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How deep can plasma penetrate into a biofilm?

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It is well known that plasma can deactivate various types of microorganisms. However, one fundamental key question has never been addressed, namely, how deep can plasma penetrate into multilayer biofilms. In this letter, *Porphyromonas gingivalis* (PG) biofilms (10 days growth, which has about 30 layers of PG cells with a thickness of about 15 μm) are treated with a cold plasma plume. It is found that the plasma can penetrate the biofilms and effectively deactivate all the bacteria in the 15 μm thick biofilms. Moreover, it was found that most of the dead cells' structures in the biofilms are not damaged. From the optical emission spectra of the plasma, it can be concluded that it is O and OH, rather than O_2^-, N_2^+, or UV emission that play the major role in the deactivation processes. © *2011 American Institute of Physics*. [doi:10.1063/1.3597622]

Atmospheric pressure nonequilibrium plasmas (APNPs) are rich in a variety of active species.[1–12] Reports show that APNPs are able to deactivate many different types of microorganisms such as bacteria, fungi, and viruses, as well as aid the coagulation of blood, enhance the proliferation of fibroblasts, and induce cancer cell apoptosis.[13–27] Therefore, their potential clinical applications including decontamination, blood coagulation, wound healing, and cancer treatment have attracted much attention. However, current research mainly focuses on the interactions of APNPs with individual cells, which are very different to realistic situations where cells form biofilms with multilayer structures. To achieve desirable effects, one fundamental key question, i.e., how deep APNPs can penetrate into the biofilms, has to be answered.

For example, many bacteria living in the human body are in the form of bacteria biofilms rather than individual cells. A biofilm is an aggregate of microorganisms in which cells adhere to each other and/or to a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also referred to as slime (although not everything described as slime is a biofilm), is a polymeric conglomeration generally composed of extracellular DNA, proteins, and polysaccharides. The microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single cells that may float or swim in a liquid medium. In addition, bacteria in the form of biofilms normally have a multilayer structure. It is much more difficult to kill microorganisms in the form of biofilms than in the form of individual cells, due to the protection afforded by the EPS and their multilayer structure. Few investigations on plasma destruction of 24 h growth biofilms have been carried out.[17,18] However, there is no information about the thickness of the biofilms. Thus, the issue of how deep APNPs can penetrate into biofilms has not yet been addressed.

In this letter, an APNP jet is used to deactivate *Porphyromonas gingivalis* (PG) biofilms, which have been incubated for 10 days. The PG are inoculated on the anaerobic solid medium which is packed into an anaerobic bag under 37 °C for 5 days. Typical individual colonies are then selected. They are diluted into 1.5×10^8 colony-forming unit (CFU)/ml suspensions with sterile physiological saline. Sterile coverslips (1 cm$\times$1 cm) are placed inside 24-well-cell plastic culture plates as substrates for bacteria biofilm growth. A 0.5 ml PG suspension culture is injected into each hole, followed by addition of 2.5 ml Bacto™ brain heart infusion (Becton, Dickinson and Co., USA). The samples are then again put into the anaerobic bag under 37 °C for 10 days to form multilayer biofilms. The brain heart infusion is replaced three times during the 10 days to make sure the bacteria have enough nutrition. After 10 days, the culture mediums are removed, and the coverslips are washed using 0.9% sterile physiological saline. The biofilms are now formed on the surface of the coverslips, which are ready for plasma treatment.

An APNP jet is used to treat the biofilms. Figures 1(a) and 1(b) are a schematic and photograph of the plasma jet device, respectively. Details about the device can be found in Ref. 15. For all the experimental results reported in this letter, an applied voltage V_a of 8 kV, a frequency of 8 kHz, and pulse width of 1600 ns are fixed. The working gasses He/O_2 (1%) with flow rate of 1 l/min are also fixed. The distance between the nozzle of the plasma jet and the coverslip is fixed at 1 cm. Samples are divided into two groups (five samples in each group)—the control group (gas flow only for 5 min) and the plasma treated group (5 min).

After treatment, all the samples are washed using 2 ml 0.9% sterile physiological saline. Then 100 ul SYTO 9 (component A) and 100 ul propidium iodide (PI, component B) staining solution are added. The SYTO 9 labeled (green fluorescence) all the living bacteria cells while PI (red fluorescence) only penetrated the ones with a damaged membrane, which makes the damaged cells fluoresce red. After staining for 15 min in a dark room, all the samples are washed again using 2 ml 0.9% sterile physiological saline, repeated three times. Immediately after that, the samples are examined us-

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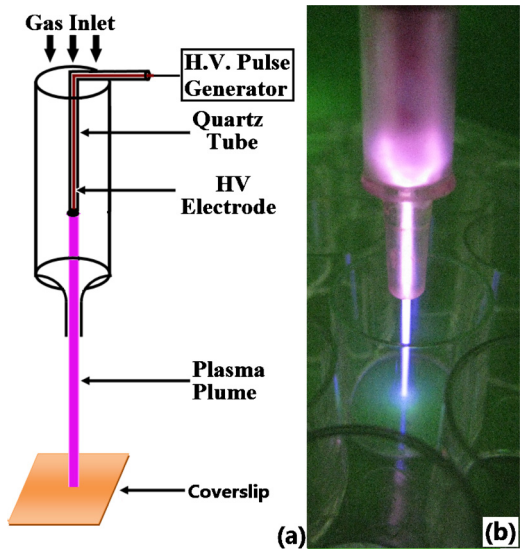

FIG. 1. (Color online) (a) Schematic of the device and (b) photograph of plasma jet acting on the PG biofilms. The coverslips full of biofilms are put inside the holes.

ing a confocal laser scanning microscope (CLSM, Olympus FV500).

Figures 2(a) and 2(b) show the three-dimensional (3D) CLSM images of the control sample and the plasma treated sample, respectively. Red stain spots in the images show the dead cells while the green spots represent the living cells. As we can see from the images, the control sample appears green while the treated sample is completely red. Therefore, we can conclude that the atmospheric pressure plasma jet can at least effectively deactivate the top surface of the PG biofilms.

As mentioned above, biofilms have multilayer structure. The question to ask, therefore is, are the bacteria under the top layer also killed? Fortunately, CLSM not only gives an image of the top layer of the biofilms but can also give images at any biofilm depth. The CLSM can scan the biofilms from the topmost surface to the very bottom, layer by layer at a preset step. Here, the CLSM scanning step is fixed at 1 μm. The total thickness of the biofilms is about 15 μm. Therefore, we get 15 layers in total for each sample. Because the dimension of a single PG cell is about 0.3 μm $\times$0.8 μm$\times$0.3 μm, there are about 30 layers of PG cells. In other words, each layer image contains about two layers of PG cells. Figures 3(a), 1–4, 3(b), and 1–4 are the images of the first, tenth, 11th, and 15th layer images of the control sample and the plasma treated sample, respectively.

As we can see from Figs. 3(a) and 1–4, there are not many dead cells from the first layer to the tenth layer for the control group. Some dead cells (red spots) start to appear on

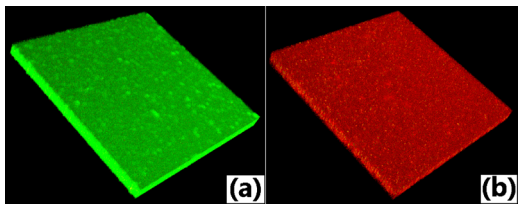

FIG. 2. (Color online) (a) 3D CLSM image of the control sample (gas flow only for 5 min) and (b) the plasma treated group sample (plasma treatment for 5 min). (a) shows the live cells and (b) shows the dead cells.

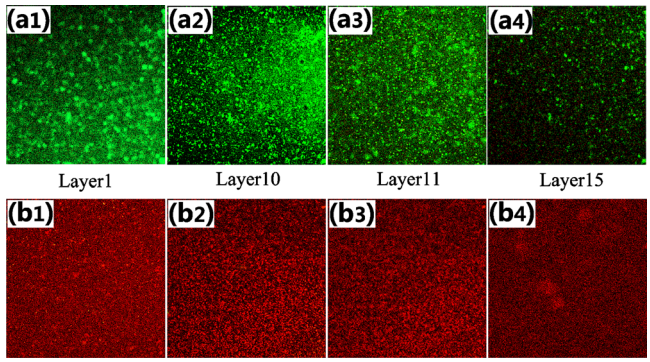

FIG. 3. (Color online) 2D CLSM images of different layers. (a1)–(a4) are the first, tenth, 11th, and 15th layer CLSM images of the control sample (gas flow only for 5 min), respectively. (b1)–(b5) are the first, tenth, 11th, and 15th layer CLSM images of the plasma treated sample (plasma treatment for 5 min), respectively. (a) shows the live cells and (b) shows the dead cells.

11th layer, more can be seen on the 15th layer. Because the gas interacts with the biofilms from the top layer but there are not many dead cells from the first to the tenth layers, it can be concluded that the dead cells that are observed from the 11th layer onwards are probably a result of natural death due to lack of nutrition. Therefore, we can conclude that the gas flow has no significant effect on the death of the PG biofilms.

For the plasma treated sample, as we can see from Figs. 3(b) and 1–4, almost all the cells are killed for all the layers. The numbers of cells start to decrease from the 11th layer. This is consistent with the control sample. However, the cells for the plasma treated sample, even the cells on the 15th layer, are all dead. Thus, we can conclude that the plasma can penetrate through the 15 μm biofilms and kill all the bacteria present.

To understand the mechanism of how the bacteria are killed, transmission electron microscope (TEM, FEI Tecnai $G^2$12, The Netherlands) images of the PG cells are taken for further analysis. Standard TEM procedures are followed. Figures 4(a)–4(c) are images of the control and treated PG cells. As we can see from Fig. 4(a), the control PG cells appear bacilliform. The cells capsule is consisted of polysaccharide or polypeptide around the cell wall. Figures 4(b) and 4(c) are two typical dead cell samples after plasma treatment. For the case of Fig. 4(b), the whole cell is broken up into pieces, the cytoplasm leaks to the extracellular material. This sample could not be dyed by either SYTO 9 or PI stain. For Fig. 4(c), the cell capsule becomes blurry. This sample could be dyed using PI, which produces the red spots on the CLSM images. Because the cell numbers between Figs. 3(a), 1, 3(a), 2, 3(b), 1, 3(b), and 2 have no obvious difference, we can conclude that, for most of the dead cells, their cell structures are not damaged.

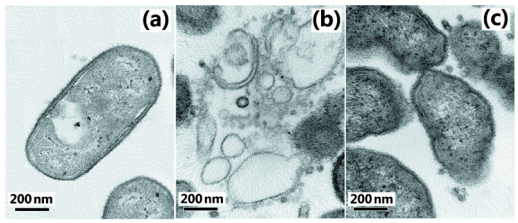

FIG. 4. (Color online) TEM images of the PG cells for (a) control and [(b) and (c)] plasma treated samples.

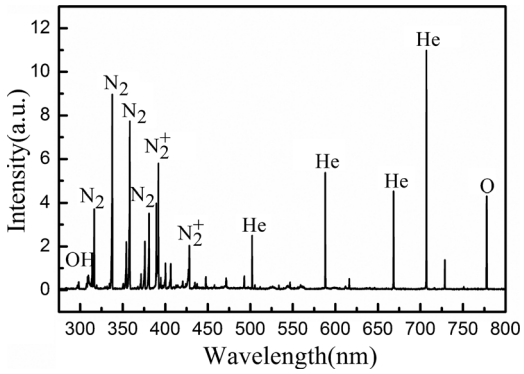

FIG. 5. Optical emission spectra of the plasma. Working gases: He/O_2 (1%), flow rate: 1 l/min.

To investigate what kind of active species are present in the plasma, a half-meter spectrometer (Princeton Instruments Acton SpectraHub 2500i) is used to measure the optical emission of the plasma plume. The operation parameters of the spectrometer (grating: 1200 g/mm, slit width: 100 μm) are unchanged. Figure 5 shows the typical emission spectra (300–800 nm) of the plasma plume. It clearly shows that excited O, OH, N_2, N_2^+, and He are presented in the plasma plume. In our previous work on the deactivation of individual bacterium,[24] it was found that the active species, including O, OH played a crucial role in the deactivation of the bacteria. These species could directly act on microorganisms, especially the outside membranes. Species O and OH could react with membranes and damage them. Since they are neutral particles, they can easily penetrate into the biofilms and kill the bacteria. Excited N_2 probably plays a minor role in the deactivation process.[24] It was found in our previous study[24] that negative O_2^- ions play a significant role in the deactivation of individual bacterium cells. However, for the deactivation of biofilms, when the top layer of the biofilms is charged with negative ions, it is difficult for the negative ions to penetrate into the biofilms due to a repulsive force among the charged particles. So it can be concluded that the negative ions probably play a minor role in the deactivation of the biofilms. The final potential reactive agent is UV emission. However, the UV emission for our plasma jet is measured to be only approximately 0.05 mW/cm^2. Therefore, it only plays a minor role, if any, in the deactivation of the bacteria.

In conclusion, the PG biofilms (10 days growth) are treated using an APNP jet. The biofilms have a thickness of about 15 μm, which corresponds to 30 layers of PG cells. The 3D CLSM images show that the plasma can penetrate into the biofilms and effectively deactivate all the bacteria in the 15 μm biofilms. Most of the dead cells' structure are not damaged by the plasma. From the optical emission spectra of the plasma, it can be concluded that it is O and OH, rather than O_2^-, N_2^+, or UV emission that plays the major role in the deactivation processes.

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