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Inactivation of a 25.5 μm *Enterococcus faecalis* biofilm by a room-temperature, battery-operated, handheld air plasma jet

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Abstract
Effective biofilm inactivation using a handheld, mobile plasma jet powered by a 12 V dc battery and operated in open air without any external gas supply is reported. This cold, room-temperature plasma is produced in self-repetitive nanosecond discharges with current pulses of ~100 ns duration, current peak amplitude of ~6 mA and repetition rate of ~20 kHz. It is shown that the reactive plasma species penetrate to the bottom layer of a 25.5 μm-thick *Enterococcus faecalis* biofilm and produce a strong bactericidal effect. This is the thickest reported biofilm inactivated using room-temperature air plasmas.

(Some figures may appear in colour only in the online journal)

1. Introduction

Cold atmospheric-pressure plasmas (CAPPs) have widespread biomedical and health care applications in pathogen inactivation, wound healing, blood coagulation and interventional oncology, to mention just a few (Fridman *et al* 2008, Shashurin *et al* 2008, Cvelbar *et al* 2009, Keidar *et al* 2011, Kong *et al* 2009, Laroussi 2009). An overwhelming majority of the existing CAPPs rely on external power (e.g. generators, wall power, cables) and gas feed (e.g. gas bottles, valves, flow controllers) supply (Cvelbar *et al* 2009, Kong *et al* 2009, Keidar *et al* 2011). This limits their utility in mobile handheld devices for point-of-care applications, e.g. in ambulance emergency outcalls, natural disaster rescue and military combat operations, treatments in remote locations, etc. Moreover, treatment efficacy of such devices should be comparable or even superior to less mobile hospital-based equipment.

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Here we report on the generation of stable, room-temperature, atmospheric-pressure plasma jet discharges operated in open air without any external power or gas supply. This portable plasma source is handheld (named 'plasma flashlight' here) and is powered by a 12 V dc battery. We have also applied the plasma jet to effectively inactivate multilayered *Enterococcus faecalis* biofilms. *Enterococcus faecalis* is a Gram-positive facultative anaerobic bacterium, which often infects root canals during endodontic dental treatments of patients with persistent apical periodontitis.

These bacteria are among the most antibiotic- and heat-resistant pathogens which strongly resist calcium hydroxide treatment commonly used in the course of endodontic therapy (Fisher and Phillips 2009). Moreover, self-organization of bacterial colonies into (typically a few tens of micrometres thick) biofilms offers additional degrees of resistance to the treatments due to their multilayer structure, cemented by the extracellular polymeric substance (Ma *et al* 2009, Flemming

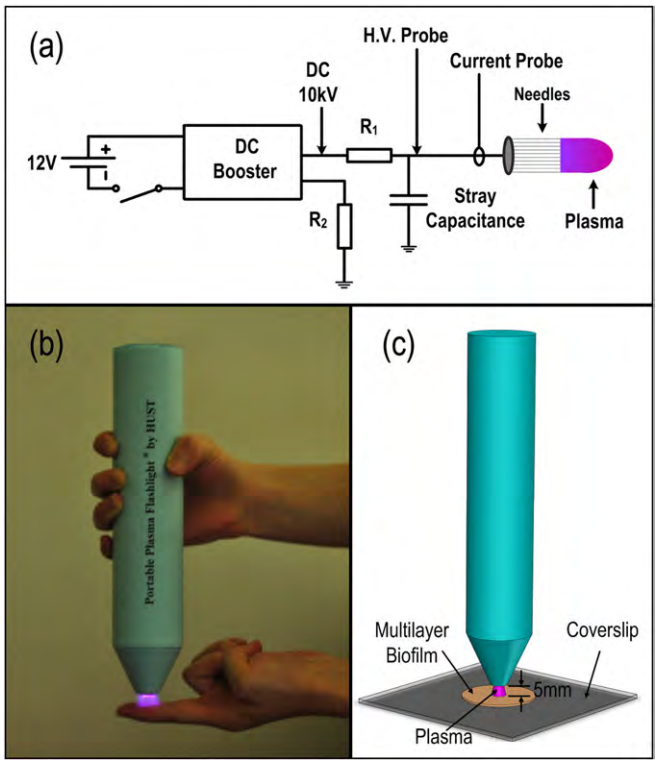

Figure 1. (*a*) Schematic of the plasma jet setup, (*b*) photograph of the portable handheld plasma flashlight device and (*c*) schematic of the biofilm treatment.

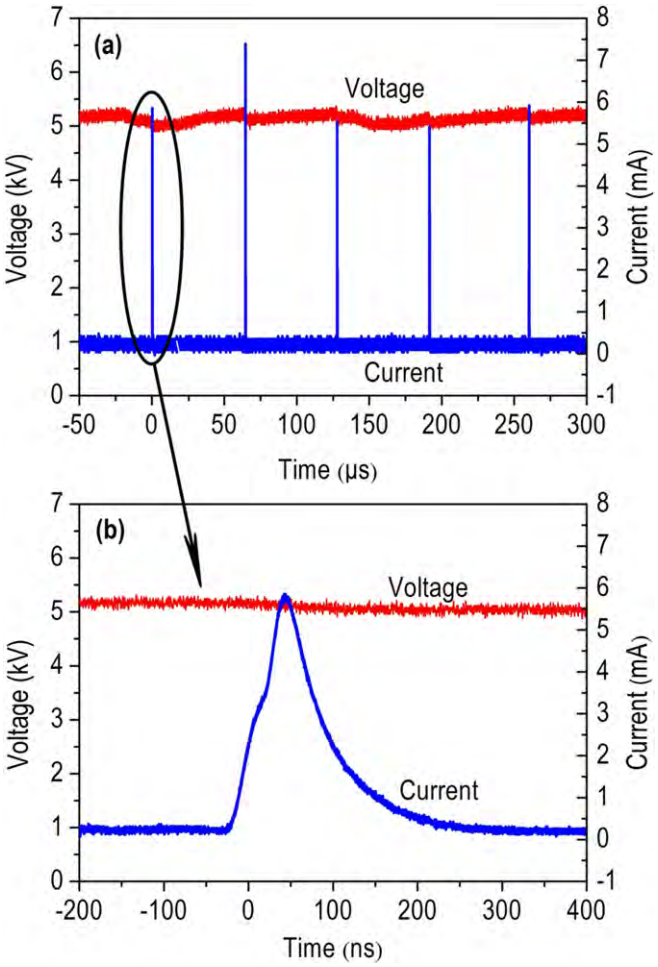

Figure 2. Current–voltage waveforms of the plasma discharge: (*a*) multiple repetitive pulses and (*b*) a single pulse.

and Wingender 2010). Here we demonstrate the efficacy of the plasma flashlight open-air discharge in a rapid, 5 min-long inactivation of an approximately 25.5 μm-thick *Enterococcus faecalis* biofilm through effective penetration of the plasma-generated reactive oxygen species (ROS) to the very bottom, 17th layer of the biofilm. These and other reactive species produced in the plasma discharge are also studied using high-resolution optical emission spectroscopy (OES).

2. Methods

Figure 1 shows the circuit diagram and a photograph of the plasma flashlight device and also a sketch of the biofilm treatment. This handheld plasma jet is driven by a 12 V battery and does not require any external generator or wall power; neither does it require any external gas feed or handling (e.g. valves, mass flow controllers, etc) system. With the input voltage of 12 V, the output voltage of the dc booster reaches 10 kV (figure 1(*a*)). An array of 12 stainless steel needles is used as an electrode. The radius of the needle tips is $\sim$50 μm. The ballast resistors R_1 and R_2 (both 50 MΩ) shown in figure 1(*a*) are used to limit the discharge current. This is made to minimize the plasma heating and electric shock effects on the human body and to make it safe to touch, as pictured in figure 1(*b*). The gas temperature of the plasma plume is measured within the 20–28 °C range, which is very close to room temperature.

The dynamics of the plasma discharges was studied using a combination of high-voltage (HV) current and voltage probes and high-resolution OES. The discharge voltage and current were measured using P6015 and CT1 HV current and voltage probes, respectively, both from Tektronix. The current and voltage waveforms are recorded by a Tektronix DPO7104 wideband digital oscilloscope, and are shown in figure 2. From figure 2 one can clearly see that the discharge appears as a periodic sequence of nanosecond repetitive pulses with a pulse repetition rate of approximately 20 kHz. Figure 2(*b*) shows the current and voltage waveforms of a typical single pulse. The current pulse has a full-width at half-maximum of $\sim$100 ns and a peak value of $\sim$6 mA. Using these current–voltage waveforms, the power dissipated into the plasma was estimated to be $\sim$60 mW.

The *Enterococcus faecalis* biofilms were prepared as follows. The dry-freeze (stored at -70 °C) *Enterococcus faecalis* is inoculated on the Bacto^{TM} brain heart infusion (Becton, Dickinson and Co., USA) ager plate at least subculture (incubated at 37 °C for 24 h) twice after thawing out and recovery. Then typical individual colonies are selected; they are diluted into 1.5×10^8 colony-forming unit (CFU/ml) suspensions with sterile physiological saline. Sterile coverslips (diameter of 12 mm and thickness of 0.17 mm) are placed inside the 24-well-cell plastic culture plates as substrates for bacterial biofilm growth. A 0.2 ml *Enterococcus faecalis* suspension culture is injected into each hole, followed

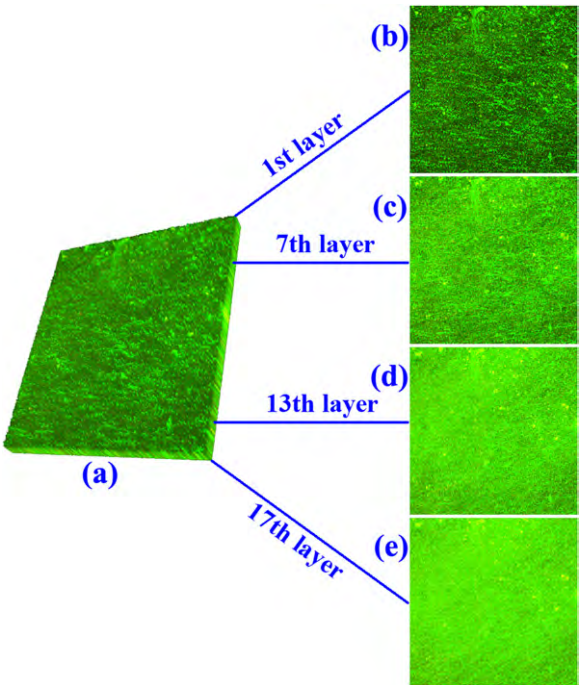

Figure 3. (*a*) 3D CLSM image and (*b*)–(*e*) 2D CLSM images of the 1st, 7th, 13th and 17th layers of the control biofilm sample, respectively. The cells are live.

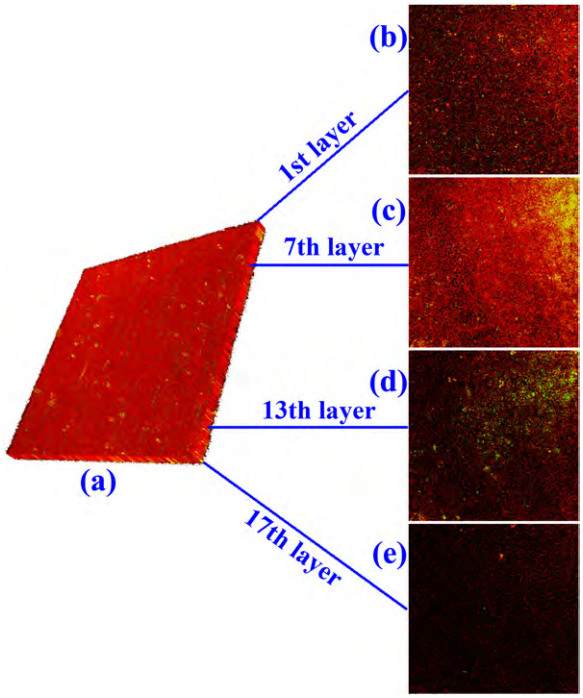

Figure 4. Same as in figure 3 for the sample treated in the plasma for 5 min. The cells are dead in all the layers of the biofilm.

by addition of 2.8 ml Bacto^{TM} brain heart infusion broth (Becton, Dickinson and Co., USA). The samples are then incubated at 37 °C for 7 days to form multilayer biofilms. The brain heart infusion is replaced twice during these 7 days to ensure adequate cell nutrition. After 7 days, the culture medium is removed, and the coverslips are washed using 0.9% sterile physiological saline. As a result, the biofilms are formed on the coverslip surface.

During the plasma treatment, the biofilms are placed about 5 mm away from the plasma flashlight nozzle, as shown in figure 1(*c*). The treatment time is fixed at 5 min for all the samples. After the treatment, all the samples are washed using 2 ml 0.9% sterile physiological saline. Then 100 μl SYTO 9 and 100 μl propidium iodide (PI) staining solution is added. The SYTO 9 green fluorescence label is used to map the living bacteria cells while the PI red fluorescence label (which can only transfuse through a damaged cell membrane) is used to trace damaged bacterial cells. 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 afterwards, the samples are examined using confocal laser scanning microscopy (CLSM, Olympus FV500).

3. Results and discussion

Figures 3(*a*) and 4(*a*) are the three-dimensional (3D) CLSM images of the control (untreated) sample and the plasma-treated sample, respectively. Figures 3(*b*)–(*e*) and 4(*b*)–(*e*) are the two-dimensional (2D) 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. The 2D images are produced through layer-by-layer CLSM scans of the biofilms from the topmost surface to the very bottom, in preset steps of 1.5 μm. The total thickness of the biofilms is about 25.5 μm. Given the sizes and shapes of the *Enterococcus faecalis* cells, each sample contains ∼17 layers.

As one can see from figures 3(*a*) and 4(*a*), the control sample appears green while the treated sample is completely red. One can thus conclude that the room-temperature air plasma can at least effectively inactivate the bacteria on the top surface of the biofilms. As mentioned above, biofilms have a multilayer structure. The question to ask, therefore, is whether the bacteria under the top layer are also inactivated? Figures 3(*b*)–(*e*) show that there is almost no trace of dead cells in the images of the 1st, 7th, 13th and the 17th layer of the control sample. On the other hand, almost all the cells are killed in all the 17 layers of the plasma-treated sample (figures 4(*b*)–(*e*)). Therefore, the plasma-generated reactive species can penetrate through the 25.5 μm-thick biofilms and exert a bactericidal effect. The biofilms treated in this experiment are 10.5 μm thicker compared with the earlier report (Xiong *et al* 2011).

To investigate the presence of reactive species in the plasma, a half-meter spectrometer (Princeton Instruments Acton SpectraHub 2500i; spectral resolution: 2 nm, grating: 1200 g mm^{-1}, slit width: 100 μm) is used to measure the optical emission of the plasma plume. Figure 5 shows typical emission spectra (in the 250–800 nm spectral range) of the plasma plume. It is clearly seen that the optical emission spectra are dominated by the excited N_2 and O species.

It is well known that ROS play a crucial role in bacterial inactivation (Liu *et al* 2010, 2011) and lead to various biological effects in the intracellular space (Yan *et al* 2012).

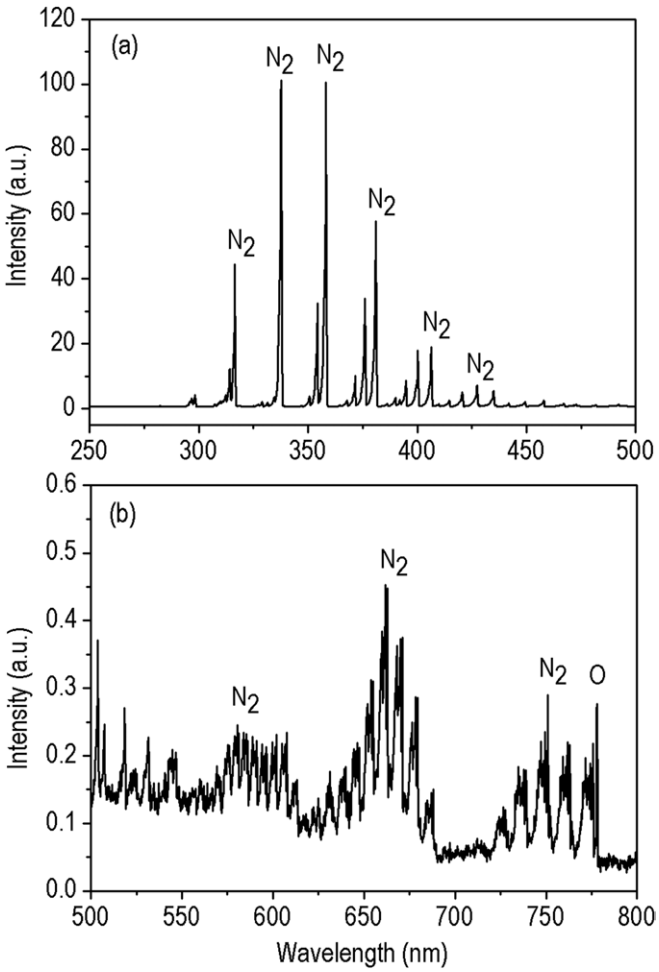

Figure 5. Optical emission spectra of the plasma: (*a*) 250–500 nm and (*b*) 500–800 nm.

These species directly act on microorganisms, especially their outer membranes, thereby acting as bactericidal agents. Moreover, due to the enhanced mobility, ROS species can easily penetrate into the biofilms and kill the bacteria deep inside them. The role of the excited N_2 species is expected to be less significant in the pathogen-inactivation process compared with ROS species (Lu *et al* 2008, Kong *et al* 2009, Laroussi 2009). The other potential bactericidal agent is UV radiation (Nosenko *et al* 2009). However, the energy flux from UV emission for our plasma jet is measured to be less than 0.05 W cm^{-2}. This is typically less compared with many other CAPP devices used in pathogen treatments (Kong *et al* 2009, Nosenko *et al* 2009). Therefore, the UV radiation indeed plays a minor role in the biofilm inactivation in these experiments. More importantly, this low level of UV exposure is an added benefit of the plasma flashlight device from the occupational health and radiation safety perspective.

4. Conclusion

In summary, we have demonstrated the effective generation of cold, room-temperature open-air plasmas using a handheld, mobile plasma jet powered by a 12 V dc battery. The plasma source does not rely on any external power or feedstock gas supplies and has the obvious advantage of utility in numerous point-of-care applications where these supplies are unavailable. Only 60 mW dc power is required to sustain the discharge, which attests on the excellent energy efficiency of the device; this is a very competitive amount of power which may ensure the future commercial applications of this device, after a reasonable scaling down. More importantly, this simple and portable plasma jet has demonstrated superior performance in inactivation of a 25.5 μm-thick *Enterococcus faecalis* biofilm, which is 10.5 μm thicker than the previously reported one. It is found that the reactive plasma species not only inactivate the top-layer cells, but also penetrate into the very bottom layer of the biofilms and kill the bacteria. These results advance our ability to effectively inactivate biofilms formed by notorious drug- and treatment-resistant pathogens. Our mobile, handheld plasma flashlight device may also be used for surface treatment/functionalization in nanotechnology, device fabrication and several other applications, where surface temperature sensitivity is an issue (Ostrikov 2011).

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