formula:

$$He + electron \rightarrow He^{*}(2^{3}S,2^{1}S) + electron$$
$$He^{*} + O_{2} \rightarrow He + O^{*} + O$$

Sterilization efficiency can be expected for a higher concentration of atomic oxygen excited via the Penning process. Increase in the oxygen flow rate causes an increase in the energy relaxation process and decrease in the population of helium in the excited state. The maximum antibacterial effect was observed at the minimum sterilization time at an oxygen ratio from 0.06 to 0.09%.[14]

Table 2 shows the result of sterilization of spore-forming bacteria *B. atrophaeus,* for different spore density. A group of 10^7 CFU remained alive after 20 min of plasma treatment, but a group of 10^5 CFU was sterilized in 20 min, and groups of lower spore density, 2.7×10^4 to 6.8×10^1, were sterilized in 5 min. Thus, necessary plasma treatment is shorter for lower densities. A similar result was observed for *G. stearothermophilus*. A group of 6.6×10^5 CFU was sterilized in 20 min, and a group of 3.3×10^4 to 8.2×10^1 CFU was sterilized in 5 min. The required plasma treatment is shorter for lower densities, and every group of a different density remained alive after dry-heat treatments at

TABLE 1: Antibacterial effect of APG

Microorganism and population (CFU)	Exposure time (s)					
	5	10	20	30	40	60
Escherichia coli ATCC8739 (4.4×10^7)	+	–	–	–	–	
Salmonella enteritidis (4.0×10^7)	+	–	–	–	–	
Staphylococcus aureus ATCC6538 (6.8×10^7)	+	+	–	–	–	–
Candida albicans ATCC10231 (5.1×10^6)	+	–	–	–	–	
Bacillus atrophaeus ATCC9372 (5.1×10^6)		+	+	–	–	–
Geobacillus stearothermophilus ATCC7953 (3.8×10^5)		–	–	–	–	–

+ Propagation of bacteria; – no propagation; gap 10mm; biological indicator glass slide carrier; RF frequency and power 13.56 MHz, 670 W; gas flow rate He 5 l/min.

TABLE 2: Sterilization of spore-forming bacteria in the Tyvek package for various spore densities

Sterilization of *Bacillus atrophaeus* ATCC9372				
Density (CFU)	**Plasma treatment (min)***			**Heat treatment**
	5	**10**	**20**	**20 min (90°C)**
1.1×10^7	+	+	+	+
5.4×10^5	+	+	–	+
2.7×10^4	–	–	–	+
1.4×10^3	–	–	–	+
6.8×10	–	–	–	+
Sterilization of *Geobacillus stearothermophilus* ATCC7953				
Density (CFU)	**Plasma treatment (min)***			**Heat treatment**
	5	**10**	**20**	**20 min (90°C)**
6.6×10^5	+	+	–	+
3.3×10^4	–	–	–	+
1.6×10^3	–	–	–	+
8.2×10^1	–	–	–	+

*RF power and frequency 670 W and 27.12 MHz; pulse width and interval 10 μs; gas O_2/He, 0.06% (He 1.5 l/min; O_2 1.0 ml/min); biological indicator carrier glass slide; gap 3mm.

90°C for 20 min. Figure 2 shows an image of a biological indicator in a sterile package, consisting of the Tyvek package, in the electrodes.

The sterilization time varied depending on the species of microorganism: *C. albicans, S. aureus, S. enteritidis,* and *E. coli.* Table 3 compares the sterilization time between the plasma treatment and dry-heat treatment at 90°C, using these biological indicators. Plasma was excited at conditions of an oxygen/helium mixture ratio of 0.06%, (He, 1.5 l/min; O_2, 1.0 ml/min), and biological indicators coated on a glass slide were exposed to the plasma in Tyvek sterile packages. A control group was settled in dry-heat treatment at equivalent gas temperature, 90°C. *E. coli* and *C. albicans* remained alive after the high-temperature treatment at 90°C for 1 min. Plasma-treated samples were inactivated in 1 min. *S. enteritidis* remained alive after dry-heat treatment for 5 min. Plasma-treated samples were inactivated in 1 min.

The temporary dependence of the surviving microbes was measured in a later experiment using the same biological indicator, *G. stearothermophilus*. In this turbidity assessment, we measured the minimum completion time required for disinfection for the systematic selection of the trial cases being complementary to each other.

Figures 3 and 4 show images of *C. albicans* and *S. aureus* recorded by an inverted

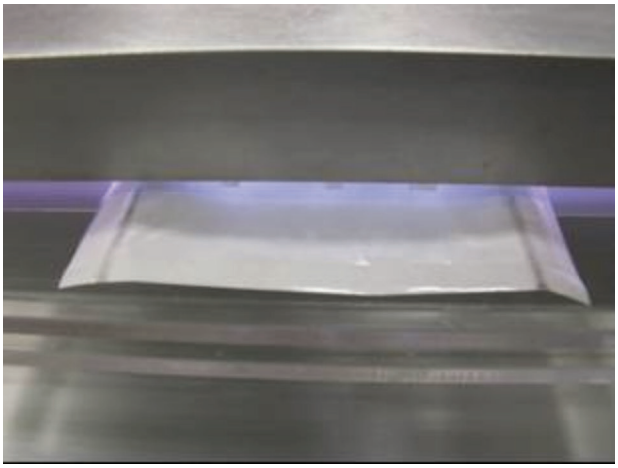

FIG. 2: Tyvek package in the electrodes

TABLE 3: Summary of the experimental comparison between the plasma and dry-heat treatment

Sterilization process (min) Species and density (CFU)	Plasma treatment*					Heat treatment (90°C)				
	1	3	5	10	15	1	3	5	10	15
Escherichia coli ATCC8739 (1.6×10^7)	–	–	–	–	–	+	–	–	–	–
Salmonella enteritidis (3.5×10^7)	–	–	–	–	–	+	+	+	–	–
Staphylococcus aureus ATCC6538 (4.7×10^7)	+	+	–	–	–	+	+	+	+	+
Candida albicans ATCC10231 (5.1×10^6)	–	–	–	–	–	+	–	–	–	–

*Biological indicator opportunistic pathogen on glass slide carrier, in Tyvek package; RF power and frequency 670 W and 27.12 MHz; pulse width and interval 10 μs; He 1.5 l/min; O_2 1.0 ml/min.

microscope system installed with digital imaging facilities (Olympus Co. Ltd.).[14] Figure 3(a) shows an image of living cells of *C. albicans*, typically 5 μm in diameter. After the plasma treatment, the spherical cellular structure of *C. albicans* was destroyed, and residue of cell leakage was partly cleaned by oxidation etching, as shown in Fig. 3(b). In this experiment, the antibacterial effect is brought by atomic oxygen excited in the helium plasma. The sterilization efficiency is also different between plasma treatment and heat sterilization.

Figure 4(a) shows an image of *S. aureus*, showing its round shape, ~1 μm in diameter, and Fig. 4(b) shows an image of a specimen exposed to atmospheric-pressure

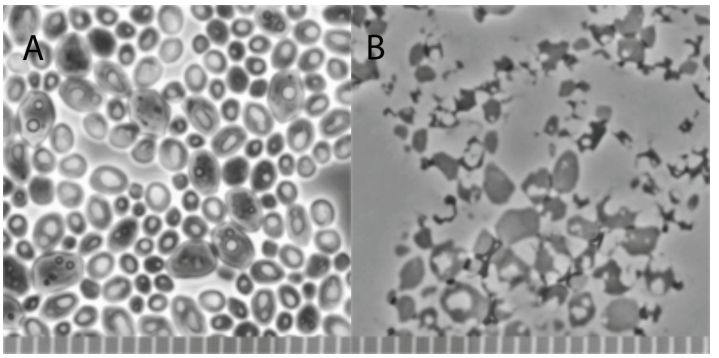

FIG. 3: Plasma sterilization and postcleaning of *Candida albicans*. Optical-microscope images of *C. albicans* (a) before, and (b) after the plasma treatment. RF power and frequency 670 W and 27.12 MHz; pulse width and interval 10 μs; duration 5 min, 5 μm/division

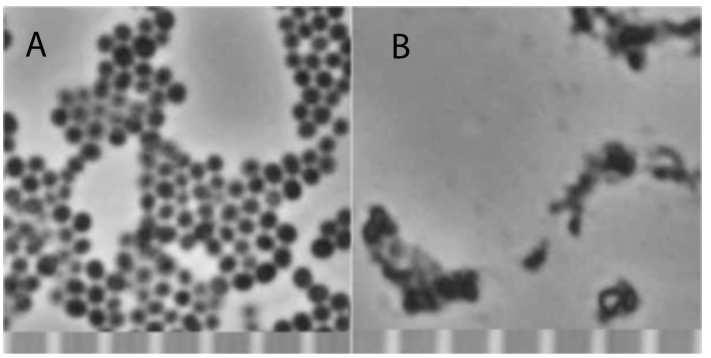

FIG. 4: Plasma sterilization and postcleaning of *Staphylococcus aureus*. Optical-microscope images of *S. aureus* (a) before, and (b) after the plasma treatment. RF power and frequency 670 W and 27.12 MHz. Pulse width and interval 10 μs; duration 5 min, 5 μm/division

glow plasma for 5 min. The sterilization process destroyed cells of *S. aureus*, and the destroyed cells are being cleaned by oxidation etching.

Finally, a decrease in the heating effect by 50 kHz frequency power induced a decrease in neutral gas temperature, to as low as 50°C, with a discharge gap of 15 mm. The antibacterial effect of low-temperature glow discharge, excited between dielectric barrier electrodes at 150 W, 50 kHz, was tested on a type of biological indicator: *G. stearothermophilus* spores, Attest 1262 from 3M. Table 4 compares the sterilization mechanisms that were tested using helium/oxygen and helium/nitrogen atmospheric discharge. Plasma-treated biological indicators were inactivated in 15 min in the case of using helium/oxygen. Meanwhile, in the case of using helium/nitrogen, sometimes biological

TABLE 4: Sterilization mechanism test using helium/oxygen and helium/nitrogen atmospheric discharge*

Flow rate (l/min)			Time (min)		
He	O_2	N_2	5	10	15
3	0.003	0	+ + +	+ + +	– – –
3	0	0.003	– + –	+ + +	+ – –

*RF power and frequency: 150 W and 50 kHz; Gas: He: 3 l/min, O_2 or N_2: 3 ml/min; Biological indicator carrier: *Geobacillus stearothermophilus* spores, Attest 1262 from 3M, filter paper direct exposure; Gap: 15 mm

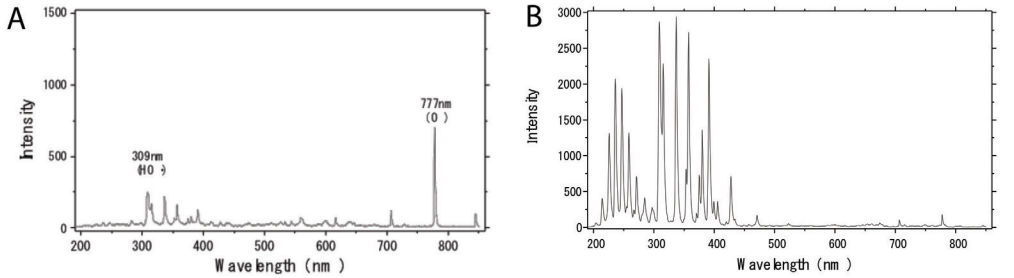

FIG. 5: Spectral emission of from atmospheric pressure glow discharge in (a) oxygen/helium mixture and (b) nitrogen/helium mixture

indicators were inactivated in 5 min and sometimes these were not inactivated in 15 min.

Figure 5 shows the spectra in cases of helium/oxygen or helium/nitrogen mixture gas discharge plasma. In the case of helium/oxygen, we also observed a strong emission at 777 nm due to the O atomic line, as shown in Fig. 5(a). In the case of helium/nitrogen, we observed several strong UV emissions, as shown in Fig. 5(b).[15]

In this experiment, two types of primary sterilization effect are pointed out. One scheme is the radical effect as observed in the emission of active oxygen and hydroxyl radicals as in Fig. 5(a), and the other important scheme is UV radiation by nitrogen, as in Fig. 5(b).

A compatibility test was carried out with the impression material vinyl-polysiloxane(VPS), which has flexibility and hydrophilic surface characteristics that provide dimensional accuracy of 20 μm after polymerization of impressions, as shown as Fig. 6. VPS allows stronger attachment of microorganisms compared to a smooth surface such as a glass slide, and it was Exafine, as shown in Fig. 6(a) and Exahigh-flex, as shown in Fig. 6(b) from GC Medical Corp. VPS allows stronger attachment of microorganisms compared to a smooth surface such as a glass slide, and it was Exafine and Exahigh-flex from

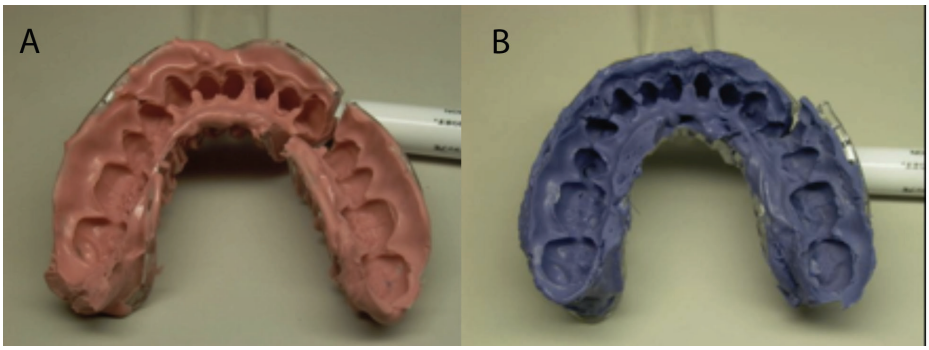

FIG. 6: (a) Plasma treatment of impression material Exafine (GC), (b) Impression material Exa-high-flex (GC)

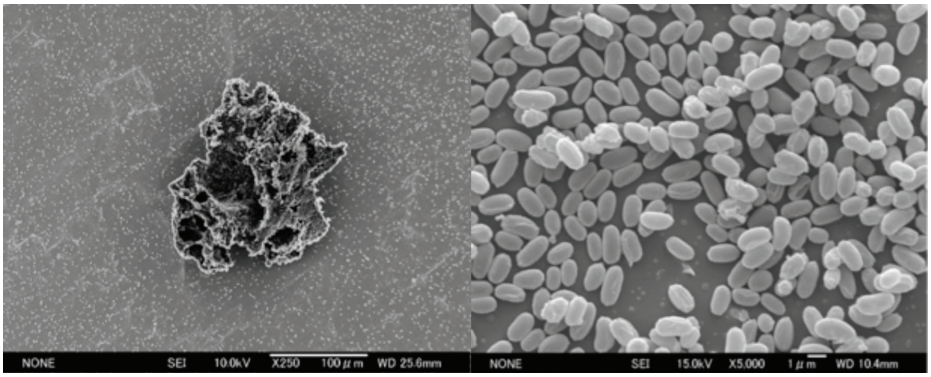

FIG. 7: Scanning electron microscope image showing impurities and cramping spores, by the courtesy of Raven Laboratory Japan

GC Medical Corp. These samples were cut out into 15 × 30 mm dimensions, including several impressions of teeth. A change in quality on the surface of the impression material after 10 mins of plasma treatment was not recognized with a result of analysis by FTIR.

IV. DISCUSSION

We have discussed problems of commercial biological indicators. A standard biological indicator may enable a global comparative study on plasma sterilization. The present stage of commercially available indicators is almost there, but still, contamination such as empty spores and other impurities can be found as shown in Fig. 7. The overlap of spores and impurities such as metallic wires, defective carbon tips, and adhesive proteins are considered as origins of tailing, i.e., the delay in sterilization time.[16,17]

V. CONCLUSION

Plasma sterilization at normal atmospheric pressure was examined using an RF generator, in the experimental comparison varying the frequency and discharge gap. The thermal effect and energy deposition of plasma particles mutually contributed to the sterilized surface of carriers. The antibacterial effect was investigated using biological indicators of Gram-stain-positive spore-forming bacteria *G. stearothermophilus* and *B. atrophaeus*, Gram-stain-positive bacteria *S. aureus*, Gram-stain-negative bacteria *E. coli and S. enteritidis*, and the yeast *C. albicans*. Sterilization by plasma showed satisfactory results for standard levels of cleanliness, excluding spore-forming bacteria in a sterile package, consisting of Tyvek nonwoven polyethylene fabric for lower densities. In our experiment, the microscopic observation indicated that the etching by oxygen radicals is necessary to explain the experimental result, because the UV sterilization leaves contracted cells.

A compatibility test was carried out with the impression material vinyl-polysiloxane (VPS), including several impressions of teeth, and after 10 min, plasma treatment was not recognized with a result of analysis by FTIR.

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