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Oral Bacterial Deactivation Using a Low-Temperature Atmospheric Argon Plasma Brush

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Summary

Objectives—To study the plasma treatment effects on deactivation effectiveness of oral bacteria.

Methods—A low temperature atmospheric argon plasma brush were used to study the oral bacterial deactivation effects in terms of plasma conditions, plasma exposure time, and bacterial supporting media. Oral bacteria of *Streptococcus mutans* and *Lactobacillus acidophilus* with an initial bacterial population density between 1.0×10^8 and 5.0×10^8 cfu/ml were seeded on various media and their survivability with plasma exposure was examined. Scanning electron microscopy was used to examine the morphological changes of the plasma treated bacteria. Optical absorption was used to determine the leakage of intracellular proteins and DNAs of the plasma treated bacteria.

Results—The experimental data indicated that the argon atmospheric plasma brush was very effective in deactivating oral bacteria. The plasma exposure time for a 99.9999% cell reduction

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was less than 15 seconds for *S. mutans* and within 5 minutes for *L. acidophilus*. It was found that the plasma deactivation efficiency was also dependent on the bacterial supporting media. With plasma exposure, significant damages to bacterial cell structures were observed with both bacterium species. Leakage of intracellular proteins and DNAs after plasma exposure was observed through monitoring the absorbance peaks at wavelengths of 280nm and 260nm, respectively.

Conclusion—The experimental results from this study indicated that low temperature atmospheric plasma treatment was very effective in deactivation of oral bacteria and could be a promising technique in various dental clinical applications such as bacterial disinfection and caries early prevention, etc.

Keywords

atmospheric gas plasmas; glow discharge; oral bacteria; sterilization

1. Introduction

As a biofilm on tooth surface, dental plaque consists of complex communities (usually colorless) of oral bacteria with hundreds of species present.^{1,2} These biofilms build up on the teeth surfaces, and if not removed regularly, can lead to one of the most prevalent diseases of mankind and dental caries, which is the localized destruction of tooth tissues by bacterial fermentation of dietary carbohydrates (Marsh and Matin 1999).³ The microorganisms that form the biofilm are almost entirely bacteria with the composition varying by tooth location in the mouth.

Streptococcus mutans (*S. mutans*) and *Lactobacillus acidophilus* (*L. acidophilus*) are Gram-positive, facultatively anaerobic bacteria,⁴ and are both highly acid-producing species. *S. mutans* is one of the most implicated bacteria in smooth surface caries and considered to be a major pathogen in dental caries.^{5–7} *L. acidophilus* has been associated with dental caries,^{8,9} and the quantity of *L. acidophilus* in saliva is still used as a direct measure of caries risk, which is known as Lactobacillus counts (Caries Test).^{10,11}

In clinical management, caries are removed by dental burs and restored with dental material. The drilling is achieved by removal of the often painful necrotic, infected and demineralized tissue. With such treatments, an excess of healthy tissue usually may be removed to ensure that the cavity is free of bacteria and often the integrity of the remaining tooth is weakened. Furthermore, the chemotherapeutic agents for caries prevention, such as chlorhexidine, will often lead to some undesirable side effects and unpleasant taste and stains.^{12–14}

Low temperature gas plasma technology has been used in industrial applications as early as a hundred years ago. In the 1960's, the first attempts were made to sterilize surfaces in low-pressure radio frequency (RF) glow discharge plasma.¹⁵ Although the healthcare industry has long needed a sterilization method that functions near room temperature, and in much shorter times, traditional methods, such as dry and moist heat, autoclave, and ethylene oxide, are widely used for many years because they are dependable and well understood. Recently, more and more newly developed sterilization approaches and techniques were introduced into the medical field. These new methods include vapor phase hydrogen peroxide, and liquid chemical sterilants (peracetic acid). In 1990's, low-pressure gas plasma sterilization systems have been approved by the U.S. Food and Drug Administration (FDA).¹⁶ However, low-pressure gas plasma sterilization methods have some drawbacks, such as limited plasma volume and the need of several vacuum cycles, which hinder their wide application in the medical field. More recently, more attention has been paid on low temperature atmospheric plasma sterilization, such as corona discharges, dielectric barrier discharges, arc jets,

atmospheric plasma jet and inductive plasma torches, which were developed since early 1990s.^{17–20}

Recently, a new low temperature atmospheric gas plasma source, low temperature atmospheric plasma brush was developed in our Plasma Research Center at the University of Missouri and Los Alamos National Laboratory.^{21,22} Such a plasma source can be ignited and sustained at very low power consumption, with plasma temperature close to human body temperature, and thus eases hand-held capability and makes direct in-vivo application possible. Therefore, one promising application for such a low-temperature atmosphere plasma brush is dental care. In this paper, low-temperature argon atmosphere plasma brush was applied to the gram-positive oral bacteria, *S. mutans* and *L. acidophilus*, which are two most implicated bacteria for the initiation and progression of dental caries, to study the effects of plasma deactivation.

2. Experimental Procedures

Materials and Organisms

Argon (99.997% purity) gas was used as the plasma gas and was purchased from the General Store of the University of Missouri-Columbia. P5 filter papers and glass slides (Fisher Scientific, St. Louis, USA) and PTFE (Polytetrafluoroethylene) films (0.1 mm in thickness) (Fuxing Fluorin Chemical Works Ltd. China) were used as the bacterial supporting media. Filter papers have porous structures, which simulate the fissures of the tooth.²⁰ Glass slide was used as smooth solid surface similar to the intact tooth surface and PTFE film was a polymer surface carrier for *S. mutans* and *L. acidophilus*. Tryptic Soy Agar and Tryptic Soy Broth (Difco Bacto, Detroit, MI, USA), Standard Methods Agar (Becton Dickson, Cockeysville, MD), and *Lactobacillus* MRS Agar (MRSA) and MRS Broth(MRSB) (Difco Bacto, Detroit, MI, USA) were used in this study. Also, two strains of bacteria, *S. mutans* ATCC 25175 and *L. acidophilus* ATCC 4356 (American Type Culture Collection, ATCC) were used in this study.

Low Temperature Atmospheric Argon Plasma Brush

The detail of this plasma source was reported previously.^{21,22} MKS mass flow controller (MKS Instruments Inc. Andover, MA, USA) was used to control argon gas flow rate. The discharge was ignited and sustained by a DC power supply (Pd 1556c, Power Design Inc. New York, NY, USA). With a relatively high gas flow that varied with the plasma chamber size, the plasma discharge formed inside the chamber could be blown out of the chamber to form a brush-shaped low temperature plasma jet, i.e., a low temperature atmospheric plasma brush. This plasma source can be operated under very low electrical power (as low as a few watts), and as a result very low plasma temperature can be achieved. As shown in Figure 1, the gas phase temperatures of such an argon atmospheric plasma, measured using an infrared camera combined with a thermocouple thermometer range from 30°C to 65°C, with the corresponding argon gas flow rate varying from 3500 sccm (standard cubic centimeter per minute, a volumetric flow rate at 273 K under 1 atm) to 500 sccm and input power from 5 W to 15 W.

Experiment Procedures

The strain of *S. mutans* was grown in Brain Heart Infusion (BHI) broth for 48 hrs (hours) at 37°C, transferred onto a Tryptic Soy Agar (TSA) plate and incubated at 37°C for 48 hrs. A single colony was grown in Tryptic Soy Broth (TSB) at 37°C for 24 hrs, and then kept at 4°C subsequently. Prior to being used for the treatment, the bacteria were transferred into 10ml TSB and allowed to grow for 24 hrs until the bacterial population density was measured between 1.0×10^8 cfu·ml⁻¹ to 5.0×10^8 cfu·ml⁻¹.

The strain of *L. acidophilus* was grown in Lactobacillus MRS Broth at 37°C in an anaerobic incubator for 48 hrs, and the bacteria were transferred onto a Lactobacillus MRS Agar (MRSA) plate and anaerobically incubated at 37°C for 48 hrs. A single colony was anaerobically grown in Lactobacillus MRS Broth (MRSB) at 37°C for 24 hrs, and kept at 4°C. Before being treated, the bacteria was transferred into a 10 ml MRSB and allowed to grow for 24 hrs until the bacterial population density was measured in the range between 1.0×10^8 cfu·ml⁻¹ to 5.0×10^8 cfu·ml⁻¹.

Filter Paper (Porous Medium)—A volume of 5 µL solution containing the bacteria were dispensed onto a sterilized filter paper (10 × 10 mm) that was used as the supporting medium, and then dried in a moderate vacuum incubator at 37°C for 20 min. The support media containing bacteria (4 mm diameter) were treated with the low-temperature atmospheric plasma brush under a selected plasma condition (10 w, 2000 sccm argon) for a preset exposure time. Then, the treated bacteria were transferred into test tubes containing 10 mL of 0.03 mol/L phosphate buffer solution (PBS), and mixed using a vortex mixer for 20 s. The pour-plate method was used to count bacterial numbers. The suspension was exponentially diluted until the cell number on the Petri dishes could be counted with under an optical microscope (usually from 300 cfu to 30 cfu). The number of survival bacteria cells was counted after incubation at 37 °C for 48 hrs.

Glass Slide (smooth solid surface medium)—A 5 µL droplet solution containing the bacterium suspension was dispensed onto a sterilized glass slide (10×10 mm) to form a liquid spot of 3 mm in diameter. The carrier was then dried in a moderate vacuum incubator at 37°C for 20 min. Under a selected plasma condition (10 w, 2000 sccm argon), the bacteria were plasma treated for a preset time. After the treatment, the bacteria were transferred into test tubes containing 10 ml PBS. The solution was then treated similar with the samples from filter paper support media, and plate counting was made after incubated at 37°C for 48 hrs.

PTFE film (polymer surface medium)—A volume of 5µL solution containing the bacteria was dispensed onto a distilled PTFE film (10×10 mm) and then dried in a moderate vacuum incubator for 20 min to form a bacteria spot with 2 mm diameter. Then the bacteria carrier was treated by the atmospheric plasma brush under a selected plasma condition for a preset duration. After being treated, the bacteria was transferred into a test tube containing 10 ml PBS, and then mixed for 20 s using a vortex and exponentially diluted. The pour-plate method was used to count bacterial survival numbers after incubation at 37°C for 48 hrs.

Effects of Plasma power input—With the filter paper as the carrier, 5 µL solutions containing the bacteria were dispensed onto sterilized filter paper disks (10×10 mm). After similar experimental procedures described above, the filter paper carrier containing bacteria were plasma treated for a preset exposure time. After plasma treatment, the bacteria were transferred into test tubes containing 10 mL phosphate buffer solution (PBS), and mixed using a vortex mixer for 20 s. The pour-plate method was used to count bacterial numbers. The suspension was exponentially diluted until the cell number on the Petri dishes could be counted under an optical microscope. The number of survival bacterial cells was counted after incubation at 37°C for 48 hr.

Effect of Gas Blowing and Heating on Cell Reduction—In order to distinguish the plasma sterilization effects from the possible gas blowing effect and heating, the survival rates of the bacterium cells were also assessed by treating the bacteria using the same argon flow rate 2000 sccm at 50°C without igniting the plasmas.

Effects of Initial Cell Density on Cell Surviving Rate—To prepare samples for the plasma treatment, the bacterial solution were diluted 10% original concentration and 1% original concentration. PTFE films as the supporting media seeded with different cell density were treated under the same plasma conditions (2000sccm Argon flow rate, 10 w input power).

All experiments for the two strains were done in triplicate parallel samples and the plate count results for cell reduction were the average counts obtained from the triplicate parallel plate counting Petri-dishes, respectively.

Morphological Examination

The effects of plasma treatment on the cell morphology and structure changes of the bacteria were examined using a Scanning Electron Microscopy (SEM) (QUANTA 600F, FEI, Holland). The bacterial solutions of the controls, i.e., untreated bacteria, and plasma treated samples were placed in the fixation and then coated with a thin layer of plasma sputtered platinum.

UV-visible Absorbance Examination

S. mutans and *L. acidophilus* seeded on various supporting media were exposed to argon plasma brush for different periods of time, and the bacterial deactivation effects of the plasma brush were assayed using a UV-visible spectrometer to monitor the leakage of intracellular proteins and nucleic acid. A volume of 10 μ L *S. mutans* or *L. acidophilus* solution was *first* seeded on a glass slide surface, dried in a moderate vacuum incubator at 37°C for 30min, and then exposed to argon plasma. Control experiments were also performed by exposing the seeded glass media to 2000sccm of argon gas (no power input) at 40°C. After plasma exposure, the bacteria were washed off from the glass slide surface by using 1 mL PBS buffer solution and collected into a 1.5 ml centrifugal tube. For each treatment condition, five test samples were prepared. These five parallel samples were collected into the same centrifugal tube to get enough protein and DNA from the leakage of intracellular contents of the bacterial cells. The treated bacterial suspensions 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 (UV-1650, Shimadzu, Japan) at wavelengths of 260 nm (for DNA absorbance) and 280 nm (for protein absorbance), respectively. All experiments were completed in duplicate groups.

3. Results and Discussion

Heat and gas blowing effects

The favorable temperature ranges for *S. mutans* and *L. acidophilus* growth are 30 °C to 47 °C and 37 °C to 45 °C, with optimal growth at human body temperature, 37 °C.²³ The lethal temperature of *L. acidophilus* is 65 °C. *S. mutans* is a typical mesophile in relation to membrane and catabolic functions and can be deactivated at temperature above 60 °C.²⁴ Based on the infrared images, the hottest spot of the argon plasma brush used in this study was less than 50 °C under the selected operating condition of 2000 sccm argon flow rate, 10 W input power.

To distinguish plasma treatment effects from the argon gas blowing and gas temperature, *S. mutans* and *L. acidophilus* were seeded onto various supporting media and exposed to the flowing argon gas (2000 sccm) at 50 °C for different periods of time and the cell surviving data is shown in Figure 2. It can be seen from Figure 2 that argon gas blowing at 50 °C did not cause obvious survival rate decrease in the number of bacteria seeded on the supporting media used in this study.

Inactivation Kinetics of Dental Bacteria

Figure 3 shows the cell surviving curve of *S. mutans* with different plasma exposure time on three different supporting media. It can be seen that the argon plasma brush is very effective in deactivating *S. mutans* and a very short plasma exposure time of less than 15 s gave a complete kill of the bacteria. Figure 4 shows the cell surviving curve of *L. acidophilus* with different plasma exposure time. It was found that longer plasma treatment time was required for deactivating *L. acidophilus* than killing *S. mutans*. There are two possible reasons for such a difference in bacterial deactivation between these two kinds of bacteria. The first is that plasma etching, oxidation and UV-radiation have been known to be the main plasma sterilization mechanisms. Such sterilization mechanisms indicate that bacterial sizes and structure would affect the plasma effectiveness and efficiency in bacterial deactivation. The bigger cell size of *L. acidophilus* ($\sim 1 \times 3 \mu\text{m}$) would have higher plasma tolerance and are harder to kill than the smaller *S. mutans* ($\sim 1 \mu\text{m}$ in diameter). In order to achieve the same intensity of injection plasma for a single cell, under the same plasma conditions, several minutes of exposure time were needed to kill *L. acidophilus*, while only tens of seconds of exposure time was needed for *S. mutans*. The second reason is that the spread area (4 mm diameters) of the seeded cells on the supporting media was limited. With similar cell population densities used in this study, such a limited area leads to more overlapped cell layers for *L. acidophilus* than that for *S. mutans*. Consequently, less *L. acidophilus* cells resided on the top layer were directly exposed under plasma than that for *S. mutans* under the similar cell population densities and plasma conditions.²⁵ Plasma species was blocked by the outer layer of the cells from reaching the underneath layer of the cells.

From Figures 3 and 4, it was also noted that most efficient deactivation for both types of bacteria was observed with filter papers as the supporting media. Based on our experimental observation, the *S. mutans* cell droplets had the biggest spread area on filter paper (4 mm in diameter) than those on the other two media. This larger spread area would provide the most cells resided on top cell layers to be exposed under plasma at the same time. Because of the highest water contact angle of PTFE surface, the smallest droplet spread area (2 mm in diameter) was observed when the cells were seeded. From Figures 3, it was observed that the plasma deactivation rate for *S. mutans* became slower in the period from 3 s to 9 s and re-accelerated after 10 s plasma exposure. This duration enabled plasma decomposed the remaining debris of the lethal cells before the underneath layer cells were directly exposed to the plasma.

Figures 5 and 6 show the cell survival rate using different initial cell densities with plasma treatment time. From Figure 5, it was found that 5 s plasma exposure was needed for complete deactivation of *S. mutans* when 10% original cell density was used as compared with the 13 s for 100% original density. When 1% original density was used for cell seeding, plasma exposure as short as 3 s was needed for a complete kill of the bacteria. Similar trends were also observed with *L. acidophilus* as shown in Figure 6. With an initial *L. acidophilus* population density was reduced to 10 % and 1%, a complete kill of the bacteria was achieved in less 90 s and 60 s, respectively.

Figures 7 and 8 show the cell survival curves of the two bacteria exposed to plasmas maintained with different plasma power inputs. It was noted that, for both oral bacteria, the plasma deactivation efficiency increased with an increase in the plasma power input. The power input and gas flow not only affected the plasma density, but also the plasma temperature as shown in Figure 1. A higher power input could result in a higher degree of ionization of the gas and thus increase the density of various plasma species,²¹ which are the reactive agents in deactivation of the bacterial cells. Therefore, the plasma deactivation efficiency was improved with high plasma power input.

Protein and DNA leakage

To investigate the plasma deactivation mechanism, 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 leakage amount of the intracellular proteins and DNAs. Figures 9 and 10 show the intensity changes of these absorbance peaks with plasma exposure time for *S. mutans* and *L. acidophilus*, respectively. It can be seen that a very short plasma exposure of 1 s could significantly increase the peak intensity for both protein and DNA absorbance, i.e., dramatic leakage of the intracellular proteins and DNAs occurred. For both bacteria, the absorbance intensity of at wavelengths of 260 nm and 280 nm showed significant increase in the first several second plasma exposures. These results suggested that there were a large amount of protein and/or nucleic acids leaked out the bacterial cells due to plasma exposure, which usually occurs when the cell membranes are damaged.

From Figures 9 and 10, it was also noted that the increase in absorbance peak intensity at wavelength of 260 nm and 280 nm leveled off with plasma exposure times of longer than 3 s. This phenomenon can be explained by a synergistic situation: 1) 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 were etched away by the plasmas, the bacterial cells resided underneath the overlapped cell layers had little chance to be directly attacked by gas plasma species. This would lead to the slowdown in cell structure damage and thus the intracellular leakage rate with longer plasma exposure as seen from Figures 9 and 10. 2) Once the intracellular substances leaked out of the cells, aerobic degradation reaction would start. Due to the existence of highly reactive plasma species and UV radiation, the protein and nucleic acid leaking out from the exposed cells would decompose quickly.²⁶ This would also lower the peak absorbance intensity at wavelengths of 260 nm and 280 nm.

SEM Investigation

SEM was used to examine the cell structural changes of both *S. mutans* and *L. acidophilus* bacteria after plasma exposure. As shown in Figure 11, plasma treatment of *S. mutans* resulted in a significant alteration in cell size and morphology changes when compared with the untreated controls. After 5 s of Argon plasma exposure, distinct cell structural damage was noticed with *S. mutans* cells. Being consistent with the cell survival curves shown in Figure 3, 9 s and 15 s argon plasma exposure of *S. mutans* resulted in a large amount of cell debris. In case of *L. acidophilus* as shown in Figure 12, damages on cells wall were found with 60 s plasma treatment. Such SEM observations are consistent with the biofluid leakage results shown in Figures 9 and 10. In other words, at the early stage of plasma exposure, the cell structural damage induced the biofluid leakage out of the damaged cells (Figures 11b and 12b). As shown in Figure 12c, more cell damage was observed a longer plasma exposure of 180 s. With further increase in the plasma exposure time of 300 s, fragments of *L. acidophilus* cells were found and the SEM image of such debris is shown in Figure 12d. Because of the cell structural damage observed in the SEM images (Figures 11d and 12d), the cell survival curves shown in Figure 3 and 4 indicate that the bacterial cell structures were completely destructed and as a result the bacteria were completely killed. In comparison with *L. acidophilus* shown in Fig. 12, the pronounced cell damages of *S. mutans* shown in Figure 11 explained more rapid and effective deactivation observed in Figure 3.

4. Conclusions

The experimental results obtained from this study demonstrate the capability and effectiveness of Low Temperature Atmospheric Argon Plasma Brush in disinfection of oral bacteria, including both *S. mutans* and *L. acidophilus*. A short plasma treatment of 13 s

could achieve a 99.9999% cell reduction for *S. mutans*. Based on SEM examination, such a plasma brush could induce significant structural damage on oral bacteria, and as a result leakage of both cellular DNAs and proteins occurred as evidenced by optical absorbance measurement at 260 nm and 280 nm, respectively. It was also noted that the plasma bacterial disinfection efficiency was also dependent on the input power of plasma, the cell supporting media, and the type of bacteria. The findings from this study indicated that low temperature atmospheric plasmas could be a promising technique in various dental clinical applications such as bacterial disinfection and caries early prevention.

5. Study Limitation and Future Work

In this study, some of experimental tests were conducted using one medium only, instead of using all three support media. The major limitation of this study is that the bacterial deactivation effects of the atmospheric plasma were investigated using replacement material surface rather than extracted human teeth with carious infection. However, the supporting media used in this study have similar surface characteristics with the common surfaces in clinical situation, such as porous structure caused by caries on tooth surfaces; smooth surfaces of enamel; dental surgical instruments which are made from PTFE material. In order to fully determine the efficiency of the plasma deactivation of oral bacteria, extracted human teeth, both decayed and intact, should be used in future studies. Future work will focus on the sterilization on the surfaces of tooth and artificial tooth and the optimization of the plasma conditions for sterilization.

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Figure 1.
Temperature change of argon atmospheric plasmas with argon flow rate at different DC power inputs.

Figure 2. Effects of gas blowing and heating on the surviving cell numbers of (a) *S. mutans* and (b) *L. acidophilus* without igniting plasmas. The conditions were 2000 sccm argon at 50 °C, and 0 W power input.

Figure 3. Plasma sterilization effect on *S. mutans* seeded on different support media. Plasma conditions were 2000 sccm argon flow rate and 10 W DC power input.

Figure 4. Plasma sterilization effect on *L. acidophilus* seeded on different support media. Plasma conditions were 2000 sccm argon flow rate and 10 W DC power input.

Figure 5. Plasma sterilization effect on *S. mutans* seeded on PTFE with different initial cell densities. Plasma conditions were 2000 sccm Argon flow rate, 10 sccm oxygen flow rate, and 10W DC power input.

Figure 6. Plasma sterilization effect on *L. acidophilus* seeded on PTFE with different initial cell densities. Plasma conditions were 2000 sccm argon flow rate, 10 sccm oxygen flow rate, and 10W DC power input.

Figure 7.
Effects of plasma power input on plasma deactivation of *S. mutans* seeded on filter paper support media. Plasma condition was 2000 sccm argon flow rate.

Figure 8. Effects of plasma power input on plasma deactivation of *L. acidophilus* seeded on filter paper support media. Plasma condition was 2000 sccm argon flow rate.

Figure 9. The change in absorbance (Abs.) intensity of intracellular protein and DNA leakage from *S. mutans* with plasma treatment time. Plasma conditions were 2000 sccm argon flow rate and 10 W DC power input.

Figure 10.

The change in absorbance (Abs.) intensity of intracellular protein and DNA leakage from *L. acidophilus* with plasma treatment time. Plasma conditions were 2000 sccm argon flow rate and 10 W DC power input.

Figure 11.

SEM images of *S. mutans* cells of (a) untreated control; (b) 5 s argon plasma treatment; c) 9 s argon plasma treatment; and d) 15 s argon plasma treatment. The support media were glass slides and plasma conditions were 2000 sccm argon flow rate and 10 W DC power input.

Figure 12. SEM images of *L. acidophilus* cells of (a) untreated control; (b) 60 s argon plasma treatment; (c) 180 s argon plasma treatment; and (d) 300 s argon plasma treatment. The support media were glass slides and plasma conditions were 2000 sccm argon flow rate and 10 W DC power input.