Inactivation of Gram-Negative Bacteria by Low-Pressure RF Remote Plasma Excited in N₂-O₂ Mixture and SF₆ Gases

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Abstract

The role of low-pressure RF plasma in the inactivation of Escherichia coli O157, Klebsiella pneumoniae, Proteus mirabilis, and Enterobacter sakazakii using N₂-O₂ and SF₆ gases was assessed. 1×10⁹ colony-forming units (CFUs) of each bacterial isolate were placed on three polymer foils. The effects of pressure, power, distance from the source, and exposure time to plasma gases were optimized. The best conditions to inactivate the four bacteria were a 91%N₂-9%O₂ mixture and a 30-minute exposure time. SF₆ gas was more efficient for all the tested isolates in as much as the treatment time was reduced to only three minutes. Therefore, low-pressure plasma could be used to sterilize heat and/or moisture-sensitive medical instruments.

Keywords: Bacteria ● Inactivation ● Low pressure ● Plasma ● Polymer

Introduction

The objective of the low-pressure plasma process is to control the generation of ions, electrons, and free radicals on a surface in order to modify its property. This process is now deemed a new attractive method in the field of sterilizing medical instruments.¹ A low-pressure, 13.56-MHz hollow cathode discharge is a very attractive device for the process and synthesis of remote plasma-aided materials.²³

Infections acquired in hospitals claim the life of one patient every 6 minutes. Escherichia, Klebsiella, Proteus, and Enterobacter species are the most common bacterial isolates that cause nosocomial infections,⁴,⁵ the treatment of which is severely hampered by antibiotic resistance.⁴ To overcome this, a great deal of research has been carried out on the effect of stresses such as cold shock, UV irradiation,⁶ and ozone on various bacteria⁷ and spores⁸ and the results have shown that exposure to such stresses bring about changes in the cell structure of these microorganisms. O₂-N₂ plasma mixture is a good example of such applications in that it is an efficient source of both N and O atoms (chemically reactive species) and of UV radiation emitted by NO-excited molecules.⁸

We sought to study the inactivation potency of plasma treatment by using O₂-N₂ and SF₆ gases against local E. coli O157, K. pneumonia, P. mirabilis, and E. sakazakii bacterial isolates.
Materials and Methods

Plasma System
The experimental set-up of the HCD-L 300 system was described in detail in our previous works.\(^3\)\(^,\)\(^9\) Tables 1 and 2 summarize the plasma operation conditions using N\(_2\)-O\(_2\) mixture and pure SF\(_6\) gas, respectively.

Polymers
Polyethylene (PE), polyethylene terephthalate (PET), and polyvinyl chloride (PVC) polymers, commercially used for bio-application, were provided as films.

Micro-Organisms and Growth Conditions
Clinical local isolates were collected from patients suffering from urinary tract infection (E. coli O157 or P. mirabilis), upper respiratory tract infection (K. pneumonia), or gastrointestinal infection (E. sakazakii). Identification of the bacteria was performed by using the API20E method (bioMérieux, Charbonnieres-les-Bains, France). The isolates were grown using standard cultures (Difco, BD, Spars, MD), and the cultures were harvested in a sterile PBS and adjusted by spectrophotometry to 1.0×10\(^9\) CFU/ml. Serial dilutions of 100 µl (1.0×10\(^9\) CFU/ml) of each freshly grown isolate were placed either in 96-well microtiter plates or on three sterilized polymer foils. The plates and the foils were exposed to different experimental plasma conditions (tables 1 and 2). After treatment, the bacterial suspensions were grown on bacterial mediums. The plates were incubated for 24 hours at 37°C. All the experiments were confirmed in duplicate. Reported values were the average of each two values.

Statistical Methods
The statistical analyses were performed with SPSS statistical program (version 15). A mean value for each bacterial count was obtained by averaging the duplicate values after log conversion.

Results
The best conditions that led to the elimination of 10\(^9\) CFU/ml of each tested bacterial isolate (using O\(_2\)-N\(_2\) plasma mixture at 300 W) are shown in figures 1, 2, and 3. Figure 1 illustrates the influence of plasma pressure on bacterial count (exp. 1-6 in table 1). Minimum CFU values were seen using 1.24 mbar pressure. The effect of O\(_2\) percentage (exp. 5, 7, and 8 in table 1) in N\(_2\)-x%O\(_2\) plasma mixture is presented in Figure 2: the CFU values of E. coli O157 were

| Table 1: Experimental plasma conditions for the inactivation processes of 10\(^9\) CFU/ml of different types of bacteria using N\(_2\)-O\(_2\) plasma mixture |
|---|---|---|---|---|
| Exp | x (%) in N\(_2\)-x% O\(_2\) | Gas flow N\(_2\)/O\(_2\) (sccm) | Pressure (mbar) | Power (W) | Treatment time (min) | Z (cm) | Substrate |
|---|---|---|---|---|---|---|
| 1 | 9 | 500/50 | 0.35 | 300 | 30 | 4.5 | 96-well plate |
| 2 | 9 | 500/50 | 0.65 | 300 | 30 | 4.5 | 96-well plate |
| 3 | 9 | 500/50 | 0.95 | 300 | 30 | 4.5 | 96-well plate |
| 4 | 9 | 500/50 | 1.17 | 300 | 30 | 4.5 | 96-well plate |
| 5 | 9 | 500/50 | 1.24 | 300 | 30 | 4.5 | 96-well plate |
| 6 | 9 | 500/50 | 1.48 | 300 | 30 | 4.5 | 96-well plate |
| 7 | 5 | 500/26 | 1.25 | 300 | 30 | 4.5 | 96-well plate |
| 8 | 2 | 500/10 | 1.25 | 300 | 5 | 4.5 | 96-well plate |
| 9 | 9 | 500/50 | 1.25 | 300 | 10 | 4.5 | 96-well plate |
| 10 | 9 | 500/50 | 1.25 | 300 | 15 | 4.5 | 96-well plate |
| 11 | 9 | 500/50 | 1.25 | 300 | 20 | 4.5 | 96-well plate |
| 12 | 9 | 500/50 | 1.25 | 300 | 25 | 4.5 | 96-well plate |
| 13 | 9 | 500/50 | 1.25 | 300 | 40 | 4.5 | 96-well plate |
| 14 | 9 | 500/50 | 1.25 | 300 | 30 | 4.5 | PVC, PE, PET |

| Table 2: Experimental plasma conditions for the inactivation processes of different types of bacteria using pure SF\(_6\) plasma |
|---|---|---|---|---|
| Gas flow (sccm) | Pressure (mbar) | Power (W) | Treatment time (min) | Z (cm) | Substrate |
|---|---|---|---|---|
| 1 | 200 | 0.55 | 100 | 0.5 | 4.5 | 96-well plate |
| 2 | 200 | 0.55 | 100 | 1 | 4.5 | 96-well plate |
| 3 | 200 | 0.55 | 100 | 3 | 4.5 | 96-well plate |
| 4 | 200 | 0.55 | 100 | 5 | 4.5 | 96-well plate |
| 5 | 200 | 0.55 | 100 | 10 | 4.5 | 96-well plate |
| 6 | 200 | 0.55 | 100 | 15 | 4.5 | 96-well plate |
decreased, while O₂ percentage was increased and only 2% O₂ pressure was sufficient to completely deactivate the other types of bacteria. The influence of the time of treatment (exp. 5 and 9-14 in table 1) is demonstrated in figure 3. A 30-minute treatment was required to eliminate all the different kinds of microorganisms except *E. coli* O157, which was decreased only to 2×10² CFU/ml. According to these results, the best conditions were 4.5 cm distance from the source, 30 minutes of treatment, 9% of O₂, and 1.25 mbar pressure.

Using the above-mentioned conditions on PVC, PE, and PET polymers (exp. 15 in table 1), we observed total inactivation of all the tested microorganisms with the PVC and PE polymers. However, *K. pneumonia* was not inactivated when we used PET polymer.

Figure 4 shows the effect of SF₆ plasma on all the previously mentioned microorganisms, using 96-well plates. Total inactivation of all the tested bacteria was seen only 3 minutes after the application of SF₆. Approximately, 100% of
all the \( P. \) mirabilis isolates were eliminated within 0.5 minute after \( \text{SF}_6 \) exposure and 100% of all the \( E. \) coli O157 and \textit{Enterobacter} isolates were eliminated within one minute after \( \text{SF}_6 \) exposure. However, about 80% of the \( K. \) pneumoniae isolates were eliminated within one minute after exposure.

**Discussion**

Plasma treatment is considered a good and safe method to eliminate the decontamination of not only dental instruments but also general surgical instruments.\(^{10} \) Our results showed that the best bacterial inactivation plasma conditions were 300 W applied power, 4.5 cm distance from the source, and 1.24 mbar pressure at 9% of \( O_2 \). Philip et al.\(^{11} \) demonstrated that total inactivation of \textit{Bacillus subtilis} spores was achieved 40 minutes after plasma exposure at 100 W with 2% of \( O_2 \). Furthermore, Xu et al.\(^1 \) reported that the time needed for the
inactivation of *Geobacillus stearothermophilus* spores was 3 minutes. In another study, Xu et al. also found that 10-20% of O₂ was sufficient to inactivate these bacteria. Elsewhere, Feichtinger et al. discovered that spores numbers were reduced one second after the application of laboratory air as plasma gas. Our results agree with those reported by Xu et al., who revealed that using argon (Ar) in a plasma jet source for 10 minutes did not totally eliminate *E. coli*. According to our results, O₂-N₂ gas using a plasma source was able to totally inactivate all kinds of bacteria except *E. coli*. The inactivation effect was more pronounced when we used flat polymers as substrates. Ricard and Monna reported that N₂–5% O₂ gas mixture completely eliminated *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* bacteria 15–20 minutes after treatment. In contrast, our results demonstrated that SF₆ gas totally inactivated the bacteria in only 1-3 minutes.

**Conclusion**

Plasma inactivation using N₂-O₂ gas mixture and SF₆ gas proved promising for the inactivation of the bacterial isolates in the present study. Our findings could be helpful in many medical and industrial fields; however, further investigations are needed to integrate this technique into the field of bacteria disinfection.

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