The Effect of Air Plasma on Sterilization of Escherichia coli in Dielectric Barrier Discharge

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Abstract In this work, a Dielectric Barrier Discharge (DBD) air plasma was used to sterilize Escherichia coli (*E. coli*) on the surface of medical Polyethylene Terephthalate (PET) film. The leakage of cellular DNA and protein by optical absorbance measurement at 260 nm and 280 nm, together with transmission electron microscopy (TEM) about cell morphology were performed after sterilization to analyse inactivation mechanisms. The results indicated that the DBD air plasma was very effective in *E. coli* sterilization. The plasma germicidal efficiency depended on the plasma treatment time, the air-gap distance, and the applied voltage. Within 5 min of plasma treatment, the germicidal efficiency against *E. coli* could reach 99.99%. An etching action on cell membranes by electrons, ions and radicals is the primary mechanism for DBD air plasma sterilization, which leads to the effusion of cellular contents (DNA and protein) and bacterial death.

Keywords: DBD, air plasma, Escherichia coli, PET, sterilization

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1 Introduction

Escherichia coli (*E. coli*) is a classic opportunistic pathogen found in hospitals. The World Health Organization professes that this bacterium is one of the primary pathogens of nosocomial infection [1]. *E. coli* contributing to a large percentage of nosocomial infections ranks first in the infection rate of various gramnegative nosocomial pathogens [2,3]. In recent years, because of the multi-drug resistant mechanism of *E. coli*, infection incidents have occurred frequently, and the drug-resistance of the bacterium has gradually risen. Thus, we urgently need new sterilization methods that have advantages such as a shorter sterilization times, lower processing temperatures, that can deal with a wide range of materials and have harmless operation conditions [4,5]. Sterilization using non-thermal plasma which possesses the above-mentioned advantages represents one of the most promising technologies and alternatives to conventional sterilization methods [6∼8].

The dielectric barrier discharge (DBD) is most frequently used as a non-thermal plasma source that can be operated with different gasses at elevated pressures (up to atmospheric pressure) [9,10]. The plasma is created between two conductive electrodes connected to an ac or pulsed power source. At least one of the DBD electrodes is covered by a dielectric layer, which prevents the arc formation after breakdown. DBD discharge usually consists of a large number of short-living microchannels (filaments) that are randomly distributed over the entire area of the dielectric barrier. Despite a high breakdown voltage in gas at atmospheric pressure (several kV), the average electric current is low. Therefore, a DBD plasma can be applied directly to living tissue and open injuries without causing them damage [11,12]. During DBD plasma treatment, electrons, ions and active chemical species from the plasma reach the surface of a biologically contaminated object and can eventually lead to its sterilization [13∼16].

In this study, a parallel-plate DBD air plasma driven by a low-frequency ac power supply was applied to inactivate *E. coli* on the surface of medical PET film. The influences of this kind of DBD plasma on germicidal efficiency were investigated. The temperature before and after plasma treatment was measured for evaluating whether the heat played a role in plasma inactivation. The leakage of cellular DNA and protein as well as the cell structure of *E.coli* after plasma treatment were observed by optical absorbance measurements and a TEM technique to verify the mechanism of inactivating *E. coli* by DBD systems.

2 Experimental method

2.1 DBD plasma reactor

Fig. 1 shows the apparatus used in the experiment. It consists of a low-frequency ac power source, a DBD reactor, and impedance matching. The parallel-plate discharge was applied to obtain plasma. A quartz plate (100 mm× 2 mm) as a dielectric was used to protect the powered electrode and a glass plate (100 mm×1 mm),

another dielectric served as a sample-carrying surface. The DBD reactor was operated just in room air (no separate gases were used or added) under normal atmospheric conditions. Then, large quantities of highly reactive species were produced during the discharge process, which sterilized the bacteria. The discharge was driven by a low-frequency high-voltage ac power source (Shanghai Yisheng Electronic Corporation, China) and provided an output of a maximal peak voltage of 30 kV. For the treatment of bacteria, the distance between the bottom edge of the quartz plate and the bacteria positioned on the lower glass plate was varied in the range of $z = 3 \sim 5$ cm. Note that "z" denotes the coordinate in the downstream direction measured from the bottom edge of the upper quartz dish. During all experiments throughout this study, the frequency of the device was fixed at 100 Hz and the plasma exposure time was varied from 1 min to 5 min.

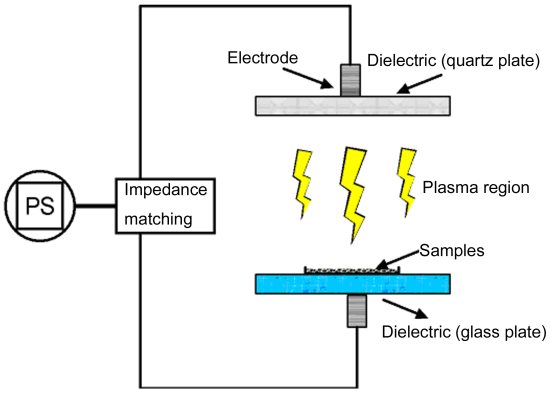


Fig.1 Schematic diagram of the experimental apparatus

In order to avoid microbial contamination and keep the experimental environment sterile, the DBD plasma reactor was placed on a superclean bench with a UV lamp. Before each experiment, the inner surface of the bench was cleaned twice by medical cotton balls with 75% alcohol and then irradiated for 2 h by a UV lamp.

2.2 Sample preparation of bacterial cells

E. coli cells were cultured on a solid surface (agar) containing a tryptic soy base, washed in saline (two times), and adjusted to a standard density (usually about a 10^6 colony-forming unit). Then the bacteria (100 μL) were transferred and spread on sterile sheets of 20×20 mm medical PET. After drying at room temperature for 1 h, the samples were exposed to the parallel-plate DBD plasma region in the planned test conditions as shown later. After the plasma treatment, the samples were washed with phosphate buffered saline (PBS), and then 1/10 diluted colonies in the saline were inoculated on a standard agar plate. After incubation of the bacteria at 37°C for 48 h, the number of colonies was counted by eye. The results were expressed in values of colony-forming units (CFUs) and the mean values were used in the bacterial inactivation curves. Germicidal efficiency was determined as follows:

$$\text{Germicidal efficiency} = (N_0 - N_t)/N_0 \times 100\%, \quad (1)$$

where N_0 and N_t are the number of colony-forming units on the control and sterilized samples, respectively.

In order to verify whether the nutrition properties of the agar media were compromised by plasma exposure or not, two standard Petri dishes with the same quantity of agar (10 mL) were prepared. One Petri dish was treated by the DBD air plasma for 7 min and the other one was determined as the control sample. After plasma treatment, both Petri dishes were inoculated with bacterial suspensions with the same concentration and incubated at 37°C for 48 h. Then the CFUs on both dishes were counted and no visual difference was detected in the bacterial growth due to agar plasma exposure.

2.3 Determination of protein and DNA leakage

The PET sheets with *E. coli* treated by plasma were oscillated and eluted in PBS. The suspensions of treated bacteria were centrifuged at 8000 rpm for 5 min to remove residual cells. The light absorbance of the supernatant was examined using a UV-visible spectrophotometer at a wavelength of 260 nm (for DNA absorbance) and 280 nm (for protein absorbance), respectively [17]. All experiments were completed in duplicate groups.

2.4 Transmission electron microscopy

The morphology of bacterial cells during the DBD air plasma exposure was visualized by transmission electron microscopy. *E. coli* was prepared for electron microscopy as follows: The untreated and plasma-treated suspension of *E. coli* cells were rinsed in PS and then centrifuged at 2700 rpm for 10 min at 4°C. The supernatant was removed and then the pellet was resuspended in 3% (v : v) gluteraldehyde solution in a 0.1 M phosphate buffer (PBS, pH 7.2) and fixed for 24 h. After the primary fixation, the cells in suspension were pelleted and the gluteraldhyde was removed, and a 1% osmium tetroxide solution (pH 7.2) was added. After 1.5 h, the cells were rinsed in PBS three times. The cells were dehydrated in a series of cold ethanol solutions for 10 min each, starting with a 10% ethanol solution continuing through 30%, 50%, 70% and 90% solutions. The cells were rinsed in 100% ethanol at 10 min intervals twice. Then, the cells were infiltrated with a solution of acetone and epon-araldite over 24 h. Following infiltration, the samples were placed in polyethylene capsules and the resin polymerized at 60°C for 48 h. These samples were removed from the capsules and trimmed. Ultrathin sections (60∼90 nm) were mounted on copper grids and stained with uranyl acetate and lead citrate.

TEM images were obtained with a Hitachi H600 transmission electron microscope.

3 Results and discussion

3.1 Germicidal effect

In order to evaluate the sterilization efficiency of the DBD air plasma, the germicidal efficiency was investigated as a function of the plasma treatment time, air-gap distance and applied voltage. As shown in Figs. 2∼4, germicidal efficiency exhibited a strong dependence on these conditions. In order to get sufficiently significant statistics, usually three samples under the same plasma conditions were treated.

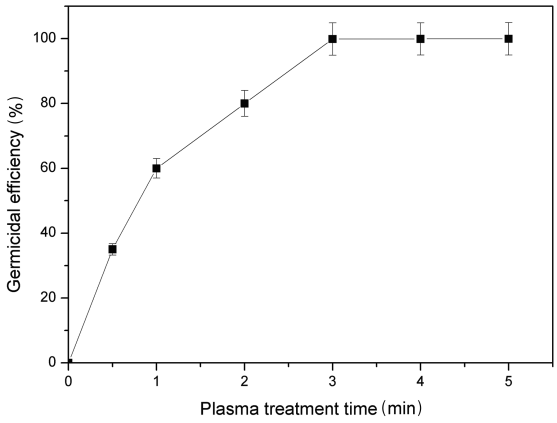


Fig.2 Effect of treatment time on *E. coli* sterilization (under the operating conditions of a 17 kV applied voltage and $z = 4$ cm)

3.1.1 Germicidal effect of different plasma treatment times

When a 17 kV high voltage was applied to the DBD and the air-gap distance was fixed at 4 cm, the inactivation curve of *E. coli* with different treatment durations were as plotted in Fig. 2. The experimental results showed that the DBD air plasma was effective in inactivating *E. coli* and a short plasma exposure time of 3 min brought about a germicidal efficiency of more than 99.9%. It was noted that the germicidal efficiency increased rapidly with an increase in the plasma treatment time up to 3 min, and then reached a plateau. This curve indicated that the efficiency of sterilization became high with an increasing treatment time within 3 min. However, a longer time had little effect on improving the germicidal efficiency after 3 min. This was because the interaction between bacteria and reaction species was almost complete within 3 min.

3.1.2 Germicidal effect of different air-gap distances

Fig. 3 shows the plots of the inactivation curve of *E. coli* against different air-gap distances. The results indicated that the germicidal efficiency was enhanced by decreasing the air-gap distance and increasing the treatment time. In other words, there was a better sterilization efficiency resulting from a smaller air-gap distance and longer treatment time. When the air-gap distance was 3 cm, the germicidal efficiency could exceed 99.99% in 3 min.

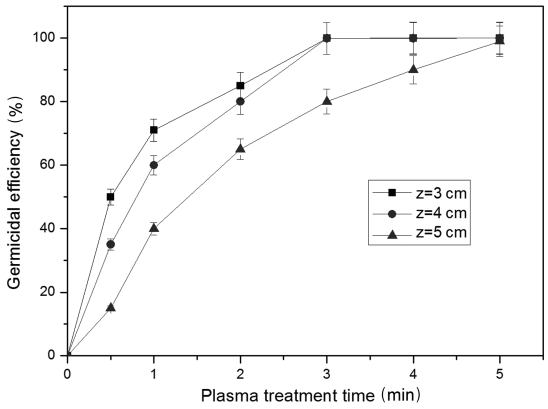


Fig.3 Effect of air-gap distances on *E. coli* sterilization (under the operating conditions of a 17 kV applied voltage)

To reach a good state of sterilization under different air-gap distances, the discharge electrode required different voltages. When the applied voltage was 17 kV, the electric field was intensified under a smaller air-gap distance, and the effect of the low-temperature plasma generated by DBD was strengthened. Moreover, the DBD plasma produced more abundant reaction species which came in close contact with the bacteria, thereby providing the bacteria with a higher energy transfer efficiency. Consequently, a higher germicidal efficiency was achievable.

3.1.3 Germicidal effect of different applied voltages

In order to investigate the effects of the applied voltage in the sterilizing process, the experiments were performed in the 13∼21 kV voltage range, as shown in Fig. 4. It can be seen that the germicidal efficiency against *E. coli* increased with an increase in the applied power input. The plasma density was positively correlated with the power supply. Increasing the applied voltage resulted in a higher degree of ionization of the gas and thus increased the density of various reaction species which were the reactive agents in the inactivation of the bacterial cells [18,19]. Hence, the plasma germicidal efficiency was improved and enhanced with a higher applied voltage. But on the other hand, when the applied voltage was too high, the glow discharge would turn into an arc discharge. And then the plasma shrank in the discharge channel and the cross-sectional area of the plasma was substantially reduced, which weakened the plasma germicidal efficiency.

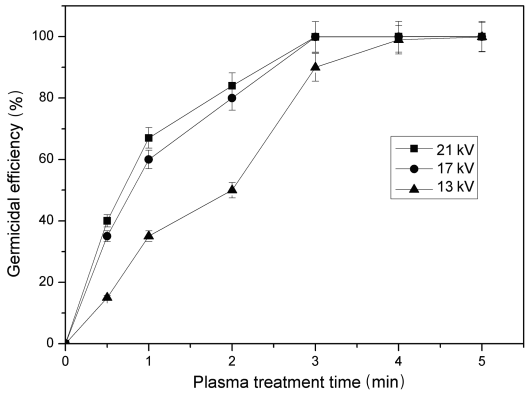


Fig.4 Effect of applied voltage on *E. coli* sterilization (under the operating conditions of $z = 4$ cm)

The germicidal efficiency rose more slowly than the rate of increase in the applied voltage. As presented in Fig. 4, when the treatment time was fixed for 3 min, the discharge-voltage increased from 13 kV to 17 kV, enabling the germicidal efficiency to increase from 90% to 99.9%. But when the discharge-voltage increased from 17 kV to 21 kV, only a 0.03% increase in the germicidal efficiency occurred. The utilization of energy was significantly weakened. No matter how much applied voltage was provided, the germicidal efficiency could reach 99.99% after 5 min plasma exposure. On the other hand, too high an applied voltage will cause damage to the cathode and result in a higher energy requirement. Hence, in consideration of the energy consumption and economical value of the voltage discharge system, a suitable applied voltage of 17 kV was chosen (stimulated by 220 V ac and without the need of any facility for additional electric voltage increase).

3.2 Sterilization mechanisms of the DBD air plasma

3.2.1 Protein and DNA leakage

The bombardment of bacteria by the reaction species in the plasma results in an etching action on cell surfaces. This etching action erodes cell material, such as crust lipoprotein and inner fat amylase of cell membranes. As a result, cell membranes rupture and the contents effuse, which leads finally to the death of the bacteria [20]. So the changes in protein and the quantity of cell DNA leakage in the suspensions of bacteria after plasma treatment are used to reflect the bactericidal rate by plasma [21]. A UV-visible spectrometer was used to monitor the absorbance peak intensities at wavelengths of 260 nm (DNA absorbance) and 280 nm (protein absorbance). The peak intensity of the absorbance is related to the amount of leakage of intracellular protein and DNA.

Fig. 5 shows the variation in the absorbance intensity peaks with plasma treatment time. It can be seen that after 3 min of plasma exposure, the peak intensity for both protein and DNA absorbance significantly increased, i.e., dramatic leakage of the intracellular protein and DNA occurred. These results suggested that a large amount of protein and/or nucleic acid leaked out of the bacterial cells due to plasma exposure, which usually occurs when the cell membranes are damaged. From Fig. 5, it is also noted that the increase in absorbance peak intensity at wavelengths of 260 nm and 280 nm reached a plateau when the plasma treatment time lasted longer than 3 min. This phenomenon can be explained by a synergistic situation: **a.** Only the top layer of the bacterial cells seeded on the media was directly exposed to plasma during the initial several seconds. Before the top layer dead cell debris was etched away by the plasma, the bacterial cells residing beneath the overlapped cell layers had little chance to be directly attacked by gas plasma species. This would lead to a slowdown in cell structural damage and thus the intracellular leakage rate with a longer plasma treatment as seen in Fig. 5. **b.** Once the intracellular substances leaked out of the cells, aerobic degradation reaction would start. Due to the existence of highly reactive plasma species, the protein and nucleic acid leaking out from the exposed cells would decompose quickly [5]. This would also lower the peak absorbance intensity at the wavelengths of 260 nm and 280 nm. These protein and DNA leakage data are highly consistent with the germicidal efficiency results shown in Fig. 2. When the treatment time was 3 min, the germicidal efficiency and the protein and DNA leakage both reached a maximum.

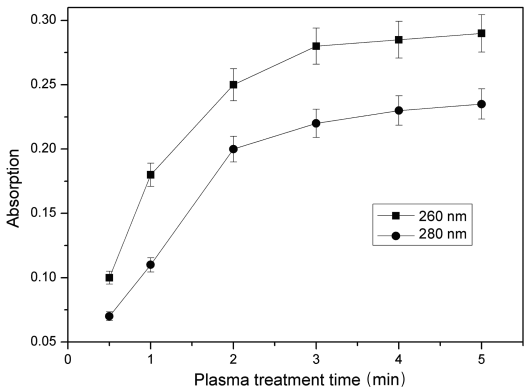
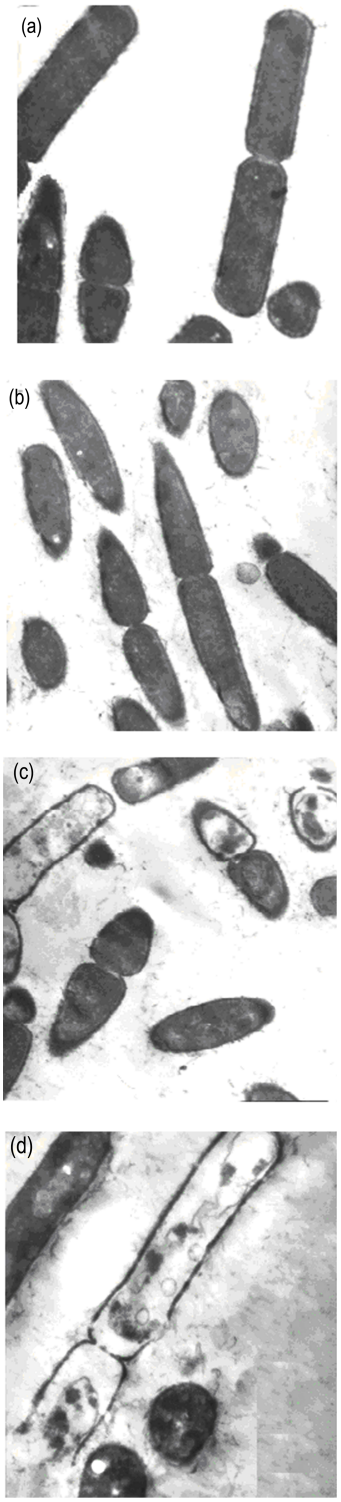


Fig.5 The variation in absorbance intensity of intracellular protein and DNA leakage (under the operating conditions of a 17 kV applied voltage and $z = 4$ cm)

3.2.2 Morphology of *E. coli*

The observation of *E. coli* made by TEM proved to be a useful way of visualizing certain modifications at a microstructural level that helps us to understand the mechanism of microbial inactivation. Normal *E. coli* cells exhibit a homogeneous microstructure in Fig. 6(a). This image shows that a healthy *E. coli* cell has a well-defined cell wall and a uniform interior material distribution, which corresponds to an inner zone full of protein and DNA molecules [22]. The lighter part of the cell is the nuclear region containing some DNA fibrils and electron-dense ribosomes [23].



(a) Before, (b) After 1 min, (c) 3 min, (d) 5 min

Fig.6 TEM micrographs of *E. coli* (10000×magnification) before and after plasma treatment(under the operating conditions of a 17 kV applied voltage and $z = 4$ cm)

Fig. 6(b) presents a sample treated with 1 min of plasma exposure. As shown, the appearance of cells is not noticeably different from that of the control. Fig. 6(c) corresponds to a sample exposed to plasma for 3 min. Essentially, most of the cells exposed to the conditions corresponding to micrograph c were inactivated. Compared with the two previous micrographs, distinct changes were observed in the bacterial cells. The nucleoid seemed to have contracted. The precipitation of DNA was obvious. In other words, a general observation of *E. coli* treated was that the cytoplasmic membrane (CM) appeared to be retracted from the outer membrane (OM) at a large scale. Fig. 6(d) illustrates bacterial cells after exposure to plasma for 5 min. A significant number of empty cell envelopes were observed. The overall density of the nuclear material was low compared with the previous micrographs and the cell envelopes appeared severely convoluted, hanging loosely around the cell instead of fitting snugly. Some of the bacteria appeared to be disrupted, and fragments of the lysed cells were observed. The cytoplasmic content might be released during plasma sterilization. This means that the damage created by plasma causes the cytoplasmic membrane to dislocate, subsequently leading to a loss of structural integrity and a leakage of intracellular content. Cavitational collapse produces an alteration of membrane integrity, which can result in a disruption of the bacterial membranes. It is reasonable to speculate that a loss of integrity of the membranes caused by plasma treatment may lead to the loss of their viability.

3.2.3 Experimental temperature

There are optimal temperatures for microorganisms, cultivation or growth. The favorable temperature range for the growth of *E. coli* is 30°C to 37°C. *E. coli* is a typical mesophilous bacterium and can be deactivated at a temperature above 43°C.

In this study, the temperature was measured using a K-type thermocouple. It can be seen from Fig. 7 that the temperature increased with an increase in treatment time and decreased with an increase in gap spacing when the applied voltage was 21 kV. After 5 min of plasma treatment, the increment in temperature was 22.8°C, 20.9°C and 18.9°C, respectively. The final temperatures were all below 43°C and could not lead to the inactivation of *E. coli*.

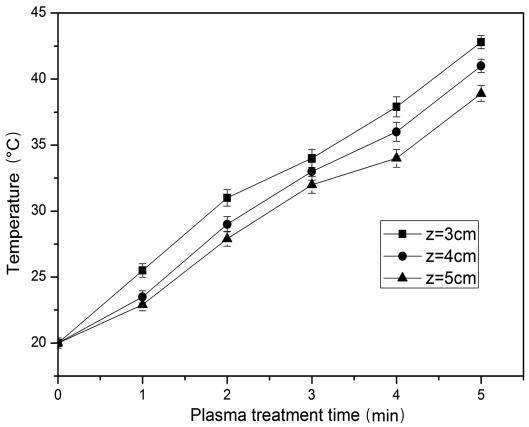


Fig.7 The temperature during the plasma treatment (under the operating conditions of a 21 kV applied voltage)

From these results of protein/DNA leakage and cell morphology, the most probable mechanism of inactiva-

tion by the DBD plasma is considered as follows. In principle, plasma inactivation may be induced by reactive species, ultraviolet (UV) photons, electrons, positive ions and negative ions. Reactive chemical species are abundant in a gas discharge [24,25]. These reactive species could be neutral, positive and negative atomic oxygen (O, O^+, O^-); positive and negative molecular oxygen (O_2^-, O_2^+); ozone (O_3); and neutral, positive and negative hydroxyl (OH, OH^+, OH^-), depending on the working gases. These species cause localized damage of the bacterial cell wall and under the chemical activities of the radicals a progressive disintegration of the bacterium takes place from the outside to the inside structure. The contents of the bacteria and the cell surface are attacked by radicals produced in the plasma region and their contents are leaked from spots of localized damage. Then the partially and totally empty cells are largely deformed, being unable to keep their original form and leading to death. Moreover, in the atmospheric-pressure plasma, most of the generated UV radiation is absorbed by air molecules and does not play a significant part in the disinfection process [26].

4 Conclusion

The experimental results obtained from this study demonstrate the capability and effectiveness of DBD air plasma in the sterilization of *E. coli*. It was noted that the plasma germicidal efficiency was dependent on the plasma treatment time, the air-gap distance, and the applied voltage. Within 5 min of plasma exposure, a 99.99% germicidal efficiency could be achieved for *E. coli*. Based on TEM examination, such DBD air plasma could induce significant structural damage to bacteria, and then the resultant leakage of cellular DNA and protein occurred as evidenced by optical absorbance measurement at 260 nm and 280 nm, respectively. The findings of this study indicate that the sterilization of *E. coli* is attributed to various active species produced by the plasma, and this DBD air plasma could be a promising technique in applications for biological and medical materials contaminated with microorganisms.

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