Sterilization Efficacy of a Below 40°C Plasma Processes Assessed by a Biological Indicator

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Abstract: Biodegradable biopolymers have been widely used in biomaterials for tissue engineering. Sterilization is a process by which a product can be made free of contamination from microorganisms such as bacteria, yeasts, and viruses. When sterilizing biodegradable scaffolds, the sterilization technique chosen must maintain the structural and biochemical properties of the scaffolds to ensure that the scaffolds can fulfill their intended purposes post-sterilization. In addition, in response to the development of customized, rapid and accurate medical devices via 3D printed biomaterials, sterilization of such devices by low-temperature hydrogen peroxide (H₂O₂) gas plasma (HPGP) will provide new capabilities not only for rapidly manufacturing customized medical devices but also for producing sterile end-use parts. This device uses a below 40°C temperature plasma with H₂O₂ activated by ultraviolet light irradiation processes to evaluate the sterilization efficacy with a biological indicator. The results demonstrated several parameter combinations for HPGP that can achieve sterilization at temperatures below 40°C. Therefore, the parameters associated with this HPGP device have the potential to provide a sterilization process applicable for future development of biopolymer devices manufactured by 3D printing.

Keywords: Biological Indicator; Plasma; Hydrogen peroxide; Sterilization

Introduction

Traditional sterilization methods include steam sterilization [1], dry heat [2], gamma ray [3], electron beam [4], and ethylene oxide (EO) sterilization [5]. Steam sterilization, carried out in a temperature range of 120-130°C, is not expected to affect the molecular structure of polymers but can lead to post-crystallization and physical aging effects on a material [6]. For polymers intended for use as an implant or for other biomedical applications, a safe and nontoxic sterilization method must be developed [7].

Gas plasma sterilization [8] is a safe and low-cost sterilization method compared to traditional sterilization methods such as high-pressure steam sterilization and EO sterilization. Plasma is known as the fourth state of matter, and it is an electrically energized matter in a gaseous state composed of charged particles, free radicals, and some radiation [9]. In a glow discharge at low pressures, the discharge is sustained by ionization of gas molecules via collision with accelerated electrons. Typical electron density (or plasma density) of the glow discharge at low pressure is referred to as “non-equilibrium plasma” or “cold plasma” [10]. In the gas plasma sterilization procedure, the working gases can

be air, Ar, O₂, CO₂, N₂/O₂, or Ar/O₂ mixtures [11]. Hydrogen peroxide (H₂O₂) is commonly used to increase the levels of reactive oxygen species to promote the sterilization efficacy [12].

Unfortunately, most thermoplastics and hydrogels widely used in biomedical applications, particularly polylactic acid (PLA), polyglycolic acid (PGA), poly(lactide-co-glycolide) (PLGA), etc., will not survive a standard autoclave cycle. In addition, while sterilization with γ -radiation is effective, it causes drastic changes to the biochemical properties of these biomaterials. Holy et al. [13] reported that low-temperature radio-frequency glow discharge (RFGD) plasma sterilization is the most suitable method for sterilizing polyester devices for tissue-engineering applications because it does not alter the 3D morphology of such scaffolds. Scaffolds treated by RFGD plasma showed no change in morphology and limited change in molecular weight (Mw). Furthermore, the degradation profile of RFGD plasma-treated samples most closely resembled that of control samples. New methods that sterilize polyester scaffolds without affecting them chemically and morphologically would be optimal for subsequent in vivo use of 3D scaffolds.

Therefore, we designed a new low-temperature (below 40°C) plasma system for such a sterilization procedure and evaluated its sterilization efficacy via a biological indicator by examination of various sterilization process parameters, such as H₂O₂ import time, plasma power, treatment time, processing cycles, and sterilization time. 3D printing is the process of generating physical 3D objects by sequentially depositing layers of material in different shapes. In the medical field, the prototyping capabilities of 3D printing have made it a practical tool for medical professionals to quickly fabricate customized patient implants and models of patient anatomy for pre-surgical planning [14] [15]. It is worth mentioning that implanted 3D biomaterials require low-temperature, fast and effective sterilization. One of the more important elements of 3D printing medical materials is in time to afford them to patients. Here, we detail our work demonstrating that a low-temperature hydrogen peroxide gas plasma (HPGP) process appears to be sufficient for ensuring sterility for the biological indicator *Bacillus atrophaeus*.

Materials and Methods

The customized plasma sterilization system was set up by Taiwan's Industrial Technology Research Institute (ITRI). The low-temperature H₂O₂ plasma system is shown in Fig. 1. The cylindrical chamber was filled with the required reactive atmosphere, and direct current (DC) electrodes [16] were placed at the opposite ends of the

cylinder and activated to achieve the plasma state. The atomized H₂O₂ (30%, J.T. Baker, USA) gas comes from an ultrasonic oscillator (ITRI, Taiwan) because ultrasonic transducers can be used as atomizers due to the vibration characteristics of the transducer. However, two mechanisms were combined by various researchers for atomization, breaking up of capillary waves at the liquid surface and cavitation. [17] To enhance the effective ionization of the reaction gas, the H₂O₂ needs to first be activated by ultraviolet (UV) light. Next, the reactive atmosphere can be introduced into the empty chamber. The gas flow was controlled by a mass flow controller at 100 ~ 300 sccm. Before the plasma treatment procedure, the vacuum was adjusted approximately 8×10^{-2} Torr to allow the reactive oxygen species generated from the H₂O₂ induced by the plasma to diffuse into the samples inside for sterilization. The chamber was also designed with a transparent window in the center for sample observation, allowing us to easily observe the plasma state and the samples.

Figure 1. The device of the low-temperature plasma system.

The sterilization procedure performed using a low-temperature plasma is described as follows. The H₂O₂ solution was atomized and introduced to the chamber as the reactive oxygen species [18]. The chamber was vacuum-pumped to a pressure of 8×10^{-2} Torr, and then the reactive gas was introduced into the system at a pressure of approximately 1.5×10^{-1} - 6×10^{-1} Torr for plasma activation at powers of 100, 200, and 300 W. Subsequently, plasma treatment was performed for 0.5, 1, and 2 min. To increase the levels of reactive oxygen species, the pressure relief valve was opened to allow the chamber pressure to increase to 7.6×10^2 torr, and the procedure for introducing H₂O₂ into the chamber was repeated several times. However, there are only four variation parameters: H₂O₂ import time, plasma power, treatment time, and processing cycles. Therefore, this sterilization procedure experiment method used the L9 array of the Taguchi method and used up to 4 factors

with 3 levels to calculate the sterilization time. All of the sterilization efficiency parameters design are shown in Table 1.

Aliphatic polyesters such as PLA, PGA, PLGA, and polycaprolactone (PCL) have been developed, and their glass transition temperatures are generally less than 60°C. It is important to ensure that the low-temperature plasma sterilization process is below 40°C to prevent significant changes to their physical and chemical properties. Therefore, the plasma sterilization process considered to have a significant effect on the physicochemical properties of the biopolymer due to overheating at high power. For this experiment, the plasma activation was set at powers of 100, 200, and 300 W. It is worth mentioning that the current low temperature setting below 40°C is the basic requirement due to consideration of the glass transition temperature problem. However, even if the biopolymer retains its original physical and chemical properties, further analysis and confirmation are still required. Temperature indicator paper can be used as needed to monitor temperature during sterilization. Therefore, we obtained the chamber temperature during sterilization with Therman™ temperature measurement paper (THERMAX, UK), as it is suitable for the temperature range used in our experiments. The distance between the electrodes and position of the temperature monitor paper is shown in Fig. 2.

Figure 2. Electrode distance and position of monitor temperature papers in the low-temperature plasma system.

Bacillus atrophaeus [19] at a bioburden amount larger than 10^6 was used as the biological indicator (BI; Mesa Labs, USA) [20] for evaluating sterilization efficacy. The BI was placed in a sterilization bag (Tyvek®, DuPont,

USA) and then into the plasma chamber for sterilization before being placed in a vial filled with tryptic soy broth (TSB; Merck Millipore, German) medium and incubated at 30°C with a shaking speed of 130 rpm for 7 days. A sketch of the biological indicator used to confirm sterilization efficacy is shown in Fig. 3.

BI is Incubated for 7 days in TSB medium at 30°C

Figure 3. Sketch of the biological indicator used to confirm sterilization efficacy.

After BI incubation, the TSB medium, called the original inoculum, was inoculated with *Bacillus atrophaeus* bacteria. This original inoculum was then diluted to various concentrations. The dilutions were used to coat tryptone soy agar (TSA) plates (Creative Media Plate, Taiwan). TSA is mainly used as an initial growth medium for the purpose of observing colony morphology, developing a pure culture and achieving sufficient growth for further biochemical testing and culture storage. TSA contains digests of casein and soybean meal. The combination of casein and soy peptones renders the medium nutritious by supplying organic nitrogen, particularly amino acids and longer-chained peptides. Sodium chloride is added to maintain the osmotic equilibrium and agar is the solidifying agent.

Each plate received 1 ml of its respective dilution for coating. The coated plate was then incubated at 30°C for 24 hours and observed for colony-forming unit (CFU) counting. The CFU is a measure of viable bacterial or fungal cells. In direct microscopic counts (cell counting using haemocytometer) all cells, dead and living, are counted, but CFU measures only viable cells. For convenience, the results are given as CFU/mL (colony-forming units per milliliter) for liquids.

However, this biological indicator standard curve was used to evaluate the sterilization efficacy and is shown in Fig. 4. The CFU/ml can be calculated using the formula: $\text{cfu/ml} = (\text{no. of colonies} \times \text{dilution factor}) / \text{volume of culture plate}$. For example, suppose the plate of the $\times 10^5$ dilution yielded a count of 4 colonies. The number of bacteria in 1 ml of the original inoculum sample can be calculated as follows: $\text{Bacteria/ml} = (4) \times (10^5) = 4 \times 10^5$.

Figure 4. BI standard curve to evaluate sterilization efficacy.

Results and Discussion

The biological indicator was placed in the vacuum chamber, and Fig. 5 shows a photo of the activation state of the low-temperature plasma in a hydrogen peroxide atmosphere. The glow and purple color demonstrated that the plasma was successfully activated. The temperature in the center of the chamber was below 40°C, achieving the requirement for a low-temperature plasma. The BI bioburden without plasma treatment is shown in Fig. 6. Each plate contained 1 ml of a dilution used for coating and was incubated for 24 hours. The original inoculum was diluted at various concentrations to build a standardized curve for colony-forming unit (CFU) counting.

Figure 5. Photo of activation state for the low-temperature plasma in a hydrogen peroxide atmosphere.

Figure 6. BI bioburden with various dilutions without plasma treatment. (n=3)

Results showed the plate of the $\times 10^6$ dilution yielded a count of 14 colonies. The number of bacteria in 1 ml of the original inoculum sample can then be calculated as follows: Bacteria/ml = $(14) \times (10^6) = 1.4 \times 10^6$. The results correspond with the information provided by the manufacturer.

All of the sterilization efficiency test results are shown in Table 1 and each data is a result of three repetitions. Photos of typical samples are shown in Fig. 7. Sample no. 2 and 4 showed successful sterilization under 1.4×10^4 Bacteria/ml for coating on TSA plates and 24 hours incubation. On the other hand, samples no. 5 and 6 showed sterilization failure under 1.4×10^4 Bacteria/ml. The experimental parameters were designed similar to those of the Taguchi methods [21]. The experimental parameters include H_2O_2 import time, plasma power, treatment time, processing cycles, sterilization time and sterilization effect. Sample no. 3 failed because the temperature in the center of the chamber exceeded 40°C, indicating the lack of a low-temperature plasma. The higher temperature was induced by the energy accumulation resulting from the high power (300 watts) and longer sterilization time (40 min).

Figure 7. BI bioburden with $\times 10^2$ dilutions with plasma treatment. (n=3)

After plasma treatment, samples no. 1, 5 and 6 show no apparent sterilization efficacy and still respectively showed bacterial growth of 1.6×10^6 , 2.9×10^6 , and 9.6×10^6 CFU. However, samples no. 2, 4, 7, 8 and 9 show sterilization efficacy based on the observation of no bacteria colonies on their respective agar plates. Among the samples that demonstrated sterilization efficiency, samples no. 4, 7, and 8 all had a sterilization time above 30 min, and thus results in wasted energy. Furthermore, sample no. 9 used high power (300 watts) but a shorter sterilization time (5 min). The high power results in an instantaneous reaction, thus producing fast, efficient sterilization. These parameters would be suitable for materials that require rapid sterilization, such as polymers [22].

Sample no. 2 had moderate power (200 watts) and

Table 1. Parameters and sterilization efficiency of the low-temperature plasma systems.

No.	H ₂ O ₂ Import (min)	Plasma Power (watts)	Treatment Time (min)	Processing Cycles (times)	Sterilization Time (min)	Front · Middle · End (T<40 °C)	Sterilization Effect (CFU count)
1	2	100	0.5	1	2.5; (2+0.5)×1	L / L / L	1.6 x 10 ⁶
2	2	200	1	5	15; (2+1)×5	H / L / H	Pass
3	2	300	2	10	40; (2+2)×10	H / H / H	N.A.
4	3	100	1	10	40; (3+1)×10	H / L / H	Pass
5	3	200	2	1	5; (3+2)×1	H / L / H	2.9 x 10 ⁶
6	3	300	0.5	5	17.5; (3+0.5)×5	H / L / H	9.6 x 10 ⁶
7	4	100	2	5	30; (4+2)×5	H / L / H	Pass
8	4	200	0.5	10	45; (4+0.5)×10	H / L / H	Pass
9	4	300	1	1	5; (4+1)×1	H / L / H	

Note: Pass means no bacteria colony on the agar plate.

sterilization time (15 min), so the temperature was still below 40°C after 5 sterilization cycles. This caused the reactive oxygen species to fully diffuse into the samples. This would be more suitable for materials with complex microstructures, such as scaffolds for tissue engineering [23] or narrow tubes [24].

Conclusion

It is very important to choose an appropriate sterilization technique that both effectively sterilizes heat-sensitive biomaterials and maintains their structural and biochemical integrity. In addition, more complex items such as tissue engineering scaffolds and customized 3D printed medical biomaterials need timely and effective sterilization. The results of the low temperature plasma sterilization efficacy, as determined by the biological indicator, demonstrated optimal processes (samples no. 2, 4, 7 and 8) at below 40°C plasma with H₂O₂ activated by ultraviolet light. However, even if the biopolymers retain their original physical and chemical properties, further analysis and confirmation is still required. These low-temperature hydrogen peroxide (H₂O₂) gas plasma (HPGP) processes could become the sterilization method of choice for biopolymer devices and 3D printed custom medical devices in the future.

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