Removal and sterilization of biofilms and planktonic bacteria by microwave-induced argon plasma at atmospheric pressure

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\textit{New Journal of Physics} 11 (2009) 115022 (11pp)

Received 28 February 2009
Published 26 November 2009
Online at http://www.njp.org/
doi:10.1088/1367-2630/11/11/115022

Abstract. Microbial biofilms are a functional matrix of microbial cells, enveloped in polysaccharides, enzymes and virulence factors secreted by them that can develop on indwelling medical devices and biomaterials. Plasma sterilization has been widely studied in recent years for biological applications. In this study, we evaluated the possibility of removal and anti-recovery of biofilms by microwave-induced argon plasma at atmospheric pressure. We observed that all bacterial biofilms formatted by Gram-negative and Gram-positive bacteria are removed in less than 20 s, and the growth inhibitions of planktonic bacteria within biofilms are also confirmed by plasma exposure for 5 s. These results suggest that our plasma system can be applied to medical and biological fields where the removal of biofilms and their debris is required.

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1. Introduction

Microbial biofilms are a structural matrix consisting of microbial cells attached to an inert or living surface and polysaccharides, enzymes and virulence factors secreted by them (Sutherland 2001). It may be exposed to people requiring medical devices and pose public health problems and it is clear from epidemiologic evidence that biofilm-associated diseases are related to detachment of cells or cell aggregation on biomedical devices, production of endotoxins, resistance to the host immune system, and resistance to antimicrobial agents (Donlan 2001, Donlan and Costerton 2002).

Therefore, sterilization and removal of biofilms for medical devices and biomaterials are an important process in medical and biological fields, because planktonic bacteria can initiate biofilm formation on its surface. However, removal and sterilization of biofilms are complicated, because bacteria within biofilms are more resistant to antimicrobial agents.

Among the sterilization techniques, antibacterial properties by plasma have been reported in many studies and the plasma sterilization has been considered as an alternative method due to its remarkable advantage of not producing toxic residues after treatment (Inc 2001, Poncin-Epaillard and Legeay 2003, Sladek et al 2007).

In particular, microwave-induced plasma at atmospheric pressure has many advantages, such as the lack of the necessity for expensive vacuum equipment, low cost and simple systems and easy operation (Moon et al 2002). It produces specific active agents, such as ultraviolet (UV) photons, ozone and short-living reactive radicals, e.g. oxygen radical and hydroxyl radical.

For that reason, we investigated the removal efficiency of the most common biofilms produced by *Escherichia coli* (*E. coli*), *Staphylococcus epidermidis* (*S. epidermidis*) and methicillin-resistant *Staphylococcus aureus* (MRSA) using our self-designed microwave-induced argon plasma system at atmospheric pressure in this study.

2. Materials and methods

2.1. Microorganisms

*E. coli* ATCC 8739 and *S. epidermidis* ATCC 12228 were obtained from the American Type Culture Collection (Rockville, MD, USA) and MRSA was isolated from clinical patients in...
Yonsei Medical Center at Seoul, Korea (Lee et al. 2005). After that, the strains were stored frozen at $-70^\circ\text{C}$ before the experiment was performed. The bacterial strains were grown on the standard methods agar (Becton, Dickinson and Company, Sparks, MD, USA) at $37^\circ\text{C}$ for 15 h. The bacterial strains were maintained at $4^\circ\text{C}$ prior to use the experiment.

2.2. Biofilms development on slide glass under static condition

The cut glasses were washed with tryptic soy broth (TSB, Becton, Dickinson and Company, NJ), with distilled water and then with a 20-fold diluted TSB. For the formation of bacterial biofilms on the slide glasses by modified method as described by Assere et al. (2008), the bacterial strains were cultured with TSB at $37^\circ\text{C}$ for 18–20 h. Cultured bacteria were resuspended with TSB and the optical density (OD) was adjusted to $0.1 \pm 0.05$ at 600 nm, which equals a bacterial cell count of $10^8 \text{CFU ml}^{-1}$. A tenfold dilution of bacterial suspension was inoculated on the sterilized glasses placed in a 12-well plate and incubated at $37^\circ\text{C}$ in a humidified atmosphere for 24 h. After the incubation, the bacterial suspension was discarded. Then, the slide glasses were rinsed with sterilized distilled water to remove the unattached bacteria and dried at room temperature for 1 h.

2.3. Treatment of microwave-induced argon plasma

As previously described (Lee et al. 2005, Park et al. 2003, 2004, 2007a, 2007b), a self-designed microwave-induced argon plasma was used in this study. Briefly, this system consisted of 2.45 GHz magnetron power supply (1 kW), an applicator including a tuning section and the nozzle made of quartz (figure 1(a)). The microwave was introduced through a WR-284 copper waveguide with internal cross section dimensions of 72 mm $\times$ 24 mm. The plasma generated at the end of a nozzle was formed by an interaction between the high electrical field, which is generated by the microwave power supply, the waveguide aperture and the gas nozzle. The electric field intensity around the nozzle was calculated by a high frequency structure simulator (HFSS) code simulation as previously described (Park et al. 2003). Argon was used as a working gas for this plasma system, which was chosen for its inertness, and the gas flow rate is approximately 1001 min$^{-1}$ at 8 kgf cm$^{-2}$. The intensity of UV light generated by plasma was observed indirectly by using a radiometer/photometer (IL1400A, International Light, Inc, Newburyport, MA) with a solar blind vacuum photodiode and the temperature of surface treated with plasma was measured directly using a thermo label (Thermo label 5E, Nichiyu Giken Kogyo Co, Ltd, Osaka, Japan) as previously described (Lee et al. 2005, Park et al. 2003).

For evaluating the removal efficiency of bacterial biofilms, the slide glasses with bacterial biofilms were placed in front of a nozzle in the plasma system and exposed to plasma for 1, 5, 10, 15 and 20 s, respectively. For observing the morphologies of the biofilms by scanning electron microscopy (SEM), the plasma treated and untreated slide glasses were fixed in was fixed in phosphate buffer containing 3% buffered glutaraldehyde and lyophilized, and coated with an ultra-thin layer of gold/platinum by ion sputter (E1010, Hitachi, Tokyo, Japan) and observed by SEM (S-800, Hitachi) (figure 1(b)).

2.4. Quantitative assay for recovery of the biofilms and planktonic bacteria

After the plasma treatment, the slide glasses were incubated in 1 ml of TSB at $37^\circ\text{C}$ for 24 h and the growth of planktonic cells was determined by an automatic microplate reader (Versamax,
Molecular Device, Sunnyvale, CA) at a wavelength of 600 nm. Quantitative analysis of the biofilms was performed by crystal violet staining (Jackson et al 2002). The slide glasses with bacterial biofilms were rinsed with sterilized distilled water and stained with 0.2% crystal violet (dissolved in 2% ethanol) for 5 min. The stained biofilms were eluted with 95% ethanol and the absorbance was determined at a wavelength of 600 nm using a microtiter plate reader as described above. The recoveries of the biofilms and planktonic bacteria were represented as percent from average absorbance of the untreated control and plasma-treated group. Data shown are from two separate experiments, respectively, and were analyzed statistically by calculating means and SD of the means performed in quadruplicate.
3. Results and discussions

Although it is accepted that biofilms are ubiquitous in nature, the significance of biofilms in clinical settings, especially with regard to their role in device-related infections, is often underestimated (Lindsay and von Holy 2006).

Biofilm formation on medical devices and medical implants has even led to the characterization of a new infectious disease called chronic polymer-associated infection and it can be thought of as a virulence factor—a bacterial strategy that contributes to its ability to cause an infection (Hall-Stoodley et al 2004).

For these reasons, microbial sterilization and removal of biofilms are important in the medical and biological fields and a more rapid and less damaging method of sterilizing for various materials than conventional sterilization methods (Park et al 2003) is needed. Therefore, we evaluated the possibility of removal and anti-recovery of biofilms by microwave-induced argon plasma at atmospheric pressure (Lee et al 2005, Park et al 2003, 2004, 2007a, 2007b).

Biofilms on indwelling medical devices may be composed of Gram-positive or Gram-negative bacteria or yeasts. The Gram-positive S. epidermidis and S. aureus and the Gram-negative E. coli were commonly isolated from medical devices, which may originate from the skin of patients or healthcare workers or from tap water (Donlan 2001). As shown in figure 2, regrowth of planktonic bacteria was not observed with plasma treatment for 5 s in all bacterial biofilms used in this study. In other words, because of the inactivation of weakly attached bacteria within biofilms by plasma, those were not grown in fresh media. In the process of biofilm development, the first step is attachment of planktonic (free-floating) bacteria by electrostatic attraction and physical forces. Mampel et al (2006) suggested that biofilm formation is due to growth of planktonic bacteria rather than to growth of sessile bacteria and extrabiofilm bacterial replication is essential for biofilm formation.

When bacterial biofilms were incubated in fresh media after the plasma treatment, sessile bacteria within biofilms were not recovered or reformed to biofilms (figure 3). In addition, all bacterial biofilms formatted by Gram-negative and Gram-positive bacteria in this study were removed in less than 20 s. In our previous study (Lee et al 2005, Park et al 2007a), we have already shown that plasma treatment can completely sterilize E. coli and MRSA after 1 s and can inactivate lipopolysaccharide (LPS) after 10 s. From the results of this study and our previous study, we confirmed that higher plasma exposure time is required to completely remove and inhibit biofilms.

Biofilms associated with bacteria have different growth rates and resistance to antimicrobial agents from the planktonic bacteria, therefore biofilms are much more difficult to remove from surfaces than freely suspended organisms (Sutherland 2001).

Extracellular polysaccharide matrices within biofilms are heterogeneous, varying in hydration with depth from the surface. Those extracellular products are able to block antibacterial effect and penetration of antibiotics, oxidizing biocides such as isothiazolones, halogens and quaternary ammonium compounds, etc (Fraise et al 2004).

Figure 4 shows the SEM images of plasma-treated sessile bacteria on biofilms. In the plasma-treated sessile bacteria on biofilms (figures 4(b, c); (e, f); (h, i)), damaged and ruptured morphologies can be observed in comparisons with the untreated group (figures 4(a, d and f)). As shown in figures 4(b), (e) and (h)) treated with plasma for 5 s, smaller holes and extensive leakage of cell constituents were observed in sessile bacteria. In general, the bacterial membrane leakage is rapid following the exposure to biocidal agent according to the classic
Figure 2. Recovery efficiency of planktonic cell on biofilms of *E. coli* (a), *S. epidermidis* (b) and MRSA (c). Bacterial biofilms treated with microwave-induced argon plasma for 1, 5, 10, 15 and 20 s, respectively. After the bacterial biofilms were incubated in fresh TSB at 37°C for 24 h, the re-growth of planktonic bacteria was determined by an automatic microplate reader at a wavelength of 600 nm. The results shown were expressed as percent of plasma treated absorbance per untreated control absorbance.

New Journal of Physics 11 (2009) 115022 (http://www.njp.org/)
Figure 3. Recovery efficiency of biofilms of *E. coli* (a), *S. epidermidis* (b) and MRSA (c). Bacterial biofilms treated with microwave-induced argon plasma for 1, 5, 10, 15 and 20 s, respectively. After the bacterial biofilms were incubated in fresh TSB at 37°C for 24 h, development of biofilms was assayed by crystal violet staining assay. The results shown were expressed as percent of plasma treated absorbance per untreated control absorbance.

These might explain the comparison with SEM images in figure 5 that completely removed planktonic and sessile bacteria within the biofilms after the plasma treatment for 20 s.

The mechanism of microorganisms inactivation by plasma is not fully understood, however, there are three processes that are being considered; (a) destruction by UV irradiation of the genetic material of the microorganism, (b) erosion of the microorganism, atom-by-atom, through intrinsic photodesorption breaking chemical bonds in the microorganism material and leading to formation of volatile compounds from atoms intrinsic to the microorganism and (c) erosion of the microorganism, atom-by-atom, through etching from the adsorption of reactive species from the plasma on the microorganism (Moisan et al 2002).
Figure 4. SEM micrographs of biofilms onto slide glasses treated with plasma; *E. coli* (a–c), *S. epidermidis* (d–f) and MRSA (g–i). (a, d and g, untreated control groups; b, e and h, plasma treated for 5 s; c, f and i, plasma treated for 20 s).

These processes were also confirmed as shown in figures 4 and 5, and the UV intensity generated by the plasma ranged from 72 mW cm$^{-2}$ (minimum) to 96 mW cm$^{-2}$ (maximum) at a wavelength of 254 nm, which killed the microorganisms. The figures show that there was a relatively high level of UV emission in the microwave-induced argon plasma, and that the UV radiation generated was involved in sterilizing the microorganisms. Furthermore, a relatively
Figure 5. SEM micrographs of recovered biofilms of MRSA onto slide glasses, which were treated with plasma and then incubated at 37°C for 24 h. (a) untreated control; (b) plasma treated for 5 s; (c) plasma treated for 20 s. Lower figures are magnified sections of the upper figures.

A high level of UV light generated by the plasma enhanced the etching and the erosion effects of the bacteria and their biofilms to volatile compounds, as a result of slow combustion using oxygen atoms or radicals emanating from the plasma (Moisan et al 2002, Park et al 2003, 2004).

In addition, the temperature of the surface treated with plasma was measured to be about 170°C for 20 s and 300°C for above 1 min. This heat generated by the plasma was dry heat. To sterilize microorganism, the recommended treatment of dry heat is maintenance at 160°C for 2 h (Fraise et al 2004). In other words, biofilm removal can be assumed to be caused by plasma not by the heat generated in plasma production.

4. Conclusion

In conclusion, the inhibition of growth and formation of biofilms by plasma at atmospheric pressure might be due to the inactivation of planktonic bacterial growth that may be required for biofilm formation, and to the suppression of colonization of sessile bacteria on the surface required for biofilms reformation.

New Journal of Physics 11 (2009) 115022 (http://www.njp.org/)
In this study, we confirmed that our plasma system was not only effective on the inactivation of planktonic bacteria and their growth, but was also able to remove the biofilms from the surface of the objects being sterilized. Therefore, this study suggests that our plasma system can be applied to medical and biological fields where the removal of biofilms and their debris is required, although further studies are required to characterize the physicochemical properties and changes of plasma-treated biofilms.

Acknowledgments

This study was supported by the Nano-Bio R&D program (Platform technologies for organ/tissue regeneration (Regenomics), Grant No. 2005-00009) of the Korea Science & Engineering Foundation (KOSEF).

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New Journal of Physics 11 (2009) 115022 (http://www.njp.org/)
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