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Fundamental Study on Novel Biological Indicator Using DNA-labeled Microbeads for Evaluating Nonthermal Plasma Sterilization

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Abstract—Nonthermal atmospheric-pressure discharge plasma is considered important for sterilization. Reactive species, such as active oxygen species, radicals, and nitrate ions, generated by the discharge plasma damage the target bacterial cell wall/membrane and DNA. Several plasma sterilization methods have been proposed, including dielectric barrier discharge (DBD). To achieve effective sterilization, it is necessary to evaluate their characteristics using many parameters. This study aims to demonstrate a proof-of-concept of a novel biological indicator for plasma sterilization. A biological indicator is used to verify sterilization outcomes. Furthermore, we employ DNA-labeled microbeads as biological indicators for the rapid visualization of plasma sterilization. This is based on our recently developed method for visual detection of DNA molecules. If plasma-derived factors cause the degradation of the DNA attached to the microbeads, this can be confirmed by visualization. Herein, we present the correlation between sterilization and visualization in the case of DBD. This method offers a rapid evaluation of plasma sterilization because it easily and quickly determines the sterilization capability of the plasma.

Index Terms—Chemical and biological sensors, Biological indicator, DNA degradation, Plasma diagnosis, Plasma sterilization

I. INTRODUCTION

Biological applications of nonthermal discharge plasma under atmospheric pressure, such as dielectric barrier discharge (DBD) and corona discharge have been widely studied in various fields [1-6]. In nonthermal plasma, many types of reactive chemical species, ultraviolet light, and electric fields are generated, which influence biological targets. Sterilization is an important application of the nonthermal plasma. It is considered that oxidization by the reactive species generated by the reactions in humid air, such as O_3, NO_3^-, OH^-, $OH^\cdot$, and $O^\cdot$, is the main factor of sterilization. Additionally, ultraviolet light, electric-field-induced destruction, and shock waves can assist in sterilization. In addition to the direct exposure of nonthermal plasma to the targets, plasma-treated water, called plasma-activated water, has been studied [7, 8]. Sterilization using discharge plasma directly and indirectly (plasma-activated water) does not require chemical reagents such as sodium hypochlorite or hydrogen peroxide, which require careful handling and safe storage. Plasma sterilization is performed under atmospheric pressure at room temperature, whereas autoclave sterilization employs high-pressure and high-temperature steam.

Several methods have been proposed for plasma discharge. Their characteristics depend on the voltage sources (dc, ac, pulse, waveforms, and frequencies), electrode structure, plasma type (DBD, corona, and jet), and atmospheric gas (humidity and additional gases such as He and Ar). Bacteria such as *Escherichia coli* (*E. coli*) and *Bacillus subtilis* are generally used to evaluate the sterilization capability of each method. The evaluation requires several days for cultivation and a well-trained person for aseptic manipulation to ensure accuracy.

Chemical and biological indicators such as autoclaving and chemical vapor sterilization are used to monitor a sterilization process [9]. Chemical indicators, paper-based materials in which chemical reagents reacting to specific sterilization matters are impregnated, are employed. After sterilization, the chemical indicators change color to indicate whether the process is completed. Bacteria are used as biological indicators and change color depending on the bacterial viability. The color change of the chemical indicator indicates that the target was exposed to a chemical reagent or a temperature sufficient to sterilize it completely. Biological indicators show that color change takes several hours to days due to cultivation. Because chemical indicators show a single sterilization factor, and biological indicators require a long time, they are unsuitable for evaluating sterilization using discharge plasma. Therefore, plasma sterilization requires a quick evaluation because there are many parameters for generating

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the discharge plasma.

Chemical indicators for plasma sterilization have been commercialized [10]. In our study, the colors of the chemical indicators change with plasma exposure. However, the color change did not reflect sterilization. Although sterilization was incomplete, the color changed completely (data not shown).

This study proposes an effective biological indicator based on DNA degradation caused by plasma discharge. It is well-established that the reactive species generated by discharge plasma damage DNA molecules [11, 12]. Kurita *et al*. evaluated plasma jets using DNA breaks [13, 14]. The DNA breaks were detected using fluorescent dyes. While these methods are effective for evaluating the effect of the discharge plasma, they require a specific apparatus to measure fluorescence.

In this study, a direct eye DNA detection method, recently developed by our group, is utilized to detect DNA degradation by discharge plasma. DNA detection methods were initially invented for the rapid detection of pathogens to be used for nucleic acid amplification, such as polymerase chain reaction (PCR) [15, 16]. This method utilizes magnetic microbeads. The amplified DNA is attached to magnetic microbeads, and the DNA-labeled microbeads are suspended in deionized water. Subsequently, the mixture is poured onto a hydrophilic glass plate. When the glass plate is placed on a permanent magnet, the DNA-labeled magnetic microbeads agglomerate at the point of strongest magnetic field on the glass. If the magnetic microbeads are not labeled with DNA, they are uniformly adsorbed onto the glass surface because of their hydrophilicity. Differences in the behavior of the magnetic microbeads with and without DNA molecules under a magnetic field can be easily distinguished through direct observation.

This study aimed to demonstrate a proof-of-concept for evaluating discharge plasma sterilization using a direct eye DNA detection method. DBD with a conducting water electrode was used to sterilize the bacteria (*E. coli*). DBD was also used to prepare plasma-activated water. The plasma-activated water reacted with the DNA-labeled microbeads. The aggregation of the magnetic microbeads was observed under a magnetic field. If the DNA on the microbeads degraded, it was adsorbed and spread on the glass surface. Otherwise, the microbeads agglomerate. The sterilization capabilities of DBD and agglomeration were compared. As a result, the potential to evaluate the sterilization capability of DBD plasma by the direct eye DNA detection method was demonstrated when the addition of radical scavengers controlled DNA degradation.

II. EXPERIMENTAL

A. DBD Sterilization

A DBD reactor utilizing a liquid electrode, as shown in Fig. 1, was used for the sterilization experiments. The acrylic reactor consisted a fluid reservoir into which several milliliters of the electrolyte solution (phosphate-buffered saline, PBS) was poured. The dimensions of the bottom of the reservoir were 50 mm × 30 mm. The tip of the high-voltage wire connected to the high-voltage source was soaked in the electrolyte solution. The thin bottom wall of the reservoir (2 mm thick) served as a dielectric barrier. The electrolyte solution served as a flexible high-voltage electrode, resulting in the electrode contacting the inside walls of the reservoir without any gaps that might cause a breakdown. To maintain a stable DBD, the voltage source was tuned to generate sinusoidal voltages of 16 kV_P and 21 kHz.

A plastic dish (1 cm in diameter) with a plate culture medium was placed on a grounded metal plate for the sterilization experiments. The acrylic reactor was then placed in a dish. The distance between the bottom of the reactor and culture medium surface was adjusted to 1 mm.

The sterilization experiments were performed as follows. *E. coli* was used as the target. Cultured bacteria were serially diluted in deionized water, and 100 μL each of the bacteria were placed at six points on the plate culture medium. The concentration of the bacterial solution was determined by the standard colony-forming unit method. After drying the bacterial suspension in the culture medium, the DBD reactor was placed in a dish. DBD was then applied for 0 s, 10 s, 30 s, and 60 s. The dishes, including the plate culture medium, were then moved into an incubator, and cultured at 37˚C overnight. Sterilization was evaluated by examining the bacterial growth at each spot on the plate culture medium. If no growth was observed, complete sterilization was indicated. In contrast, if white spots, such as colonies, appeared, then sterilization was incomplete. If the size of the white spot was smaller than that of the control sample without DBD, it indicates partial sterilization.

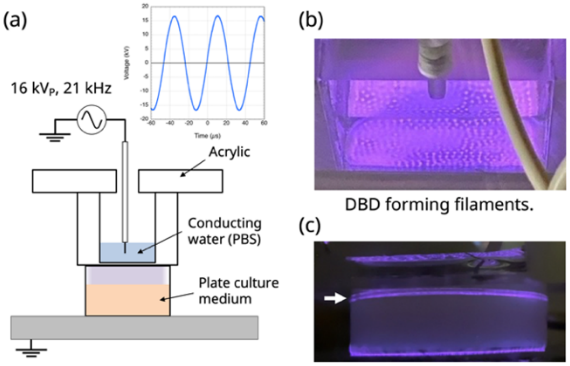

Fig. 1. DBD setup for the sterilization. (a) Schematic illustration of the experimental setup. The waveform is the applied voltage. (b) Top view photo of DBD. Self-assembled filaments were formed. (c) Side view photo of DBD. The white arrow indicates the region of the gap where DBD occurred.

B. Diagnosis of DBD Plasma using DNA-labeled Microparticles

A similar setup for bacterial sterilization was used to demonstrate the proof-of-concept of plasma sterilization using the DNA-labeled microbeads. The acrylic liquid DBD reactor was placed above a well plate with a reservoir diameter of 33.5 mm. A grounded stainless-steel mesh (10 mesh, 0.4 mm in wire diameter) was placed between the DBD reactor and well plate. The distances from the stainless mesh to the bottom of the DBD reactor and well plate were adjusted using 2 mm and 5 mm silicone rubber sheets, respectively. A test liquid of 1 mL of deionized (DI) water or TE (10 mM Tris-HCl, 1 mM EDTA, pH8.0) solution was poured into the well. DBD was generated between the reactor bottom and the stainless-steel mesh. The setup is shown in Fig. 2. The reactive species were anticipated to move to the test liquid, which was plasma-activated water. After DBD exposure for the appropriate time, the test liquid was recovered, applied to the DNA-labeled microbeads, and incubated for 1 min. The DNA-labeled microbeads were tested using the direct-eye detection method.

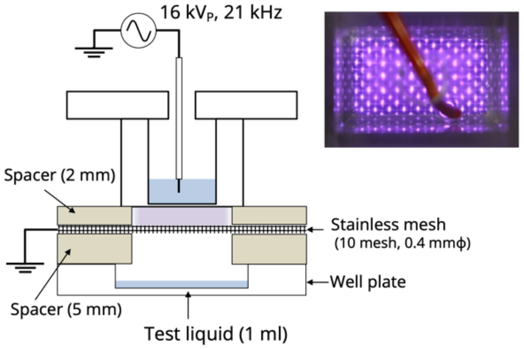

Fig. 2. Experimental setup of the DBD for preparing the plasma-activated water. The top view photo of DBD is also shown.

The DNA-labeled microbeads were prepared using 796 bp DNA modified with biotin on 5'-terminus and streptavidin-labeled magnetic microbeads (2.8 μm in diameter). Details of direct eye detection are described in our previous studies [15, 16]. In our previous studies, the direct eye detection method was used to determine the presence of amplified DNA by PCR for the rapid detection of pathogens. In this study, DNA degradation was evaluated using this technique.

After incubation in a plasma-activated solution, the DNA-labeled microbeads were suspended in deionized water. The microbead suspension was then placed on a hydrophilic concave glass plate. The glass plate was subsequently placed on a permanent magnet. If the DNA on the microbeads is not damaged, the microbeads aggregate at the bottom of the concave shape. Otherwise, the microbeads are widely spread at the bottom if the DNA is degraded. The difference between aggregation and widespread distribution can be easily distinguished by the naked eye. DBD sterilization and DNA-labeled microbead-based plasma diagnosis were compared.

III. RESULTS AND DISCUSSION

A. DBD Plasma Sterilization Characteristics

The DBD generated using this setup is shown in Fig. 1. The setup was designed as a portable sterilization device. It consists of a liquid reservoir with a high-voltage liquid electrode and a dielectric bottom that can be placed on any surface with a spacer, such as a desk, floor, or handrail. A grounded metal-mesh electrode was used in this setup, as shown in Fig. 2. Furthermore, we used a DBD generator to demonstrate the proof-of-concept of the evaluation method using DNA-labeled microbeads.

In this setup, self-assembled filaments were generated between the bottom of the reactor and the surface of the plate culture medium. The streamer-like filaments generated by our DBD setup were thicker than those generated by an ordinal DBD and moved into the gap. Therefore, we considered each filament to be caused by the streamer assembly. The detailed mechanism of self-assembled filament generation is not clear; however, it will be clarified in our future investigations. The plate culture medium was in a gel form, meaning that more than 90% of its weight was water. This may be involved in self-assembly, such as the phenomena observed in dc glow discharges on liquid surfaces [17].

Fig. 3 shows part of the DBD sterilization results. The spots dropped without DBD exposure were white after overnight cultivation, indicating bacterial growth. The white spots in the DBD exposure samples became smaller or disappeared. Spots smaller than those in control samples indicated incomplete or partial sterilization. The absence of white spots indicated the complete sterilization of the bacteria in the dropped solution. Table 1 summarizes the DBD sterilization results at various exposure times. In our DBD set-up, 30 s of exposure was sufficient to sterilize 10^9 cfu/mL of bacteria (*E. coli*). Note that 10-s exposure samples showed that the higher the concentration of the bacteria, the more effective the sterilization. This could be due to the thickness of the self-assembled filaments, indicating the inhomogeneous nature of the DBD. When we used a nanosecond pulsed generator as the voltage source (16 kV$_P$, 21 kHz), it caused a homogeneous DBD in the gap, indicating the presence of many thin streamers. In this case, 10-s exposure samples exhibited results opposite to those of the sinusoidal wave voltage. Samples of 10^5–10^7 cfu/mL were sterilized completely, whereas 10^8 and 10^9 cfu/mL samples exhibited incomplete sterilization.

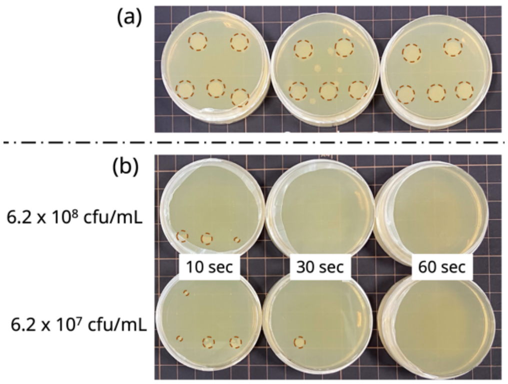

Fig. 3. Part of the DBD sterilization results. (a) Samples without DBD exposure. (b) Samples exposed by the DBD for indicated times. Red dotted circles indicate the white spots that appeared after cultivation.

Table 1. Results of the DBD sterilization

DBD exposure time (s)	*E. coli* concentration (cfu/mL)				
	6.2 × 10^5	6.2 × 10^6	6.2 × 10^7	6.2 × 10^8	6.2 × 10^9
0 (control)	×	×	×	×	×
10	×	×	×	○	○
30	○	○	○	○	○
60	○	○	○	○	○

"○" and "×" indicate complete and incomplete sterilizations. Each result was the sum of two independent experiments. "○" means no growth occurred at a total of 12 spots.

B. Direct Eye Evaluation of Capability of DBD Plasma Sterilization

Fig. 4 shows the responses of DNA-labeled magnetic microbeads after incubation in plasma-activated solutions. When DI water was used as the test liquid, the magnetic microbeads were widely spread on the bottom of the concave glass plate, even though the exposure time was 10 s, indicating that almost all DNA was degraded by the reactive species in the plasma-activated solution. When TE was used, aggregation occurred in all tested samples. This is due to the radical-scavenging properties of TE [11]. This suggested that the reactive species generated by the DBD plasma were deactivated in the TE solution. A dilute TE solution was used to adjust for deactivation of the TE solution. When we used 1,000 times diluted TE, agglomeration varied with time. In this case, the change in agglomeration was comparable to the temporal change during DBD sterilization.

The method using DNA-labeled microbead visualized the sterilization capability of the plasma device. The obtained image can be digitalized using binary image processing [15].

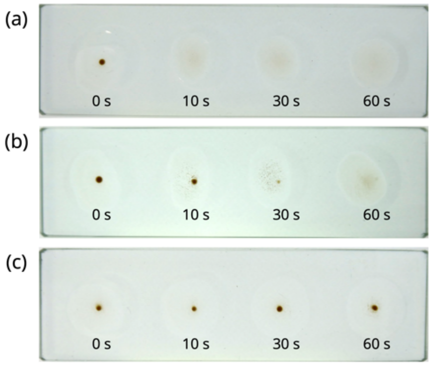

Fig. 4. DNA-labeled magnetic microbeads accumulation after incubating with the plasma-activated water. (a), (b), and (c) indicate the samples of DI water, 1/1000-diluted TE, and TE, respectively. “0 s” to “60 s” indicate the time of the DBD exposure. “0 s” indicates the control sample without the DBD exposure.

IV. CONCLUSION

In this study, to demonstrate the proof-of-concept of a new biological indicator for evaluating plasma-based sterilization using DNA-labeled magnetic microbeads, the sterilization properties of our DBD reactor were compared with the DNA degradation of plasma-activated water prepared using this reactor. The DBD reactor sterilized 10^9 cfu/ml of bacteria within 30 s. When 1/1000 TE was used to prepare plasma-activated water, the DNA degradation properties were comparable to the sterilization characteristics of the DBD reactor. Adjustment of DNA breakage by adding radical scavengers, such as diluted TE, can be applied to various combinations of DNA and microbeads. Optimizing the method involves considering the DNA length and microbead diameter as the parameters.

The results shown in Fig. 4 demonstrated the effectiveness of the proposed method. The DBD used for sterilization (Fig. 1) and plasma-activated water (Fig. 2) exhibited differences. However, the plasma-activated water produced by the upper part of the sterilization setup, which can be used independently, demonstrated sterilization capabilities when used with DNA-labeled microbeads.

The proposed new evaluation method requires a 1 min incubation with plasma-activated water and is determined directly by the eye. Given that many types of plasma sterilization methods have been proposed, the proposed biological indicator using DNA-labeled microbeads provides an easy and quick evaluation.

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